Identification and Development of Fetal Epigenetic Markers for Non-invasive Prenatal Diagnosis

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Chemical Pathology

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ABSTRACT

Identification and development of fetal epigenetic markers for non-invasive prenatal diagnosis

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The discovery of fetal-derived circulating nucleic acids in maternal plasma has opened up new opportunities for non-invasive prenatal diagnosis. This non-invasive means of obtaining fetal genetic materials is safer than invasive tissue-sampling procedures, which are associated with a small but finite chance of fetal loss. However, the detection of fetal DNA in maternal plasma is complicated by the co-existence of a large background of maternal DNA. Over the past decade, the detection of fetal DNA in maternal plasma has evolved from dependency on discriminative genetic markers, such as Y-chromosome-specific loci or paternally-inherited polymorphisms, to detection of circulating RNA, fetal-specific methylation or by massively parallel sequencing. Fetal-specific methylation, or fetal epigenetic marker, does not require prior knowledge of the sex or polymorphic status of the fetus and thus can be applied in essentially all pergnancies. This thesis focuses on the development of this kind of marker for non-invasive monitoring and detection of pre-eclampsia and fetal aneuploidies.

Fetal epigenetic markers were developed based on differential methylation patterns between fetal and maternal tissues. To evaluate their application in pregnancy-associated disorders, the first part of this thesis describes the use of a reported fetal epigenetic marker, *RASSF1A*, to measure the fetal DNA concentrations in maternal plasma of pre-eclamptic subjects versus gestational-age-matched controls. This was achieved by a strategy based on the specific removal of unmethylated maternal DNA by methylation-sensitive restriction enzymes and the detection of digestion-resistant hypermethylated fetal DNA. Concentrations of hypermethylated *RASSF1A* in third-trimester (28 – 40 weeks) maternal plasma was significantly higher in pre-eclampsia than those in uncomplicated pregnancies. This finding demonstrates that fetal epigenetic marker has the potential for the non-invasive prenatal assessment of pre-eclampsia regardless of the sex or the polymorphic status of the fetus.

The second part of this thesis describes a systematic search for potential epigenetic markers for pre-eclampsia and the second commonest fetal aneuploidy, trisomy 18. Numerous approaches for methylation profiling are described, including locus-specific approaches, such as methylation-specific polymerase chain reaction (MSP), bisulfite sequencing, a mass spectrometry-based platform (the Epityper assay), and a genome-wide approach, namely methylated DNA immunoprecipitation coupled with tiling array analysis (MeDIP-chip).

Using MeDIP-chip, I systematically searched the entire chromosome 18 (>351,500 CpG sites), and identified 178 regions (3,040 CpG sites) that are differentially methylated between the placenta and maternal blood cells. I obtained quantitative

methylation data on 26 regions (370 CpG units) by the Epityper and bisulfite sequencing, and then selected the most promising locus for further characterization in maternal plasma. Thus, the third part of this thesis describes the identification and validation of these markers, and their detection in maternal plasma. An intergenic region between *VAPA* and *APCDD1* (the *VAPA-APCDD1* DNA) was found to be methylated in the placenta, but not maternal blood cells. The methylated *VAPA-APCDD1* DNA was detected in maternal plasma only during pregnancy, but not after delivery, and its concentrations in maternal plasma correlated significantly with those of an established fetal genetic marker.

The final part of this thesis presents the development of an approach called epigenetic-genetic (EGG) chromosome dosage for the detection of trisomy 18. I measured the concentrations of the novel methylated fetal epigenetic marker, *VAPA-APCDD1* DNA, located on chromosome 18, relative to those of a fetal genetic marker, *ZFY*, located on chromosome Y. Thereby, I have demonstrated that it is feasible to infer the relative dosage of fetal chromosome 18 by analysing maternal plasma obtained from the first to third trimesters, and to non-invasively detect fetal trisomy 18 with a sensitivity of 88.9% and a specificity of 96.3%.

This thesis illustrates different strategies for methylation profiling and presented two examples of applying DNA methylation for the non-invasive prenatal assessment of pregnancy-associated disorders, such as pre-eclampsia, and fetal chromosomal aneuploidies, such as trisomy 18. I envision that a similar strategy could be developed for other pregnancy-related diseases to broaden the application of epigenetic markers in non-invasive prenatal diagnosis.

摘要

傳統上採用的入侵性產前診斷方法有機會引至流產。自從科學家在母體血漿中發現屬於胎兒的游離脫氧核糖核酸 (DNA)之後,這一安全獲取胎兒遺傳訊息的重大發現爲無創性產前檢查提供了新方向。然而，由於在母體血漿中亦同時存在著大量來自母親的游離 DNA，科學家一直努力開發不同類型的胎兒標誌物,用以區分母體血漿中的胎兒和母體 DNA。 早期最爲廣泛研究應用的胎兒基因標誌物是 Y 染色體序列或遺傳自父親的單核苷酸多態性，但這些標記只適用於部分胎兒。爲了更廣泛地將無創性產前檢查應用到更多的孕婦身上,研究開發與性別或多態性無關的胎兒標誌物尤其重要。其中一個方法是利用表觀遺傳學的概念，研究母體與胎兒某些基因所呈現的不同甲基化狀態，從而在母體血漿中檢察胎兒的基因訊號。

本論文第一部分著重評估已知的表觀遺傳學胎兒基因標誌物用於無創性產前診斷的成效。這個表觀遺傳學標誌物處於 *RASSF1A* 基因的第一個外顯子，可以用於定量檢測母體血漿中胎兒游離 DNA 的濃度。研究結果發現，這個基因標誌物在妊娠高血壓症的孕婦的血漿濃度明顯比對照組高。是次研究證明表觀遺傳學標誌物對於妊娠高血壓症的無創性產前診斷有一定的用途。由於這個基因標誌並不受制於胎兒的性別或多態性，相比起基於 Y 染色體序列開發的胎兒基因標誌物，它在無創性產前診斷方面的用途將更廣泛。

論文第二部分則集中論述開發新的表觀遺傳學標誌物。其中一部份利用甲基特異性聚合酶鏈反應 (methylation-specific polymerase chain reaction，MSP)

及 EpiTYPER™ DNA 甲基化分析技術 [利用基質輔助鐳射解析/電離飛行時間質譜原理(Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry，MALDI-TOF MS)]，比較患有妊娠高血壓症的孕婦與對照組之間 15 個候選基因在胎盤的甲基化表現。初步研究結果顯示兩組之間並沒有明顯分別;另一部份利用甲基化 DNA 免疫沉澱方法（Methylated DNA Immunoprecipitation，MeDIP）加上嵌合晶片，進行高通量的全基因組甲基化定量分析，比較正常孕婦胎盤與血細胞 18 號染色體的甲基化表現。結果顯示，18 號染色體上有 178 個區域的甲基化表現在兩組之間有明顯分別，而這些區域有機會被開發成胎兒標誌物。因此，論文的第三部分論述利用 EpiTYPER™和亞硫酸鹽基因測序法對其中 26 個區域進行有系統的驗證，並評估這些候選標誌物用於定量檢測母體血漿中胎兒游離 DNA 的準繩度。

論文的最後部分論述如何利用其中一個最有前途的甲基化胎兒標誌物在母體血漿中檢測胎兒是否患有 18 號染色體三體症（愛華氏綜合症）。結果顯示，利用數碼化聚合酶鏈反應 （digital PCR）定量檢測 18 號染色體以及 Y 染色體兩個胎兒標誌物在母體血漿中的相對濃度，能夠在妊娠首三月期的母體血漿中成功檢測愛華氏綜合症，敏感度及特異性分別高達 88.9%及 96.3%。

總括而言，本論文闡述了如何利用不同的甲基化定量分析方法研發更多胎兒標誌物，並以妊娠高血壓症和愛華氏綜合症作爲例子，具體證明甲基化胎兒標誌物在無創性產前診斷方面的應用。我寄望這些經驗將會幫助開發更多無創性產前檢測方法，讓更多的孕婦受惠。

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Conference abstracts:

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CONTRIBUTORS

I declare that I am the main contributor to the work described in this thesis. Under the supervision of Professor Dennis Lo, I was responsible for the processing of the samples, the design and development of the assays, experimental work, data analyses and interpretation, and writing of the manuscript unless otherwise specific here: The *BstUI*-mediated real-time quantitative PCR duplex assay for *RASSF1A* and *β-actin* described in Chapter 4 were developed by Professor Allen Chan and Ms S. W. Yeung. Professor Allen Chan has prepared the verification protocol for the single molecule detection limit test in Appendix II. The *ZFY/X* duplex assay described in Chapter 6 was originally developed by Professor Stephen Chim and Mr. Tristan Shing. The samples used in this thesis were recruited with the assistance of Professor T. K. Lau, T. N. Leung and T. Y. Leung, Dr Mary H.Y. Tang and Dr Elizabeth Lau.

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LIST OF ABBREVIATIONS

ABL	*C-abl oncogene 1, receptor tyrosine kinase*
APCDD1	*Adenomatosis polyposis coli down-regulated 1*
AFP	α-fetoprotein
B4GALT6	*β-1,4-galactosyltransferase 6*
β hCG	*β subunit of human chorionic gonadotropin*
CDH1	*E-cadherin*
Cq	Quantification cycle
CTDP1	*Carboxy-terminal domain, RNA polymerase II, polypeptide A phosphatase, subunit 1*
CV	Coefficient of variation
CVS	Chorionic villus sampling
DAPK	*Death-associated protein kinase 1*
ddNTP(s)	Dideoxynucleotide triphosphate(s)
DEPC	Diethyl pyrocarbonate
EAR	Epigenetic allelic ratio
EDTA	Ethylenediaminetetraacetic acid
EGG	Epigenetic-genetic (chromosome dosage analysis)
ERBB2	*V-erb-b2 erythroblastic leukemia viral oncogene homolog 2*
FAM	6-carboxyfluorescein
GSTP1	*Glutathione S-transferase pi*
HBEGF	*Heparin-binding EGF-like growth factor*
hCG	Human chorionic gonadotropin
HLCS	*Holocarboxylase synthetase*
hPL	*Human placental lactogen*

HSD11B2	*11b-hydroxysteroid dehydrogenase type 2*
IGB	Integrated Genome Browser
IGFBP3	*Insulin-like growth factor binding protein-3*
IPTG	Isopropyl β-D-thiogalactopyranoside
IQR	Interquartile range (IQR)
KIT	*V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog*
LDLR	*Low-density lipoprotein receptor messenger*
LOD	Limit of detection
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MAT	Model-based Analysis of Tiling array
MeDIP-chip	Methylated DNA immunoprecipitation coupled with tiling array analysis
MET	*Met proto-oncogene (hepatocyte growth factor receptor*
MGB	Minor-groove binding
MI	Methylation index
MSF	Methylated site frequency
MSP	Methylation-specific PCR
MSRE(s)	Methylation-sensitive restriction enzyme (s)
MYC	*V-MYC myelocytomatosis viral oncogene homolog*
NOS2A	*Inducible NO synthase (iNOS)*
NT	Nuchal translucency
p16	*Cyclin-dependent kinase inhibitor 2A*
PAPP-A	*Pregnancy-associated plasma protein A*
PCR	Polymerase chain reaction
PET	Pre-eclampsia

PLAC4	*Placenta-specific 4*
qMSP	Quantitative MSP
qPCR	Quantitative PCR
RASSF1A	*Ras association domain family 1A*
RhD	Fetal Rhesus D
RT-PCR	Reverse-transcriptase-PCR
SAP	Shrimp alkaline phosphatase
SERPINB5	*Serpin peptidase inhibitor, clade B (ovalbumin), member 5*
SERPINB2	*Serpin peptidase inhibitor, clade B (ovalbumin), member 2*
SLR	Signal log ratio
SNP	Single nucleotide polymorphism
SRY	*Sex-determining region Y*
T18	Trisomy 18
TAMRA	6-carboxytetramethylrhodamine
TAS	Tiling Array Software
TSPY1	*Testis-specific protein, Y-linked 1*
UNG	Uracil-N-glycosylase
VAPA - APCDD1	Intergenic region between the genes *VAPA* and *APCDD1*
VAPA	*Vesicle-associated membrane protein*
VEGFA	*Vascular endothelial growth factor A*
VIC	4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein
VLDLR	*Very-low-density lipoprotein receptor messenger*
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
ZFY	*Zinc finger protein, Y-linked*
ZNF516	*Zinc finger protein 516*

Section I BACKGROUND

This section briefly reviews the methods that are conventionally used for performing prenatal diagnosis, their limitations, and the subsequent development via the analysis of cell-free fetal DNA in maternal plasma.

Chapter 1 Prenatal Diagnosis

1.1. Demand for prenatal diagnosis

Prenatal screening or diagnosis is an integrated part of modern obstetrics care. Early detection of fetal complications is important for medical practitioners to arrange timely medical intervention. For example, in some conditions such as fetal chromosomal aneuploidies, the fetus might be born with severe cardiac defects and so would require immediate medical support upon delivery. In severe cases, the mother would be counselled for terimination of pregnancy. It allows the parents to get psychologically and financially prepared for the affected newborn. Nowadays, the mode of family planning has changed. People tend to have a smaller family and postponed childbearing. As the incidence of fetal aneuploidies increases with maternal age (Akesson et al. 1966; Driscoll et al. 2009), there is a growing need for safe and accurate prenatal diagnosis.

1.2. Conventional methods for prenatal diagnosis

Fetal chromosomal aneuploidy is the main reason for most pregnant women seek prenatal diagnosis (Driscoll et al. 2009), which may cause severe birth defects or early childhood death. The commonest autosomal aneuploidy that survive to birth is trisomy 21 (Down syndrome), which affects 1 in 800 births. There is an observable correlation of advanced maternal age with the incidence of fetal chromosomal aneuploidies, and therefore historically, pregnant women aged 35 or above were offered genetic counseling and invasive diagnostic tests (Driscoll et al. 2009).

1.2.1. Invasive procedures

The most definitive approach to assess a fetus's genetic health would be to directly examine the tissues that contain fetal genetic materials, such as the amniotic fluid or chorionic villi.

The collection of amniotic fluid was done by using a needle to puncture the uterus to aspirate the fluid from the amniotic cavity. This procedure, called amniocentesis, is usually performed at around 16 weeks of gestation, when sufficient viable fetal cells could be relatively safely obtained for analysis (Alfirevic et al. 2003). On the other hand, chorionic villus sampling (CVS) was done via transabdominal or transcervical approaches to aspirate the chorionic villi from the placenta. In contrast to amniocentesis, this procedure is usually feasible starting from the 10th week of gestation. After collection, the tissues might be subjected to karyotyping.

The major limitation of these invasive approaches is that they are associated with around 1 – 2 % chance of miscarriage (Alfirevic et al. 2003). There is also a risk of infection and leakage of the amniotic fluid, which might affect the development of the fetal lung.

To achieve a balance between the risk of losing the fetus and the detection of fetal trisomy, the maternal age of 35 has been used as the cut-off for offering invasive diagnostic tests. However, there were still around 75% of infants with Down

syndrome born to women younger than the age of 35 (Loncar et al. 1995). In view of that, a number of non-invasive screening options have been developed since the 1970s, such that all pregnant women can choose to perform a screening test prior to invasive diagnosis.

1.2.2. Non-invasive alternatives

Many prenatal screening programs would include two main parts: (1) physical anatomy of the fetus by ultrosonography; and (2) maternal serum tests to measure the levels of pregnancy-associated hormones in the maternal blood.

1.2.2.1 Ultrasonographic examination

Starting from the 6th week of gestation, the developing embryo can be visualized by an ultrasound scan. This procedure confirms the gestational age of the fetus, which is crucial for the choice of subsequent screening tests, and reveals the size and anatomy of the fetus. In particular, during the 11th – 14th weeks, nuchal thickness scan might be offered to assess the amount of fluid behind the neck of the fetus, a parameter also known as the nuchal translucency (NT). Around 65 – 85% of trisomic fetuses tend to present a significantly higher NT thickness (Adekunle et al. 1999; Witters et al. 2007). However, a similar elevation is also observed in up to 13% of normal fetuses. Therefore, the NT measurement alone is not sufficient for screening of chromosomal abnormalities.

1.2.2.2. Maternal serum screening

Screening by markers from maternal serum is usually performed during the second trimester (15th – 28th week of gestation) (Spencer 1999). A number of hormones are useful maternal serum markers for predicting the risk of fetal chromosomal aneuploidies. These markers include α-fetoprotein (AFP), unconjugated estriol, human chorionic gonadotropin (hCG) and inhibin *A*.

Many studies have been carried out to evaluate the predictive accuracy of these markers, either used on their own or in combination with each other. The triple test, which measures the levels of AFP, estriol and hCG, is widely used during the second trimester and could detect 72% of affected pregnancies (Wald et al. 1988; Wald et al. 1995). Later on, inhibin A was also introduced to the panel and the measurement of the four markers is known as the quadruple test (Aitken et al. 1996; Benn et al. 2003; Christiansen et al. 2005). Pregnancies affected by trisomy 21 are usually associated with higher levels of hCG and inhibin A while lower levels of AFP and estriol (Benn et al. 2002; Driscoll et al. 2009). The quadruple test has a detection rate of 80% at a fixed false positive rate of 5%, which is superior to the triple test (Malone et al. 2005). It is thus widely used in many clinical centers (Benn et al. 2002; Borrell et al. 2004).

Serum marker screening is also available during the first trimester (until 14th week). For example, measurements of PAPP-A (pregnancy-associated plasma protein A) and hCG could be combined with the measurement of nuchal translucency to achieve

a detection rate of 83% for trisomy 21 (Borrell et al. 2004; Wald et al. 2003). Integrating these two first-trimester and second-trimester screenings could further enhance the detection rate to over 90% (Malone et al. 2005).

1.2.3. Limitation of conventional methods

Invasive procedures are associated with a small chance of miscarriage, while non-invasive alternatives such as ultrasonographic examination, nuchal translucency and maternal serum biochemical screening, offer non-ideal diagnostic accuracy because they can only assess epiphenomena associated with the disease. To directly examine the core genetic abnormalities, such as the chromosome dosage imbalance in fetal trisomy, scientists have explored the possibility of obtaining the genetic materials of the fetus in a non-invasive manner.

1.3. Circulating fetal genetic materials for non-invasive prenatal diagnosis

1.3.1. Circulating fetal cell analysis

Since the 1960s, scientists began to search for fetal nucleated cells in the blood of pregnant women (Walknowska et al. 1969). Various attempts revealed the presence of fetal-derived lymphocytes (Herzenberg et al. 1979), nucleated erythrocytes (Bianchi et al. 1990), and trophoblasts (Beroud et al. 2003) in the maternal circulation. They could be enriched from the maternal circulation by fluorescence-activated cell sorting (Herzenberg et al. 1979), microdissection (Cheung et al. 1996), magnetic-based separation systems (Bianchi et al. 2002) and

size-dependent isolation (Beroud et al. 2003). Later work demonstrated that such transfer of genetic materials between the fetus and the mother is likely a bilateral trafficking process (Lo et al. 1996). The fetal cells that were released into the maternal circulation could persist for as long as 27 years postpartum, and appeared to be able to further divide and migrate to maternal organs (Bianchi 2000; Bianchi et al. 1996). This phenomenon, often regarded as fetal-maternal microchimerism, has been suggested to have significant clinical implications in autoimmune diseases (Evans et al. 1999; Gannage et al. 2002; Lambert et al. 2003; Maloney et al. 1999; Nelson 1999). Scientists then attempted to use this non-invasive source of fetal genetic materials to develop non-invasive diagnostic tests. For example, point mutations in single-gene disorders such as sickle cell anaemia and β-thalassemia could be detected based on genotyping the fetal cells isolated from the maternal blood (Beroud et al. 2003; Cheung et al. 1996; Sekizawa et al. 2001a). As the concentration of fetal cells were found to be higher in aneuploid pregnancies (Bianchi et al. 1997), fetal cell analysis has been proposed as a second-step in prenatal screening following maternal serum test (Bianchi et al. 2002).

1.3.2. Limitations of fetal cell analysis

In maternal blood, fetal cells exist in the order of one cell per milliliter (Bianchi et al. 1997). Its low abundance hinders its application for prenatal diagnosis. In a large-scale clinical project, the National Institute of Child Health and Development Fetal Cell Isolation Study, researchers from different centers concluded that technological advances are needed before fetal cell analysis could be clinically applied as part of a non-invasive screening marker for prenatal assessment (Bianchi et al. 2002).

1.3.3. Circulating cell-free fetal DNA analysis

A significant development caused a paradigm shift of the field in 1997 when Lo *et al.* found that apart from fetal nucleated cells, extracellular DNA of fetal origin is also detectable in the plasma of pregnant women (Lo et al. 1997). By using standard polymerase chain reaction (PCR), Lo and co-workers detected a Y-specific gene, *DYS14*, in the plasma of 24 out of 30 pregnant women who bear male fetuses (Lo et al. 1997). This detection rate is much higher than that of fetal cells isolated from a similar volume of whole blood (Bianchi et al. 1997; Lo et al. 1997; Lo et al. 1998b). This finding has opened up new opportunities for the development of safe and simple non-invasive prenatal diagnostic tests.

1.3.4. Historical overview of the identification of circulating nucleic acids

The first description of circulating DNA in human subjects dated back to about 50 years ago, in 1948 by Mendel and Métais (Mendel et al., 1948). During the 1960s – 1980s, a number of studies revealed that an increased levels of circulating DNA were found in the plasma and serum of patients with systemic lupus erythematosus and cancer (Koffler et al. 1973; Leon et al. 1977; Stroun et al. 1977; Tan et al. 1966). In 1989, Stroun and Anker proposed that the circulating DNA in cancer patients might actually come from tumor cells (Stroun et al. 1989). A few years later, their hypothesis was proven by a number of publication which demonstrated that tumor-derived genetic alterations are detectable in the plasma and serum of cancer patients (Chen et al. 1996; Nawroz et al. 1996; Sorenson et al. 1994). Since then, scientists began to explore the potential of using cell-free nucleic acids as non-invasive biomarkers.

These exciting findings inspired Lo *et al.* to investigate whether cell-free fetal DNA could also be detected in the maternal circulation (Lo et al. 1997). Extensive research efforts were then devoted to characterize circulating fetal DNA in maternal plasma.

1.3.5. Biological characteristics of circulating fetal DNA

Soon after the first detection of circulating fetal DNA in maternal plasma, scientists began to investigate the changes of fetal DNA concentrations in maternal circulation at different gestational ages. In 1996, the timely introduction of the real-time quantitative PCR technology provided an ideal tool for such a purpose (Heid et al. 1996). This technique allows the quantification of specific DNA targets with a high precision. Using this technique, Lo *et al.* developed two assays for plasma fetal DNA analysis, one targeted at the *SRY* (*sex determining region Y)* gene on the Y-chromosome for the quantification of fetal DNA, and another one targeted at the *β-globin* gene for the quantification of total maternal and fetal DNA (Lo et al. 1998b; Lo et al. 1999d). Their results revealed several important characteristics of circulating fetal DNA.

1.3.5.1 Correlation of plasma fetal DNA levels with the progression of pregnancy

Circulating fetal DNA is present at higher concentrations than fetal nucleated cells in maternal circulation, and their concentrations increased as pregnancy advances (Lo et al. 1998b). During the first trimester, there is a mean of 25.4 genome equivalents / mL plasma of fetal DNA. This concentration increases to a mean of 292 genome equivalents / mL plasma during the third trimester. Remarkably, when compared to fetal cells, the concentrations of fetal DNA in maternal plasma and serum are around 20 times higher than that in the cellular fraction of maternal blood at the same

gestational age (Lo et al. 1998b). They are detectable as early as the seventh week of gestation and increase sharply during the last 8 weeks of pregnancy. The fact that such concentrations of circulating fetal DNA are detectable without the need of any extra enrichment procedures greatly simplifies the procedures of prenatal genetic analysis.

1.3.5.2 Low fractional concentration of fetal DNA in maternal circulation

The fractional concentrations of fetal DNA out of the total plasma DNA were rather low. It constituted a mean fractional concentration of 3.4% and 6.2% in maternal plasma during the first and third trimesters, respectively (Lo et al. 1998b). Later report from the same group revealed that with the use of a more precise quantification platform, the median concentrations of fetal DNA at the corresponding trimesters are actually two times higher (Lun et al. 2008a). Despite that, the signals from the fetus are still largely overwhelmed by the background plasma DNA contributed by the mother. This characteristic has significant impact in the subsequent direction of research in the field. Since then, many efforts have been devoted to overcome the large interfering signals from the maternal DNA. More details would be discussed in later chapters.

1.3.5.3 Rapid turnover of circulating fetal DNA

The concentrations of fetal DNA dropped very rapidly after delivery of the fetus at a mean half-life of 16.3 minutes (range 4-30 minutes) (Lo et al. 1999d). Although another study has reported detectable fetal DNA in maternal circulation decades after delivery (Invernizzi et al. 2002), the results of that particular report could not be reproduced by other research groups (Benachi et al. 2003; Johnson-Hopson et al.

2002; Smid et al. 2003).

The demonstrated rapid turnover of fetal DNA in maternal plasma has two implications: one is that diagnostic tests based on analysis of circulating fetal DNA would be less susceptible to false-positive results arising from prior pregnancies. It is advantageous over the use of fetal cells, which could persist in maternal circulation for years post-partum (Bianchi et al. 1996); Another one is that a large quantity of fetal DNA must be liberated into the maternal circulation to maintain the observed high concentrations during pregnancy. This raised the question of the source of the fetal DNA.

1.3.5.4 Placenta as the major source of circulating fetal DNA

There have been a number of speculations about the origin of circulating fetal DNA. As plasma DNA has been known as a marker of cell death (Fournie et al. 1995; Fournie et al. 1993), it has been speculated that circulating fetal DNA might be released from the dying fetal cells that are present in the maternal circulation. However, the work by Zhong *et al.* revealed that in pregnancies complicated with preterm labor, concentrations of circulating fetal DNA were significantly elevated without any significant elevation in fetal erythroblasts (Zhong et al. 2002). In another study, Angert *et al.* evaluated the effects of time after phlebotomy on the quantity of circulating fetal DNA in blood samples from pregnant women who have just undergone termination of pregnancy (Angert et al. 2003). They hypothesised that, if cell-free fetal DNA are indeed released from fetal cells, the death of apoptotic fetal cells in the tube would result in increase of the levels of cell-free fetal DNA over time. However, they found that the levels of cell-free fetal DNA remain stable over a

24 hour period after phlebotomy (Angert et al. 2003). These results suggested that circulating fetal DNA in maternal plasma may come from a source other than haematopoietic fetal cell.

Scientists then speculated about whether circulating fetal DNA may be liberated from the placenta (Bianchi 1998). This hypothesis is supported by several pieces of evidence:

First, as described earlier, the quantitative kinetics of circulating fetal DNA in the maternal circulation correlates with the growth of the placenta: their levels increase when pregnancy advances (Chan et al. 2003; Honda et al. 2002; Lo et al. 1998b), and drop rapidly upon delivery of the fetus (Lo et al. 1999d).

Second, in pregnancies where the placenta is associated with specific genetic alterations, such changes are also detectable in maternal plasma. For example, in the case when the fetus possesses placenta-specific mosaicism, the same alterations are detectable in both the placenta and circulating fetal DNA (Masuzaki et al. 2004). Similarly, in some special cases of pregnancy where the male-specific Y-chromosome signals are detectable in all fetal tissues but not in cytotrophoblastic cells, such Y-specific signal is also absent in the maternal circulation (Flori et al. 2004).

Third, investigators have also found that mRNA transcripts from placental-expressed gene, namely *human placental lactogen (hPL)* and the *β subunit of human chorionic gonadotropin (βhCG)*, are readily detectable in maternal plasma (Ng et al. 2003).

One therefore speculates that, like cell-free fetal mRNA, cell-free fetal DNA in maternal plasma may also be released predominantly from the placenta. Scientists later have found more definitive evidence when they demonstrate that placental epigenetic signatures [unmethylated *SERPINB5, serpin peptidase inhibitor, clade B (ovalbumin), member 5*] are detectable in maternal plasma (Chim et al. 2005).

Fourth, *Alberry et al.* studied the concentrations of circulating fetal DNA in maternal plasma of pregnancies that were diagnosed with anembryonic pregnancies. They found that in the case where a placenta is present but the fetus is absent, there are no significant differences in the levels of circulating fetal DNA in plasma when compared with normal pregnancies (Alberry et al. 2007).

Taken together, these reports suggest that the placenta may be the major source of circulating fetal DNA in maternal circulation.

1.3.6. Clinical applications of circulating fetal DNA analysis

Over the years, many studies have been carried out to demonstrate the clinical applications of cell-free fetal DNA for non-invasive prenatal diagnosis.

1.3.6.1 Fetal gender determination

In pregnancies involving male fetuses, Y-chromosome-specific signals are detectable in maternal circulation starting from the 7th week of gestation (Lo et al. 1998b). Therefore, by using a simple PCR that targets at a Y-specific marker, it is feasible to confirm the gender of a male fetus (Costa et al. 2001; Lo et al. 1997; Smid et al. 1999). This information would be particularly useful for the prenatal management of

pregnant women who carry the gene for X-linked genetic disorders (Costa et al. 2002). Furthermore, diagnostic tests may be offered to women carrying male fetuses only to avoid unnecessary invasive procedures that could potentially lead to miscarriage.

1.3.6.2 Detection of fetal genetic conditions

One of the important clinical complications that require prenatal monitoring is the determination of the fetal rhesus D (*RHD*) status. The Rh blood-group system is one of the major reasons for the development of hemolytic diseases in newborns. When the father is heterozygous for the *RHD* gene while the mother is *RHD*-negative, there is a 50% chance that the fetus is *RHD*-positive. Non-invasive prenatal determination of the *RHD* status could be achieved by extracting fetal DNA from the plasma of *RHD*-negative pregnant women (Faas et al. 1998; Finning et al. 2002; Lo et al. 1998a). *RHD*-negative fetues would be excluded from further investigation via invasive procedures (Finning et al. 2002). Another example is the prenatal detection of β-thalassemia, which is one of the commonest autosomal recessive single-gene disorders (Weatherall 1997). Analysis of fetal DNA allows prenatal exclusion of fetal inheritance of paternally-transmitted mutations, such that the need for definitive diagnosis by invasive procedures could be eliminated by half (Chiu et al. 2002b; Ding et al. 2004b; Li et al. 2005). Other examples include the prenatal detection of myotonic dystrophy (Amicucci et al. 2000) and congenital adrenal hyperplasia (Chiu et al. 2002a) via detection of the disease-specific allele in the maternal circulation. Apart from being more easily isolated than fetal cells, results obtained with cell-free fetal DNA is more reliable than fetal cell analysis because it is less susupectible to false positive results introduced by persistency of fetal cells from previous

pregnancies (Lo et al. 1998a).

1.3.6.3 Quantitative aberrations of fetal DNA in fetal aneuploidy and pregnancy-associated diseases

Early studies revealed that the concentrations of cell-free fetal DNA in maternal plasma or serum are elevated in pregnancies involving aneuploid fetuses (Lee et al. 2002; Lo et al. 1999a; Wataganara et al. 2003). Previous study demonstrated that by combining second-trimester quadruple test with plasma fetal DNA analysis would increase the detection rate from 81% to 86% at a 5% false-positive rate (Farina et al. 2003).

On the other hand, quantitative aberrations of circulating fetal DNA have been reported in a variety of pregnancy-associated disorders. For examples, in pregnancies complicated with pre-eclampsia, the concentrations of *SRY* DNA were elevated 5-fold compared to uncomplicated controls during the third trimester (Lo et al. 1999b). Other reports also revealed an elevation of fetal DNA in maternal plasma before the onset of clinical symptoms in pre-elcamptic pregnancies, suggesting the predictive values of circulating fetal DNA (Leung et al. 2001b; Levine et al. 2004). Besides pre-eclampsia, quantitative aberrations of circulating fetal DNA have also been described in preterm labor (Farina et al. 2005; Leung et al. 1998), and other pregnancy-associated disorders (Sekizawa et al. 2002; Sekizawa et al. 2001b; Sugito et al. 2003).

1.3.7. Limitation of detecting circulating fetal DNA via fetal genetic markers

The major challenge for the development of non-invasive diagnostic tests is that fetal

DNA only constitutes around 3 – 10% of the total amount of plasma DNA in the maternal circulation (Lo et al. 1998b; Lun et al. 2008a). Many applications typically rely on the specific detection of fetal DNA is based on genetic markers, such as Y-chromosome-specific loci like the *SRY* or *DYS14* gene sequences, or paternally-inherited polymorphisms which are either absent or different in the maternal genome (Chiu et al. 2002b; Leung et al. 1998; Lo et al. 1999b; Saito et al. 2000; Tang et al. 1999b),

However, there is certain limitation associated with the use of fetal genetic markers. First, a Y-specific target restricts the detection of fetal DNA only to pregnancies involving male fetuses. Furthermore, negative test results would need to be interpreted with extra precautions, as a lack of Y-chromosome signals in the maternal plasma would either imply that the fetus was female; or that the fetal DNA proportion was below the detection limit of the assay or was not being extracted from the plasma properly (Finning et al. 2008). Secondly, although scientists have attempted to detect a female fetus based on the detection of paternally inherited polymorphisms (Tang et al. 1999b), such markers (Houfflin-Debarge et al. 2000; Lo et al. 1998b) require prior knowledge of the polymorphic status of the parents, and could only apply to a subset of individuals who possessed the concerned polymorphism.

Therefore, it would be desirable to develop a universal fetal DNA marker that allows differentiation of the fetus and the mother, and yet are independent of the gender or polymorphoric status of the fetuses. In this regard, placental epigenetic signatures has been explored and applied for detecting fetal DNA from maternal plasma.

Chapter 2 The development of universal fetal markers based on epigenetics

2.1. The potential of epigenetic profiles as non-invasive biomarkers

Epigenetic changes refer to the molecular changes that affect gene expression without changing the sequence context. They are stably transmitted through cell division and are potentially reversible (Beck et al. 1999; Callinan et al. 2006; Laird 2005). DNA methylation is one of the most widely studied epigenetic changes, particularly in human cancers, in which an aberrant DNA methylation pattern is usually associated with disrupted gene expression (Ehrlich 2002; Jones et al. 2007). In 1999, it was first demonstrated that the specific DNA methylation signatures of tumor DNA are detectable in the plasma of cancer patients, which raised possibilities of using them as non-invasive biomarker (Lo et al. 1999c; Wong et al. 1999). Soon after such developments, various attempts have been made to detect fetal DNA from maternal plasma based on differential methylation patterns between the fetus and the mother (Chan et al. 2006; Chim et al. 2005; Poon et al. 2002).

2.2. Fetal-specific DNA-methylation signatures

2.2.1. Parent-of-origin-specific methylation pattern

DNA methylation is one of the epigenetic modifications that regulate the establishment of genomic imprinting in humans (Driscoll et al. 1992; Li et al. 1993). Fetal epigenetic markers may be developed based on an imprinted region, in which the DNA methylation patterns are inherited in a parent-of-origin-specific manner

(Barton et al. 1984). Theoretically, if a pregnant woman has inherited the methylated copy of an imprinted region from her father, when she passs this copy onto her growing fetus, it would become unmethylated. The methylation status of this region should then be distinguishable between the fetus and the mother in an allele-specific manner. In 2002, Poon et al. proved this hypothesis by targeting an imprinted region between the *insulin-like growth factor 2* and *H19* genes (Schneid et al. 1993) and confirmed the results by genotyping a biallelic polymorphism within the differentially methylated region (Poon et al. 2002). Using this method, for the first time, the detection of a stretch of DNA that a fetus has inherited from the mother was made possible.

An imprinted region, however, is not fetal-specific *per se*, and thus the differentiation of fetal- and maternal-derived sequences would need to be supplemented with other polymorphic markers. A marker that can be applied universally regardless of the gender or polymorphic status of the fetus is needed. Investigators then explored another approach that is based on fetal-specific methylation profile.

2.2.2. Placenta-specific methylation patterns

Previous studies have suggested that the human placenta carry a specific pattern of DNA methylation with respect to other somatic tissues (Chiu et al. 2007; Fuke et al. 2004; Gama-Sosa et al. 1983; Maccani et al. 2009; Novakovic et al. 2008; Novakovic et al. 2009; Novakovic et al. 2010). Many researchers in the field believe that the majority of the fetal nucleic acids in the maternal plasma are derived from the placenta (Bianchi 1998; Flori et al. 2004; Masuzaki et al. 2004; Ng et al. 2003),

while the maternal counterpart is predominantly derived from the maternal hematopoietic cells (Lui et al. 2002a). Therefore, theoretically, a genomic region that is differentially methylated between the placenta and the maternal blood cells can be adopted to differentiate placenta-derived fetal DNA from the maternal background DNA in plasma. This strategy was first used in 2005, when a region on the *SERPINB5* gene promoter was found to be hypomethylated in the placenta while heavily methylated in the maternal blood cells (Chim et al. 2005). The authors also demonstrated that the unmethylated version of the *SERPINB5* gene sequences was detectable in maternal plasma throughout the course of pregnancy, and its level dropped significantly after delivery. This marked the first report of a universal fetal marker that can be used in all pregnancies. Other examples of fetal epigenetic markers that were developed based on placenta-specific methylation pattern include the first exon of *RASSF1A (Ras association domain family 1A)* gene (Chan et al. 2006) and the gene promoter of *HLCS (holocarboxylase synthetase)* (Tong et al. 2010). Both of them are hypermethylated in the placenta while predominantly unmethylated in the maternal blood cells.

The main advantage of detecting fetal DNA based on placenta-specific methylation pattern rather than allele-specific methylation pattern is that no prior knowledge of the fetal or parental polymorphic status is needed. This feature allows the development of a single, simple test to determine the presence of fetal DNA in the maternal plasma with improved simplicity and coverage.

2.3. The detection of fetal epigenetic markers in maternal plasma

In general, to detect fetal epigenetic markers in maternal plasma, the first step is to

differentiate methylated and unmethylated sequences via bisulfite modification of the template DNA or differential cleavage by methylation-sensitive restriction enzymes. The fetal-specific methylation pattern is then quantified by, for example, quantitative methylation-specific PCR, quantitative real-time PCR or other methods.

2.3.1. Differentiation of methylation patterns by bisulfite-dependent approaches

For qualitative measurement, the bisulfite-converted DNA can be differentially amplified by MSP, which specifically amplifies the methylated or unmethylated fetal-derived DNA (Chiu et al. 2007). Alternatively, the methylation status can also be ascertained by direct sequencing after bisulfite conversion (Poon et al. 2002). One can confirm the detection specificity by genotyping a polymorphic site within the target region by direct sequencing or primer-extension PCR (Chim et al. 2005; Poon et al. 2002).

For quantitative measurement, bisulfite-converted DNA can be quantified by quantitative MSP (qMSP), in which a fluorescence probe is present and its signal is compared against a series of calibration standards. This method has been used to quantify methylated biomarkers in the plasma of cancer patients (Lo et al. 1999c). The work by Chim *et al.* has demonstrated that using qMSP, one is able to detect a fetal-specific methylation pattern in maternal plasma from early gestation, during which the concentration of circulating fetal DNA is less abundant than that in the later stage of pregnancy (Lo et al. 1998b). Alternatively, it is also possible to quantify the bisulfite-converted target with Methyl-BEAMing (Li et al. 2009; Li et al. 2006) in plasma.

However, the major drawback of this technique is that the chemical bisulfite is known to degrade > 90% of the template DNA (Grunau et al. 2001). This is undesirable for the detection of fetal DNA, which is already of minor proportion in maternal plasma, particularly during early gestation. Such degradation would affect the robustness of detection and thus the accuracy of the quantification. Therefore other approaches have been developed to bypass the use of bisulfite.

2.3.2. Differentiation of methylation patterns by methylation-sensitive restriction enzymes

Many restriction enzymes are sensitive to methylation at bases in their recognition sequence. The cleavage may be blocked completely, but sometimes the effect may be partial, depending upon the duration of digestion or the amount of enzymes that is used (McClelland et al. 1994). For example, the cleavage by *Bst*UI or *Hpa*II is completely blocked by CpG methylation at their recognition sites. For markers that are hypermethylated in the placenta, like *RASSF1A* and *HLCS*, if there are recognition sites of methylation-sensitive restriction enzymes within the differentially methylated regions, one could treat the plasma DNA with such enzymes to remove the unmethylated maternal DNA, leaving the digestion-resistant (methylated) fetal DNA for detection by quantitative methods such as real-time PCR (Chan et al. 2006) or digital PCR (Tong et al. 2010). More details about these two quantitative methods will be discussed in Chapter 3.4.4. For markers that are hypomethylated in the placenta, like *SERPINB5*, after treatment with the appropriate enzymes, one could amplify the short fragments of cleaved unmethylated DNA by using stem-loop primers (Tong et al. 2007), a technique that has been developed for amplifying microRNA (Chen et al. 2005). Comparing with bisulfite conversion, this

digestion-based method introduced less damage to the input plasma DNA.

2.3.3 Factors that affect the robustness of detection

The robustness of detection of fetal epigenetic markers may be affected by a number of factors: First, it depends very much on the percentage of methylation in the placenta and its difference from that of the maternal blood cells. The more divergent the two are, the better is the differentiation. The percentage of methylation can be ascertained by cloning and bisulfite sequencing (Chim et al. 2008; Chiu et al. 2007). Second, in general, detection methods that do not depend on the use of bisulfite are more efficient than those that depend on it (Chan et al. 2006; Tong et al. 2010). The loss of template DNA due to the degradation by bisulfite would need to be compensated by, for example, increasing the amount of input plasma DNA (Tong et al. 2006) or using a more sensitive analytical platform (Li et al. 2009).

2.4. Fetal epigenetic markers for non-invasive prenatal assessment

2.4.1 Determination of fetal gender and fetal DNA proportion in maternal plasma samples

Fetal epigenetic markers can be used as qualitative and quantitative markers to indicate the presence of fetal DNA, or to quantify the amount of fetal DNA, in a particular maternal plasma sample. For example, as a qualitative marker, it can be used to detect the false negative results obtained based on genetic markers when doing fetal rhesus D genotyping, fetal gender determination or the detection of paternally-inherited polymorphisms (Chan et al. 2006). This application addresses a

problem associated with the use of fetal genetic markers discussed in Chapter 1.

As a quantitative marker, it can be used to determine the cell-free fetal DNA concentration in a maternal plasma sample. Such information is useful for interpreting the results of diagnostic tests performed using plasma DNA, for example, the measurement of the relative mutation dosage in maternal plasma for the prenatal diagnosis of single gene disorders (Lun et al. 2008b), or the measurement of the proportional representation of different chromosomes in maternal plasma using massively parallel DNA sequencing (Chiu et al. 2008; Fan et al. 2008).

2.4.2. Quantitative analysis of plasma fetal DNA in pregnancy-associated disorders

Previous studies have shown the association between quantitative aberrations of circulating fetal DNA with the development of pregnancy-associated disorders (Levine et al. 2004; Lo et al. 1999b). Such aberrations can also be detected using epigenetic markers, for example, by detecting the hypomethylated *SERPINB5* sequences in the maternal plasma from pre-elcamptic pregnancies (Chim et al. 2005). However, the involvement of bisulfite conversion in such an approach would result in a significant degradation of plasma DNA, which might hinder application for prenatal diagnosis early in pregnancy. Although such a limitation might be compensated by using a large volume of maternal plasma, it would adversely increase the complexity of the approach for the prenatal monitoring of pre-eclampsia. Bisulfite-independent strategies for the detection of fetal epigenetic markers would be highly desirable and is one of the emphases of this thesis.

2.4.3. Detection of fetal chromosomal aneuploidies

A number of reports have demonstrated the feasibility of using fetal epigenetic

markers for the prenatal detection of fetal trisomy. For example, Tong et al. has adopted *SERPINB5*, which is on chromosome 18, to deduce the fetus's trisomy 18 status non-invasively by an approach called epigenetic allelic ratio (EAR). This approach is based on the detection of a polymorphic site within the fetal-derived hypomethylated *SERPINB5* sequences. When the fetus has an extra copy of chromosome 18, the resultant allelic imbalance can be detected in maternal plasma using this approach. The drawback of this approach is that it utilises bisulfite and thus a large volume of plasma is needed to compensate for the degradation of the template DNA.

To overcome this problem, the same group then developed another approach, which is called epigenetic-genetic (EGG) chromosome dosage analysis (Tong et al. 2010). They have chosen trisomy 21 as a model to evaluate this approach, using the hypermethylated *HLCS* sequences on chromosome 21 as the fetal epigenetic marker. They measured the concentration of hypermethylated *HLCS* sequences in maternal plasma to infer the concentration of the fetal chromosome 21 and compared it to the concentration of a fetal genetic marker on chromosome Y. For a pregnant woman carrying a trisomy 21 fetus, the relative dosage of fetal chromosome 21 in maternal plasma has been shown to be elevated compared to that derived from a pregnant woman carrying a euploid fetus. The authors showed that the EGG analysis is useful for the prenatal detection of fetal trisomy 21 by testing maternal plasma collected from as early as the first trimester (Tong et al. 2010). This EGG approach could potentially be applied to detect other fetal chromosomal aneuploidies, as long as there are suitable fetal epigenetic markers on the aneuploid chromosome that would allow the digestion-based detection strategies to be applied.

2.5. The search for fetal epigenetic markers

Since the report of the first fetal epigenetic markers, the search for more markers has been under way (Chim et al. 2008; Old et al. 2007; Tong et al. 2010). Most of these initial efforts have been focused on chromosome 21, which is involved in the commonest fetal trisomy, trisomy 21. The availability of fetal epigenetic markers on chromosomes 18 and 13, the two chromosomes involved in the second and third commonest fetal trisomies, are relatively limited. Moreover, the technologies of the time favor the search to be focused on specific regions of the genome, such as the promoter regions that are associated with the genes of interest, or CpG islands in which extreme methylation patterns in the maternal blood cells have been described (Chim et al. 2008; Old et al. 2007; Tong et al. 2010).

The recent introduction of genomic tiling array and massively parallel sequencing allows scientists to study the human genome in unprecedented depth and scale (Ishkanian et al. 2004; Schuster 2008). It is expected that the search for new fetal epigenetic markers will extend to the entire genome. In fact, investigators have already begun to launch genome-wide search for new fetal epigenetic markers, hoping to extend the application to cover trisomy 18 and 13 (Chu et al. 2009; Papageorgiou et al. 2009). However, given the complexity of such high-coverage studies, it is expected that any new markers being identified would need to be evaluated via a systematic scheme to verify their clinical potential.

2.6. Aims of thesis

The presence of fetal DNA in the plasma of pregnant women has opened up new

possibilities for non-invasive prenatal diagnosis. The development of fetal epigenetic markers has extended the application of cell-free fetal DNA analysis to essentially all pregnancies, regardless of the gender of the fetus or its polymorphic status. However, two major challenges persist: one is that the issue of degradation caused by bisulfite conversion may adversely affect the diagnostic accuracy; another one is that the availability of fetal epigenetic markers on chromosome other than chromosome 21 that may be involved in aneuploidies is relatively limited. Investigations into these two aspects are therefore highly valuable for the development of the field.

The first focus of this thesis is on the development of fetal epigenetic markers for non-invasive prenatal assessment of an important pregnancy-associated disorder, pre-eclampsia. Chapter 4 describes the use of a previously reported fetal epigenetic marker, the hypermethylated *RASSF1A* gene, for the quantitative analysis of fetal DNA in maternal plasma obtained from pre-eclamptic pregnancies. In order to avoid the use of bisulfite which would pose degradation to the precious fetal DNA in maternal plasma, a strategy based on the detection of hypermethylated DNA molecules that are resistant to restriction enzyme-digestion is described. The concentrations of digestion-resistant hypermethylated *RASSF1A* sequences are compared between pre-eclamptic pregnancies and gestational-age matched controls in which both male and female fetuses were involved. This study demonstrates the robust detection of fetal DNA via a bisulfite-independent strategy, and the application of such a strategy in the non-invasive prenatal assessment of pre-eclampsia.

The second focus of the thesis is on the search for new fetal epigenetic markers that are either disease-specific or fetal-specific. Chapter 5 describes a quantitative method to compare the methylation profiles of placental tissues obtain from pre-eclamptic and normal pregnancies, aiming to identify methylation patterns that are specific to pre-eclampsia. Candidate genes including those that have been previously reported to be down-regulated in pre-eclamptic pregnancies were selected for the analysis. Chapter 6 describes a genome-wide search for fetal epigenetic markers on chromosome 18 by methylated DNA immunoprecipitation coupled with tiling array analysis (MeDIP-chip). A systematic evaluation scheme for the identification and validation of these markers would be discussed. The fetal-specificity of these markers would be demonstrated by postpartum clearance analysis and correlation of their detection with a Y-chromosome-specific marker in pregnancies bearing male fetuses. The detection of these makers does not require bisulfite conversion and is especially advantageous to be used during early-gestation, when the amount of fetal DNA in maternal plasma is very limited. Chapter 7 presents the development of an approach called epigenetic-genetic chromosome dosage for the detection of fetal trisomy 18. The approach employs digital PCR to co-amplify the hypermethylated fetal epigenetic marker and a fetal genetic marker on the Y-chromosome, the *ZFY* gene to infer the relative dosage of chromosome 18 with respect to the Y-chromosome. The relative chromosome dosage of fetal chromosome 18 and chromosome Y would be compared to determine a normal reference interval. The sensitivity and specificity of this approach to detect trisomy 18 would be evaluated.

Conclusions and future prospects would be given in Chapter 8.

Section II Materials and methods

This section outlines the methods used for the work described in this thesis. It focuses on the collection and processing of clinical samples, and the molecular techniques for identification and detection of fetal epigenetic markers.

Chapter 3 Methods for the identification and quantification of fetal epigenetic markers

3.1. Sample preparation

3.1.1. Patient consent

Pregnant women attending the Department of Obstetrics and Gynaecology at the Prince of Wales Hospital, Hong Kong, or the Prenatal Diagnostic and Counselling Department at the Tsan Yuk Hospital, Hong Kong, or the Harris Birthright Research Centre for Fetal Medicine, at the King's College Hospital, London, UK were recruited with informed consent. Plasma from the UK was harvested, kept frozen and sent to Hong Kong in batches on dry ice. Ethical approval was granted by the various institutional review broads.

3.1.2. Collection of placental tissues

Placental tissues were collected after elective caesarean section. Placental tissues were cut into small pieces and rinsed with diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, MO)-treated water and stored in polypropylene tubes at –80 °C. Samples of CVS were collected from pregnant women during their visits for invasive diagnostic procedures. The samples were stored in RNAlater (Ambion, Austin, Texas, USA) and stored at –20 °C.

3.1.3. Collection of maternal plasma and blood cells

Maternal peripheral blood samples (10 - 20 mL) were collected just before the performance of obstetrical procedures for termination of pregnancy or elective

caesarean delivery, or normal vaginal delivery. The blood samples were stored in tubes containing ethylenediaminetetraacetic acid (EDTA) to prevent blood clotting, because the clotting process would result in release of cell-free DNA that would introduce technical biases to the analysis of circulating DNA (Lui et al. 2002b).

3.2. Sample processing and extraction of DNA

3.2.1. Processing of samples

Maternal peripheral blood samples were centrifuged at 1,600g for 10 min at 4°C (Centrifuge 5810R, Eppendorf, Hamburg, Germany) to separate the plasma from the peripheral blood cells. The plasma portion was re-centrifuged at 16,000g for 10 min (Centrifuge 5415R, Eppendorf) to further pellet the cells. The peripheral blood cell portion was re-centrifuged at 2,500g for 5 min to remove residual plasma (Chiu et al. 2001). The processed samples were stored in polypropylene tubes at –20 °C until DNA extraction.

3.2.2. Extraction of DNA from placental tissues

DNA was extracted from CVS and placental tissues using the QIAamp tissue kit (Qiagen, Hilden, Germany) according to the tissue extraction protocol recommended by the manufacturer. Briefly, around 25 mg of placental tissues were first treated with proteinase K (Qiagen) in the presence of a lysis buffer. The process was performed at 56°C until lysis was completed. The lysate was then loaded into the QIAamp Mini spin column. After one round of centrifugation, the DNA bound to the membrane was washed with two different wash buffers provided by the

manufacturers to remove residual contaminants and to remove the protein and other contaminants that might inhibit PCR and the downstream extraction process. The DNA was then eluted into 100 - 200 μL water and stored at –20 °C until further processing.

3.2.3. Extraction of DNA from blood cells

DNA was extracted from peripheral blood cells using either the Nucleon Extraction and Purification Kit with the BACC2/3 protocol, or the QIAamp tissue kit (Qiagen), according to manufacturer's instructions. The procedures of the QIAamp tissue kit for blood extraction were the same as those for tissue extraction, except that the Qiagen proteinase would be used for blood extraction instead of proteinase K. For the Nucelon extraction BACC2/3 protocol, the 200 – 800 μL blood samples were subjected to cell lysis with the reagent provided by the manufacturer. The lysate was then deproteinised in sodium perchlorate. Then, chloroform and nucleon resin were added to the lysate in the described order. After centrifugation, two phases were formed: the upper aqueous phase that contains DNA and the lower organic phase that contains proteins. The two phases would be separated by the resin. The DNA-containing upper aqueous phase was collected and centrifuged to pellet the DNA. The pellet was washed with absolute ethanol and eluted in 100 - 200 μL water. The extracted DNA was stored at –20 °C until further processing.

3.2.3. Extraction of DNA from plasma

DNA was extracted from maternal plasma with the QIAamp DSP DNA Blood Mini Kit (Qiagen). The procedures of the QIAamp DSP DNA blood Mini kit for plasma DNA extraction were the same as those for blood extraction, except that QIAamp

Mini Spin columns were stored at 4°C before the extraction process. Slight modifications had been made to the recommended protocol: 400 μL of plasma, instead of the recommended 200 μL, was added into each DSP Spin Column for DNA extraction in order to obtain more DNA for subsequent analysis. The total plasma involved for extraction varied from 800 μL to 3200 μL. The extracted DNA was stored at –20 °C until further processing.

3.3. Identification of fetal epigenetic markers

3.3.1. Bisulfite conversion

In this thesis, DNA methylation specifically refers to the addition of a methyl group to the 5' position of cytosine residues to form 5-methyl-cytosine. In the human genome, it predominantly takes place at the cytosine in the context of CpG dinucleotides (Holliday et al. 1993). The process of bisulfite conversion changes unmethylated cytosine residues into uracil, leaving methylated cytosine unchanged (Frommer et al. 1992). The modification could then be detected by molecular techniques.

The modification involves a reagent called sodium bisulfite, which deaminates cytosine residues in single-stranded DNA to uracil residues via the formation of an intermediate 5,6-dihydrocytosine-6-sulfonate. Subsequent PCR amplification would further change the uracil to thymine. In the 1980s, Wang and co-workers have found that, under specific conditions, 96% of unmethylated cytosine would be converted into uracil while only 2 - 3% of 5-methylcytosine (methylated cytosine) would be converted (Wang et al. 1980). Such difference in the rate of conversion allows

scientists to adopt this modification to differentiate methylated and unmethylated cytosines.

For the work described in this thesis, bisulfite conversion was performed with the EZ DNA Methylation Conversion Kit (Zymo Research, Orange, CA). Briefly, up to 1 μg genomic DNA was mixed with 5 μL of M-Dilution buffer and sterilized water and made up to a volume of 50 μL. The sample was incubated at 37℃ for 15 minutes. After that, 100 μL of freshly prepared CT Conversion Reagent was added to the mixture, which was then incubated at 50℃ for 16 hours in the dark. After the incubation, the mixture was chilled on ice for 10 minutes and then mixed with 400 μL M-Binding Buffer. The mixture was then loaded onto a purifying column and then subjected to centrifugation at 16,000g for 30 seconds. After that, 200 μL M-Wash Buffer was added to the column and centrifuged again. Desulphonation was done by adding 200 μL M-Desulphonation Buffer to the column and incubation was performed at room temperature for 15 minutes. The column was then centrifuged at 16,000g for 30 seconds. The column was then washed twice with 200 μL M-Wash Buffer and centrifuged at 16,000 g for 30 seconds and 1 minute, respectively. The bisulfite-converted DNA was eluted from the column by 20 μL water and stored at -20℃ until further processing.

After bisulfite modification, the DNA methylation could be assessed by different techniques. In this thesis, mainly three techniques were used, namely methylation-specific PCR (MSP), Epityper (mass spectrometry-based analysis) and bisulfite sequencing.

3.3.2. Methylation-specific PCR (MSP)

MSP was first described in 1996 (Herman et al. 1996). It involves a set of primers that bind specifically to methylated or unmethylated bisulfite-converted DNA. The preferential binding to the template was achieved by designing the PCR primers to target the cytosine residues if methylation-specific binding is desired or to target the thymine residues if unmethylated-specific binding is desired. Successful amplification was confirmed by gel electrophoresis.

The primers for MSP were designed using the MethPrimer software which is a free computer program available online (Li et al. 2002) (http://www.urogene.org/methprimer/index1.html). The primer should cover at least one CpG site. Preferably, the C or T residues that distinguish the methylated and unmethylated sequences were placed at the 3' end of the primers. Specificity of the primers was checked against the bisulfite-converted human genome with the Bisearch online database (Tusnady et al. 2005) (http://bisearch.enzim.hu/). All assays were run in a reaction volume of 25 µL consisting of 1X buffer II, 200 µmol/L of each dNTPs, 200 nmol/L of each primer, and 1U of AmpliTaq Gold (Details are summarized in Table 3.1). The PCR product was subjected to size-separation by gel electrophoresis in a 3% agarose gel, at 150V for 15-20 minutes. A 100-bp DNA ladder was included to indicate the size of the fragments. Genomic DNA artificially methylated by the *SssI* DNA methyltransferase was bisulfite-converted and amplified together with the tested samples as a positive control for each MSP assay.

One advantage of MSP is that it is simple to perform and yield results quickly. Moreover, Herman *et al*. reported that MSP is sensitive for detection down to 0.1% methylated alleles of a given CpG island-associated locus (Herman et al. 1996). Many studies have adopted it as a screening tool for studying the aberrant methylation patterns associated with cancer (Aoki et al. 2000; Frazier et al. 2003; Gutierrez et al. 2004; Klump et al. 2003; Sirchia et al. 2000; Spugnardi et al. 2003).

The main disadvantage of MSP is that it only interrogates a few CpG sites in each reaction. When one is interested to know the methylation status of a long stretcg of DNA containing multiple CpG sites, multiple MSP assays are designed. To obtain methylation data of multiple CpG sites of a region in a single assay, other profiling methods are needed. One technique, often being regarded as the "gold standard" for quantitative methylation analysis, is cloning and bisulfite sequencing.

3.3.3. Cloning and bisulfite sequencing

Analysis of DNA methylation by cloning and bisulfite sequence was first described in 1992 (Frommer et al. 1992). Bisulfite conversion changes unmethylated cytosine residues to uracil residues, while leaving methylated cytosine unchanged. In contrast to MSP, the primers flank the CpG sites being interrogated without overlapping with them. Thus, their binding to the DNA does not depend on the methylation status at the primer regions. The amplified DNA would then be subjected to chain-termination Sanger sequencing (Sanger et al. 1977).

The primers for bisulfite sequencing were designed using the MethPrimer

programme (Li et al. 2002). Specificity of the primers was checked using the Bisearch search tool (Tusnady et al. 2005). The primer should cover as minimal CpG site as possible, but if necessary, the CpG cytosines on the prime binding sites would be replaced by degenerated bases [C/T replaced by Y (pyrimidine) on the forward strand and A/G replaced by R (purine) on the reverse strand] to prevent biased amplification of either methylated or unmethylated version of the target region.

DNA extracted from maternal blood cells or placental tissues was bisulfite-converted using the EZ DNA Methylation Conversion Kit (Zymo Research). The eluted DNA was then amplified with the bisulfite-specific primers in a reaction volume of 25 μL consisting of 1X buffer II, 3 mM $MgCl_2$, 200 μmol/L of each dNTPs, 200 nmol/L of each primer, and 1U of AmpliTaq Gold polymerase. Details of the reaction mixtures and amplification profiles are summarized in Table 3.1.

After PCR amplification, the product would be TA-cloned into the pGEM-T Easy vector with the pGEM T-Easy Cloning Kit (Promega, Madison WT) according to manufacturer's protocol. Ligation was done by incubating around 3.5 μL of PCR product with 1X Rapid Ligation Buffer, 50 ng of pGEM-T Easy Vector, 3 Weiss units of T4 DNA Ligase in a 10 μL reaction at 16 ℃ overnight.

Three microliters of ligated product were incubated with 40 – 50 μL of *Escherichia coli* strain JM109 (Promega) and chilled on ice for 20 minutes. The cells were heat-shocked at 42℃ for 45 seconds. Around 950 μL of super optimal broth,

catabolite repression (SOC) medium (Invitrogen, Carlsbad, CA) was added to the cells and incubated at 37 ℃ for 1.5 hours with general shaking. Subsequent centrifugation at 1,000 g for 10 minutes pelleted the cells, which were then resuspended in 100 μL of SOC medium and plated onto agar plates in the presence of the antibiotic ampicillin. The plates were incubated at 37 ℃ overnight. The vector contained an ampicillin-resistance gene, such that only cells containing the vector would survive. Positive cells were screened by the blue/white screening system. The vector contained a *lacZ* promoter. When IPTG (isopropyl β-D-thiogalactopyranoside), which was the inducer of the *lacZ* promoter, was added to the plates, the *E.coli* would produce β-galactosidase and hydrolyze a dye called X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) giving a blue color. However, if a piece of DNA had been inserted into the vector and disrupted the *lacZ* reading frame, β-galactosidase would not be produced and the bacterial clones would remain white. Therefore, the clones with inserts would appear white while the clones without insert would appear blue. White clones were picked randomly and incubated in 10 μL water at 95 ℃ for 5 minutes.

The insert of each colony was amplified using the primers specified for this vector, T7 (5' –TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAGAA-3'), in a reaction volume of 25 μL consisting of 1X buffer II, 4 mM $MgCl_2$, 200 μmol/L of each dNTPs, 100 nmol/L of each primer, and 1U of AmpliTaq Gold polymerase. Details of the reaction conditions and thermal profile of colony PCR are summarized in Table 3.1.

Sequencing reactions would be performed using the BigDye Terminator Cycle Sequencing v1.1 kit (Applied Biosystems). Reactions were set up in a 20 μL reaction, in the presence of 2.5X BigDye, 1X Sequencing Buffer, 320 nM SP6 primers with 2 μL of the colony PCR product. The thermal profile is given in Table 3.1. DNA was then purified with ethanol precipitation and dissolved in 10 μL of HiDi formamide (Applied Biosystems) and denatured at 95℃ for 5 minutes. Sequencing was performed on an automated capillary electrophoresis system ABI PRISM® 3100 Genetic Analyser (Applied Biosystems) Data Collection Software v1.0.1. The data were output from the Sequencing Analysis software 3.7. The output chromatogram would be analysed using the SeqScape software v2.5 (Applied Biosystems). Only data with > 99% completeness of bisulfite conversion were scored. This was checked by the absence of non-CpG cytosine. A "C" residue at CpG dinucleotide was scored as methylated, while a "T" as unmethylated. A minimum of 8 clones were scored for each sample.

The methylation levels of each sample would be given by the methylated site frequency (MSF), calculated by dividing the number of methylated CpG sites by the total number of CpG sites across all clones and all CpG sites in a PCR amplicon. The methylation status at each individual CpG site was given by the methylation index (MI), calculated by dividing the number of methylated CpG sites over both methylated and unmethylated CpG sites.

The main disadvantage of cloning and bisulfite sequencing is the tedious procedures. From PCR amplification, cloning, sequencing to sequence analysis, the whole process would normally take 3 - 5 days. Moreover, the complicated procedures have

also limited the throughput of the analysis: only a limited number of samples could be handled at any one time. Thus, other methods that allow more rapid and efficient analysis would be highly desirable. Preferably, such methods should also provide quantitative methylation data. One option would be the *Epityper*.

Table 3.1. Reaction conditions and thermal profiles for MSP and bisulfite sequencing assays

MSP reaction conditions

	Final concentration	Thermal profile		
10X buffer II	1X			
$MgCl_2$	3mM			
dNTP	200μM	95℃	10 min	
Forward primer	200nM	95℃	40s	40 cycles
Reverse primer	200nM	Specific annealing temperatu	45s	
AmpliTaq Gold	1U	72℃	45s	
Bisulfite converted DNA	50 ng	72℃	7 min	
Total reaction volume	25 μL			

Bisulfite PCR reaction conditions

	Final concentration	Thermal profile		
10X buffer II	1X			
$MgCl_2$	3mM			
dNTP	200μM	95℃	10 min	
Forward primer	200nM	95℃	40s	40 cycles
Reverse primer	200nM	Specific annealing temperatu	45s	
AmpliTaq Gold	1U	72℃	45s	
Bisulfite converted DNA	50 ng	72℃	7 min	
Total reaction volume	25 μL			

Colony PCR assays

	Final concentration	Thermal profile		
10X buffer II	1X	95℃	10 min	
$MgCl_2$	4 mM	95℃	1 min	10 cycles
dNTP	200μM	55℃-0 5℃ per cycle	1 5 min	
Forward primer	100nM	72℃	1 min	
Reverse primer	100nM	95℃	1 min	18 cycles
AmpliTaq Gold	1U	50℃	1 5 min	
Template DNA	Colony	72℃	1 min	
Total reaction volume	25 μL	72℃	7 min	

Sequencing reaction

	Final concentration	Thermal profile		
Big Dye v 3 1	2 5X	96℃	10s	25 cycles
5x BigDye sequencing buffer	1X	50℃	5s	
Primer (SP6)	250nM	60℃	4 min	
Water	variable			
Total reaction volume	10 μL			

3.3.4. EpiTYPER (MALDI-TOF mass spectrometry-based)

The Epityper platform is a quantitative DNA methylation analytical platform that utilizes MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) analysis of base-specific cleavage products (Ehrich et al. 2005).

Following bisulfite conversion, the DNA was amplified by a set of PCR primers that bind to both methylated and unmethylated DNA. A T7 promoter sequences was added to the 5' end of the reverse primer. PCR products were *in vitro* transcribed into RNA by T7 RNA & DNA polymerase. RNase A would cleave the subsequent DNA products at either cytosine or uracil ribonucleotides. By incorporating cleavage-resistant dCTP to the reaction, uracil-specific cleavage could be achieved, while incorporating cleavage-resistant dTTP (an uracil residue corresponds to a thymine residue on the complementary strand) to the reaction, cytosine-specific cleavage could be achieved. The cleavage process would generate CpG-containing fragments, or CpG units, whose sizes would be dependent on the methylation status of the CpG sites (i.e., CpG or TpG for methylated and unmethylated CpGs, respectively, after bisulfite conversion). C/T changes would be reflected as G/A changes on the complementary strand, and hence would result in a mass difference of 16 Da (Daltons) for each CpG site (Ehrich et al. 2005). The products were then cleaned up, and resolved and quantified with a MALDI-TOF mass spectrophotometer (MassARRAY Analyser Compact). The reaction condition and thermal profile of Epityper assay are summarized in Table 3.2.

MALDI-TOF MS is a popular platform for characterization of proteins or other biomolecules (Glish et al. 2003). It can also be used for studying genomic DNA sequence polymorphisms (Ding et al. 2004a; Tang et al. 1999a; Tost et al. 2002). In a MALDI system, amplified DNA products co-crystallized with the matrix before subjected to laser irradiation. The matrix is made up of crystallized molecules that would absorb most of the laser energy to partially protect the analytes. The matrix transfers some of its energy to the analytes and ionises them. The ionized analytes were then accelerated by an electric field in a vacuum flight tube. The velocity of the ions during the flight depends on the mass-to-charge ratio. A mass difference as small as 16 Da could be readily resolved by the system.

Figure 3.1 illustrates the principle of Epityper analysis.

The Epityper platform is able to assess methylation levels in a range between 10 - 90% with a standard deviation of 5% (Coolen et al. 2007; Ehrich et al. 2005). It is also able to detect methylation status of up to 82% of all CpG sites within a target region as long as 600 bp (Ehrich et al. 2005). This method allows high-throughput analysis of DNA methylation patterns and yet provides close to single CpG site-resolution data. Our laboratory has evaluated that the methylation data obtained with Epityper are consistent with those obtained with cloning and bisulfite sequencing. An example using the *RASSF1A* gene promoter is shown in Figure 3.2.

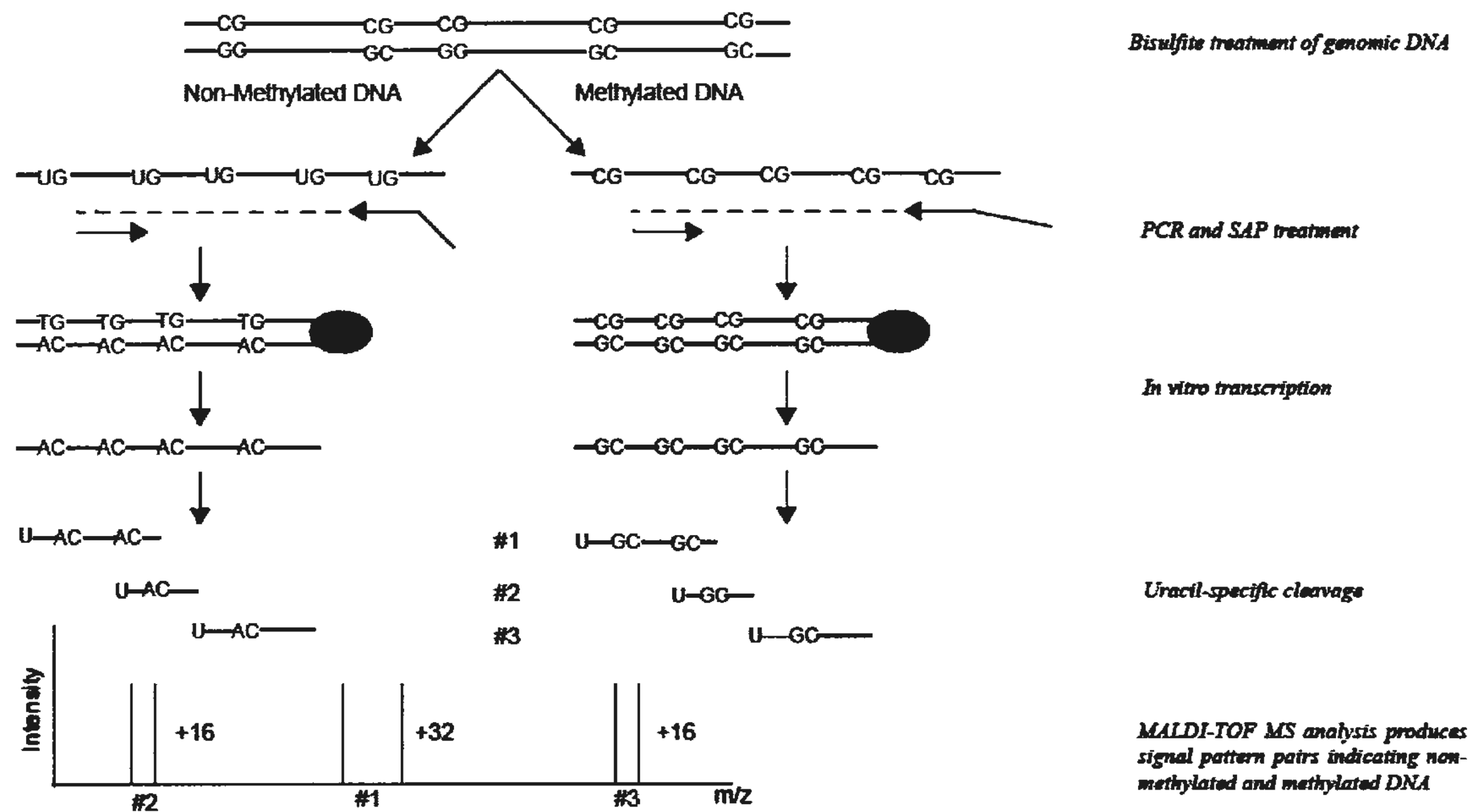


Figure 3.1. Analysis of methylation by base-specific cleavage and MALDI-TOF MS.

Figure 3.1. Analysis of methylation by base-specific cleavage and MALDI-TOF MS.

Bisulfite-converted DNA is amplified by using primers that flank the CpG sites of interest. One primer would be tagged with a T7 promoter sequence, such that the PCR product would be transcribed into a RNA molecule, which is then cleaved specifically at the cytosine or the uracil residues. The cleaved products would carry different masses according to the methylation status of the CpG sites within each fragment, which would then be resolved with a MALDI-TOF MS. In this figure, the PCR product is transcribed from the complementary strand and cleaved at an uracil. A methylated molecule carries a cytosine which appears as GC on the complementary strand. Conversely, an unmethylated molecule carries a thymine which appears as AC on the complementary strand. The change of sequences from G to A would result in 16-Da mass shifts. The mass shifts would be 32 Da between a fragment with 2 methylated CpG sites and the same fragments with 2 unmethylated CpG sites. By detecting the mass shifts, the methylation status of the CpG sites could be deduced. The ratio of the peak areas of corresponding mass signals can give an estimation of the methylation levels. Therefore, this platform would allow analysis of DNA mixtures without cloning the PCR products.

(Information adopted from Ehrich et al. 2005 Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. PNAS 102(44): 15785-90.)

Table 3.2 Reaction conditions and thermal profiles for Epityper assays

Epityper assay

PCR amplification

	Final concentration	Thermal profile		
10X Hot Star Buffer	1X			
dNTP	200μM	94℃	15 min	
Forward primer	200nM	94℃	20s	45 cycles
Reverse primer	200nM	Specific annealing temperatu	30s	
Hot Star Taq	0.2 unit	72℃	1 min	
Total reaction volume	5 μL	72℃	3 min	

Dephosphorylation

	Volume per reaction	Thermal profile	
RNase-free water	1.7μL	37℃	40 min
Shrimp alkaline phosphatase (SAP)	0.3μL	85℃	5 min
Total reaction volume	2 μL		

This mixture is added to the product of the PCR

In vitro transcription and Rnase A cleavage

	Final concentration	Thermal profile	
RNase-free water	variable	37℃	3 hours
5X T7 Polymerase Buffer	0.64X		
T-cleavage mix (or C-cleavage)	0.24μL		
DTT	3.14mM		
T7 RNA & DNA Polymerase	22 units		
RNase A	0.09 mg/mL		
Total reaction volume	5 μL		

Sample conditioning

Add 20 μL water and 6 mg Clean Resin to each well in a 384-well setting. Rotate for 10 minutes and spin down for 5 minutes at 3,200g. The samples are then ready to be dispensed onto the SpectroCHIP® bioarray.

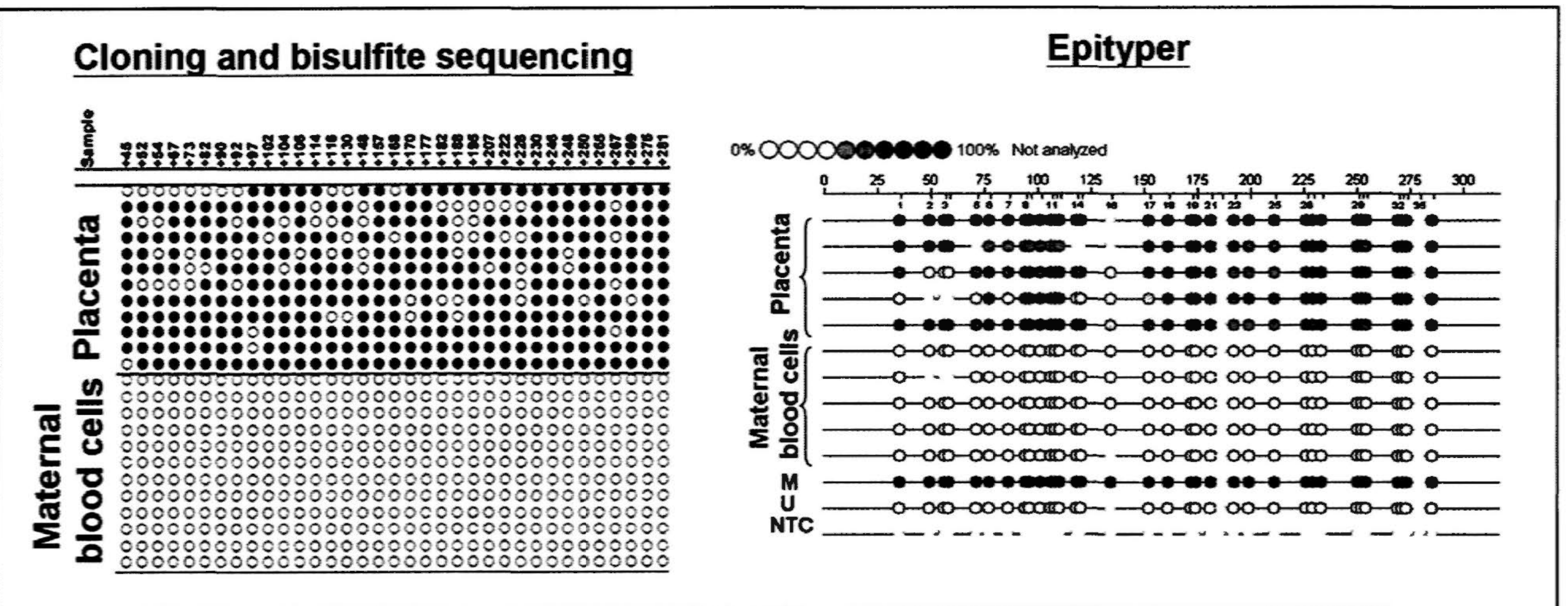


Figure 3.2. **Methylation data of *RASSF1A* exon 1 in placenta and maternal blood cells obtained with cloned bisulfite sequencing (left panel) and Epityper (right panel).**

Figure 3.2. Methylation data of *RASSF1A* exon 1 in placenta and maternal blood cells obtained with cloned bisulfite sequencing (left panel) and Epityper (right panel).

Data obtained from two methods on the first exon of *RASSF1A* in the placenta and maternal blood cells.

Left panel Each column represents one CpG site and each row represents one DNA molecule randomly picked from a tissue sample. A filled circle represents a methylated CpG site, and an empty circle represents an unmethylated CpG site.

Right panel The methylation levels determined by the Epityper are shown on a color scale. Each circle represents the data for one CpG site, and each row represents one sample. The numbers from 0 to 300 indicate the nucleotide position from the start of the amplicon, while the smaller numbers beneath them indicate the positions of the CpG sites from the start of the amplicon.

All the Epityper assays described in this thesis were performed with the standard MassCLEAVE protocol (Sequenom), which consists of four steps: (1) PCR amplification of bisulfite-converted DNA; (2) treatment by shrimp alkaline phosphatase (SAP), which dephosphorylates unincorporated dNTPs such that they cannot further participate in the subsequent reactions. (3) *In-vitro* RNA transcription with T7 DNA & RNA polymerases and base-specific cleavage by RNase A in a single reaction. The cleavage process happened on the reverse (complementary) strand of the template DNA. Uracul (U) or cytosine (C)-specific cleavage is achieved as previously described (as uracil actually refers to thymine (T) on the original DNA template, the U-specific cleavage is also known as "T-specific cleavage"). (4) Samples conditioning with resin such that the PCR products could be analysed by the MALDI-TOF mass spectrometer.

It is worth noting that in a CpG-rich region, C-specific cleavage will often result in relatively smaller fragments than T-specific cleavage. Those small fragments are more likely to be smaller than the lowest mass that can be detected by mass spectrometer (the detectable masses range from 1500 Da to 7000 Da). According to the experience of Ehrich and co-workers, the T-specific cleavage alone would provide the methylation data of ~82% of the CpG sites within a region of interest. Adding the C-specific cleavage would further increase such information to 89% (Ehrich et al. 2005). In this thesis, I performed only the T-specific cleavage reaction for large-scale screening to conserve costs.

The design of primers was performed using the EpiDesigner (v.2.0) computer programme provided by the Sequenom, or the MethPrimer programme. Both of these programmes can be accessed freely through the internet.

Several points need to be considered for the design of an Epityper assay:

First, since Epityper involved base-specific cleavage of the amplicon sequence, certain loci with an abundance of A- or G-residues would result in mass fragments that would be too short for mass spectrometry. Therefore, one would need to consider if the T-cleavage would result in CpG-containing fragments within the detectable mass range i.e. 1500-7000 Da on the MassARRAY Analyser Compact (Sequenom).

Second, certain loci contained repetitive DNA or paralogous sequences that would result in amplification of multiple, non-specific PCR products. Therefore, the

specificity of the primers were checked using the bisulfite-converted human genome predicted *in silico* by the Bisearch search tool as described.

Third, for some sequences, the mass of cleavage products within the same assay might overlap with each other, and thus some CpG sites might not be informative. Therefore, one should check with the EpiDesigner program for the number of informative CpG units within the designed amplicon.

3.3.5. Methylated DNA immunoprecipitation followed by tiling array analysis (MeDIP-chip)

Methylated DNA immunoprecipitation (MeDIP) enriches methylated DNA in a sample by an antibody that recognizes 5-methylcytosine (5mC) (Weber et al. 2005). The enriched product would then be subjected to analysis with a high-throughput analytical platform, such as DNA microarrays (Hatada et al. 2006; Lippman et al. 2005; Weng et al. 2009). The choice of DNA microarrays would depend on the specific purpose of study: if one is interested in the patterns of promoter methylation, one could hybridize the enriched samples to a microarray with probes design to target the predicted promoter regions (Hatada et al. 2006). If one would like to obtain a methylation map of the entire genome, one could hybridize the enriched samples to a genomic tiling array (Lippman et al. 2005). The resolution of tiling arrays would depend on the lengths and the spacing of the probes (Mockler et al. 2005).

The procedures of MeDIP consist of four main steps: sonication, immunoprecipitation, amplification and terminal labeling (Mohn et al. 2009; Thu et al. 2009; Vucic et al. 2009). Briefly, each DNA sample was sonicated to small

fragments (300 – 1000 bp) and denatured for 10 min at 95 °C, and immunoprecipitated by an antibody to 5-methylcytosine. The subsequent product was amplified and labeled using a dsDNA terminal labeling kit (Affymetrix, Santa Clara, California, USA) and hybridized to the GeneChip® Human Tiling 2.0R Arrays (Affymetrix). Arrays were washed and scanned according to Affymetrix standard procedures. In addition, a control DNA sample without immunoprecipitation (no-IP control) would also be processed in parallel with the immunoprecipitated DNA samples.

3.3.6. Statistical analysis

For the studies described in this thesis, all the statistical analyses would be performed with the SigmaStat 3.0 software (SPSS). In general, the Mann-Whitney rank sum test would be used to detect statistical significance, if any, between individual groups, such as comparing the methylation levels of two individual groups of samples (Chapter 4 and 5). The Wilcoxon's sign rank test would be used for paired samples before and after specific treatment or procedures, such as before and after delivery of the fetus (Chapter 6). In general, a difference with P value < 0.05 was considered statistically significant.

3.4. Methods for quantification of fetal DNA

3.4.1 Quantitative real-time PCR

Quantitative PCR and RT (reverse-transcriptase)-PCR were first described in the 1990s to quantitatively analyse nucleic acids (both for RNA and DNA) (Fasco et al. 1995; Kellogg et al. 1990; Mulder et al. 1994). The idea of real-time monitoring of

the amplification of DNA was first described in 1993 (Higuchi et al. 1993). However, most of these initial detection methods require post-PCR manipulations that were prone to contamination. Moreover, the sample throughput of these methods was limited, and thus not suitable for diagnostic applications (Heid et al. 1996).

In 1996, Heid *et al.* developed a method to allow "real-time" quantification of PCR products during the process of amplification (Heid et al. 1996). This quantitative real-time PCR method is based on the involvement of a dual-labeled fluorescent TaqMan® probe. The 5' end of the probe would be labeled with a fluorescent dye (called a reporter dye) such as FAM (6-carboxyfluorescein) or VIC (4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein), while the 3' end would be labeled with a quenching dye, such TAMRA (6-carboxytetramethylrhodamine). When the reporter and the quencher are in close proximity of one another, the fluorescence of the reporter would be suppressed by the quencher dye Förster-type energy transfer. The sequences of the probe are specially designed to be complementary to that of the target sequences. If the probe is bound to the target sequences during the extension step of the PCR, the fluorescent probe would be cleaved by the 5' – 3' nucleolytic activity of the DNA polymerase (Holland et al. 1991). Once the probe is cleaved, the emission spectra of the reporter would no longer be suppressed by the quencher and could be subsequently detected (Heid et al. 1996). Therefore, the increase in fluorescence signals would be proportional to the increase of the amount of target sequences during amplification. A standard calibration curve was established using serial dilutions of genomic DNA of known concentrations. To express the results as copy numbers, a conversion factor of 6.6 pg of DNA per cell is used. The copy numbers of the target sequences are determined by comparing the specific

quantification cycle (Cq) against that of the standard curve. Theoretically, if the PCR has achieved 100% efficiency, a 2-fold increase in the quantity of the target would result in a shift of one Cq. The dynamic range is usually established at around 3 copies / reaction to 10,000 copies / reaction (Lo *et al.* 1998b).

Because of its accuracy and simple experimental procedures, qPCR was soon adopted in the analysis of circulating fetal DNA in maternal plasma. For example, it has been used to investigate the quantitative changes and the biology of circulating fetal DNA during the course of pregnancy (Chan et al. 2004; Lo et al. 1998b; Lo et al. 1999d) and the aberrant quantitative changes of circulating fetal DNA associated with pre-eclamptic pregnancies (Lau et al. 2002; Leung et al. 2001b; Lo et al. 1999b).

Quantitative analysis of DNA by qPCR offers a number of advantages:
First, it is done in a closed-tube system and does not require post-PCR sample handling. This feature minimises the potential of carryover contamination. Second, qPCR offers a wide dynamic range of over five orders of magnitude and thus allows the detection of a large range of starting template concentrations (Heid et al. 1996; Lo et al. 1998b). Third, the system allows high-throughput quantification of 96 to 384 samples per plate in a single PCR run. All of these features are important for the development of a diagnostic test that could be efficiently implemented into the clinical setting.

It is crucial to avoid contamination when using qPCR for diagnostic purposes, because the presence of a trace amount of contaminating molecules would be

sufficient to generate a false positive result (Lo et al. 2006). To avoid carryover contamination from other experiments, the TaqMan® qPCR system allows incorporation of dUTP instead of dTTP during PCR, such that PCR products containing dUs that were carried over from previous experiments could be degraded by uracil-N-glycosylase (UNG) prior to new amplification (Longo et al. 1990). The qPCR thermal profile usually starts with incubation at 50℃ for 2 minutes to activate the UNG before the activation of the polymerase at 95℃ for 10 minutes. The reaction would then be cycled for the denaturation of template DNA and the extension of the PCR products. To further minimise the risk of contamination, other measures such as the preparation of the PCR master mix in a separated area are also crucial (Lo et al. 2006).

The major limitation of qPCR is that it is only robust enough to differentiate a relatively large quantitative difference of 2-fold or more. To detect a quantitative difference of less than 2-fold, alternative methods that allow more sensitive quantification would be needed. Digital PCR would be one option.

3.4.2 Digital PCR

The term digital PCR describes a general concept of quantifying the total number of starting template molecules in a sample via multiple analyses on highly diluted samples, direct counting of PCR products and determination of the initial concentrations by Poisson statistics. This concept was first described in 1992 by Sykes et al., who adopted a two-stage nested PCR system to achieve precise quantification of diluted samples (Sykes et al. 1992). In 1997, *Kalinina* and

co-workers developed a fluorescence-based detection method using the TaqMan® probe chemistry which enable a faster and simpler experimental setup (Kalinina et al. 1997). Soon after that, scientists began to explore its potential for molecular genetic analysis (Vogelstein et al. 1999; Zhou et al. 2001).

The procedures of digital PCR involve multiple analyses on highly diluted nucleic acids such that the majority of positive signals were derived from a single template molecule (Vogelstein et al. 1999). In that situation, the distribution of template molecules to each reaction well would follow the Poisson distribution. The individual template molecules would then be directly counted, giving a signal of "0" or "1" (and hence the name "digital"). Calculating the proportion of positive signals among the total number of PCRs would give an estimation of the actual template concentrations in the original sample. Such direct counting permits a high analytical precision to detect quantitative difference of less than 2-fold (Vogelstein et al. 1999). In a report by Chang et al., the authors described digital PCR as a means to "transform the exponential and analog signals from conventional PCR to linear and digital ones" (Chang et al. 2005). It has been shown to be useful for detecting mutations present in tumor samples (Chang et al. 2005; Zhou et al. 2001) or plasma of cancer patients (Chang et al. 2002; Diehl et al. 2005; Yung et al. 2009; Zimmermann et al. 2008).

The high precision of digital PCR is also useful for quantitative analysis of fetal DNA in maternal plasma. For example, in the detection of fetal trisomy 21, an approach called RNA-SNP has been proposed. It involved the detection of a single nucleotide polymorphism on the *PLAC4* (*placenta-specific 4*) gene. *PLAC4* mRNA

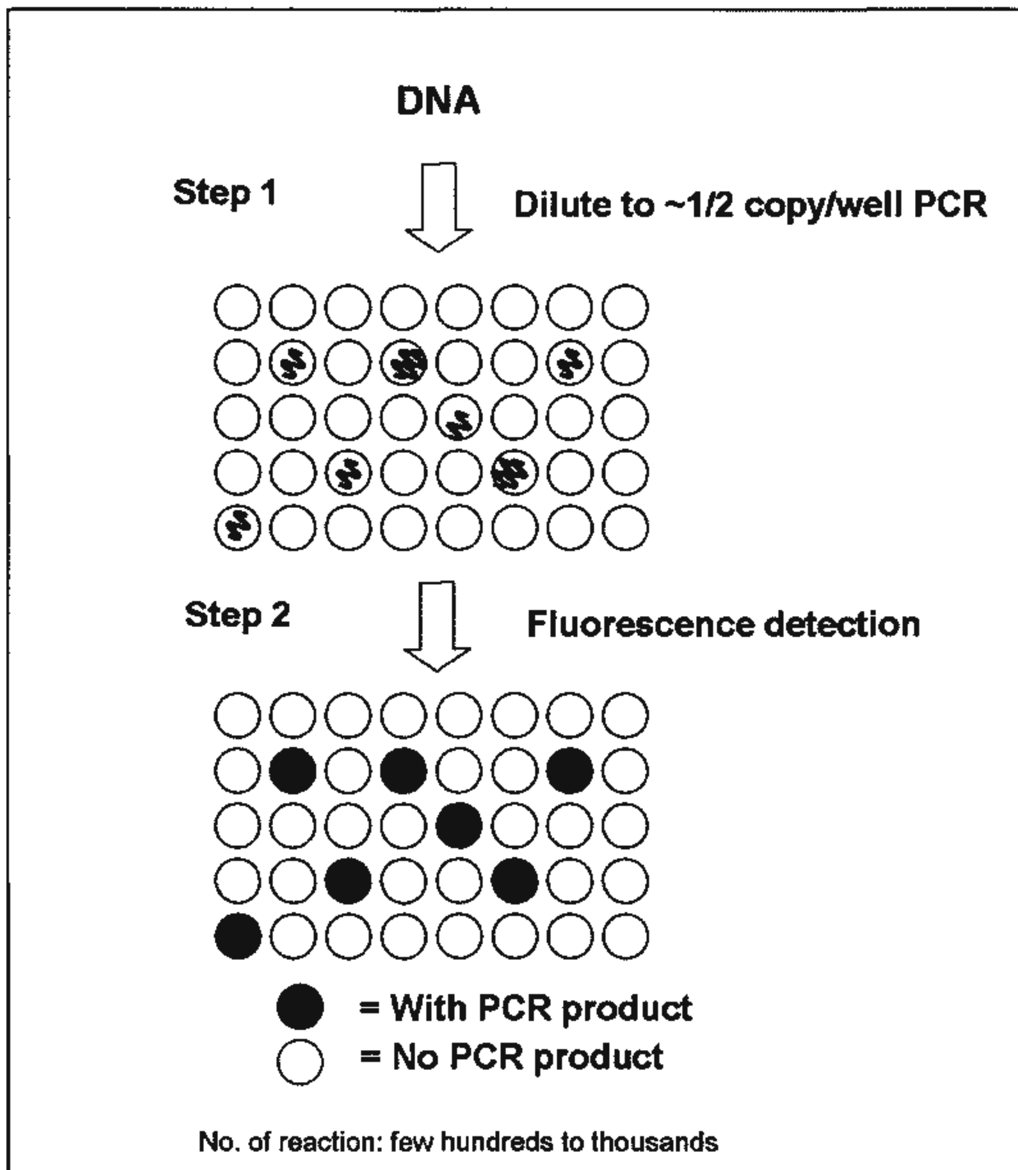


Figure 3.3. Figures illustrating the principle of quantification with digital PCR

Template molecules are diluted and subjected to multiple PCR reactions. Precise quantification is achieved by direct counting of the template molecules. Such quantification is not dependent on a calibration curve, and thus it is sensitive to detect less then 2-fold differences.

is specifically transcribed from chromosome 21 of the fetus (Lo et al. 2007c). Lo *et al.* reasoned that when the fetus carries an extra copy of the *PLAC4* gene on chromosome 21, a slight allelic imbalance between the heterozygous alleles should be detectable in the maternal plasma (Lo et al. 2007c). This approach was initially developed based on mass spectrometry analysis, while later work has shown that such slight allelic imbalance was also detectable via digital PCR (Lo et al. 2007b). Alternatively, the same team also showed that the overrepresentation of the total (fetal and maternal-derived) chromosome 21 sequences with respect to another reference chromosome in maternal plasma is also detectable in maternal plasma with the use of digital PCR (Lo et al. 2007b; Tsui et al. 2010). Apart from fetal trisomies, in the case of a fetal monogenic disease, Lun *et al.* showed that digital PCR allows detection of the presence of the maternal mutant allele in the fetal genome despite the coexistence of a high maternal DNA background in maternal plasma (Lun et al. 2008b).

In Chapter 7, I will describe how the high precision of digital PCR could be utilized to accurately detect the relative chromosome dosage of a fetal epigenetic markers on the aneuploid chromosome with respect to a fetal genetic marker on a reference chromosome, and how this information could be used to infer the aneuploidy statue of the fetus in maternal plasma.

Section III

The development of fetal epigenetic markers for prenatal assessment of pre-eclampsia

This section consists of two chapters. Chapter 4 describes an evaluation of a previously known fetal epigenetic marker for the non-invasive prenatal assessment of pre-eclamptic pregnancies. Chapter 5 describes an attempt to search for new DNA methylation markers for pre-eclampsia.

Chapter 4 Quantitative analysis of hypermethylated *RASSF1A* gene sequences in maternal plasma in pre-eclampsia

4.1. Introduction

Pre-eclampsia is a multisystem disorder affecting around 2.5 – 3.0 % of pregnancies worldwide (Redman *et al.*, 2005). Its major clinical presentations include hypertension and the presence of a significant amount of protein in the urine (proteinuria) of a pregnant woman. In some cases, it might also lead to the development of multiple maternal and fetal complications, and even maternal mortality (15 – 20% in developed countries) and premature delivery of the fetus (15 – 67%) (Sibai et al. 2005) (Figure 4.1).

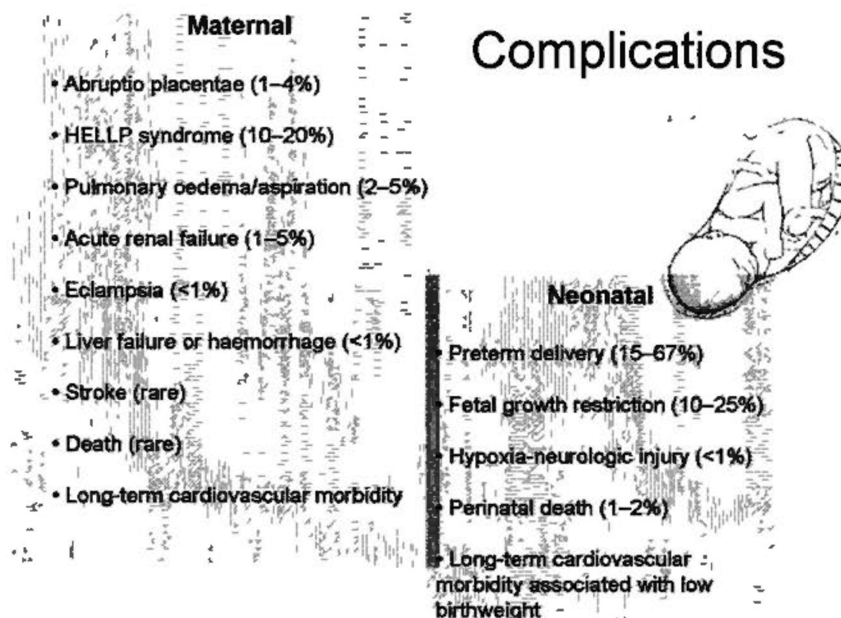


Figure 4.1. Maternal and fetal complications in pre-eclampsia

Information adopted from *Pre-eclampsia, Sibai* et al. 2005 *Lancet* 365(9461):785-99

The pathological mechanisms behind pre-eclampsia are not fully understood, but it is generally believed that distorted placentation is the main contributing factor to the development of various maternal and fetal complications. Poor placentation is characterized by inhibited invasion of the trophoblast cells to the uterioe wall and distorted remodeling of the spiral arteries. The limited invasion of the placenta would lead to restricted capacity of the uteroplacental circulation (Redman et al. 2003). The resultant placental ischemia is associated with the release of pro-inflammatory placental debris into the maternal circulation, causing damage to the maternal vascular endothelium via inflammatory response (Huppertz et al. 1998; Redman et al. 2003).

Despite extensive efforts devoted to understanding the pathological mechanisms of pre-eclampsia, unfortunately, delivery of the fetus remains the only treatment for pre-eclampsia. Pre-eclampsia is therefore one of the leading causes of premature delivery worldwide (Lindheimer et al. 2006), and the increased need for neonatal intensive care because of premature infants has created significant economic health burdens (Hanson et al. 2005; Lindheimer et al. 2006; Zandi-Nejad et al. 2006). Thus, early detection and timely management of the disease are important goals in prenatal care.

Currently, the diagnosis of pre-eclampsia is mainly based on the detection of clinical symptoms such as elevated blood pressure and proteinuria (Brown et al. 2000; Sibai 2003). Clinical symptoms usually develop during the second and third trimesters, while early-onset pre-eclampsia that develops before 34 weeks of gestation are often asymptomatic. As previously mentioned, placental ischemia as a result of poor

placentation would lead to release of placental factors into the maternal circulation. Among these factors, studies have found that placental trophoblasts and fetal erythroblasts are detected at an elevated concentration in the maternal circulation in pre-eclampsia when compared with normal pregnancies (Chua et al. 1991; Holzgreve et al. 1998).

Such observation prompted Lo *et al.* to investigate whether the transfer of cell-free fetal DNA would also be affected in pre-eclampsia. In 1999, Lo *et al.* reported that there is a 5-fold increase in the median concentrations of the male-specific *SRY* gene in the maternal plasma of pre-eclamptic women during the third trimester (Lo et al. 1999b). Subsequent work further showed that such elevation could be detected before presentation of clinical symptoms (Leung et al. 2001b; Levine et al. 2004). These results demonstrated the potential of cell-free fetal DNA analysis as a marker for the monitoring or early prediction of pre-eclampsia. However, the use of Y-chromosomal markers could only be applied to pregnancies bearing male fetuses. To broaden the population coverage, the potential of fetal epigenetic markers have been explored.

Using unmethylated *SERPINB5* as a universal circulating fetal DNA markers, Chim *et al.* have demonstrated that there is a 5.7-fold elevation of unmethylated *SERPINB5* sequences in third-trimester maternal plasma from pre-eclamptic pregnancies (Chim et al. 2005). However, the analysis was based on real-time quantitative methylation-specific PCR involving bisulfite-conversion which frequently degrades up to 96% of DNA (Grunau et al. 2001). This would limit the sensitivity of the assay as cell-free fetal DNA accounts for a minor proportion of the

total circulating DNA in maternal plasma (Lo et al. 1998b).

To further improve the robustness of detection, an analytic method that bypasses the use of bisulfite is preferred. As discussed in Chapter 3.4, one possible method is to target a fetal epigenetic marker that is hypermethylated in the placenta while unmethylated in the maternal blood cells, such that one could use methylation-sensitive restriction enzymes to digest the unmethylated maternal DNA (Chan et al. 2006). The gene promoter of *RASSF1A* has been found to be hypomethylated in blood cells and hypermethylated in the placenta (Chiu et al. 2007). A methylation-sensitive restriction digestion assay was thus developed to detect the fetal-derived hypermethylated *RASSF1A* sequences in maternal plasma (Chan et al. 2006).

The assay involved digestion by *Bst*UI, a restriction enzyme that cuts unmethylated CGCG sites, but leaves the methylated counterpart uncut. *Bst*UI treatment thus digests hypomethylated *RASSF1A* sequences derived from maternal blood cells, but spares placental-derived hypermethylated *RASSF1A* sequences intact for subsequent detection with qPCR. As an internal control, a similar assay quantifying unmethylated *β-actin* sequences has been included to confirm complete enzyme digestion. The procedure involves overnight enzymatic digestion followed by real-time PCR quantification, procedures which are significantly less tedious than the cloned bisulfite sequencing method for the analysis of methylation status (Chan et al. 2006).

In order to evaluate the application of such a universal fetal epigenetic marker in

maternal plasma for prenatal monitoring, the current study was undertaken to reveal whether fetal-derived hypermethylated *RASSF1A* would show a similar quantitative aberration to that of Y-chromosome fetal markers in pre-eclamptic pregnancies.

4.2. Methods

4.2.1. Sample collection and processing

Pregnant women attending the Department of Obstetrics and Gynaecology at the Prince of Wales Hospital, Hong Kong were recruited with informed consent , as approved by the institutional ethics committee. Pregnancies affected by pre-eclampsia were recruited between November 2002 and February 2007. Control pregnancies were recruited between January 2003 and February 2007. The details of the gestational age of each subject and the respective time of collection are summarized in Table 4.1. Pre-eclamptic pregnancies included mild and severe cases. Mild pre-eclampsia was defined as having a systolic blood pressure between 140 and 160 mmHg or a diastolic blood pressure of more than 90 mmHg on at least two occasions after 20 weeks of gestation, with the presence of proteinuria defined as more than 0.3 g/day. Severe pre-eclampsia was defined as a systolic pressure of more than 160 mmHg or a diastolic pressure of more than 110 mmHg on at least two occasions after 20 weeks of gestation with the presence of severe proteinuria defined as > 5 g/day.

Placental tissues and maternal peripheral blood cells were collected and processed as described in Chapter 3.2.

4.2.2. Methylation-sensitive restriction enzyme-mediated qPCR

DNA was extracted from 400 μL of plasma according to the procedures described in Chapter 3.2 and 35 μL of plasma DNA was digested with 100 U of a methylation-sensitive restriction enzyme, *Bst*UI, in 1X NEB buffer 2 at 60 °C for 16 h.

DNA was extracted from placental tissues according to the procedures described in Chapter 3.2 and 50 ng of extracted placental DNA was digested with 100U of *Bst*UI enzyme in 1X NEB buffer 2 at 60 °C for 16h.

RASSF1A and *ß-actin* sequences were amplified simultaneously by a duplex assay (Chan et al. 2006). The sequences of the primer and probes for each target are summarized in Table 4.2 and the reaction profiles are summarized in Table 4.3. All reactions were run in duplicate, and the mean quantity was taken. A DNA construct containing 1 copy each of the *RASSF1A*, *SRY*, and *ß-actin* amplicons was established as the quantitative standard for the 3 assays. For the plasma samples, 10 μL of enzyme-treated DNA or 5 μL of untreated DNA was used as the PCR template. For the placental samples, 1 μL of enzyme-treated DNA or 3 ng of untreated DNA was added to each PCR.

4.2.3. Evaluation of the methylation profiles of RASSF1A in pre-eclamptic and normal placentas by cloning and bisulfite sequencing

500 ng to 1 μg of placental tissue DNA was bisulfite-converted by the EZ DNA Methylation Kit (Zymo Research) according to manufacturer's protocol. Converted

DNA was subjected to PCR amplification with the forward primer: 5'-GGGGAGTTTGAGTTTATTGAGTTG-3' and reverse primer: 5'-CTACCCCTTAACTACCCCTTCC-3'. PCR products were cloned and sequenced as described in Chapter 3.3. The obtained sequences were aligned and analysed using the software SeqScape (Applied Biosystems). After confirming the sequences were completely converted, the CpG sites were scored, based on the criteria that cytosines on the sequencing trace indicated methylated residues while thymines indicated unmethylated residues.

The extent of *RASSF1A* hypermethylation for each placental tissue sample was expressed as MSF while the methylation status at each individual CpG site was given by the MI (Chapter 3.3.3)

4.2.4. Statistical Analyses

Statistical comparisons were performed using SigmaStat v.3.0.1a (SPSS). In general, a P-value of <0.05 was considered statistically significant. The exception was the comparison of the MI obtained by cloned bisulfite sequencing of placental tissues from normal and pre-eclamptic pregnancies. As 35 CpG sites were studied, after Bonferroni correction, a P-value<0.0014 was needed for statistical significance.

Table 4.1.
The gestational age of the pre-eclamptic and normal subjects involved in the study and their respective time of collection.

PET	Gestational age (week)	Time of Collection	Normal	Gestational age (week)	Time of Collection
Case 1	36 4/7	2006	Case 1	35 5/7	2006
			Case 2	36 5/7	2007
Case 2	37 4/7	2004	Case 3	37 5/7	2004
			Case 4	37 6/7	2004
Case 3	38	2007	Case 5	38 1/7	2007
			Case 6	38 4/7	2007
Case 4	38	2002	Case 7	38	2003
			Case 8	38 1/7	2004
Case 5	38 4/7	2002	Case 9	38 2/7	2003
			Case 10	38 4/7	2004
Case 6	39	2003	Case 11	39 3/7	2003
			Case 12	38 6/7	2004
Case 7	39	2006	Case 13	39	2006
			Case 14	39	2006
Case 8	39 6/7	2002	Case 15	40 2/7	2003
			Case 16	39 1/7	2004
Case 9	40 3/7	2004	Case 17	40	2004
			Case 18	40 6/7	2004
Case 10	41 3/7	2006	Case 19	41 4/7	2006
			Case 20	41 1/7	2006
Range	36 - 41		Range	35 - 41	
Median	38.8		Median	38.7	

Table 4.2.

Summary of primer and probe sequences for real-time quantitative PCR (qPCR) duplex assay.

Locus	Chromosome	Oligo	Primer sequences	Annealing Temperature
RASSF1A	3	Forward Primer	5 -AGC CTG AGC TCA TTG AGC TG-3	60℃
		Reverse primer	5'- ACC AGC TGC CGT GTG G -3'	
		Probe*	5'- FAM- CCA ACG CGC TGC GCA T -MGB - 3'	
β-actin	7	Forward Primer	5' - GCG CCG TTC CGA AAG TT - 3'	60℃
		Reverse primer	5' - CGG CGG ATC GGC AAA - 3'	
		Probe#	5' - VIC- ACC GCC GAG ACC GCG TC -MGB - 3'	

*The fluorescent TaqMan probes contained FAM as reporter and non-fluorescent quencher (MGBNFQ) as quencher.
#The fluorescent TaqMan probes contained VIC as reporter and non-fluorescent quencher (MGBNFQ) as quencher.
MGB, minor-groove binding

Table 4.3.

Summary of the reaction conditions for the enzyme digestion and the real-time quantitative PCR (qPCR) duplex assay.

Reaction conditions

Restriction enzyme digestion

	Volume per reaction
NE Buffer 2	5 µL
BstU I	10 µL
Water	variable
DNA	variable
Total	50 µL

Overnight (16 hours) incubation at 60℃

Real-time quantitative PCR

Duplex		Final concentration			
	Water	variable			
	2X TaqMan Universal master	1X	50℃	2 min	
RASSF1A	Forward primer	300nM	95℃	10 min	50 cycles
	Reverse primer	300nM	95℃	15s	
	Probe (FAM)	84nM	60℃	1 min	
Beta-actin	Forward primer	450nM			
	Reverse primer	450nM			
	Probe (VIC)	126nM			
	DNA (tissue or plasma)	variable			
	Total	50 µL			

4.3. Results

4.3.1. Quantitative analysis of RASSF1A DNA in plasma of pre-eclamptic pregnant women

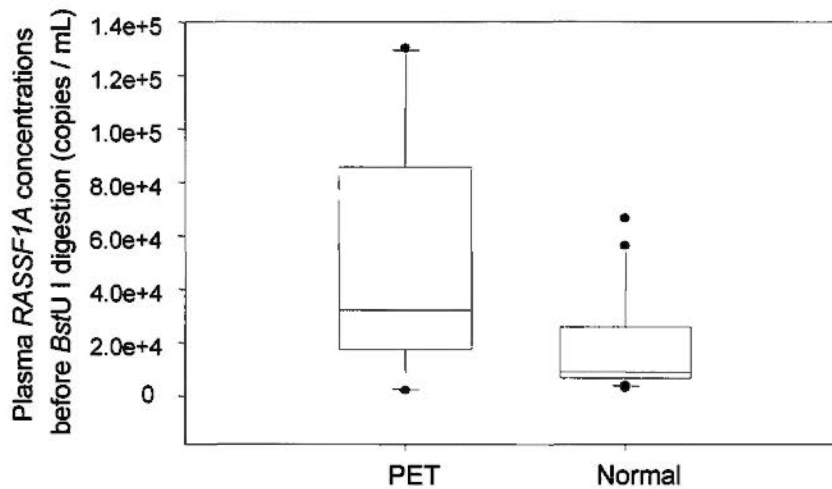
Maternal blood samples were collected from 10 pre-eclamptic (median gestational age: 39 weeks; range: 36 to 41) and 20 normal pregnancies (median gestational age: 39 weeks; range: 35 to 41). Among the 10 pre-eclamptic cases, 8 cases were diagnosed as severe and 2 as mild. *RASSF1A* sequences were detected in all maternal plasma samples before and after enzyme digestion. *β-actin* sequences were only detected before digestion, which confirmed complete *Bst*UI digestion. Before *Bst*UI digestion, the median *RASSF1A* concentrations were 32,143 copies / mL (interquartile range (IQR) 20,074 to 72,807) and 8,970 copies / mL (IQR: 6,485 to 22,652) for pre-eclamptic pregnancies and controls, respectively (Figure 4.2A). These figures represented a 3.6-fold elevation in pre-eclamptic pregnancies (Mann-Whitney Test, P-value = 0.023). After *Bst*UI digestion, the median *RASSF1A* concentrations were 3,220 copies / mL (IQR: 743 to 4,662) and 743 copies / mL (IQR: 347 to 1,890) for pre-eclamptic pregnancies and controls, respectively (Figure 4.2B). This represented a 4.3-fold elevation in pre-eclamptic pregnancies (Mann-Whitney Test, P-value = 0.023). We further expressed the post-digestion *RASSF1A* concentration as a fraction of the total *RASSF1A* DNA concentration obtained without *Bst*UI digestion which corresponded to 6.1% (IQR: 3.7 %–12.9 %) and 6.6 % (IQR: 4.85 %–12.4 %) in the plasma of pre-eclamptic and control pregnancies, respectively, with no statistically significant difference (Mann-Whitney Test, P-value = 0.982) (Table 4.4).

Table 4.4.

Summary of the plasma concentration of *RASSF1A* DNA in pre-eclamptic and normal pregnant women before and after digestion.

		Copies / ml				
		Pre-Digestion		Post-Digestion		
		RASSF1A	*ß-actin*	*RASSF1A*	*ß-actin*	Fraction of post-digestion *RASSF1A* over pre-digestion
PET	Case 1	20074	3104	743	0	3.7%
	Case 2	124833	51390	7891	0	6.3%
	Case 3	28284	1913	7226	0	25.5%
	Case 4	1909	719	459	0	24.1%
	Case 5	72807	14177	1793	0	2.5%
	Case 6	36001	4673	4654	0	12.9%
	Case 7	21903	2630	2261	0	10.3%
	Case 8	129978	30140	4662	0	3.6%
	Case 9	72278	33783	4180	0	5.8%
	Case 10	8025	1800	438	0	5.5%
Normal	Case 1	6425	588	825	0	12.8%
	Case 2	28919	215	4164	0	14.4%
	Case 3	14512	10530	1004	0	6.9%
	Case 4	34437	14555	660	0	1.9%
	Case 5	5056	10	317	0	6.3%
	Case 6	7073	71	227	0	3.2%
	Case 7	35441	755	1806	0	5.1%
	Case 8	6680	25	354	0	5.3%
	Case 9	8402	36	341	0	4.1%
	Case 10	3648	3	167	0	4.6%
	Case 11	2897	544	395	0	13.6%
	Case 12	16386	608	1973	0	12.0%
	Case 13	6545	188	1226	0	18.7%
	Case 14	6548	286	354	0	5.4%
	Case 15	13534	2430	300	0	2.2%
	Case 16	4321	1342	516	0	11.9%
	Case 17	66334	31792	4985	0	7.5%
	Case 18	56050	18579	4589	0	8.2%
	Case 19	9538	12	2654	0	27.8%
	Case 20	13718	5	834	0	6.1%

A)



B)

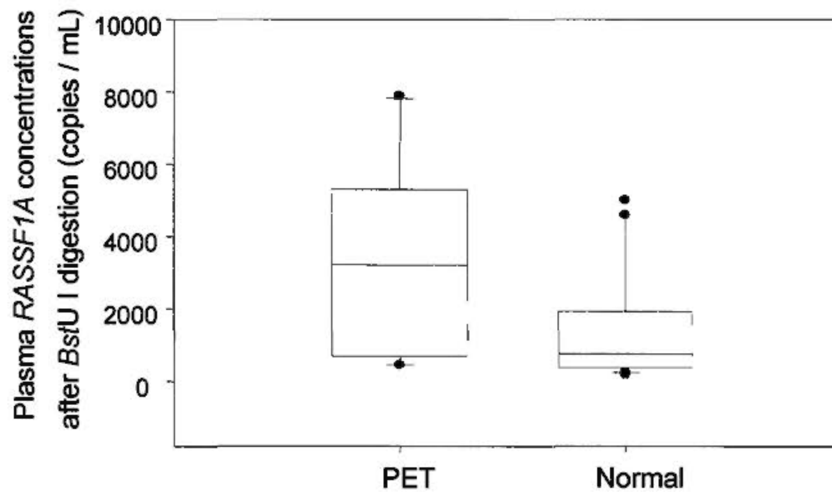


Figure 4.2. Plasma concentration of *RASSF1A* DNA in pre-eclamptic and normal pregnant women (A) before and (B) after digestion

Box plots of the concentrations of *RASSF1A* before (A) and after (B) *Bst*UI enzyme digestion in plasma from 10 pre-eclamptic pregnant women and 20 normal pregnant women. The upper and lower whiskers represent the 90th and 10th percentiles, respectively. The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The median is indicated by the line in each box. Outliers are indicated by filled circles.

4.3.2. Quantitative analysis of RASSF1A DNA in placental tissues of pre-eclamptic pregnant women

Placental tissues were available from 5 pre-eclamptic and 10 normal pregnancies in the cohort described above (median gestational age: 40 weeks; range: 38 to 41 for both groups). The median *RASSF1A* concentrations were 534 copies / ng (IQR: 402 to 941) and 690 copies / ng (IQR: 518 to 794), respectively, for the pre-eclamptic and control pregnancies after *Bst*UI digestion (Table 4.5). Before *Bst*UI digestion, *RASSF1A* concentrations were 971 copies / ng (IQR: 645 to 1,498) and 1,072 copies / ng (IQR: 781 to 1,118) (Figure 4.3), respectively, for the pre-eclamptic and control pregnancies. There was no statistically significant difference between the pre-eclamptic and control cases before or after enzyme digestion (Mann-Whitney Test, P-Value = 0.759 after digestion and 0.951 before digestion). The fraction of post-digestion *RASSF1A* concentrations over total corresponded to 59.2% (IQR: 54.4%–68.6%) and 71.4% (IQR: 48.9%–77.6%) in the placentas of pre-eclamptic and control pregnancies, respectively, with no statistically significant difference (Mann-Whitney Test, P-Value = 0.426).

Table 4.5.

Summary of the concentration of *RASSF1A* DNA in pre-eclamptic and normal placental tissues before and after digestion

			Copies / ng			
			Pre-Digestion		Post-Digestion	
		Gestational age (week)	*RASSF1A*	*ß-actin*	*RASSF1A*	*ß-actin*
PET	Case 3	38	971	954	423	0
	Case 7	39	1618	1290	939	0
	Case 8	39 6/7	570	298	338	0
	Case 9	40 3/7	1459	1084	945	0
	Case 10	41 3/7	670	385	534	0
Normal	Case 5	38 1/7	1110	1200	708	0
	Case 6	38 4/7	963	593	432	0
	Case 13	39	1059	637	518	0
	Case 14	39	568	365	439	0
	Case 15	40 2/7	781	332	714	0
	Case 16	39 1/7	1086	759	842	0
	Case 17	40	1732	1582	1321	0
	Case 18	40 6/7	690	159	673	0
	Case 19	41 4/7	1118	738	531	0
	Case 20	41 1/7	1194	1228	794	0

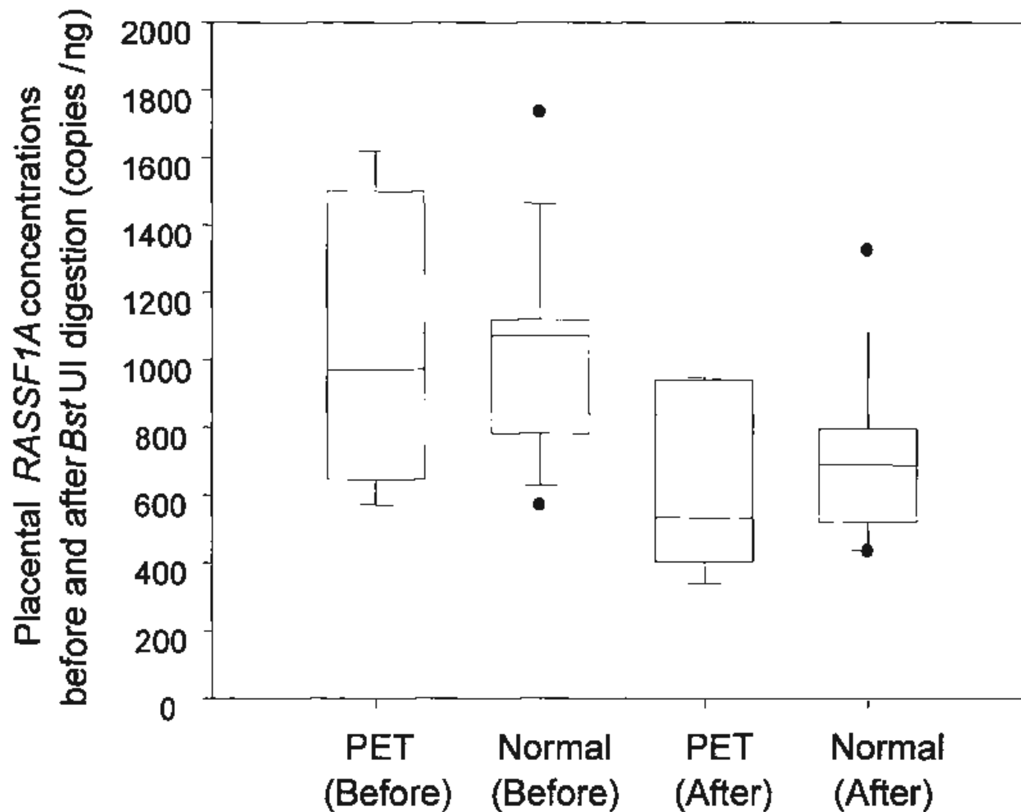


Figure 4.3. Quantitative analysis of *RASSF1A* DNA in placental tissues of pre-eclamptic pregnant women

Box plots of the placental concentrations of *RASSF1A* before and after *Bst*UI enzyme digestion in 5 pre-eclamptic pregnant women and 10 normal pregnant women. The upper and lower whiskers represent the 90th and 10th percentiles, respectively. The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The median is indicated by the line in each box. Outliers are indicated by filled circles.

4.3.3. Lack of difference in methylation profile of RASSF1A between pre-eclamptic and normal placental tissues

To further confirm the lack of difference in methylation profile of the 5' CpG island of *RASSF1A* in pre-eclamptic placental tissues, 5 third-trimester pre-eclamptic (median gestational age: 37 weeks; range: 37 to 39) and 5 normal (median gestational age: 38 weeks; range: 37 to 40) placentas were studied by cloned bisulfite sequencing (Figure 4.4). 20 clones were scored for each sample. The MSF ranged from 0.480 - 0.704 for the normal and 0.229 - 0.583 for the pre-eclamptic cases, with no significant difference (Mann-Whitney Test, P-Value = 0.548) (Figure 4.5), and the MI ranged from 0.42 – 0.64 for normal and 0.25 – 0.53 for pre-eclamptic subjects with no significant difference at each CpG site (Chi-square Test, $P > 0.0014$ for every comparison) (Figure 4.6).

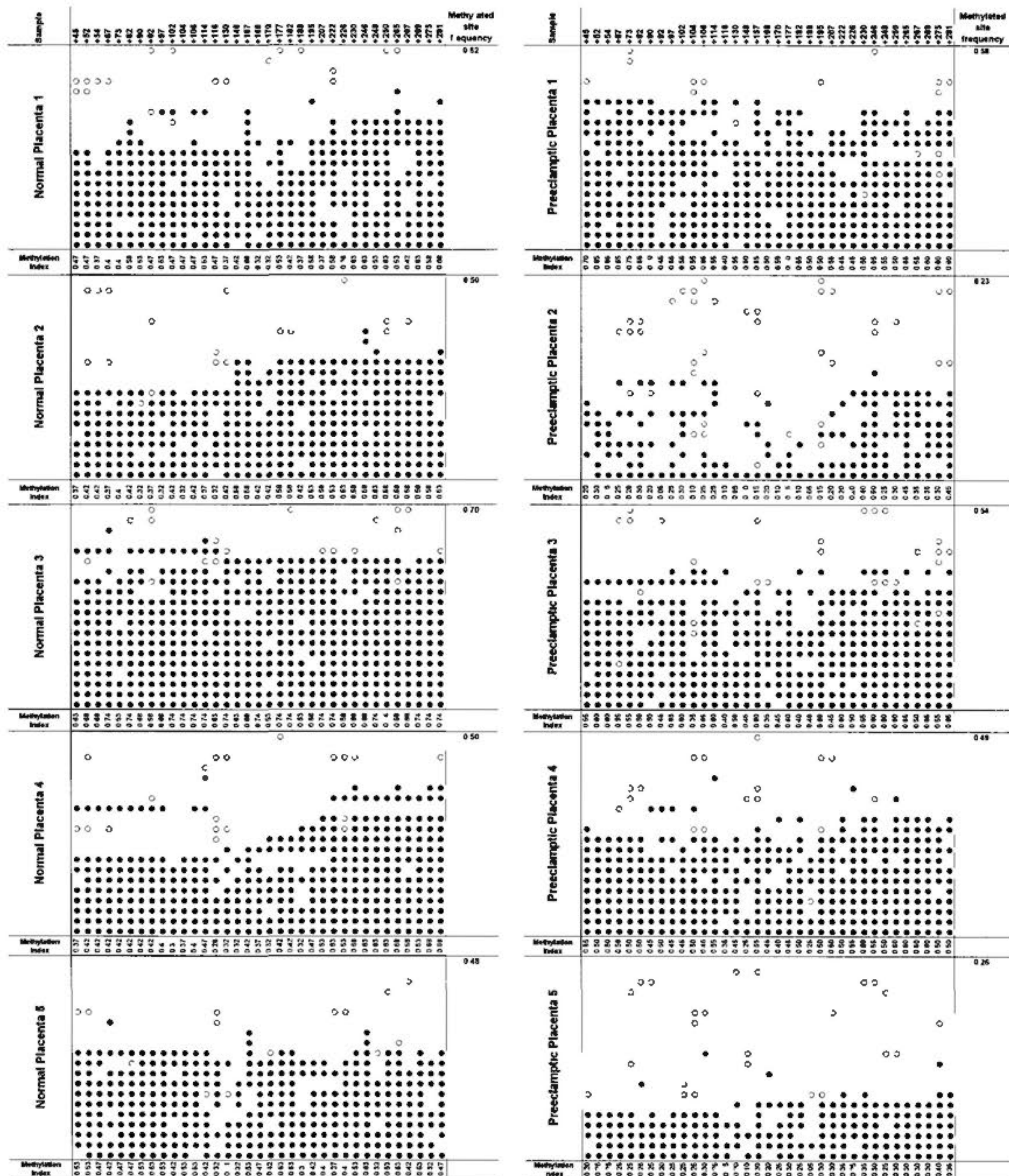
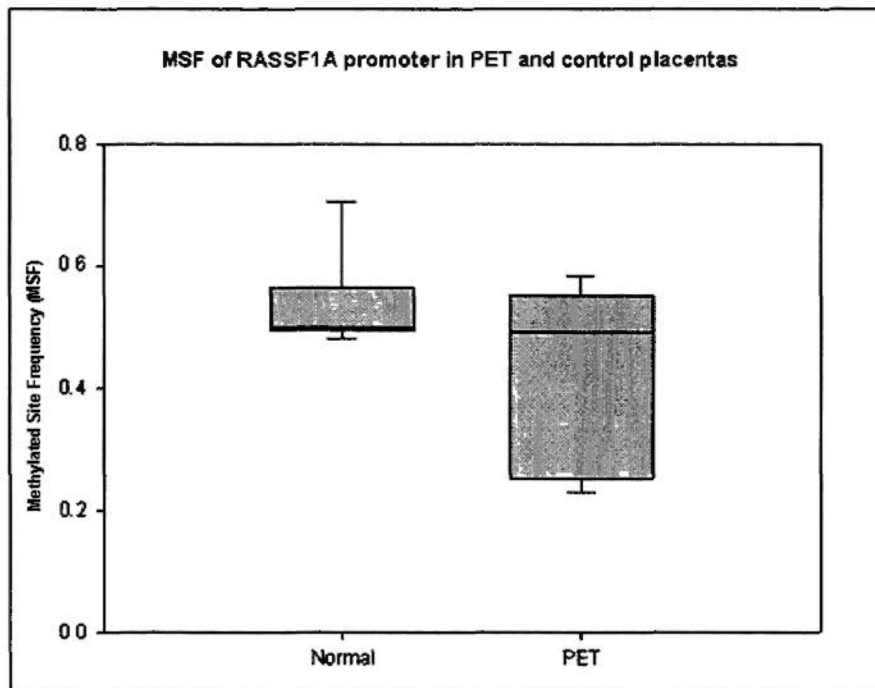


Figure 4.4. Methylation profile of *RASSF1A* between pre-eclamptic and normal placental tissues

Figure 4.4 Methylation profile of *RASSF1A* between pre-eclamptic and normal placental tissues

Cloning and bisulfite sequencing of the 5' CpG island of *RASSF1A* in third trimester normal (n = 5) and pre-eclamptic placental tissues (n = 5). After PCR amplification using primers specific for bisulfite-converted DNA, the amplified products were cloned and sequenced in order to obtain the methylation pattern for individual DNA molecules within the pool of bisulfite-converted genomic DNA. The methylation profile of a genomic location could therefore be identified by this method. **Circles** along one column represent one CpG site (**filled circles**, methylated; **open circles**, unmethylated). Each row represents one clone. 20 clones were analysed for each CpG sites. The extent of methylation of each sample was represented by the MSF while the extent of methylation of each CpG site was represented by the MI. The closer the value of MSF or MI is to 1, the higher the extent of methylation is. The analysed CpG sites are named according to the *RASSF1* (*Homo sapiens*) GenBank accession NM_007182 with the start codon of its protein coding sequence as position +1. MSF: the methylated site frequency; MI: methylation Index



Mann-Whitney test p = 0.548

Figure 4.5. Summary of the MSF of *RASSF1A* in preelcamptic and normal placentas

Box plots of the MSF of the same 5 pairs of placentas. Mann-Whitney test revealed that there is no significant difference between the two groups of MSF (P-value = 0.548). The upper and lower whiskers represent the 90th and 10th percentiles, respectively. The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The median is indicated by the line in each box. Outliers are indicated by filled circles.

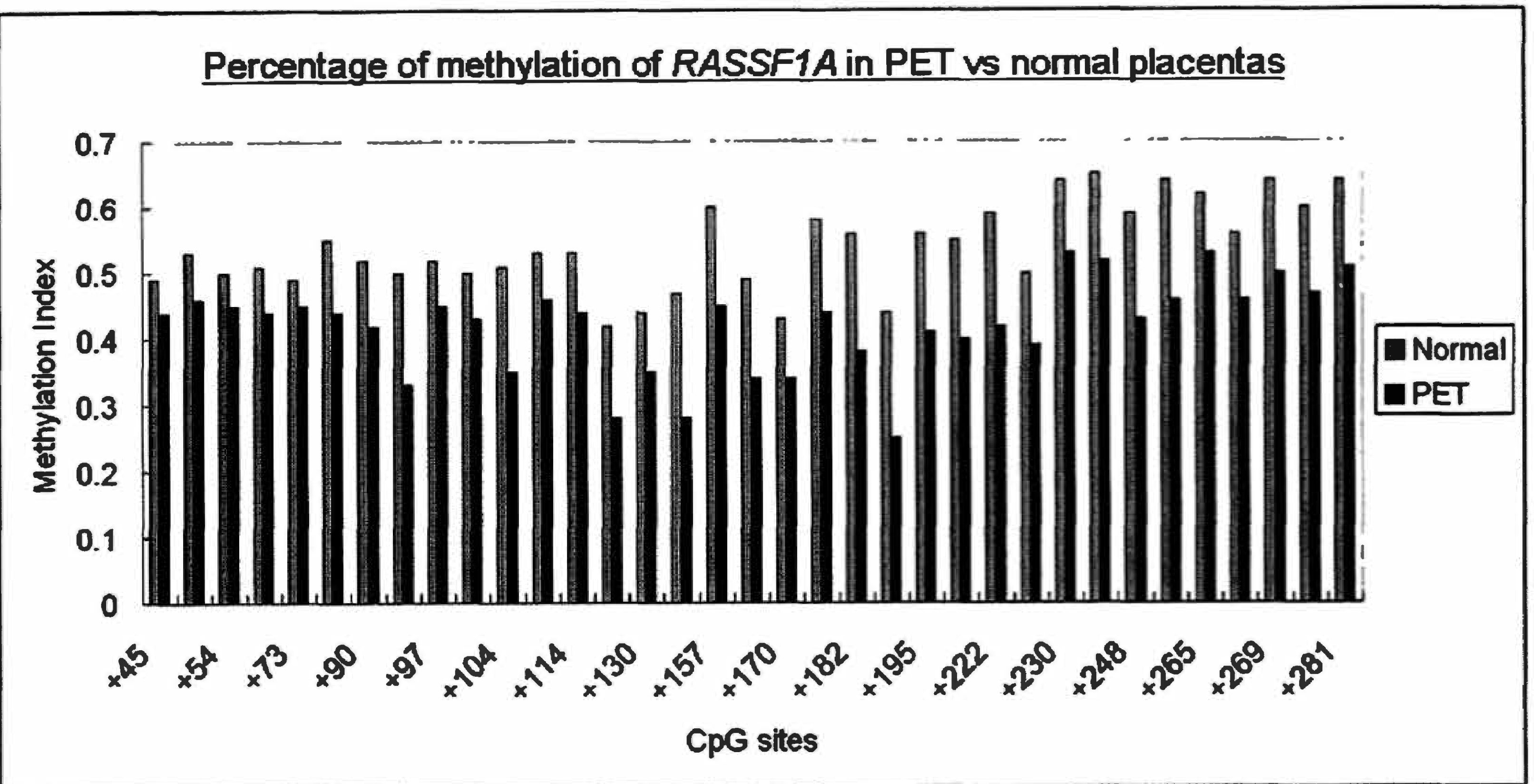


Figure 4.6. Summary of the MI of *RASSF1A* in preelcamptic and normal placentas

The average MI of 5 normal placentas were compared with the average MI of 5 PET placentas at each position of CpG site. There is no significant difference at each CpG site (Chi-square Test, $P > 0.0014$ for every comparison).

4.4. Discussion

In this study, concentrations of *RASSF1A* in maternal plasma were determined in normal and pre-eclamptic pregnancies involving both female and male fetuses, thus demonstrating the gender-independent nature of this marker. *RASSF1A* is a tumour suppressor gene that is frequently inactivated by promoter hypermethylation in at least 37 tumour types including lung, breast, prostate, neuroblast and kidney (Agathanggelou et al. 2005; Hesson et al. 2007). Among the many reports to date, placental tissues seem to be the only non-malignant tissue where *RASSF1A* hypermethylation has been demonstrated (Chiu et al. 2007). Here we explored the use of this placenta-specific epigenetic signature for prenatal applications.

In Chapter 4.3.1, the results indicate that there are 3.6-fold and 4.3-fold elevations in the median plasma *RASSF1A* concentration before and after *Bst*UI digestion, respectively, among pre-eclamptic subjects. It suggested that both total (i.e. fetal plus maternal) *RASSF1A* concentrations and fetal-derived hypermethylated *RASSF1A* were elevated significantly in plasma of pre-eclamptic women. When the post-digestion plasma *RASSF1A* concentration was expressed as a fraction of the total *RASSF1A* concentration, we observed no significant difference between pre-eclamptic and control pregnancies. It implies that in addition to the placenta-derived hypermethylated *RASSF1A* sequences, there is an elevation of hypomethylated *RASSF1A* concentrations in maternal plasma of pre-eclamptic pregnancies. We postulate that these hypomethylated molecules are mainly derived from maternal blood cells which have previously been shown to be completely unmethylated (Chiu et al. 2007) and partly from unmethylated areas of the placenta. The latter contributed to a much less significant portion because of the densely

methylated profile of *RASSF1A* in the placenta as previously reported (Chiu et al. 2007). These observations agree with previous reports of elevations of both circulating total and fetal Y-chromosomal DNA in pre-eclamptic pregnancies (Zhong et al. 2001;Farina et al. 2004a).

The elevation in total plasma DNA concentrations might be due to a variety of reasons: First, pre-eclampsia involves systematic maternal inflammatory response, including leukocyte and endothelial cell activation (Redman et al. 1999; Borzychowski et al. 2006; Germain et al. 2007). Such inflammatory factors might damage cellular membranes and result in elevated apoptosis (Roberts et al. 2001; Fatouros et al. 2006). As plasma DNA has been known as a marker of cell death (Lichtenstein et al. 2001), the induced apoptotic events might explain the elevation of the total plasma DNA concentrations. Second, it has been suggested that the kidney and liver play a crucial role in the clearance of circulating plasma DNA (Emlen et al. 1984; Lo et al. 1999d). As the development of pre-eclampsia might involve pathologic changes in these two organs, such changes might affect the removal of plasma DNA by these organs from the circulation (Lo *et al.* 1999d). Furthermore, a previous study has reported a significant correlation between total plasma DNA concentrations and liver enzyme activities (Lazar et al. 2009). Liver enzyme activities partially reflect the level of hepatocellular necrosis, and an increase in cellular necrosis might result in the elevation of circulating plasma DNA concentrations. For example, in pregnancies complicated with HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome, the total plasma DNA concentrations are significantly higher than in pre-eclamptic pregnancies without factors of the HELLP syndrome (DiFederico et al. 1999).

The potential clinical implication of these findings is that either total *RASSF1A* or fetal hypermethylated *RASSF1A* in maternal plasma might serve as gender-independent markers for prenatal monitoring of pre-eclampsia. The latter, in particular, is more preferred because of its fetal origin. Fetal DNA exists predominantly in extracellular form in maternal plasma and thus is less significantly affected by blood-processing procedures or the duration of storage than maternal DNA (Angert et al. 2003; Chiu et al. 2001). In the current study, every pre-eclamptic sample used for the restriction assay was matched with two normal samples in terms of gestational age and duration of storage. Among the 10 pre-eclamptic cases, 8 cases were diagnosed as severe and 2 as mild. It would be a subject of interest for future studies to address the correlation of maternal plasma hypermethylated *RASSF1A* concentration with severity of the disease (Swinkels et al. 2002).

In Chapter 4.3.2, I investigated if the pre-eclampsia-associated elevation in hypermethylated *RASSF1A* in maternal plasma was contributed by altered methylation profile of *RASSF1A* in the pre-eclamptic placentas. I studied third-trimester placental DNA and found that the median digestion-resistant *RASSF1A* concentration in the pre-eclamptic placentas was not significantly different from the normal placentas, nor was the fraction of digestion-resistant *RASSF1A* over total concentration. In Chapter 4.3.3, I further confirmed the absence of change in the placental tissue methylation profile by cloned bisulfite-sequencing. DNA from gestational age matched pre-eclamptic and normal placentas revealed *RASSF1A* hypermethylation pattern with no difference in MSF nor MI.

These data suggest that the elevation of hypermethylated *RASSF1A* concentration in

maternal plasma of pregnancies with pre-eclampsia is unlikely the result of an altered methylation profile of *RASSF1A*. Instead, the observation may be caused by increased liberation of circulating fetal DNA from the placenta or impaired clearance from the plasma in pre-eclamptic women (Lau et al. 2002). One of the possible mechanisms for cell-free fetal DNA release is placental apoptosis (Bianchi 2004) and pre-eclamptic placenta has been demonstrated to be associated with a higher apoptotic rate (DiFederico et al. 1999; Leung et al. 2001a). Reduced clearance of circulating fetal DNA in pre-eclampsia, on the other hand, might be due to pathological changes involving the maternal kidney and liver, which have been proposed to be the main organs for clearing circulating DNA (Tsumita et al. 1963; Emlen et al. 1984;). Repeating the study in patients of early pre-eclampsia before renal or liver function abnormalities arises would be useful for testing such a hypothesis. Further studies would also be needed to investigate whether the currently reported quantitative aberration of hypermethylated *RASSF1A* in maternal plasma may be detectable at the pre-symptomatic stage of pre-eclampsia.

In the current study, the demonstration of quantitative aberrations of hypermethylated *RASSF1A* in maternal plasma of pre-eclampsia represents a potentially useful non-invasive approach which is gender- and polymorphism-independent for the prenatal assessment of pre-eclampsia. The clinical utility of this approach would need to be assessed in larger cohorts, cases with different disease severity and those in early stages of pre-eclampsia.

Chapter 5 Methylation profiling of pre-eclamptic placentas

5.1. Introduction

In the previous chapter, I have demonstrated that fetal-specific epigenetic signature has potential to be used as a universal fetal marker to measure fetal DNA in the maternal circulation for the prenatal monitoring of pre-eclampsia.

In this chapter, I aim to search for DNA methylation signatures that are associated with pre-eclampsia. The goal is to develop a disease-specific DNA methylation marker for this disorder.

The current study focused on comparing the methylation profiles of pre-eclamptic placentas with uncomplicated pregnancies. The placenta was chosen for two reasons: First, as discussed in Chapter 4.1, pre-eclampsia developed probably because of the poor invasion of the trophoblasts. The invasion process is regulated by the expression of a specific group of genes (Ohlsson 1989; Strickland et al. 1992; Zhou et al. 2003). It would be interesting to investigate whether DNA methylation of these genes plays a role in trophoblast invasion. Second, as discussed in Chapter 1.3.5, the placenta has been shown to be the major source of circulating cell-free fetal DNA in the maternal plasma (Chim et al. 2005). Thus, it is expected that the aberrant methylation patterns in pre-eclamptic placenta, if any, would also be detectable in the maternal circulation. I am particularly interested in looking for markers that are

hypermethylated in the pre-eclamptic placenta relative to those from uncomplicated pregnancies, so that the digestion-based detection strategy could be applied to achieve the most efficient detection of those markers in maternal circulation.

Previous work has demonstrated that the placenta possessed a specific methylation profiles compared with other somatic tissues (Fuke et al. 2004; Gama-Sosa et al. 1983). It is speculated that epigenetics might be important for the development of the placenta (Ferguson-Smith et al. 2006; Maccani et al. 2009). DNA methylation that happens at close proximity to the transcriptional start site of a gene has been regarded as one of the important epigenetic mechanisms that regulate gene expression (Baylin et al. 2000; Jones et al. 2002; Jones et al. 2001; Laird 2005; Lister et al. 2009). This study aims to investigate the DNA methylation levels of the promoter or the first exon of 17 genes that may play important roles in the placenta.

Two groups of genes were studied:

(1) Seven genes which were shown to be down-regulated in pre-eclamptic placentas relative to uncomplicated controls, including *IGFBP3 (Insulin-like growth factor binding protein-3)* (Han et al. 2006), *VLDLR (Very-low-density lipoprotein receptor messenger)* (Murata et al. 1996), *LDLR (Low-density lipoprotein receptor messenger)* (Murata et al. 1996), *HSD11B2 (11b-hydroxysteroid dehydrogenase type 2)* (Hardy et al. 2002), *NOS2A (Inducible NO synthase)* (Faxen et al. 2001), *HBEGF (heparin-binding EGF-like growth factor)* (Imudia et al. 2008; Leach et al. 2002), *VEGFA (vascular endothelial growth factor A)* (Cirpan et al. 2007; Cooper et al.

1996);

(2) Ten genes that were shown to suppress or promote tumor growth. Previous studies suggested that the invasive behavior of trophoblast and that of tumor cells share a lot of similarities (Bischof et al. 2001; Janneau et al. 2002; Soundararajan et al. 2004; Strickland et al. 1992). I have chosen five tumor suppressor genes that are previously known to be unmethylated in the normal placenta, namely *p16 (Cyclin-dependent kinase inhibitor 2A), CDH1 (E-cadherin), DAPK (Death-associated protein kinase 1)* (*GSTP1 (Glutathione S-transferase pi)* (Chiu et al. 2007; Xue et al. 2004), *and SERPINB5 (serpin peptidase inhibitor, clade B [ovalbumin], member 5)* (Chim et al. 2005). I have also chosen five oncogenes that were previously known to be highly expressed in normal placenta during pregnancy, namely *MYC (v-MYC myelocytomatosis viral oncogene homolog)* (Diebold et al. 1991; Roncalli et al. 1994), *ABL (c-abl oncogene 1, receptor tyrosine kinase)* (Park et al. 1992) , *KIT (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog)* (Sharkey et al. 1994), *MET (met proto-oncogene)* (Prat et al. 1991; Somerset et al. 1998) and *ERBB2 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2)* (Jokhi et al. 1994).

I aim to compare their methylation patterns between pre-eclamptic and normal placentas. The complete gene list is summarized in Table 5.1.

Table 5.1. Candidate genes chosen for the methylation profiling of pre-eclamptic and normal placental tissues.

Gene symbol	Gene	Chromosomal location (cytogenetic band)
Down-regulated in PET placenta		
IGFBP3	*Insulin-like growth factor binding protein-3*	7p13-p12
VLDLR	*Very-low-density lipoprotein receptor messenger*	9p24
LDLR	*Low-density lipoprotein receptor messenger*	19p13.3
HSD11B2	*11 beta-hydroxysteroid dehydrogenase type 2 (11-beta-HSD2)*	16q22
NOS2A	*Inducible NO synthase (iNOS)*	17q11.2-q12
HB-EGF	*Heparin-binding EGF-like growth factor*	5q23
VEGFA	*Vascular endothelial growth factor A*	6p12
Oncogenes		
MYC	*v-myc myelocytomatosis viral oncogene homolog*	8q24.21
ABL	*c-abl oncogene 1, receptor tyrosine kinase*	4q24
KIT	*v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog*	4q11-q12
MET	*met proto-oncogene (hepatocyte growth factor receptor)*	7q31
ERBB2	*v-erb-b2 erythroblastic leukemia viral oncogene homolog 2*	17q21.1
Tumor suppressor genes		
p16	*Cyclin-dependent kinase inhibitor 2A*	9p21
CDH1	*E-cadherin*	16q22.1
DAPK	*Death-associated protein kinase 1*	9q34.1
GSTP1	*Glutathione S-transferase pi*	11q13
SERPINB5	*serpin peptidase inhibitor, clade B (ovalbumin), member 5*	18q21.3

5.2. Methods

5.2.1. Sample collection and processing

Pregnant women attending the Department of Obstetrics and Gynaecology at the Prince of Wales Hospital, Hong Kong were recruited with informed consent after ethnical approval of the study by the institutional review board. Third-trimester placental tissues were obtained from pregnant women affected by pre-eclampsia as well as uncomplicated pregnancies. The definition of pre-eclamptic pregnancy was the same as described in Chapter 4.2. Tissue samples were processed and subjected to DNA extraction according to the procedures described in Chapter 3.3.

5.2.2. Methylation-specific PCR (MSP) and Epityper analysis

One mircogram of placental tissue DNA from third trimester pre-eclamptic and uncomplicated pregnancies were bisulfite-converted by the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's protocol. MSP and Epityper assays were performed as described in Chapter 3.3. All the genes analysed with MSP were subjected to size-separation with gel electrophoresis in 3% agarose gel, at 150V for 15-20 minutes. A 100-bp DNA ladder was included to indicate the sizes of the fragments. Genomic DNA artificially methylated by the *SssI* DNA methyltransferase was bisulfite-converted and amplified together with the tested samples as a positive control in every reaction. For *p16, CDH1, DAPK, GSTP1*, maternal blood cells were used as unmethylated control, as previous studies indicated the regions analysed were unmethylated in maternal blood cells (Chiu *et al.* 2007). No template control was included as negative control. For every gene candidate, Epityper assays were performed when either (1) the sequence context of the region of interest was too CG-rich that the design of MSP primers was difficult; or (2) the positive control in

the MSP assays failed to yield positive amplification after repeated optimization.

The primers for MSP were designed using MethPrimer with the "MSP primer" function (Chapter 3.3.2), and the primers for Epityper were designed using EpiDesigner (Sequenom v2.0), or MethPrimer with the "bisulfite sequencing primer" function (Chapter 3.3.3). The specificity of primers was checked against the Bisearch search tool (Chapter 3.3.2). For the genes *p16, CDH1, DAPK, GSTP1*, the primers for both methylated-specific assays and unmethylated-specific assays were adopted from previously published sequences (Chiu et al. 2007). For other genes, the methylated-specific assays were designed for MSP analysis. All of the assays were designed to target at the regions 1.5 kb upstream or downstream of the transcriptional start site as predicted by the database of the UCSC human genome 2009 assembly (hg19). The primer sequences of each assay, their corresponding annealing temperature for MSP and Epityper reactions, their corresponding final $MgCl_2$ concentrations for MSP reactions were listed in Table 5.2.

5.3. Results

5.3.1. Systematic evaluation of methylation profiles in the pre-eclamptic placentas

I began the analysis with the four tumor suppressor genes, *p16, CDH1, DAPK, GSTP1* that were previously known to be unmethylated in the normal placentas (Chiu et al. 2007). Both methylated-specific and unmethylated-specific assays were preformed on DNA extracted from five pairs of gestational-age-matched pre-eclamptic and normal placentas from the third trimester. The results showed that both groups of placental DNA were unmethylated in the regions analysed (Figure

5.1A).

I then designed methylated-specific MSP assays for two oncogenes *MET* and *ERBB2*, as well as four genes that were shown to be down-regulated in the pre-eclamptic placenta, *HDS11B2*, *IGFBP3*, *VLDLR* and *LDLR*. Two pairs of third-trimester placenta from pre-eclamptic and control pregnancies were tested. The MSP results revealed that these 6 genes were not methylated in both pre-eclamptic and control groups in the regions analysed (Figure 5.1B).

I then attempted to design Epityper assays for the remaining candidate genes. I first compared the methylation data obtained with MSP and Epityper on the oncogene *MYC*. The positions of the two assays relative to the gene are shown in Figure 5.2. Analysis with both analytical platforms revealed that the regions analysed by the two platforms were not methylated in both pre-eclamptic and control groups (Figure 5.2).

Subsequently, I analysed *HBEGF*, *VEGF*, *NOS2A*, *SERPINB5*, *KIT* and *ABL* with Epityper. Two pairs of third-trimester placenta of pre-eclamptic and control pregnancies were tested. The results showed that, except *NOS2A*, all of these genes were not methylated in both pre-eclamptic and control pregnancies in the region analysed (Figure 5.3). *NOS2A* were shown to be predominantly methylated. The methylation profiles were not different between the pre-eclamptic and normal placentas for all 6 genes analysed.

Table 5.2. Primer sequences and PCR parameters for MSP and Epityper assays.

Genes symbol		Platform	M / U-specific	Direction	Primer sequences (5' to 3')	Annealing temperature(°C)	Final concentration of $MgCl_2$ (mM) in PCR
IGFBP3		MSP	M-specific	Forward	TTTAATTGGAGATTTGCGGTGTAGTAATAC	62	1
				Revese	ATTTAAATCTCAAAAAACCAAAACACTACG		
VLDLR	Assay 1	MSP	M-specific	Forward	GGTTTTCGTGAGTTTTAGGCGAAC	60	1
				Revese	AACGAAAAAAAAACCGAAAAAAACG		
	Assay 2	MSP	M-specific	Forward	GGTATTATTTAGGCGGGTATTATGGGTAC	60	1
				Revese	AAAAAACGAAAAAAACCGAAAAACG		
LDLR	Assay 1	MSP	M-specific	Forward	AAGGATTGGAGTGGGAATTAGAGTTTTAC	60	1
				Revese	AAAAACAAAACGACGATCCAACG		
	Assay 2	MSP	M-specific	Forward	GGTGAGGGATCGTATTTTTTTTGAGAC	60	1
				Revese	AAAATACAAAAATTAAACGAACGTAATAATTAAACG		
	Assay 3	MSP	M-specific	Forward	TAGGGGTTTCGATTCGTTTTGC	60	1
				Revese	ACAAAATAAAACTCAAACTTCCGCG		
	Assay 4	MSP	M-specific	Forward	TTTTTGTTATAAAGGCGACGATAAGTTTC	60	1
				Revese	CCGAAAAATAACTACCCCACCG		
HSD11B2		MSP	M-specific	Forward	GGATTTTGTTTCGGTTTTTTTTAAATC	60	1
				Revese	CTAAAACGCGAAAAAAACACTCG		
NOS2A		Epityper	T-cleavage	Forward	AGGAAGAGAGATTTTTTAGATGTTGAAAGTGAGGTTATG	57	-
				Revese	CAGTAATACGACTCACTATAGGGAGAAGGCTAACAATCAAACCAAAAAAAAAACCTATAC		
HB-EGF	Assay 1	Epityper	T-cleavage	Forward	AGGAAGAGAGGATAGGGATGGAGATTTTTGGTATG	60	-
				Revese	CAGTAATACGACTCACTATAGGGAGAAGGCTTTAAATATCTTATCTTACTCACTCAACCC		
	Assay 2	Epityper	T-cleavage	Forward	AGGAAGAGAGTGTTGAAGTTTTTTTTGGTTGTAGG	60	
				Revese	CAGTAATACGACTCACTATAGGGAGAAGGCTACCTCTCAAAAAATCCAAATCTACC		
VEGFA	Assay 1	Epityper	T-cleavage	Forward	AGGAAGAGAGGGATTTTAAGGGGGAGGGTTTA	60	-
				Revese	CAGTAATACGACTCACTATAGGGAGAAGGCTAAAAATTACTCTACTTCCCCAAATCA		
	Assay 2	Epityper	T-cleavage	Forward	AGGAAGAGAGTTTTTGTTTATTGGTTTTTGGTTTT	60	-
				Revese	CAGTAATACGACTCACTATAGGGAGAAGGCTACCTTACCTTACTACTCTACCTCCACC		
MYC		MSP	M-specific	Forward	CGAAATTTTGTTTATAGTAGCGGGC	61	1
				Revese	AAAAAAACGAATCCTAACAACGACG		
MYC		Epityper	T-cleavage	Forward	AGGAAGAGAGGGGAAGTGTTTTTAAATGGGTAGAA	60	-
				Revese	CAGTAATACGACTCACTATAGGGAGAAGGCTAAAAAACAAAACTTCTCAAAAACTTAAC		
KIT		Epityper	T-cleavage	Forward	AGGAAGAGAGTTTATATTTTAGGGGTGGAAAGGTG	60	-
				Revese	CAGTAATACGACTCACTATAGGGAGAAGGCTACCCTCCCAAACACAAACTTC		
ABL		Epityper	T-cleavage	Forward	AGGAAGAGAGTGGTGGGTTGTAAATTTAAGAAGG	60	-
				Revese	CAGTAATACGACTCACTATAGGGAGAAGGCTAACCTAAAAAAAACCCAACAACAAC		
MET		MSP	M specific	Forward	GGACGGATAGTACGCGAGGTAGATAGATAC	62	1
				Revese	CCTACCAATAACTCAACCGAACATCG		
ERBB2		MSP	M-specific	Forward	GATTTTTCGAGGAAAAGTGTGAGAAC	62	1
				Revese	CCTTCTCCTATAATTAAAAACAAACGCG		
p16		MSP	M specific	Forward	TTATTAGAGGGTGGGGCGGATCGC	60	1
				Revese	GACCCCGAACCGCGACCGTAA		
			U-specific	Forward	TTATTAGAGGGTGGGGTGGATTGT	60	2.5
				Revese	CAACCCCAAACCACAACCATAA		
DAPK1		MSP	M-specific	Forward	GGATAGTCGGATCGAGTTAACGTC	60	3
				Revese	CCCTCCCAAACGCCGA		
			U-specific	Forward	GGAGGATAGTTGGATTGAGTTAATGTT	60	2.5
				Revese	CAAATCCCTCCCAAACACCAA		
GSTP1		MSP	M-specific	Forward	AGTTGCGCGGCGATTTC	60	3
				Revese	GCCCCAATACTAAATCACGACG		
			U-specific	Forward	TTTTTGGTTAGTTGTGTGGTGATTTT	60	2.5
				Revese	CACTCCACCCCAATACTAAATCACA		
CDH1		MSP	M-specific	Forward	TTAGGTTAGAGGGTTATCGCGT	60	2
				Revese	TAACTAAAAATTCACCTACCGAC		
			U-specific	Forward	TAATTTTAGGTTAGAGGGTTATTGT	60	2
				Revese	CACAACCAATCAACAACACA		
SERPINB5		Epityper	T-cleavage	Forward	AGGAAGAGAGGAATGGAGATTAGAGTATTTTTTGTGTTAT	60	-
				Revese	CAGTAATACGACTCACTATAGGGAGAAGGCTACTTCCAAAAAACCTCCAACATAT		

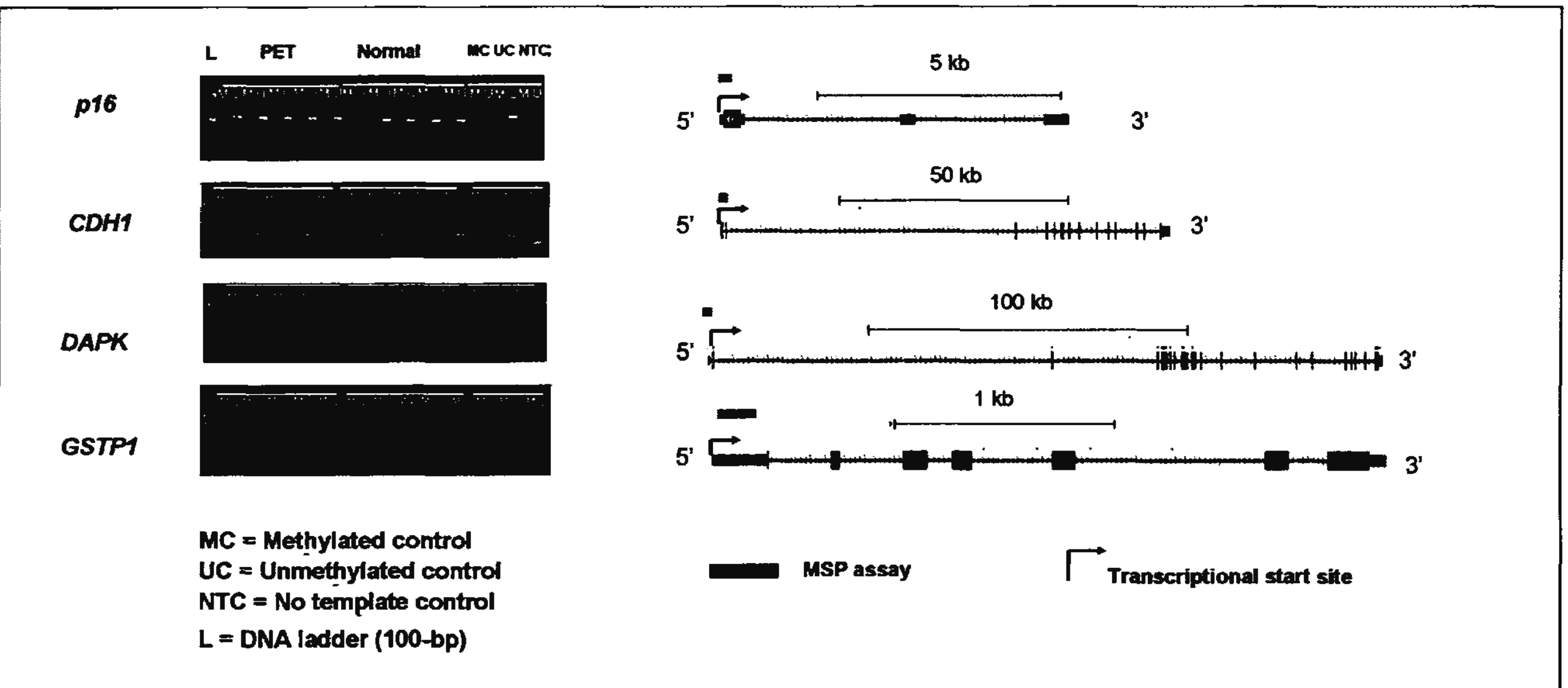


Figure 5.1A. Methylation-specific PCR (MSP) analysis in pre-eclamptic (PET) and normal placental tissues. The positions of the assays relative to the transcriptional start site of the genes are shown. M, methylated-specific reaction; U, unmethylated-specific reaction; MC, methylated control; UC, unmethylated control; NTC, no template control; L, 100-bp DNA ladder.

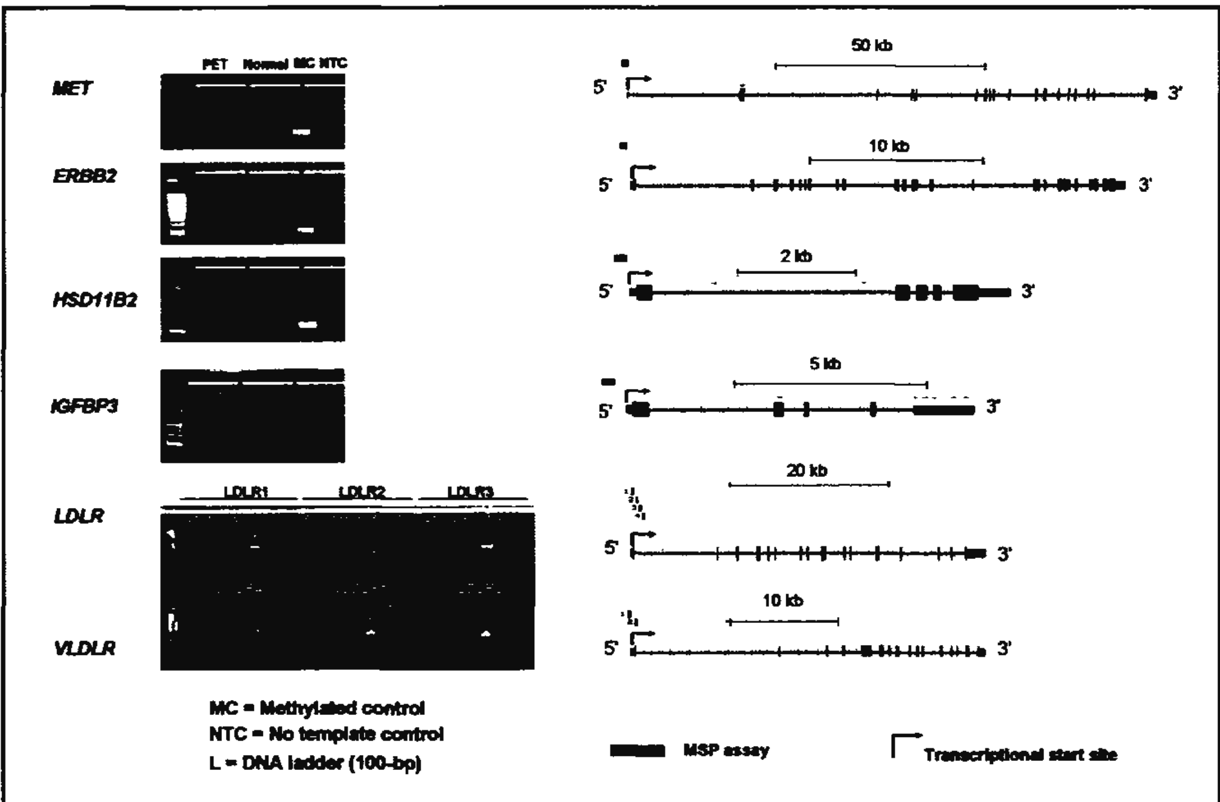


Figure 5.1B. Methylation-specific PCR (MSP) analysis in pre-eclamptic (PET) and normal placental tissues. The positions of the assays relative to the transcriptional start site of the genes are shown. M, methylated-specific reaction; MC, methylated control; NTC, no template control; L, 100-bp DNA ladder.

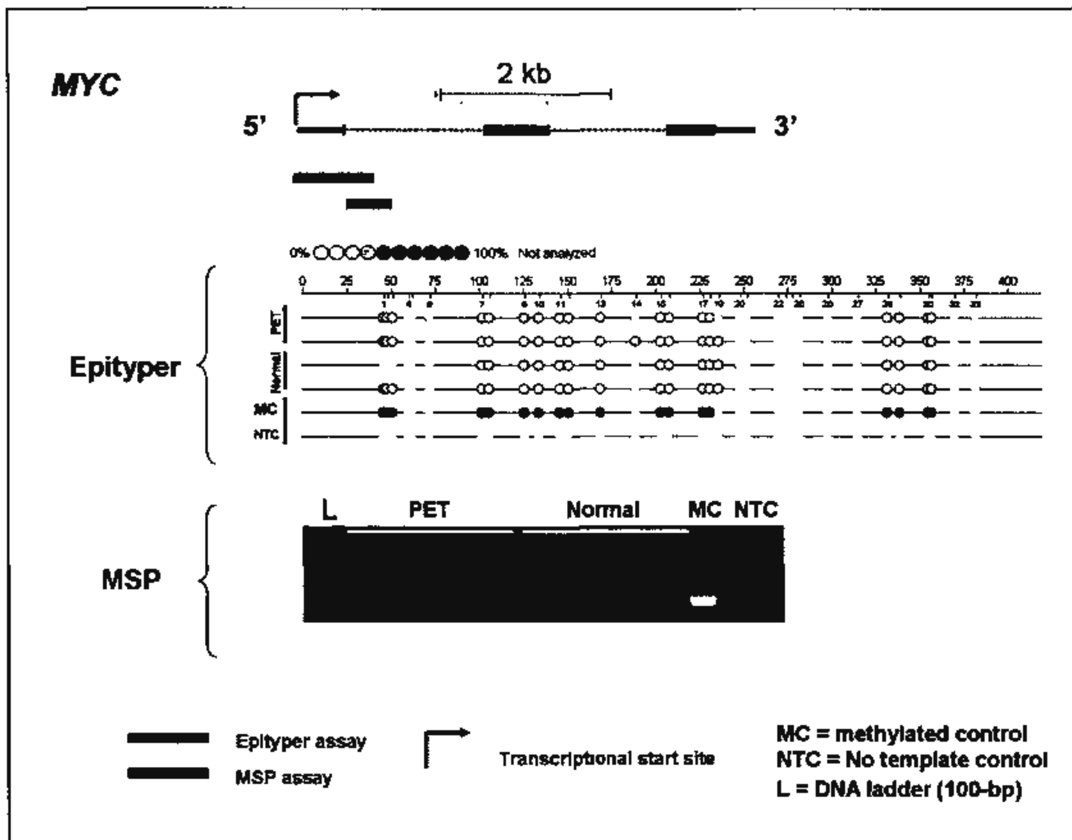


Figure 5.2. Methylation data of *MYC* obtained with MSP and Epityper. The positions of the Epityper (middle panel) and MSP assays (bottom panel) with respected to the transcriptional start site of the *MYC* gene are shown. For Epityper, the methylation levels of each CpG unit (circle) are shown on an intensity scale. Each row represents one sample. Open circles represent CpG sites that could not be analysed by Epityper. M, methylated control; NTC, no template control; L, 100-bp DNA ladder.

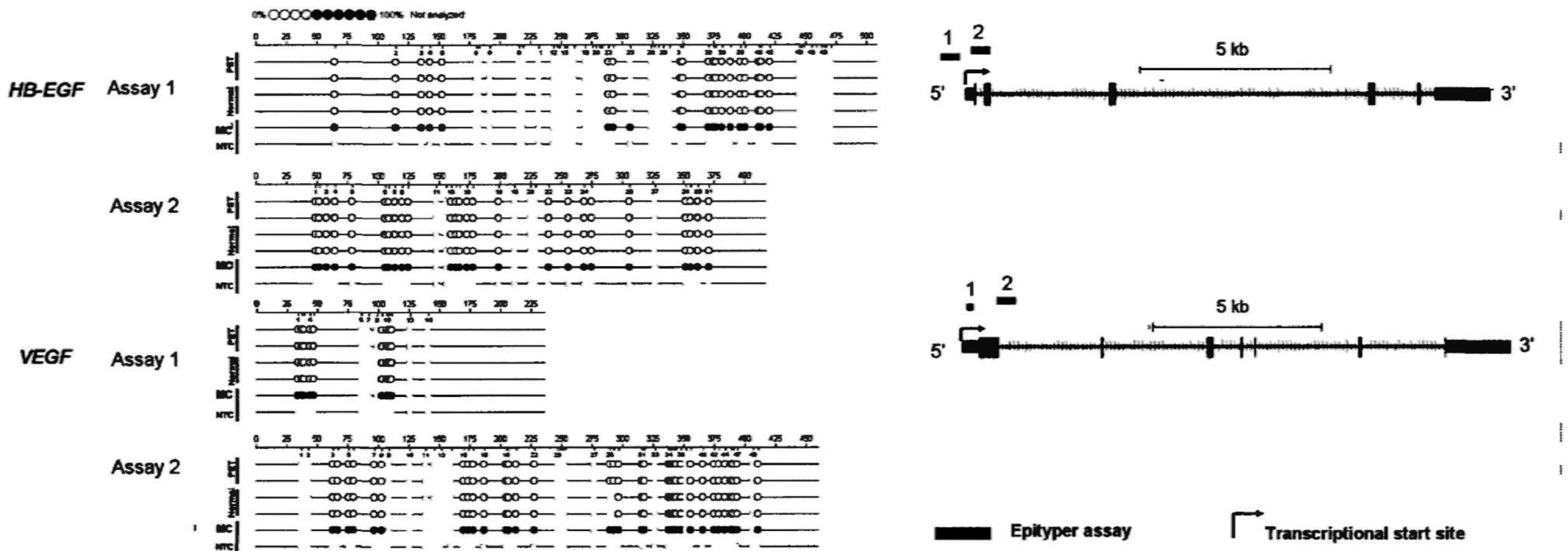


Figure 5.3A. Epityper analysis in PET and normal placental tissues. The positions of the assays relative to the transcriptional start site of the genes are shown. For figure legends of Epityper please refer to Figure 5.2. M, methylated control; NTC, no template control.

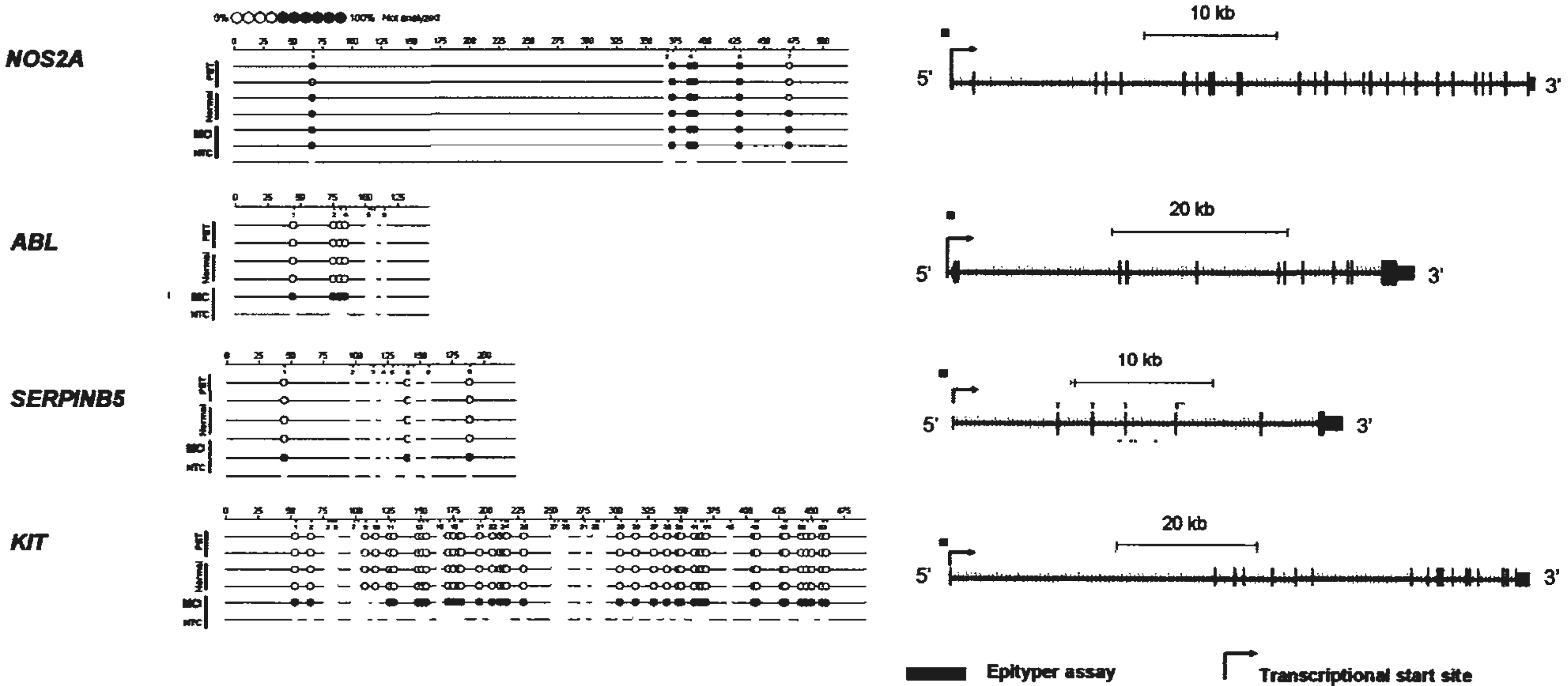


Figure 5.3B. Epityper analysis in PET and normal placental tissues. The positions of the assays relative to the transcriptional start site of the genes are shown. For figure legends of Epityper please refer to Figure 5.2. M, methylated control; NTC, no template control.

5.4. Discussion

As discussed in Chapter 4.1, the cause of pre-eclampsia is still not fully understood. Previous studies suggested that this disorder is generally associated with limited invasion of the trophoblasts (Roberts et al. 2005), placental hypoxia (increased oxidative stress) (Redman et al. 2005; Soleymanlou et al. 2005), disrupted angiogenesis (Sane et al. 2004; Selvaggi et al. 1995; Stepan et al. 2006) and increased apoptosis (DiFederico et al. 1999; Leung et al. 2001a).

Among the genes involved in the current study, seven genes have been previously shown to be down-regulated in the pre-eclamptic placenta, which include *IGFBP3* (Han et al. 2006), *VLDLR* (Murata et al. 1996), *LDLR* (Murata et al. 1996), *HSD11B2* (Hardy et al. 2002), *NOS2A* (Faxen et al. 2001), *HBEGF* (Imudia et al. 2008; Leach et al. 2002) and *VEGFA* (Cirpan et al. 2007; Cooper et al. 1996). Previous publications revealed that these genes were involved in the process of cell invasion, the regulation of hemodynamics in the body, as well as control of oxidative stress. For example, the gene products of *VLDLR* and *LDLR* are involved in fetomaternal lipid metabolism (Wittmaack et al. 1995); *NOS2A* regulates vascular resistance and angiogenesis (Escudero et al. 2008); HSD11B2 is involved in trophoblast differentiation (Causevic et al. 2007; Hardy et al. 2002); IGFBP-3 promotes the anti-apoptotic effect of IGF (insulin growth factor) (Martin et al. 1999; Michell et al. 1997); HBEGF induces trophoblast differentiation and prevents apoptosis (Leach et al. 1999); while VEGFA is a growth factor which is crucial for angiogenesis, particularly for blood vessel formation (Bogic et al. 2001; Hamai et al. 1998) . I therefore reasoned that in pre-eclampsia, where these important

physiological developmental processes are altered in the placenta, the DNA methylation patterns of these specific regulators might be different from that of the normal placental cells.

On the other hand, previous studies have shown that certain oncogenes are expressed in the placenta under spatio-temporally regulation (Diebold et al. 1991; Dungy et al. 1991; Janatpour et al. 1999; Ohlsson 1989; Roncalli et al. 1994). For example, the proto-oncogene *MYC* plays a role in proliferation of trophoblast, especially during the first trimester (Diebold et al. 1991; Roncalli et al. 1994). Another important regulator of the invasion of trophoblast and other epithelial cells, *C-met (MET)*, is also highly expressed in the placenta (Prat et al. 1991; Somerset et al. 1998). *C-abl (ABL)* and *c-kit (KIT)* are continuously expressed during the course of pregnancy. C-*abl* participates in many developmental processes (Park et al. 1992) while *c-kit* regulates placental growth with other haematopoietic growth factors (Sharkey et al. 1994). *ERBB2* is an important tyrosine kinase receptor that is highly expressed by invasive extravillous trophoblasts in normal placenta (Jokhi et al. 1994).

It was suggested that the precise regulation of the expression of these genes are important for the invasiveness and proliferation of trophoblast at specific stages of placental development (Janneau et al. 2002). DNA methylation is one of the important regulators to these specific expression profiles. Recent reports have demonstrated that the expression of certain tumor suppressor genes in the placenta are regulated by promoter-associated DNA methylation (Chiu et al. 2007; Novakovic et al. 2008; Xue et al. 2004). I therefore hypothesised that in the case of

pre-eclampsia where the process of trophoblast invasion is disrupted, these genes might be down-regulated and their promoter region and the first exon might become aberrantly methylated.

However, the results from MSP and Epityper suggested that all of the genes studied are not differentially methylated between the pre-eclamptic and normal placentas within the region analysed. There are a number of possible explanations:

First, in a technical perspective, the MSP assays could only interrogate a few CpG sites located on the PCR primers, while the Epityper assay could only interrogate the CpG sites within around 600 bp. Recent research has suggested that CpG sites within close proximity, such as those within the same CpG island, could possess distinct methylation patterns (Zhang et al. 2009). The possibility that there might be differentially methylated CpG sites locating outside of the studied CpG sites could not be ruled out.

Second, in a biological perspective, the current study only focused on comparing the methylation levels in the placenta obtained from the third trimester. Further evaluation with placental tissues of the earlier trimesters would provide more comprehensive information about the change of placental epigenetic profiles in pathological conditions. On the other hand, DNA methylation is not the only mechanism that regulates gene expression (Gheorghe et al. 2010; Maccani et al. 2009). Post-translational modification of histones by acetylation, methylation, ubiquitylation or phosphorylation (Jones 2002; Jones et al. 2007) or

post-transcriptional silencing by non-coding microRNA (Khraiwesh et al. ; Saito et al. 2006; van Rooij et al. 2007) are also important epigenetic gatekeepers that control gene expression. Therefore, it is possible that the significant down-regulation of specific genes in the pre-eclamptic placenta might be due to mechanisms other than DNA methylation.

Most developmental processes are complicated and involve precise regulation of multiple genes. High-throughput analytical platforms developed over the past decade have enabled scientists to study multiple genes or even the whole genome in a single experiment. For DNA methylation analysis, recent techniques allow rapid analysis of methylation profiles in a genome-wide scale. In particular, genomic tiling arrays (Ishkanian et al. 2004) and massively-parallel sequencing (Brenner et al. 2000) allow scientists to study the human genome at an unprecedented depth and scale. In combination with various enrichment techniques, for example, antibody-mediated enrichment of methylated fragments by MeDIP (described in Chapter 3.3.5), McrBC fractionation (Stewart et al. 2000), and differential amplification via HELP (*Hpa*II tiny fragment enrichment by ligation-mediated PCR) (Hatada et al. 2006; Khulan et al. 2006), these technologies have extended the study of epigenetic events from the promoter regions or CpG islands to the entire human genome (Ball et al. 2009; Beck et al. 2008; Callinan et al. 2006; Fazzari et al. 2004; Lister et al. 2009).

In the next chapter, I would describe the use of one of these newly emerged high-throughput techniques, MeDIP-chip, to systematically search for fetal-specific DNA methylation patterns on chromosome 18. I would demonstrate that the DNA

methylation markers identified by this technique are of high potential for the measurement of fetal DNA in maternal plasma. In Chapter 7, I would further demonstrate the clinical application of these markers for the non-invasive prenatal diagnosis of fetal chromosomal aneuploidies. It is envisioned that a similar strategy might be adopted to expand the search for disease-specific DNA methylation markers for pre-eclampsia.

Section IV

Chromosome-wide search for fetal epigenetic markers on chromosome 18

This section consists of two chapters. Chapter 6 describes a systematic search for new fetal epigenetic markers on chromosome 18. The detection rate and fetal-specificity of the new markers in maternal plasma would be evaluated. Chapter 7 describes a strategy to apply the newly identified markers to the non-invasive prenatal diagnosis of fetal trisomy 18.

Chapter 6 Identification of potential fetal epigenetic markers on chromosome 18

6.1. Introduction

In Chapter 2.3, I have demonstrated the clinical application of epigenetic markers as qualitative and quantitative markers for detecting fetal DNA in maternal plasma. As I have discussed in Chapter 3.4, DNA degradation by bisulfite treatment in methylation analysis is detrimental to the detection of fetal epigenetic markers in maternal plasma. To bypass bisulfite treatment, a detection strategy that combines methylation-sensitive restriction enzymes and quantitative real-time PCR has been developed to digest unmethylated DNA molecules (Chan et al. 2006). The robustness of this approach has been demonstrated in a number of studies. For example, in Chapter 4, I have shown that the quantitative aberrations of the fetal-derived hypermethylated *RASSF1A* sequences in the maternal plasma of pre-eclamptic pregnancies could be robustly detected using this digestion-based detection method. Moreover, as I have discussed in Chapter 2.3.3, the recent report of the EGG approach by *Tong* et al. has also adopted the digestion-mediated detection strategy to compare the dosage of a fetal epigenetic marker on the aneuploid chromosome relative to a fetal genetic marker on a reference chromosome (Tong et al. 2010).

These promising results have prompted me to explore whether the EGG approach could be used to detect the second commonest fetal trisomy, trisomy 18. However, this requires a fetal epigenetic marker located on chromosome 18 that is methylated

in the placenta while unmethylated in maternal blood cells. At the time of this writing, the availability of such markers is relatively limited, since most of the earlier searches have been focused on chromosome 21 (Chim et al. 2008; Old et al. 2007; Tong et al. 2010).

In Chapter 5, I have described a candidate gene approach to search for pre-eclampsia-specific DNA methylation patterns. Such an approach, however, is inevitably associated with relatively limited genomic coverage and throughput. To expand the coverage, in this chapter, I have adopted a recently emerged high-throughput methylation profiling platform to search for potential fetal epigenetic markers on the entire chromosome 18. Given the large amount of data, a systematic and cost-effective scheme is needed to (1) confirm the high-throughput data on an alternative analytical platform; (2) evaluate the detection rate of the potential markers in maternal plasma; (3) confirm if they are fetal-specific; and (4) assess if they could detect trisomy 18 via the EGG approach.

As explained in Chapter 1.3.5.4 and Chapter 2.2.2, the predominant sources of fetal and maternal DNA in maternal plasma are the placenta and maternal blood cells, respectively. Therefore, the first stage of this study focused on a chromosome-wide methylation profiling by MeDIP (methylated-DNA immunoprecipitation) coupled with genomic tiling array (MeDIP-chip) to identify chromosome 18 regions that are differentially methylated between the placenta and maternal blood cells. I focused on the search for loci that are methylated in the placenta and unmethylated in maternal blood cells. After that, the second stage focused on the validation of the identified markers with quantitative methylation assays, followed by an evaluation to select the

most promising candidates for marker development. The third stage focused on the evaluation of an identified marker with regard to its detection and clearance in maternal plasma, and the correlation of its concentration with a Y-chromosome-specific fetal marker in maternal plasma. Figure 6.1 summarises the workflow of the study.

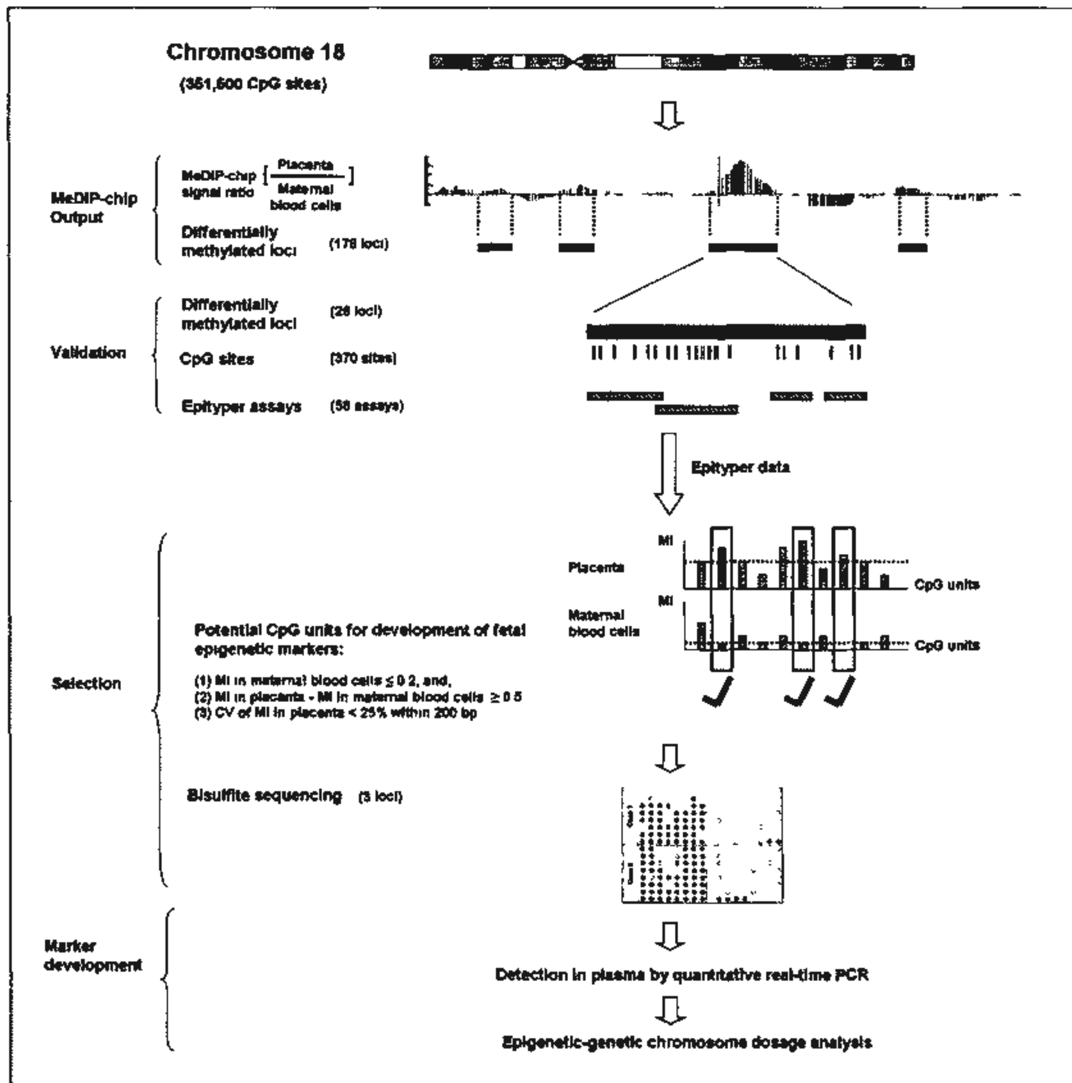


Figure 6.1. Workflow of the identification, detection and application of fetal epigenetic markers. (Legends on next page)

Figure 6.1. Workflow of the identification, detection and application of fetal epigenetic markers.

Methylation profiles of first-trimester euploid placenta (n = 5) and maternal blood cells (n = 5) were determined by MeDIP-chip analysis. The placenta and maternal blood cells are the predominant sources of fetal and maternal DNA, respectively, in maternal plasma. Thus, differentially methylated regions (loci) between these two tissues contain potential fetal epigenetic markers. One hundred and seventy-eight differentially methylated loci were identified via bioinformatic analysis of the MeDIP-chip data. Twenty-six loci were analysed with Epityper assays to validate the methylation levels. Methylation status of each CpG units was expressed as the Methylation Index (MI). A CpG unit contains one or more CpG sites that were analysed by Epityper. Potential CpG sites for developing fetal epigenetic markers were selected according to the criteria described. The methylation status of the 3 most promising loci was confirmed with bisulfite sequencing. A quantitative real-time PCR assay was developed to detect the most promising locus from maternal plasma. This marker was subsequently applied to detect fetal trisomy 18 in maternal plasma via the EGG approach. MeDIP-chip: methylated DNA immunoprecipitation coupled with tiling array.

6.2. Methods

6.2.1. Sample collection and processing

Samples were obtained from women attending the Department of Obstetrics and Gynaecology at the Prince of Wales Hospital, Hong Kong, or the Prenatal Diagnostic and Counselling Department at the Tsan Yuk Hospital, Hong Kong, or the Harris Birthright Research Centre for Fetal Medicine, at the King's College Hospital, London, UK. Plasma from the UK was harvested, kept frozen and sent to Hong Kong in batches on dry ice. Informed consent was obtained from all the recruited pregnant women as described in Chapter 3.1. The study was approved by the respective institutional review boards. Placental tissues and CVS samples were recruited among women undergoing termination of pregnancy and elective cesarean delivery, respectively. Placental villus were taken from the area around 3 cm below the umbilical cord insertion. Placental tissues were processed and subjected to DNA extraction as described in Chatper 3.2.

DNA was extracted from 1.6 mL of third trimester or 3.2 mL of first- and second-trimester maternal plasma with the procedures described in Chapter 3.2 and eluted in 50 μL of water.

6.2.2. MeDIP-chip analysis

The non-repetitive sequences of human chromosome 18 comprise 351,500 CpG sites in 74.7 million bases (Fazzari et al. 2004). We performed MeDIP-chip with five pairs of first-trimester euploid placentas and maternal blood cell samples. For each sample, 200 μg of DNA were sonicated and immunoprecipitated by antibody against

5-methylcytosine and processed as described in Chapter 3.3. The genomic tiling array was designed using the NCBI build 36 version of the human genome assembly.

6.2.3. Bioinformatic analysis of MeDIP-chip dataset

The data were analysed with two complementary bioinformatic algorithms, namely the Tiling Array Software (TAS version 1.1) (Cawley et al. 2004) and the Model-based Analysis of Tiling array software (MAT) (Johnson et al. 2006).

For TAS, quantile normalization of probe signals by two-sample analysis was performed (Cawley et al. 2004). An "estimate of fold enrichment" of immuniprecipitated (IP)-DNA was calculated as the pseudomedian signal of all IP-sample/no-IP control ratios within a 800-bp sliding window. For each sample, the signal for each locus was normalized to give an average value of 1.00 for all loci identified on the same array. A signal log ratio (SLR) between the median signal of the 5 placentas and that of the 5 maternal blood cell samples was calculated. We expected to obtain a positive SLR for regions that are hypermethylated in the placenta, relative to blood cells.

The same MeDIP-chip dataset was also analysed by MAT. For MAT, instead of quantile normalization, probe standardization was performed, which predicted the baseline probe behavior by a model that accounts for the effect of probe sequence context and genome copy number on its signal (Johnson et al. 2006). The algorithms

calculated the P-value to determine if there was a significant difference between the signals of the two groups.

I compared the array intensity signals of the placenta relative to maternal blood cells. I determined the optimal parameter settings by comparing the output data at each setting against the methylation data of 22 previously known fetal epigenetic markers on chromosome 21 (Chim et al. 2008).

The following settings were established for the analysis by MAT and TAS:

For MAT, the bandwidth was set as 400 bp, the maximum gap as 300 bp and minimum probe as 10; while for TAS, a threshold setting was set as 1.9, the maximum gap as 300 bp and minimum run as 180 bp. With these settings, MAT and TAS correctly determined the methylation status of 18 and 11 markers, respectively, out of the 22 markers on chromosome 21 (Figure 6.2). I then used these to analyse the data for chromosome 18. TAS identified 2210 loci on chromosome 18. To prioritize, I focused on those with a median SLR > 0.4 between the placenta and maternal blood cells. For MAT, 110 loci on chromosome 18 were identified to be hypermethylated in the placenta.

6.2.4. High-resolution validation of methylation levels by Epityper

The gold standard for verifying methylation quantitative data is cloning and bisulfite sequencing, as it provides methylation data of every single CpG site. However, the procedure of bisulfite sequencing is tedious and labor-intensive (Frommer et al.

1992). To increase the efficiency and throughput, at the initial stage, I validated the candidates with the Epityper (Sequenom), which is much faster than bisulfite sequencing and yet allows high-throughput analysis of methylation with a resolution close to single CpG sites (Ehrich et al. 2005). Epityper assays were performed and designed as described in Chapter 3.3.4.

Since the analysis by Epityper involves base-specific cleavage of the amplicon sequences, certain loci with an abundance of T-residues would result in fragments with masses that lie below the detection limit of the mass spectrometry system used. For some amplicons, the masses of cleavage products might overlap with each other, and thus some CpG sites might not be informative. Moreover, certain loci contain repetitive sequences which do not result in a unique PCR product. Furthermore, the optimal length of PCR amplicon for Epityper assays is between approximately 150-600 bp. This was exceeded by the average length of the 178 loci identified, which was on average 1188 bp, ranging from 207 bp to 5174 bp.

Therefore, the following considerations were taken when I chose the candidates for assay design: (i) after the T-specific cleavage, the amplicon should result in CpG-containing fragments within the detectable mass range, i.e. 1500-7000 Da; (ii) the PCR should result in a unique amplification from the bisulfite-converted human genome, as predicted *in silico* by Bisearch (http://bisearch.enzim.hu/); (iii) the PCR primers should be free of CpG sites within their binding sites, have an annealing temperature of > 57℃, and should yield an amplicon length of 150-600 bp; (iv) the amplicon should not contain any genomic variants, such as copy number variations, of a reported frequency higher than 1%. This was checked using the Database of

Genomic Variants (http://projects.tcag.ca/variation/) (Iafrate et al. 2004). Taking into account the above considerations, I designed Epitpyer assays for a total of 26 loci (Table 6.1). The primer sequences and specific annealing temperatures are listed in Table 6.2.

Prioritising the candidates based on the potential success of the Epityper assay design would enhance the efficiency of the validation process, but might miss certain highly differentially methylated regions. Nonetheless, the results of my preliminary validation showed that this prioritisation strategy is efficient in revealing potential candidates for biomarker development. For those loci that cannot be easily analysed by the Epityper, one might design bisulfite sequencing assay to determine their methylation status.

6.2.5 High-resolution methylation analysis by cloning and bisulfite sequencing

Among the potential markers, the more promising candidates are chosen for further evaluation. The selection criteria for these candidates would be discussed in Chapter 6.3. Their methylation levels at single-CpG resolution would be confirmed with cloning and bisulfite sequencing.

Details of the principle and the procedures have been described in Chatper 3.3. The sequencing data were analysed using the SeqScape software (Applied Biosystems). I ensured the bisulfite conversion achieved > 99% completeness in all the regions I tested. The methylation status at each CpG site was expressed as the MI, which was calculated by dividing the number of methylated clones with the total number of

clones for each sample. The primer sequences used for bisulfite sequencing for each particular locus were the same as those used for the Epityper analysis.

6.2.6. Detection of novel fetal epigenetic markers in maternal plasma by quantitative real-time quantitative PCR

Previous data suggested that in the maternal plasma, fetal DNA was predominantly derived from the placenta while the maternal background was predominantly from maternal blood cells (Lui et al. 2002a; Ng et al. 2003). I therefore hypothesised that the fetal-derived hypermethylated sequences should be resistant to enzyme digestion and thus detectable in maternal plasma.

In order to illustrate that the identified marker could be robustly detected in maternal plasma by the digestion-based method, a qPCR assay was designed to target at the the most promising potential marker, namely *VAPA-APCDD1 region 1*. In the assay design, the primer and probes were strategically positioned such that the unmethylated CpG sites in maternal DNA, but not the methylated CpG sites, would be specifically cleaved by the relevant restriction enzymes.

6.2.6.1 Removal of unmethylated DNA by methylation-sensitive restriction enzymes

The enzyme digestion protocol involved incubation of DNA samples with 20 U each of *Hpa*II and *Hin*P1I (New England Biolabs), in 1X NEBuffer 1 at 37 °C for 2 h.

The assay was first validated with placental and maternal blood cells samples. For

each sample, 50 ng of DNA extracted from the placentas or maternal blood cells and incubated with the enzymes specified above. A mock digestion, where 50% glycerol was added instead of the enzymes, was run in parallel with each digestion of the same DNA template as a negative control to ensure any change in the quantity of targeted DNA after digestion was due to restriction digestion.

The assay was then used to investigate the post-partum clearance pattern of digestion-resistant *VAPA-APCDD1 region 1* in pre- and post-delivery maternal plasma. DNA was extracted form 0.8 mL maternal plasma and eluted into 50 μL water. 35 μL was then taken out and subjected to enzyme digestion in the same condition described above.

6.2.6.2 Real-time qPCR

After methylation-sensitive restriction enzyme digestion, the digested products were subjected to conventional qPCR on the ABI 7300 HT sequence detection system (Applied Biosystems). Each qPCR reaction contained 1X TaqMan® Universal PCR Master Mix (Applied Biosystems). Final concentrations of 1500 nmol/L of each forward and reverse PCR primers, and 250 nmol/L dual-labeled fluorescent TaqMan® probe was added into each reaction. The probes contained FAM (6-carboxyfluorescein) as reporter and BHQ1 (Black-hole quencher 1) as quencher (Integrated DNA Technologies). Details of the primer and probe sequences are given in Table 6.3. The detailed digestion protocols and qPCR thermal profiles are given in Table 6.4.

For DNA extracted from placental and blood cell samples, 5 μL of enzyme-treated DNA or 50 ng of untreated DNA were used as PCR template. For the plasma samples, 10 μL of enzyme-treated DNA or 5 μL of untreated DNA were added to as PCR template in a total reaction volume of 50 μL using 96-well reaction plates (Applied Biosystems).

To evaluate the completeness of digestion, I developed a control qPCR system based on a deliberately selected region on the *ß-actin* gene, which I have confirmed to be (i) completely unmethylated in both placenta and maternal blood cells from the first trimester (Figure 6.11E) ; and (ii) contains similar number of recognition sites and type of the methylation-sensitive REs as the target markers. Every sample that I tested would be subjected to this control qPCR assay to ensure that, after digestion, no signals would be detected for both the placenta and maternal blood cells.

The specificity of the primers used in the assays was confirmed by "In-Silico PCR" and the "BLAT" search function on the UCSC genome browser (http://www.genome.ucsc.edu). A calibration curve for each assay was derived by serial dilutions of known concentrations of genomic DNA extracted from a male peripheral blood cell sample, which was quantified by NanoDrop ND-1000 (Thermo Fisher Scientific Waltham, MA), using the DNA-50 setting. It was then used to construct the quantitative standards with a linear dynamic range from 3 copies per reaction to 10,000 copies per reaction. The limit of detection (LOD) for the two assays was 3 copies per reaction, which was equivalent to 45 copies/mL plasma DNA detection. Any signals detected below that limit were considered undetectable.

For the *VAPA-APCDD1 region 1* assay, twenty duplicated reactions (40 replicates) were performed at the LOD concentration and 100% of the wells were positive with a median quantification cycle of 39.1 (range: 38.2 – 40.8). For each of the plasma or tissue samples, duplicated reactions were performed and the mean quantity was used for calculation. Multiple water blanks (no template control) were included in every PCR run.

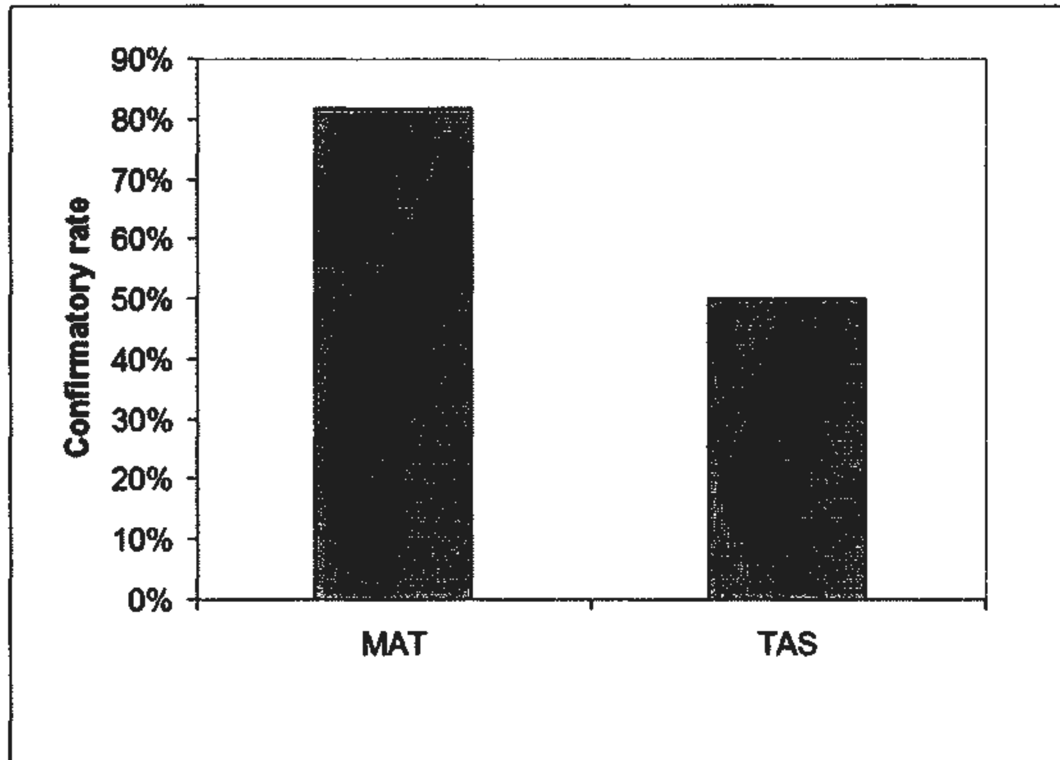


Figure 6.2. Confirmatory rates of the two data-mining algorithms, MAT and TAS

Previously known fetal epigenetic markers identified with the gold standard, bisulfite sequencing. The MAT algorithm confirmed 82% (18 loci) while the TAS algorithm confirmed 50% (11 loci) of the 22 loci. TAS, Tiling Array Software; MAT, Model-based Analysis of Tiling array.

Table 6.1 List of loci identified with MeDIP-chip analysis and chosen for validation with Epityper.

Algorithm	Locus ID	Chr[a]	Start[a]	End[a]	Median signal log ratio
TAS	TAS.18.0675	18	19035725	19036654	0.544
	TAS.18.0841	18	27486040	27487378	1.053
	TAS.18.1887	18	72227405	72228513	0.731
	TAS.18.0689	18	19366692	19367798	0.663
	TAS.18.1426	18	54206447	54207599	0.777
	TAS.18.2311	18	75587713	75589075	1.676
	TAS.18.2336	18	75691060	75693161	1.303
Algorithm	**Locus ID**	**Chr[a]**	**Start[a]**	**End[a]**	**MAT p-value**
MAT	MAT.18.0067	18	21181970	21183936	1.25E-36
	MAT.18.0069	18	72226945	72228900	2.66E-29
	MAT.18.0071	18	27485628	27487511	6.12E-26
	MAT.18.0090	18	7360625	7361926	1.26E-09
	MAT.18.0091	18	72971053	72972347	4.06E-09
	MAT.18.0094	18	10022563	10023955	9.35E-11
	MAT.18.0096	18	72292238	72293387	4.38E-09
	MAT.18.0097	18	75542484	75543900	2.10E-10
	MAT.18.0098	18	54075774	54077141	1.59E-10
	MAT.18.0070	18	75199542	75201601	3.00E-29
	MAT.18.0073	18	59789377	59790633	3.63E-11
	MAT.18.0074	18	75579432	75582090	3.37E-17
	MAT.18.0081	18	75589375	75591012	8.22E-15
	MAT.18.0083	18	75613672	75615203	9.46E-11
	MAT.18.0085	18	7614484	7615728	1.56E-08
	MAT.18.0087	18	45434883	45436312	1.34E-10
	MAT.18.0095	18	27244690	27246325	5.00E-12
	MAT.18.0101	18	75137431	75138826	9.62E-11
	MAT.18.0102	18	44403655	44404868	2.63E-09

[a]Genomic locations are defined according to the Human genome March 2006 assembly (NCBI36/hg18) of the UCSC Genome Browser (www.genome.ucsc.edu)

Table 6.2. Primer sequences for Epityper assays for the validation of loci identified with MeDIP-chip.

Locus ID	Assay ID	Genomic locations* (Assay)	Forward primer sequence	Forward primer Tm (°C)	Reverse primer sequence	Reverse primer Tm (°C)	Amplicon size (bp)	PCR annealing temperature (°C)
MAT 18 0067	MAT 18 0067 1	chr18 21182171 21182565	5 aggaagagagTTTTGGTTTTTTTATGATTTAAGTGTGA 3	60	5 cagtaatacgactcactatagggagaaggctTCCTATCAACTACTTAAACAACTCCCA 3	61	395	60
MAT 18 0067	MAT 18 0067 3	chr18 21182939-21183331	5 aggaagagagGGGGAAATTTAGATTTAGGGTTTGA 3	62	5 cagtaatacgactcactatagggagaaggctATTCACTCCAAAAACAAATAAACCA 3	60	393	60
MAT 18 0067	MAT 18 0067 4	chr18 21183295-21183691	5 aggaagagagTATTTGGATTTTTTAGGTTTTGGGT 3	60	5 cagtaatacgactcactatagggagaaggctCAACATCCTCTTCCTCCTAACTACC 3	61	397	60
MAT 18 0069	MAT 18 0069 1	chr18 72226774 72227119	5 aggaagagagTTTAGAAATTTAGAGAGGTTAGGTGGG 3	61	5 cagtaatacgactcactatagggagaaggctCAAACAACCCACCTACAAAAAATAAC 3	61	346	60
MAT 18 0069	MAT 18 0069 5	chr18 72228673-72229092	5 aggaagagagTTTAGGGGGATGTATGAGGAGTT 3	60	5 cagtaatacgactcactatagggagaaggctCCAATACCTCTACCCAAAAATAACC 3	60	420	60
MAT 18 0070	MAT 18 0070 1	chr18 75199349-75199737	5 aggaagagagTGAGAATATGTGAGGAAAGTTTTTTGA 3	60	5 cagtaatacgactcactatagggagaaggctTAAAAAACACCCCCTCTACCTTAAC 3	60	389	60
MAT 18 0070	MAT 18 0070 2	chr18 75199565-75199915	5 aggaagagagTTGAAGTTGGTATAGTTTTGATTTTTGA 3	60	5 cagtaatacgactcactatagggagaaggctAACCATCCTTCTTAATAACTTCCCC 3	61	351	60
MAT 18 0070	MAT 18 0070 3	chr18 75201049-75201496	5 aggaagagagTGGTTTTTTTTAGTGTTTAAGATTTGTG 3	60	5 cagtaatacgactcactatagggagaaggctCCAAAATTCATAACAAAACCCATCT 3	61	448	60
MAT 18 0071	MAT 18 0071 1	chr18 27485473-27485875	5 aggaagagagTTTGAAGTTTTAGTTATTGGGGAGG 3	61	5 cagtaatacgactcactatagggagaaggctACTACTCAATCCTTCCTAACCATCC 3	60	403	60
MAT 18 0071	MAT 18 0071 3	chr18 27486289-27486672	5 aggaagagagTTAGGTTTTTGATTTGGTGATTATGA 3	60	5 cagtaatacgactcactatagggagaaggctAATAACCCTTTCACAATTCCTCACT 3	60	384	60
MAT 18 0071	MAT 18 0071 4	chr18 27487302 27487698	5 aggaagagagGTGGTGGTTTATGTTTGTTTTTTAGTAGT 3	60	5 cagtaatacgactcactatagggagaaggctAAAACCCAATTCTAATAACACCTCTCT 3	60	397	60
MAT 18 0073	MAT 18 0073 1	chr18 59789200-59789578	5 aggaagagagGGGGATGAGTAGATTGTTTAGAGTT 3	58	5 cagtaatacgactcactatagggagaaggctCTAAAACCTCTCCATTCCAAAAAAC 3	61	377	60
MAT 18 0074	MAT 18 0074 1	chr18 75579998-75580427	5 aggaagagagTTTTTTAAGTTAGGAGTAGGGGTGG 3	60	5 cagtaatacgactcactatagggagaaggctAAATAAAAAACCTCAAATTCATCCC 3	60	430	60
MAT 18 0081	MAT 18 0081 1	chr18 75590129-75590425	5 aggaagagagGGATTGTTGAGTTGTGTATTTGGA 3	60	5 cagtaatacgactcactatagggagaaggctAAATCACCCCTTATCATAACCATAAA 3	60	297	60
MAT 18 0081	MAT 18 0081 2	chr18 75589510-75589941	5 aggaagagagGGATTTTATTTTGAGGGTGAATGAT 3	61	5 cagtaatacgactcactatagggagaaggctCAAACAACTCTTCACCAAACATCTA 3	60	432	60
MAT 18 0081	MAT 18 0081 3	chr18 75589856-75590155	5 aggaagagagTTGATTTTTTTTATGTGGGATTTATG 3	59	5 cagtaatacgactcactatagggagaaggctACCTCCAAATACACAACTCAACAAT 3	60	300	60
MAT 18 0083	MAT 18 0083 1	chr18 75614643-75615009	5 aggaagagagTTATAAGAGGAAGTTGAATGAAGAGGA 3	60	5 cagtaatacgactcactatagggagaaggctCAACACACCTAAACCTTTAACACCT 3	60	367	60
MAT 18 0085	MAT 18 0085 1	chr18 7615501 7615744	5 aggaagagagTATGATTTTTTTGAATGAAAAGGTTGG 3	62	5 cagtaatacgactcactatagggagaaggctCCTAACCAACATAACAAAACCCAAT 3	61	244	60
MAT 18 0085	MAT 18 0085 2	chr18 7614556 7614914	5 aggaagagagTTATTGTAGTTTTGATTTTTTGGGTTTAA 3	61	5 cagtaatacgactcactatagggagaaggctAAACTCACTACACCTACACCAAACTTACT 3	60	359	60
MAT 18 0085	MAT 18 0085 3	chr18 7614853 7615232	5 aggaagagagTGTGTAGAGTTTTTTAGAATAGTGTTGGG 3	60	5 cagtaatacgactcactatagggagaaggctCCTATCAAAAAACAAAAAAAACAAATTC 3	61	380	60
MAT 18 0087	MAT 18 0087 1	chr18 45435440-45435819	5 aggaagagagTGGGGTATTTTGAGTGTTTTTAATG 3	60	5 cagtaatacgactcactatagggagaaggctATCCTCAAAACCCAACAACTACTCT 3	61	380	60
MAT 18 0087	MAT 18 0087 2	chr18 45435797-45436243	5 aggaagagagAGTAGTTGTTGGGTTTTGAGGATTT 3	61	5 cagtaatacgactcactatagggagaaggctTCACTACCAAAAAAATAAATCCAACTATC 3	60	447	60
MAT 18 0087	MAT 18 0087 3	chr18 45436041-45436309	5 aggaagagagTAGTTAGGATAGTGGGAAAGGGGT 3	61	5 cagtaatacgactcactatagggagaaggctCACCCAAATAACCATCCAAATATAAA 3	61	259	60
MAT 18 0090	MAT 18 0090 1	chr18 7360799 7361094	5 aggaagagagTTTGTAAGGGAAGAAGTAGAATGTTG 3	60	5 cagtaatacgactcactatagggagaaggctAAAAATCATACACAAACAAAAAACC 3	58	296	60
MAT 18 0090	MAT 18 0090 2	chr18 7361459 7361775	5 aggaagagagTATTTAGGAGGTTTTTGGGTAGTTG 3	59	5 cagtaatacgactcactatagggagaaggctCCTAACACCACCCTAAAAATAAAAC 3	59	317	60
MAT 18 0091	MAT 18 0091 1	chr18 72971126-72971555	5 aggaagagagAGGAAATTTGTGTGGGATATTTTTG 3	61	5 cagtaatacgactcactatagggagaaggctTAACTTTTAATCCTCCTCCAAATTCTTTA 3	61	430	60
MAT 18 0091	MAT 18 0091 2	chr18 72971528-72971817	5 aggaagagagAAAGAATTTGGAGGAGGATTAAAAGTTAA 3	62	5 cagtaatacgactcactatagggagaaggctCTCCAAAAATATCACAATTCTACCCA 3	62	290	60
MAT 18 0094	MAT 18 0094 1	chr18 10022533-10022724	5 aggaagagagGGTTATTTGGGGGTAGTAGG 3	55	5 cagtaatacgactcactatagggagaaggctAAACTCAAAACTAAAACAAACACTC 3	66	192	60
MAT 18 0094	MAT 18 0094 2	chr18 10022727 10023041	5 aggaagagagTGAAAGATTGTAGAGTTTTGGGTTTA 3	60	5 cagtaatacgactcactatagggagaaggctAAAATTCATTTCTCTTTTTCTTAAACTTT 3	58	315	60
MAT 18 0094	MAT 18 0094 3	chr18 10023117 10023501	5 aggaagagagTTGTGTTTTGTTTTAAGGGATTGAG 3	61	5 cagtaatacgactcactatagggagaaggctCCCACACATACAAATACACACACAC 3	61	385	60
MAT 18 0095	MAT 18 0095 1	chr18 27244692 27244974	5 aggaagagagTGAGTTTTTTTTTGATGGTTTTTTAG 3	58	5 cagtaatacgactcactatagggagaaggctTAACCTTCCCACATTTACACAAACT 3	60	283	60
MAT 18 0095	MAT 18 0095 2	chr18 27244901 27245346	5 aggaagagagTTTGTTTGTAGTTTTTTTGGTTAGTTTT 3	59	5 cagtaatacgactcactatagggagaaggctTCCCATAATAAAACTCCTCTTCCTT 3	60	446	60
MAT 18 0096	MAT 18 0096 1	chr18 72292138-72292361	5 aggaagagagTTTTTAGTTTTGGTTTATGAATTAATTTT 3	58	5 cagtaatacgactcactatagggagaaggctAACCCCTCCTAAAAAATACCCTATC 3	60	224	60
MAT 18 0096	MAT 18 0096 3	chr18 72292847 72293275	5 aggaagagagAGGTTTATAGTTTTTAGATTGGGGTTT 3	59	5 cagtaatacgactcactatagggagaaggctAAACTTTATCATTCTCCTTCACTCTACCT 3	61	429	60
MAT 18 0097	MAT 18 0097 1	chr18 75542384-75542773	5 aggaagagagTGTGGAGGTAGATTTTAATGTTGGT 3	60	5 cagtaatacgactcactatagggagaaggctAAACCTCCTAAAACCAAAAAAACAC 3	60	390	60
MAT 18 0097	MAT 18 0097 2	chr18 75542839-75543260	5 aggaagagagTTGTTTGGAATTTGAGGTGGTTT 3	62	5 cagtaatacgactcactatagggagaaggctTCCACAAAAATACAACAAACATTTTAAAA 3	62	422	60
MAT 18 0098	MAT 18 0098 1	chr18 54075676-54076098	5 aggaagagagTATGGTTTAAGGTTAGGGGAAATTG 3	61	5 cagtaatacgactcactatagggagaaggctTCCTCAAACTAACAATCACATAAAATTC 3	60	423	60
MAT 18 0098	MAT 18 0098 2	chr18 54076639 54077085	5 aggaagagagTTGATGTGGAAGATAAAGGGATTTATATA 3	61	5 cagtaatacgactcactatagggagaaggctAACAACTACTACCTAAACTTAACACCCCT 3	60	447	60
MAT 18 0098	MAT 18 0098 3	chr18 54075378-54076667	5 aggaagagagAATGGTTAAGTGTTTTGTTTTGGTTT 3	60	5 cagtaatacgactcactatagggagaaggctTATATAAATCCCTTTATCTTCCACATCAA 3	61	290	60
MAT 18 0101	MAT 18 0101 1	chr18 75137843-75138228	5 aggaagagagAATTATGAGTTTGAATGTGTGGTTTG 3	60	5 cagtaatacgactcactatagggagaaggctATCATTTTATCCATTCAAAAATAAACACA 3	61	386	60
MAT 18 0101	MAT 18 0101 2	chr18 75137509-75137865	5 aggaagagagTTTGAGGTTTATTTTTTATGTTGATTTGT 3	61	5 cagtaatacgactcactatagggagaaggctACCACACATTCAAACTCATAATTCC 3	60	357	60
MAT 18 0102	MAT 18 0102 1	chr18 44403565-44403827	5 aggaagagagGTGGAGAATATTTGTGTGGTGTGATAT 3	61	5 cagtaatacgactcactatagggagaaggctCAACTAACCCTAACCTTCCTAAAAATAAA 3	61	263	60
TAS 18 0675	TAS 18 0675 1	chr18 19035779-19036213	5 aggaagagagGAGAGATTGAGGTAGTTAGGGTTGG 3	61	5 cagtaatacgactcactatagggagaaggctCAAAAAAAATAAATTTACCAAAATAACCC 3	61	435	60
TAS 18 0675	TAS 18 0675 2	chr18 19036564 19036776	5 aggaagagagTGGGGTTTTATTGTGTTAGTTAGGA 3	60	5 cagtaatacgactcactatagggagaaggctAAAAAAAACAAACATTTACACAACCTC 3	60	213	60
TAS 18 0689	TAS 18 0689 1	ch 18 19366727 19367158	5 aggaagagagTTTATTTAGGGGATTTATGATGGTGT 3	60	5 cagtaatacgactcactatagggagaaggctAAAATAATAAAAAACCCCTCATTTTCTT 3	60	432	60
TAS 18 0689	TAS 18 0689 2	chr18 19367361 19367738	5 aggaagagagTTTAGTATTTTGGGAGGTTAAGGTTG 3	60	5 cagtaatacgactcactatagggagaaggctACTCCCAATAAAACCATAACCAAAT 3	60	378	60
TAS 18 0841	TAS 18 0841 3	chr18 27486636-27487020	5 aggaagagagGTATGATTTTTAAAGGGTTTAGGGGT 3	60	5 cagtaatacgactcactatagggagaaggctCACACTTCAATTCATAATCACCAAA 3	60	385	60
TAS 18 1426	TAS 18 1426 2	chr18 54206716-54207138	5 aggaagagagGGAAATTTTTTTTGATGGAGAGTTG 3	61	5 cagtaatacgactcactatagggagaaggctCCACCTACCATAACAAATCACAAA 3	60	423	60
TAS 18 1426	TAS 18 1426 3	chr18 54207219-54207510	5 aggaagagagTTGAGGGTGGTTATTTTTTAGTGTG 3	61	5 cagtaatacgactcactatagggagaaggctCAAAACCAACATCAATCCTAAAATC 3	60	292	60
TAS 18 1426	TAS 18 1426 4	chr18 54207430-54207757	5 aggaagagagGGTGTTTTTTATTTGAGGTAGGATTG 3	60	5 cagtaatacgactcactatagggagaaggctTCTTACAAAACCATCCCTAAAACCT 3	60	328	60
TAS 18 1887	TAS 18 1887 1	chr18 72227205-72227564	5 aggaagagagAGGTGAGAAGTTTTATTTTTGAGGTTT 3	60	5 cagtaatacgactcactatagggagaaggctAACAAATCCCCACTCTACACAATTA 3	60	360	60
TAS 18 1887	TAS 18 1887 2	chr18 72227538-72227984	5 aggaagagagGATGGGTTTTGGTTTTAGGGTAGTA 3	61	5 cagtaatacgactcactatagggagaaggctAAACCTCAAAAATAAAACTTCTCACCT 3	60	447	60
TAS 18 1887	TAS 18 1887 3	chr18 72227960-72228363	5 aggaagagagGGGTTTGTGTTGGTGTATTTGTAAG 3	61	5 cagtaatacgactcactatagggagaaggctTACTACCCTAAAACCAAAACCCATC 3	61	404	60
TAS 18 2311	TAS 18 2311 2	chr18 75588041 75588313	5 aggaagagagGGGTTGGTGAGAATTTTGATTTT 3	60	5 cagtaatacgactcactatagggagaaggctTCCCACCTACATTTAACTAAAACACC 3	61	273	60
TAS 18 2311	TAS 18 2311 3	chr18 75588126-75588575	5 aggaagagagTTTTTTTGAGATTTTAGAGGTGGG 3	61	5 cagtaatacgactcactatagggagaaggctAATTCAAAACTTCCCAAACCTTATC 3	60	450	60
TAS 18 2311	TAS 18 2311 5	chr18 75588769-75589025	5 aggaagagagAGTGTTTTTTGGTTTTATTTTTGGTT 3	61	5 cagtaatacgactcactatagggagaaggctAAAAAACCAATCTTCTATTTCAAAAATCT 3	60	257	60
TAS 18 2336	TAS 18 2336 1	chr18 75690944-75691373	5 aggaagagagTGTATTTGGAGATTTTGTGTTTTTGT 3	60	5 cagtaatacgactcactatagggagaaggctTCCCTTACAAACATATTTCCTACCA 3	60	430	60
TAS 18 2336	TAS 18 2336 2	chr18 75692783-75693058	5 aggaagagagGGAGGAGAGTTTTTTTTTAGGATGTG 3	61	5 cagtaatacgactcactatagggagaaggctAAACCCACAAACACCTACTCTACCC 3	61	276	60
	Beta-actin	chr7 5536377 5536732	5 aggaagagagTTATTTTGYGATTTTTATTGGTAAGAGTT 3	60	5 cagtaatacgactcactatagggagaaggctAACACAAAACCTCRCCTTTACC 3	58	356	

*Genomic locations are defined according to the Human genome March 2006 assembly (NCBI36/hg18) of the UCSC Genome Browser (www.genome.ucsc.edu)

Table 6.3. Primer and probe sequences for the qPCR assays of *VAPA-APCDD1 region 1*, *ZFY* and *β-actin*.

Assay	Locus name	Chr	Oligo	Primer sequences	Tm
Digital PCR / qPCR	*VAPA-APCDD1 region 1* (*MAT.18.0094.1*)	18	Forward primer	5'- AGGCCTGCGCAGGTGG -3'	62℃
			Reverse primer	5'- CACTCGCTGAGCGTCCCC -3'	61℃
			Probe*	5'- FAM- CGCCCCGCACAGCGCTCG -BHQ1 - 3'	68℃
	ZFY	Y	Forward primer	5' - CAAGTGCTGGACTCAGATGTAACTG - 3'	59℃
			Reverse primer	5' - TGAAGTAATGTCAGAAGCTAAAACATCA - 3'	59℃
			Probe#	5' - VIC- TCTTTACCACACTGCAC -MGB - 3'	
	Beta-actin	7	Forward primer	5' - CCCCCATCTCCGGAGGC - 3'	62℃
			Reverse primer	5' - GTCTGGGCCGCAGCGG - 3'	64℃
			Probe#	5' - VIC- AGGGGCTTCTCCCGCGC -MGB - 3'	73℃

*The fluorescent TaqMan probes contained FAM (6-carboxyfluorescein) as reporter and BHQ1 (Black-hole quencher 1) as quencher
#The fluorescent TaqMan probes contained VIC (4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein) as reporter and non-fluorescent quencher (MGBNFQ) as quencher
MGB, minor-groove binding

Table 6.4. Summary of the reaction conditions for the qPCR assays of *VAPA-APCDD1 region 1*, *ZFY* and *β-actin*.

Restriction enzyme digestion

	Volume (μL)
NE Buffer 1	5
Hpa II	2
Hin PI1	2
Water	variable
DNA	variable
Total	50

2-hour incubation at 37°C

Real-time quantitative PCR

VAPA-APCDD1 (region 1)

	Final concentration
Water	variable
2X TaqMan Universal master mix	1X
Forward primer	1500nM
Reverse primer	1500nM
Probe	250nM
DNA (tissue or plasma)	variable
Total	50

Thermal profile		
50°C	2 min	
95°C	10 min	40 cycles
95°C	15s	
60°C	1 min	

The fluorescent TaqMan probes contained FAM as reporter and BHQ1 (Black-hole quencher 1) as quencher

ZFY

	Final concentration
Water	variable
2X TaqMan Universal master mix	1X
Forward primer	300nM
Reverse primer	300nM
Probe	100nM
DNA (tissue or plasma)	variable
Total	50

Thermal profile		
50°C	2 min	
95°C	10 min	40 cycles
95°C	15s	
60°C	1 min	

The fluorescent TaqMan probes contained FAM as reporter and BHQ1 (Black-hole quencher 1) as quencher

6.3. Results

6.3.1. DNA methylation profiling by MeDIP-chip revealed differentially methylated regions on chromosome 18

Using MeDIP-chip, 178 loci were found to be differentially methylated between placenta and maternal blood cells, covering 3,043 CpG sites. Among them, 140 (79%) were located within genes and the remaining 38 (21%) were intergenic (Table 6.5).

6.3.2. Quantitative methylation analysis at single-CpG resolution

Altogether, I performed 58 Epityper assays to study the methylation levels of 26 loci, involving 370 CpG units (Figure 6.3). Two first-trimester placentas and two maternal blood cell samples were tested for each assay and the MI values between the two tissues at each CpG unit were compared (Figure 6.4).

I then searched for loci that were methylated in the placenta and unmethylated in maternal blood cells, because the unmethylated maternal DNA could be removed and the methylated fetal DNA can be retained with treatment by methylation-sensitive restriction enzymes.

Table 6.5. The distribution of loci identified by MeDIP-chip in terms of specific genomic elements.

Catergory	MeDIP-chip Loci [a] (%)
Within gene	140 (79%)
Intergenic	38 (21%)
Total	**178**

[a] P value < 10^{-5} for MAT or median signal log ratio between placenta and maternal blood cells > 0.4 for TAS

In the current setup, the analysis of the MeDIP-chip only estimated the relative methylation levels between the two tissues. According to the Epityper data, some of the loci that were identified by MeDIP-chip were partially methylated in the maternal blood cells (Figure 6.5). These loci would be sub-optimal for marker development because the methylated maternal DNA would not be removed by MSRE digestion. Therefore, a set of criteria was needed to specify the selection process.

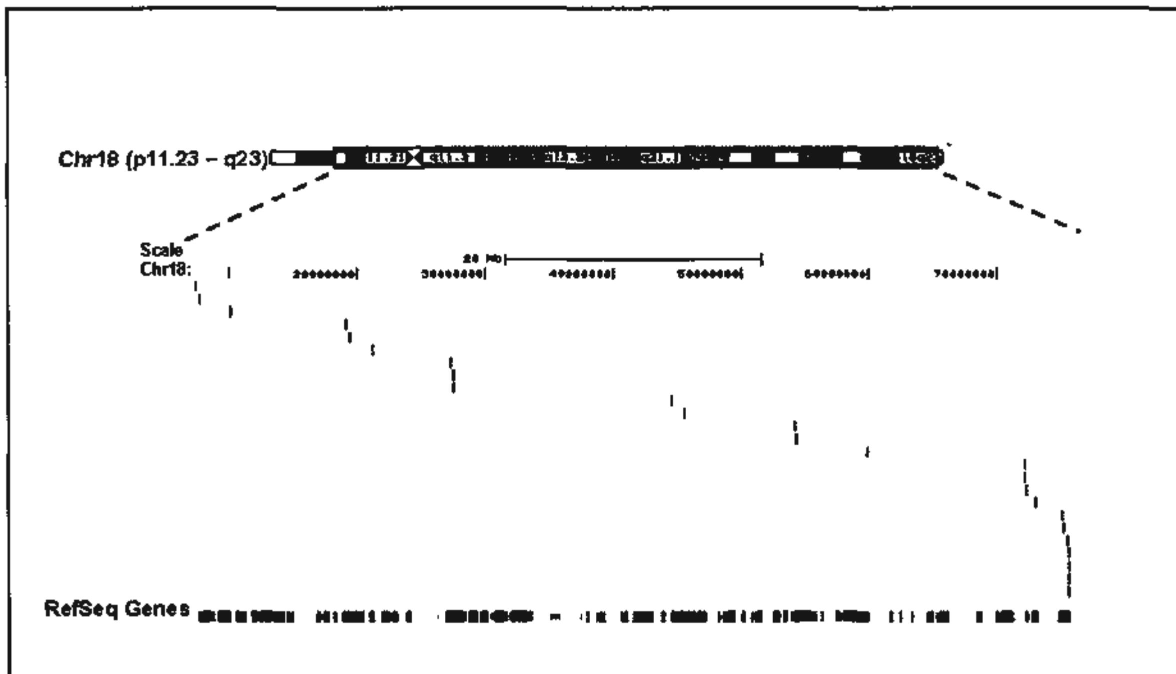
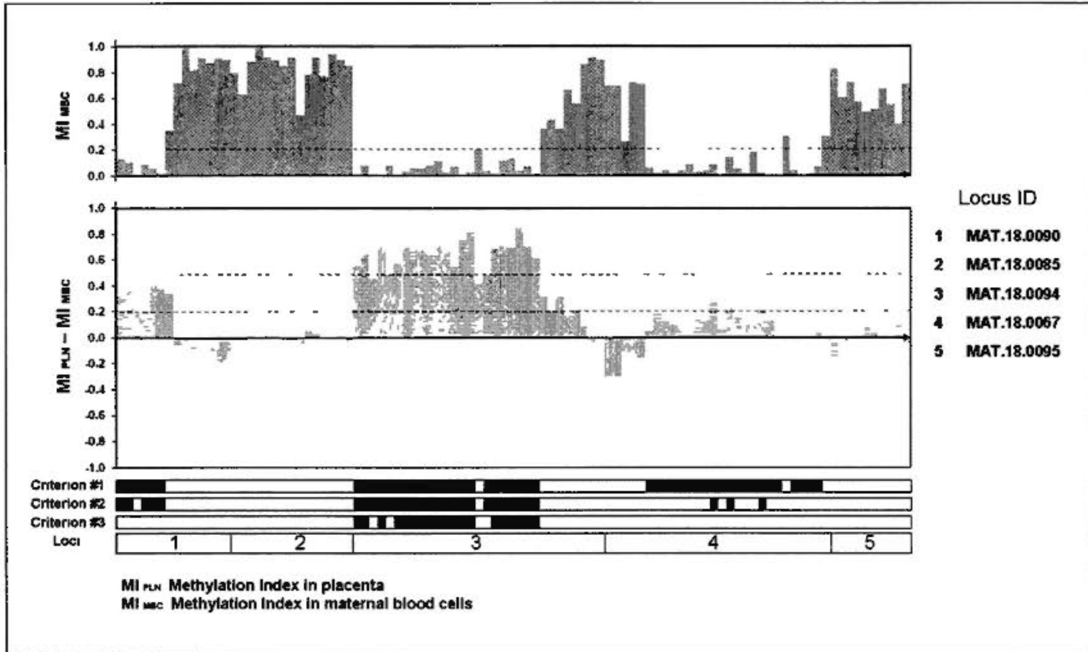


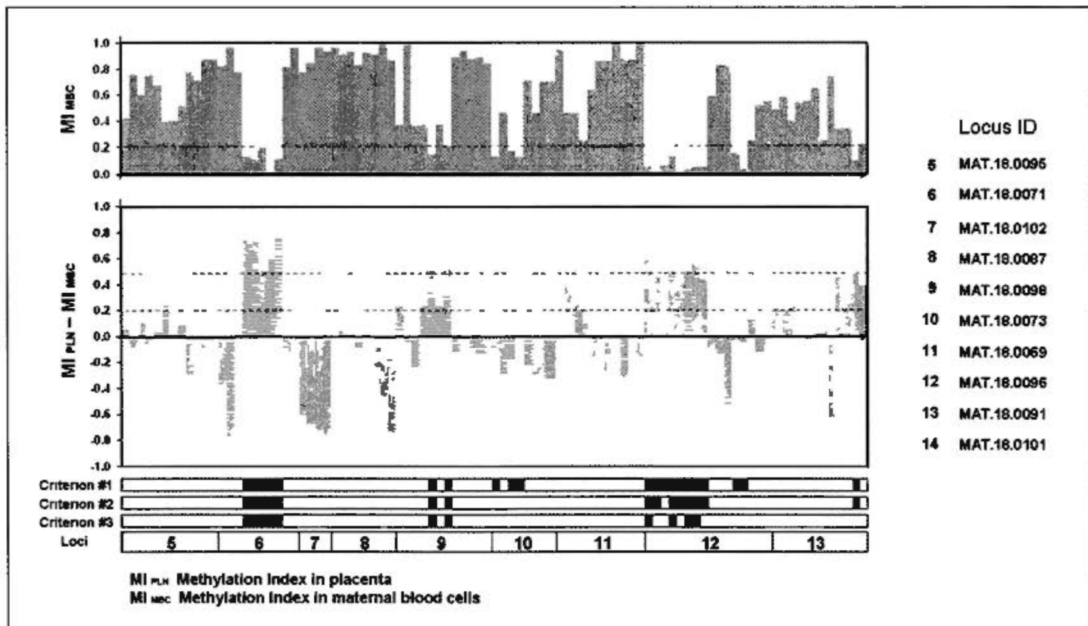
Figure 6.3. Distribution of the 26 genomic loci on chromosome 18 that were chosen from the 178 differentially methylated loci identified by MeDIP-chip. They were most efficiently covered by Epityper assays for validation. Each vertical bar represents one Epityper assay.

Figure 6.4. Summary of CpG-resolution methylation data obtained with Epityper.

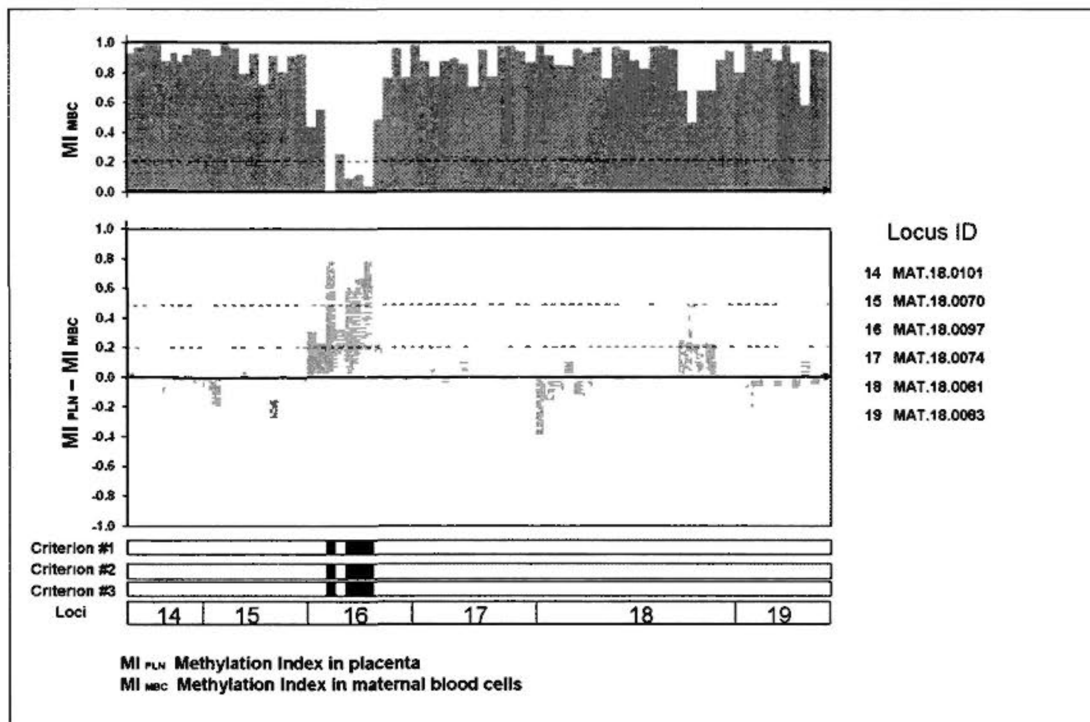
A



B



C



D

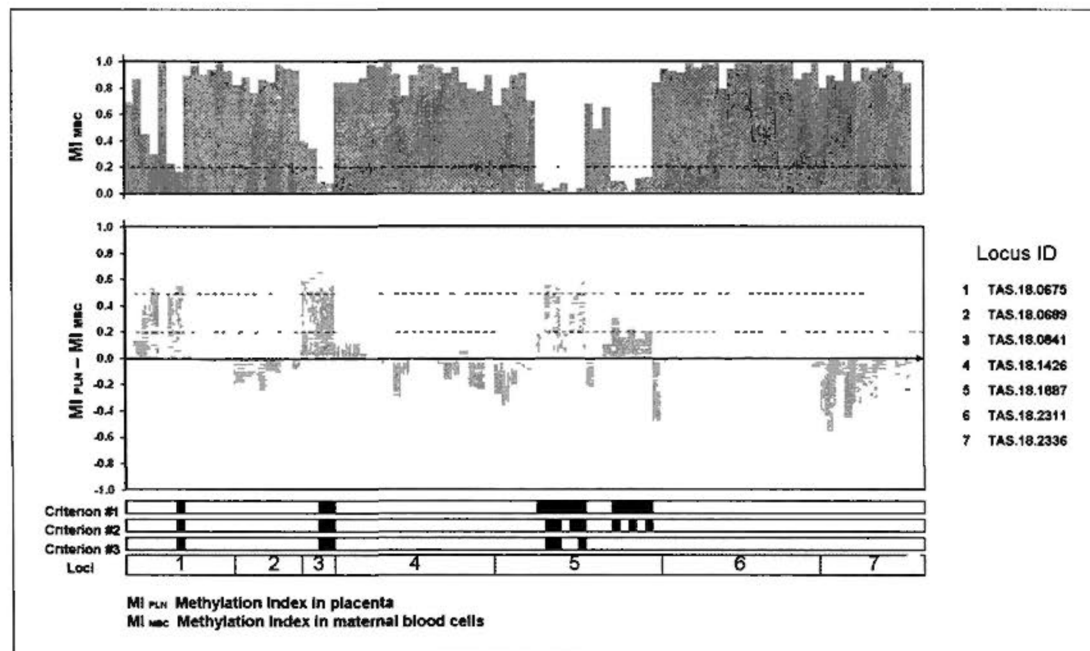


Figure 6.4. Summary of CpG-resolution methylation data obtained with Epityper.

Data of the 26 loci are shown in A-D, each of which contains 6 sub-panels.

First sub-panel. **Average methylation index (MI) in maternal blood cells.** X-axis is a categorical axis of the chromosomal locations of the CpG units within the indicated locus. When there are more than one CpG sites within a CpG unit, only the first CpG site is shown. The chromosomal locations of every CpG site are summarized in Table 6.2. The Y-axis shows the MI of each CpG unit. A CpG unit potentially useful for marker development needs to fulfill criterion #1: MI in maternal blood cells ≤ 0.2 (lower than the orange dotted line). These CpG units are highlighted as green bars in the third panel. ***Second sub-panel.*** **Differences between the average MI in the placenta and that in maternal blood cells.** The Y-axis shows the difference in MI of each CpG unit. A CpG unit potentially useful for marker development needs to further fulfill criterion #2: MI difference ≥ 0.2 (higher than the lower dotted line). These CpG units are highlighted as green bars in the fourth sub-panel. The CpG units that are most suitable for marker development must further fulfill criterion #3: MI difference ≥ 0.5 (higher than the upper dotted line). These CpG units are highlighted as green bars in the fifth sub-panel. Locus ID are shown on the right panel.

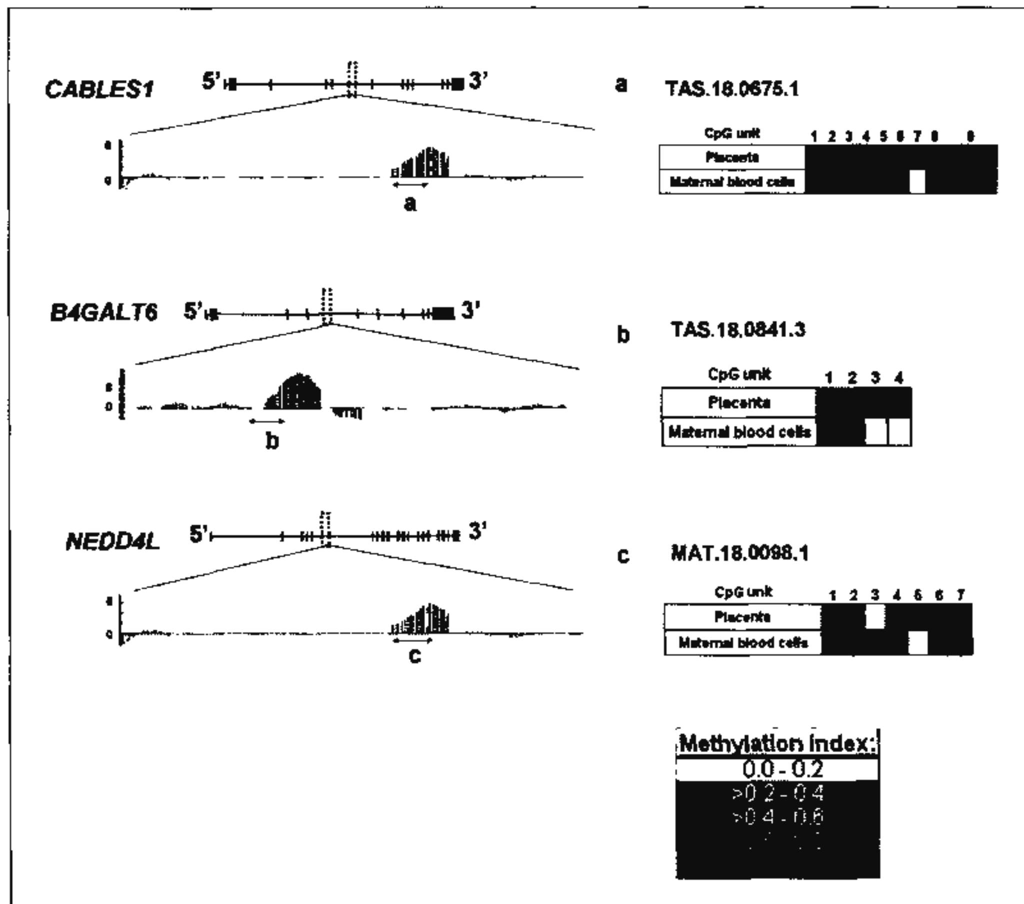


Figure 6.5. Methylation data obtained with MeDIP-chip and Epityper on 3 typical loci.

Figure 6.5. Methylation data obtained with MeDIP-chip and Epityper on 3 typical loci.

The positions of these 5 loci (dotted-line boxes) in relation to the associated genes, and their exons (blocks) and introns (lines) are shown. The genes are shown in the direction of mRNA transcription from left to right. The bar graph below each gene shows the difference in the MeDIP-chip probe signals between the placenta and maternal blood cells (Y-axis). Each vertical bar represents the signal from one probe, and its height represents the differences in the MeDIP-chip probe signals intensities between the placenta and maternal blood cells Horizontal lines with arrows at both ends indicates the position(s) of the Epityper assays that spanned the MeDIP-chip loci. The MI of the placenta and maternal blood cells as determined by the Epityper are shown on a grey scale. Letters A-C indicate the positions of the CpG units analysed by the Epityper. The chromosomal location of each CpG sites can be inferred from Table 6.2.

6.3.3. Selection of potential fetal epigenetic markers

6.3.3.1 Identification of potential CpG units for development of fetal epigenetic markers

The criteria of potential fetal epigenetic markers was modified from the previous finding of our group, in which 22 novel CpG islands on chromosome 21 have been identified as potential fetal epigenetic markers by bisulfite sequencing (Chim et al. 2008). As it was known that the methylation data obtained with the Epityper are associated with deviations at extreme values (Coolen et al. 2007), I modified the criteria to adjust for the Epityper platform.

Potential CpG units were selected according to the following criteria: (1) having a MI $\leq$ 0.20 in the maternal blood cells; and (2) having a MI difference between the placenta and maternal blood cells of $\geq$ 0.50. Among the 370 CpG units analysed by the Epityper, 40 of them fulfilled these criteria and thus could potentially be developed into robust fetal epigenetic markers (Figure 6.4).

Before further evaluation of these markers, their methylation levels in placental tissues obtained from trisomy 18 (T18) fetuses were compared with those of normal controls. The methylation levels of these 40 CpG units in five pairs of euploid and T18 placentas were determined by the Epityper. No significant difference between the two groups could be observed (the P-values of the Mann-Whitney Test are listed in Appendix I Table 1). Therefore, these CpG units were suitable to be used as quantitative fetal markers for comparing the dosage of fetal chromosome 18 between euploid and T18 pregnancies.

6.3.3.2 Identification of clusters of differentially methylated CpG sites

I further prioritized the 40 CpG units by focusing on the potential CpG units that were clustered within 200 bp of each other. It was important for the subsequent assay design to target at fetal DNA molecules which were generally smaller than 200 bp in maternal plasma (Chan et al. 2004). These clusters of potential CpG units were distributed in five different genomic regions, among which four were located on introns of genes and one was located in an intergenic region (Figure 6.6). Their chromosomal coordinates are listed in Table 6.6.

6.3.3.3 Assessment of inter-individual variations

I then assessed the inter-individual variations of the placental MI of these loci, a parameter that was important for robust detection across different individuals. Ten placentas and five maternal blood cell samples were tested to compute the coefficient of variation (CV) according to the formula:

$$CV = standard\ deviation / mean \times 100\%.$$

A placental MI with CV less than 25% was preferred. As the maternal blood cells MI were generally close to 0, the CV computed for the maternal blood cells MI was expected to be sensitive to small changes. Therefore I had focused only on the variation of the placenta MI. The methylation data of the 10 placentas and their respective CV values are summarized in Figure 6.7 and 6.8 and Appendix Table 2.

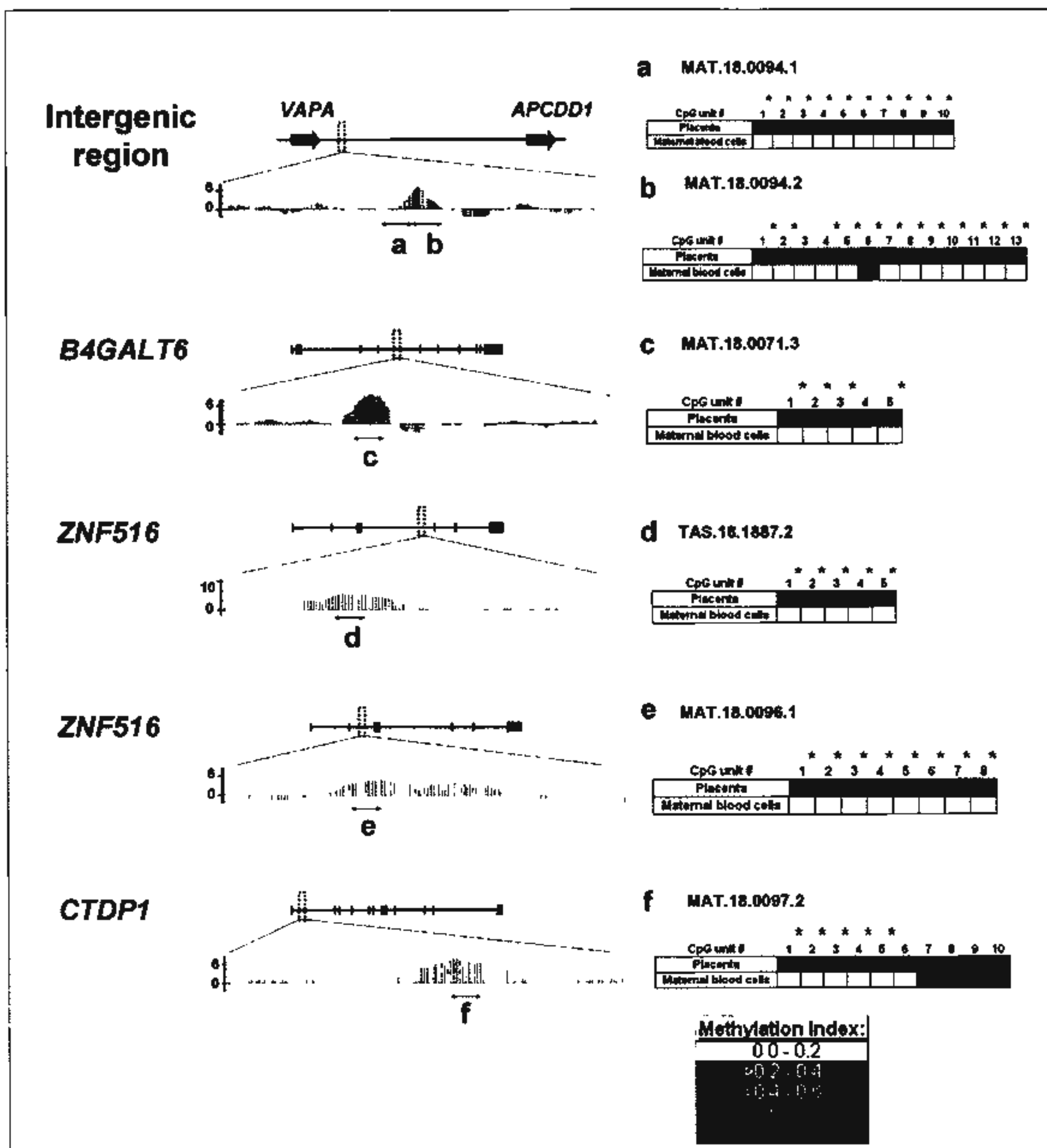


Figure 6.6. Methylation data obtained with MeDIP-chip and Epityper on the 5 most promising loci for developing fetal epigenetic markers.

Figure 6.6. DNA methylation levels by MeDIP and the Epityper on 5 promising loci for marker development. *Left panels*. The positions of these 5 loci (dotted-line boxes) in relation to the associated genes, and their exons (blocks) and introns (lines) are shown. The genes are shown in the direction of mRNA transcription from left to right. The bar graph below each gene shows the difference in the MeDIP-chip probe signals between the placenta and maternal blood cells (Y-axis). Each vertical bar represents the signal from one probe. The average inter-probe distance is 35 bp. A positive value implies higher DNA methylation level in the placenta, compared with maternal blood cells. Horizontal lines with arrows at both ends indicate the regions spanned by the Epityper assays (a-f). *Right panels*. The median of DNA methylation indices (MI) of CpG units within the Epityper assays are shown in grey scale. Asterisks indicate the CpG units with higher DNA methylation in five placentas than five maternal blood cell samples (Mann-Whitney test, $P<0.05$). The chromosomal locations and MI of each CpG unit are listed in Table 6.2.

Table 6.6. The genomic location of the 5 most promising loci for developing fetal epigenetic markers.

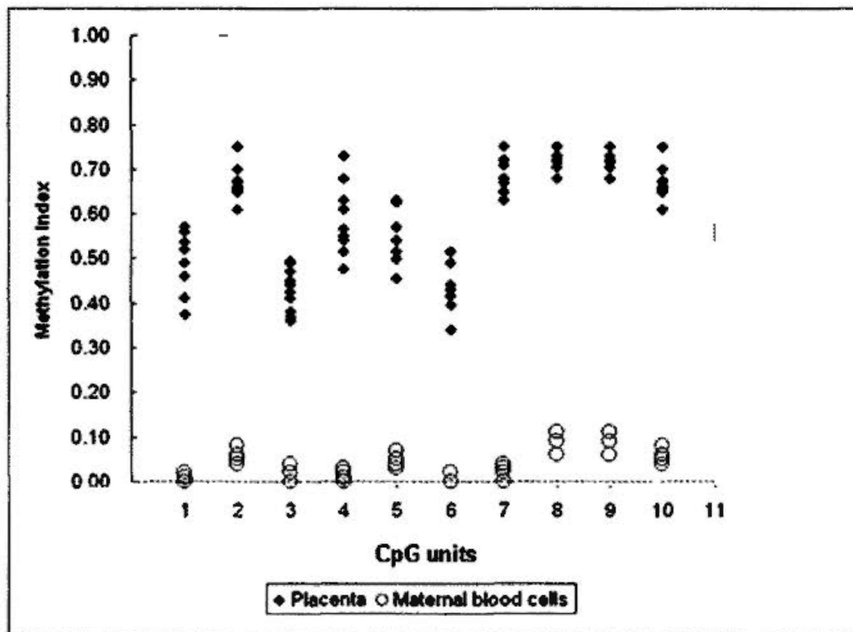
Chromosomal location on chr 18 (hg18)	Region	Gene associated with the locus		Accession No.
10022563-10023955	Intergenic	*VAPA - APCDD1**	Vesicle-associated membrane protein and adenomatosis polyposis coli down-regulated 1	NM_003574 and NM_153000
27485628-27487511	Introns	*B4GALT6*	Beta-1,4-galactosyltransferase 6	NM_004775
72227405-72228513	Introns	*ZNF516*	Zinc finger protein 516	NM_014643
72292238-72293387	Introns	*ZNF516*	Zinc finger protein 516	NM_014643
75542484-75543900	Introns	*CTDP1*	Carboxy-terminal domain, RNA polymerase II, polypeptide A phosphatase, subunit 1	NM_004715

* Intergenic region between VAPA and APCDD1

Locus ID	Assay ID	CpG units	Median MI in placenta	Median MI in maternal blood cells	Coefficienct of variation (%) of the placental MI (obtained from placental DNA of 10 individuals)
MAT.18.0094	MAT.18.0094.1 ***(VAPA-APCDD1)*** **(region 1)**	1	0.49	0.01	13%
		2	[illegible]	0.05	5%
		3	0.43	0.00	11%
		4	0.59	0.02	13%
		5	0.52	0.05	10%
		6	0.44	0.00	12%
		7	[illegible]	0.03	6%
		8	[illegible]	0.06	3%
		9	[illegible]	0.06	3%
		10	[illegible]	0.05	5%
MAT.18.0094	MAT.18.0094.2 ***(VAPA-APCDD1)*** **(region 2)**	1	[illegible]	0.10	18%
		2	[illegible]	0.02	16%
		3	[illegible]	0.05	18%
		4	[illegible]	0.00	14%
		5	[illegible]	0.01	13%
		6	[illegible]	0.23	24%
		7	0.43	0.03	43%
		8	[illegible]	0.01	29%
		9	[illegible]	0.10	18%
		10	[illegible]	0.12	21%
		11	[illegible]	0.03	21%
		12	[illegible]	0.05	35%
		13	[illegible]	0.01	20%
MAT.18.0071	MAT.18.0071.3	1	[illegible]	0.09	10%
		2	[illegible]	0.15	22%
		3	[illegible]	0.14	11%
		4	[illegible]	0.03	11%
		5	[illegible]	0.07	9%
TAS.18.1887	TAS.18.1887.2	1	[illegible]	0.12	21%
		2	0.34	0.13	47%
		3	[illegible]	0.12	21%
		4	0.58	0.06	37%
		5	0.34	0.13	47%
MAT.18.0096	MAT.18.0096.1	1	0.56	0.05	24%
		2	0.33	0.02	35%
		3	0.36	0.05	42%
		4	[illegible]	0.14	22%
		5	0.25	0.00	72%
		6	0.30	0.04	45%
		7	0.45	0.05	34%
		8	0.35	0.05	42%
MAT.18.0097	MAT.18.0097.2	1	[illegible]	0.02	15%
		2	[illegible]	0.16	32%
		3	[illegible]	0.09	24%
		4	0.57	0.08	23%
		5	[illegible]	0.05	14%
		6	0.40	0.10	24%
		7	[illegible]	0.45	21%
		8	[illegible]	[illegible]	15%
		9	[illegible]	[illegible]	8%
		10	[illegible]	[illegible]	15%

Intensity Scale:
0.0 <= MI < 0.2
0.2 <= MI < 0.4
0.4 <= MI < 0.6
[illegible]

Figure 6.7. MIs and CVs of individual CpG units of the 5 most promising fetal epigenetic markers.



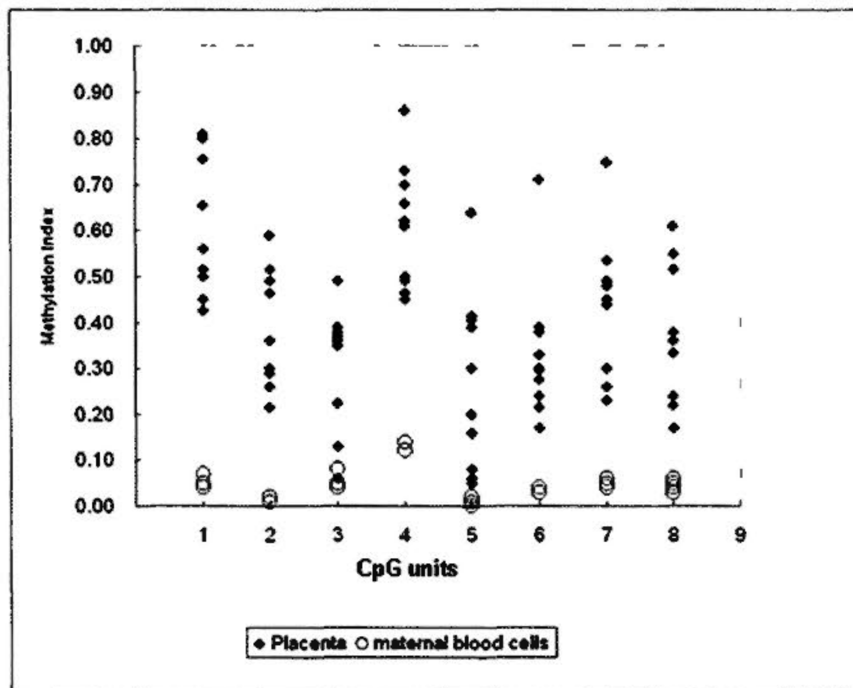


Figure 6.8. Variations of the MIs in euploid first trimester placentas (n=10) with respect to maternal blood cells.

Figure 6.7. MIs and CVs of individual CpG units of the 5 most promising fetal epigenetic markers

The median MIs were calculated from 10 euploid first trimester placentas and 5 maternal blood cell samples. At each CpG unit, the P-values of the Mann-Whitney tests were computed to compare the MIs of the two tissues. CV, coefficient of variation, of the MI in the 10 placentas. A CpG unit contains one or more CpG sites that were analysed by Epityper.

Figure 6.8. Variations of the MIs in euploid first trimester placentas (n=10) with respect to maternal blood cells for (A) a Marker with stable methylation levels, *MAT.18.0094.1* and (B) a marker with variable methylation levels, *MAT.18.0096.1*. The MIs of each case are plotted against the CpG unit.

I finally selected three loci that contains CpG units with: (1) MI ≤ 0.20 in maternal blood cells; and (2) having the MI in the placenta ≥ 0.50 than in maternal blood cells; and (3) locating within 200 bp; and (4) each with CV of MI ≤ 25% in 10 placentas. Their methylation status as determined by Epityper and bisulfite sequencing was concordant with the prediction by MeDIP-chip (Figures 6.9 and 6.10). To identify the best locus for assay development, I proceeded to examine the DNA sequences of these 3 loci for the recognition sites of MSRE.

6.3.3.4 Overlapping with methylaton-sensitive restriction enzymes (MSRE)

Among the 3 loci selected above, one was found to contain the highest number of MSRE recognition sites. I performed cloning and bisulfite sequencing on 6 pairs of first-trimester placentas and maternal blood cells. Single-CpG site methylation data from bisulfite sequencing had confirmed that this locus was predominantly methylated in the placenta but were essentially unmethylated in maternal blood cells (Figure 6.11A and 6.11B). This locus spanned two approximately 200 bp-long regions that contained seven differentially methylated CpG sites coincident with the recognition sites of MSRE, namely *VAPA-APCDD1* [region 1 (146 bp) and region 2 (230 bp)], which contained around 11 and 10 potential CpG overlapping with MSRE (Figure 6.11A and 6.11B). Both regions are intergenic loci that were located 73 kb downstream of the gene *VAPA (vesicle-associated membrane protein)-associated protein A)* and 421 kb upstream of the gene *APCDD1 (adenomatosis polyposis coli down-regulated 1)* (Figure 6.9). Region 1 of the intergenic region *VAPA-APCDD1* overlapped with 5 sites recognized by two commonly used methylation-sensitive restriction enzymes, *Hin*P1I and *Hpa*II, within 100 bp, and so was selected for further development.

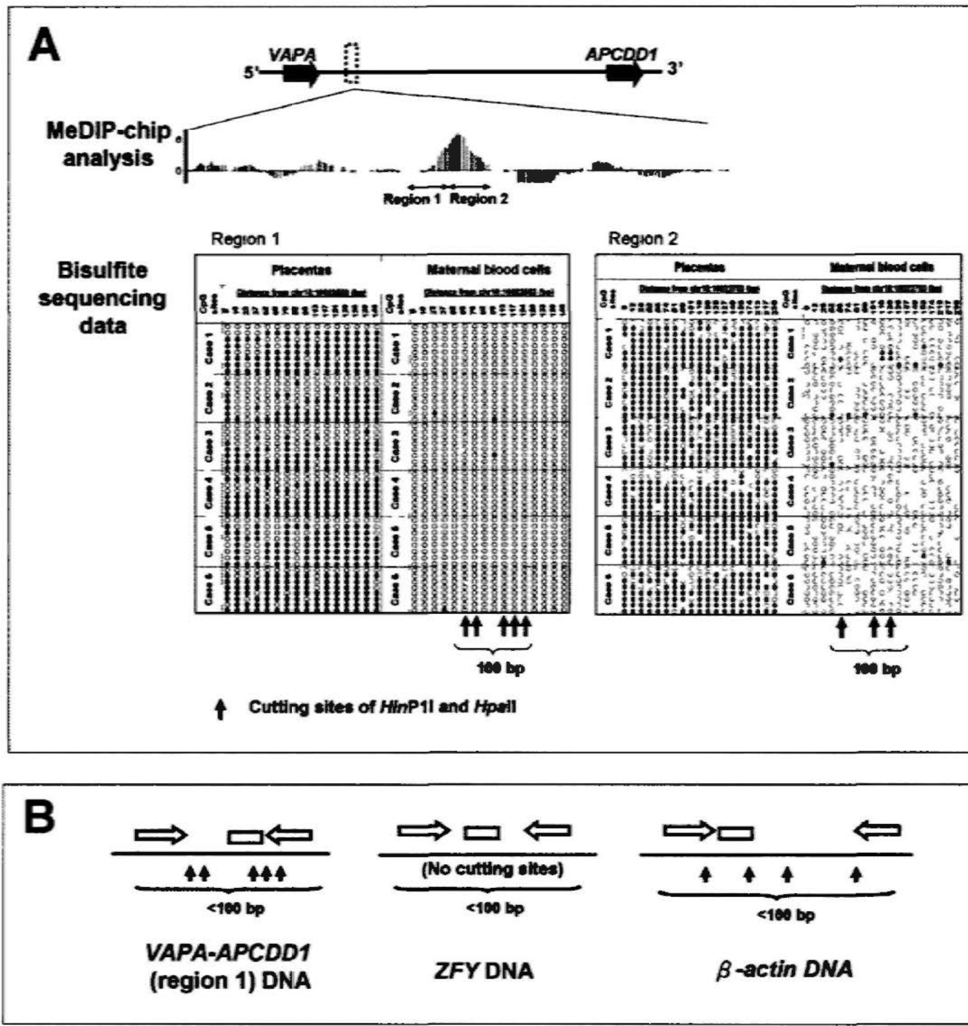

A
VAPA
APCDD1
5'
3'
MeDIP-chip analysis
Region 1
Region 2
Bisulfite sequencing data
Placentas
Maternal blood cells
Case 1
Case 2
Case 3
Case 4
Case 5
Case 6
100 bp
Cutting sites of HinP1I and HpaII
B
(No cutting sites)
<100 bp
VAPA-APCDD1 (region 1) DNA
ZFY DNA
β-actin DNA

Figure 6.9. DNA methylation levels by bisulfite sequencing in the most promising locus for marker development. (A) *Top panel.* The genomic location of one promising locus in relation to two associated genes. *Middle panel.* Two regions, which were identified by MeDIP as possessing higher methylation in the placenta relative to maternal blood cells, was analyzed by bisulfite sequencing. See Figure 2 for the legend on the bar graph for MeDIP. *Bottom panel.* Single-base DNA methylation levels by bisulfite sequencing. For each sample, 8 randomly-picked clones (rows) were scored for each CpG site (column). Filled circles, methylated CpG sites. Empty circles, unmethylated CpG sites. Upward arrows, cutting sites of the methylation-sensitive restriction enzymes *Hpa*II and *Hin*P1I. (B) Design of qPCR and digital PCR assays for region 1 of *VAPA-APCDD1* (5 cutting sites), *ZFY* (0 cutting sites), and *β-actin* DNA (4 cutting sites). Block arrows, PCR primers. Rectangle, hydrolysis probe. Information of the individual methylation levels of each CpG site and the positions of the methylation-sensitive enzymes restriction sites are shown in Figure 6.11A.

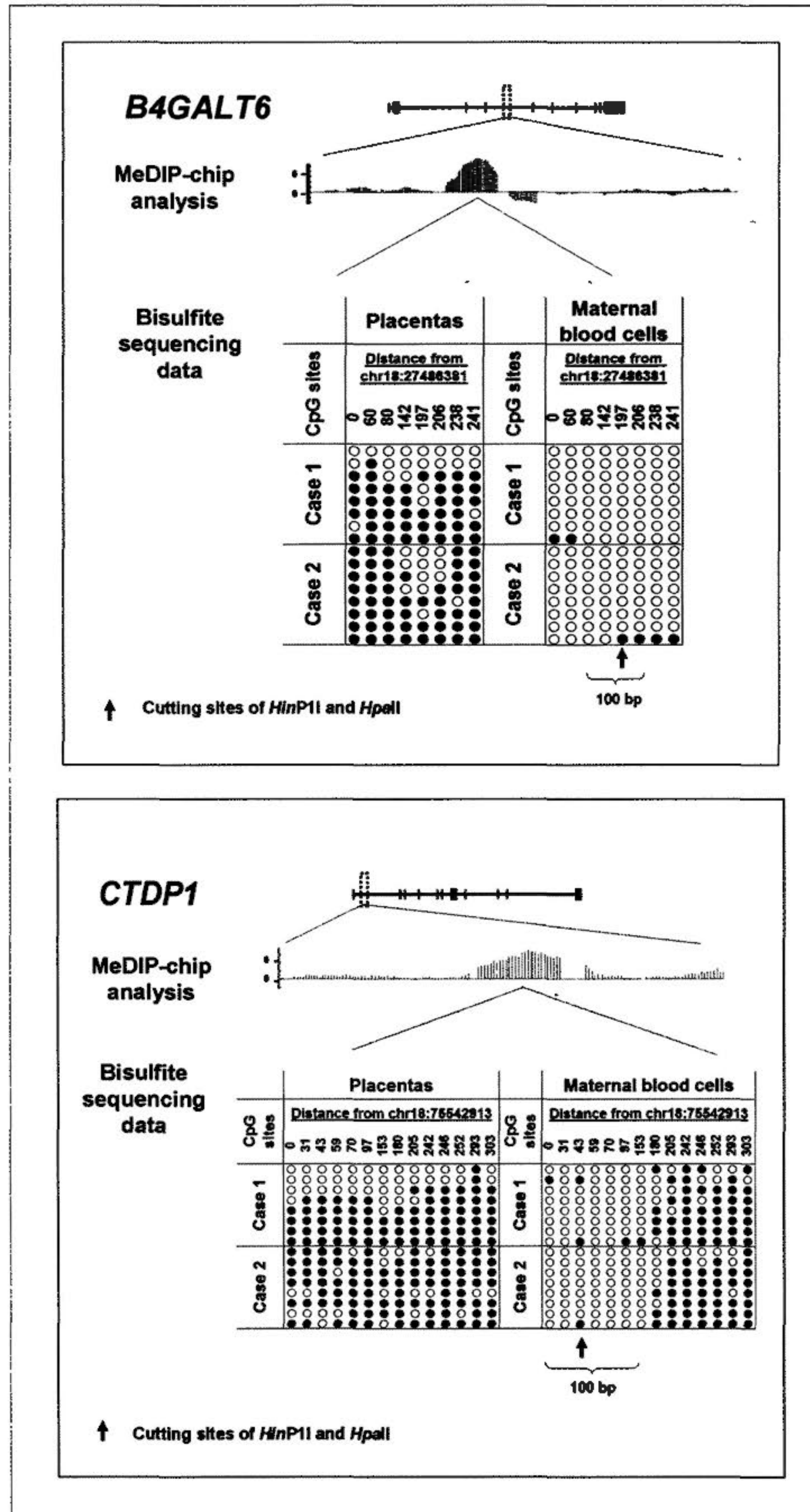


Figure 6.10. Two among the three most promising fetal epigenetic markers.

Figure 6.10. DNA methylation levels by bisulfite sequencing in two promising loci for developing fetal epigenetic markers. Data on each locus are shown in two panels, each of which contains 3 sub-panels. *Top sub-panel.* The genomic location of the promising locus in relation to the associated gene. *Middle sub-panel.* The locus, which was identified by MeDIP as possessing higher methylation in the placenta relative to maternal blood cells, was analyzed by bisulfite sequencing. See Figure 2 for the legend on the bar graph for MeDIP. *Bottom panel.* Single-base DNA methylation levels by bisulfite sequencing. For each sample, 8 randomly-picked clones (rows) were scored for each CpG site (column). Filled circles, methylated CpG sites. Empty circles, unmethylated CpG sites. Upward arrows, cutting sites of the methylation-sensitive restriction enzymes *Hpa*II and *Hin*P1I. Information of the individual methylation levels of each CpG site and the positions of the methylation-sensitive enzymes restriction sites are shown in Figure 6.11C and 6.11D.

VAPA-APCDD1 region 1

MAT.18.0094.1

Chromosomal locations of CpG sites (hg18)

Euploid placental tissues

Enzyme	CpG site	Case 1	Methylation Index	Case 2	Methylation Index	Case 3	Methylation Index	Case 4	Methylation Index	Case 5	Methylation Index	Case 6	Methylation Index	Average MI
AciI	chr18 10022553	●●●●●●○○	0.75	●●●●●○●○	0.75	●●●●●○○○	0.63	●●●●○○●○	0.63	●○○○○●●○	0.38	●○●●●●●○	0.75	0.65
	chr18 10022563	●●●○●●○○	0.63	●○○○○○○○	0.13	●●●●○○○○	0.50	●●●●○○●○	0.63	●●●●●●○○	0.75	●●●●●●○○	0.75	0.56
HpaII	chr18 10022575	●○●○○●○○	0.38	○○○○○○○○	0.00	●○●○○○○○	0.25	●●●●○○●○	0.63	●○○○●○○○	0.25	●●○●●●○○	0.63	0.35
AciI, NlaIV	chr18 10022590	●●●○○●○○	0.50	●○●○○○○○	0.25	●●●●○●○○	0.63	●●●●●○●○	0.75	●○○○●●●○	0.50	●●●○●●○○	0.63	0.54
AciI	chr18 10022605	●●●●●○●○	0.75	●○○○○○○○	0.13	●●○●○○○○	0.38	●●○○○●●○	0.50	○●●●○○○○	0.38	●●●●●●●○	0.88	0.50
HpaII, NlaIV	chr18 10022618	●●●●○○●○	0.63	●●○○●○○○	0.38	●●○○○○○○	0.25	●●●○●○○○	0.50	●●●●○○○○	0.50	●○●●●●●○	0.75	0.50
HinP1I	chr18 10022629	●●●●●●●○	0.88	●●●○●○○○	0.50	●●●●●●○○	0.75	●●●○●○○○	0.50	●●●●●●●○	0.88	●●●○●●●○	0.75	0.71
	chr18 10022645	●●●●●○●○	0.75	●●●○○○●○	0.50	●●●○○○○○	0.38	●●●○○●○○	0.50	○○○○○○●○	0.13	●●●●●●●○	0.88	0.52
HpaII	chr18 10022652	●●○●●●●○	0.75	●●○●○○○○	0.38	●●●●●○○○	0.63	●●●●●●○○	0.75	●●●●●○●○	0.75	●●○●●●●○	0.75	0.67
	chr18 10022666	○●●●●○●○	0.63	●●○●○○○○	0.38	○○●●○●○○	0.38	●●●●●●○○	0.75	●●●●●●○○	0.75	●●●●○○○○	0.50	0.56
HinP1I	chr18 10022670	●●●●●●●○	0.88	●●●●●●○○	0.75	●●●●○○○○	0.50	●●●●●●○○	0.75	●●●●●●●○	0.88	●●●●●●●○	0.88	0.77
AciI	chr18 10022677	●●○●●●●○	0.75	●●●●○●○○	0.63	●●●●●○○○	0.63	●●●●●●○○	0.75	●●●●●●○○	0.75	●●●●●●●○	0.88	0.73
HinP1I	chr18 10022682	●●●●●●●○	0.88	●●●●●●○○	0.75	●●●●●○○○	0.63	●●●●●●○○	0.75	●●●●●●●○	0.88	●●●●●●●○	0.88	0.79
HpaII, NlaIV	chr18 10022686	●●●●●●●○	0.88	●●●●●●○○	0.75	●●●●●○○○	0.63	●●●●●●○○	0.75	●●●●●●●○	0.88	●●●●●●●○	0.88	0.79
	chr18 10022692	●●●●●●●○	0.88	●●●●●●○○	0.75	●●●●●○●○	0.75	●●●●●●○○	0.75	●●●●●●●○	0.88	●●●●●●●○	0.88	0.81
	chr18 10022699	●●●●●●●○	0.88	●●●●●●○○	0.75	●●○●●○○○	0.50	●●●●●●●○	0.88	●●●●●○○○	0.63	●●●●○○●○	0.63	0.71
Methylated site frequency			0.73		0.48		0.52		0.67		0.63		0.77	

Euploid maternal blood cells

Enzyme	CpG site	Case 1	Methylation Index	Case 2	Methylation Index	Case 3	Methylation Index	Case 4	Methylation Index	Case 5	Methylation Index	Case 6	Methylation Index	Average MI
AciI	chr18 10022553	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
	chr18 10022563	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
HpaII	chr18 10022575	○○○○○○○○	0.00	○●○○○○○○	0.13	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.02
AciI, NlaIV	chr18 10022590	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	●○○○○○○○	0.13	0.02
AciI	chr18 10022605	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
HpaII, NlaIV	chr18 10022618	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
HinP1I	chr18 10022629	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
	chr18 10022645	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
HpaII	chr18 10022652	○○○○○○○○	0.00	●○○○○○○○	0.13	○○●○○○○○	0.13	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.04
	chr18 10022666	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
HinP1I	chr18 10022670	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
AciI	chr18 10022677	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
HinP1I	chr18 10022682	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
HpaII, NlaIV	chr18 10022686	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
	chr18 10022692	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
	chr18 10022699	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00	0.00
Methylated site frequency			0.00		0.02		0.01		0.00		0.00		0.01	

Figure 6.11A. Bisulfite sequencing data of *VAPA-APCDD1 region 1*.

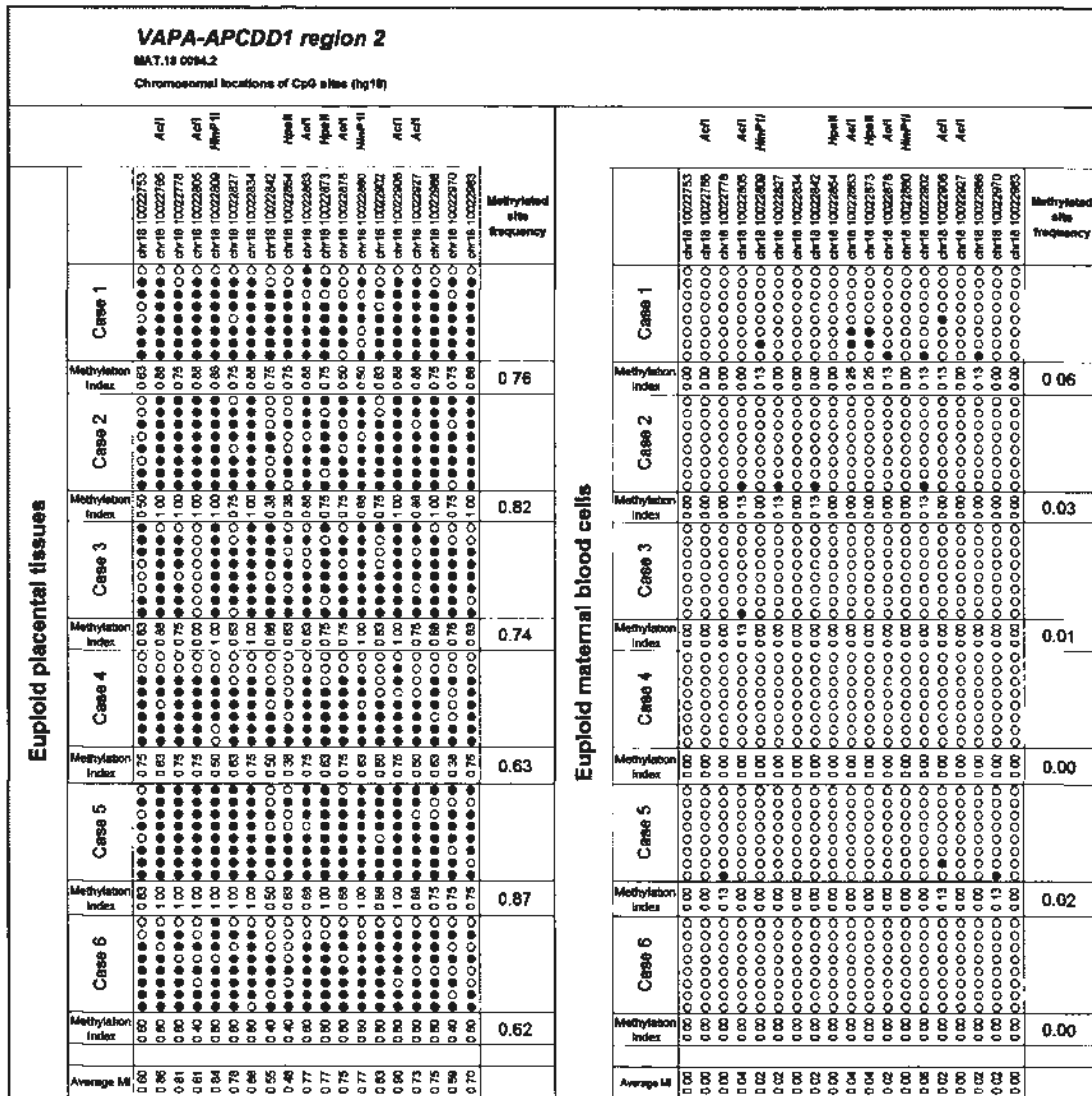


Figure 6.11B. Bisulfite sequencing data of *VAPA-APCDD1 region 2.*

MAT.18.0071.3

Chromosomal locations of CpG sites (hg18)

Restriction enzyme sites marked: *NlaIV*, *HpaII*, *AciI*

Euploid placental tissues

	chr18 27486622	chr18 27486619	chr18 27486587	chr18 27486578	chr18 27486523	chr18 27486461	chr18 27486441	chr18 27486381	Methylated site frequency
Case 1	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	●	○	
	●	●	●	●	○	○	●	●	
	●	●	●	○	●	●	●	●	
	●	●	●	○	●	●	●	●	
	○	●	●	●	●	●	●	●	
	●	●	●	●	●	●	●	○	
	●	●	●	●	●	●	●	●	
Methylation Index	0.63	0.75	0.75	0.50	0.63	0.63	0.88	0.63	0.67
Case 2	●	●	○	○	○	●	●	●	
	●	●	○	○	○	●	●	●	
	●	●	○	○	●	●	●	●	
	●	●	●	○	○	●	●	●	
	●	○	●	●	●	●	●	●	
	●	●	●	○	●	●	●	●	
	●	●	●	●	●	●	●	●	
	●	●	●	●	●	●	●	●	
Methylation Index	1.00	0.88	0.63	0.38	0.63	1.00	1.00	1.00	0.81
Average MI	0.81	0.81	0.69	0.44	0.63	0.81	0.94	0.81	

Euploid maternal blood cells

	chr18 27486622	chr18 27486619	chr18 27486587	chr18 27486578	chr18 27486523	chr18 27486461	chr18 27486441	chr18 27486381	Methylated site frequency
Case 1	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	●	●	
Methylation Index	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.13	0.03
Case 2	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	○	○	○	○	○	○	○	○	
	●	●	●	●	○	○	○	○	
Methylation Index	0.13	0.13	0.13	0.13	0.00	0.00	0.00	0.00	0.06
Average MI	0.06	0.06	0.06	0.06	0.00	0.00	0.06	0.06	

Figure 6.11C. Bisulfite sequencing data of MAT.18.0071.3.

MAT.18.0097.2

Chromosomal locations of CpG sites (hg18)

Euploid placental tissues

Chromosomal location of CpG site	Enzyme	Case 1 Methylation Index	Case 2 Methylation Index	Average MI
chr18 75542913		0.50	0.75	0.63
chr18 75542944	TaiI	0.63	0.75	0.69
chr18 75542956	HpaII	0.63	0.75	0.69
chr18 75542972		0.63	0.75	0.69
chr18 75542983		0.63	0.88	0.75
chr18 75543010		0.63	1.00	0.81
chr18 75543066		0.38	0.50	0.44
chr18 75543093		0.50	0.88	0.69
chr18 75543118		0.63	0.75	0.69
chr18 75543155		0.75	0.75	0.75
chr18 75543159		0.75	1.00	0.88
chr18 75543165	NlaIV	0.75	0.88	0.81
chr18 75543206		1.00	0.88	0.94
chr18 75543216	HpaII, NlaIV	0.75	0.88	0.81
Methylated site frequency		0.65	0.81	

Euploid maternal blood cells

Chromosomal location of CpG site	Enzyme	Case 1 Methylation Index	Case 2 Methylation Index	Average MI
chr18 75542913		0.13	0.00	0.06
chr18 75542944	TaiI	0.00	0.00	0.00
chr18 75542956	HpaII	0.25	0.13	0.19
chr18 75542972		0.00	0.00	0.00
chr18 75542983		0.00	0.00	0.00
chr18 75543010		0.13	0.00	0.06
chr18 75543066		0.13	0.00	0.06
chr18 75543093		0.50	0.38	0.44
chr18 75543118		0.63	0.88	0.75
chr18 75543155		1.00	0.88	0.94
chr18 75543159		0.75	0.75	0.75
chr18 75543165	NlaIV	0.75	0.88	0.81
chr18 75543206		0.88	0.63	0.75
chr18 75543216	HpaII, NlaIV	0.88	1.00	0.94
Methylated site frequency		0.43	0.39	

Figure 6.11D Bisulfite sequencing data of MAT.18.0097.2.

ß-actin

Chromosomal location of CpG site (hg18)

Euploid placental tissues

Enzyme	Chromosomal location of CpG site (hg18)	Case 1	Methylation Index	Case 2	Methylation Index	Case 3	Methylation Index
	chr7 5536443	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536449	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HpaII	chr7 5536458	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HinP1I	chr7 5536463	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
BstUI	chr7 5536465	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	chr7 5536472	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HinP1I	chr7 5536476	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536478	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	chr7 5536484	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HinP1I	chr7 5536486	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	chr7 5536492	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	chr7 5536500	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HpaII	chr7 5536520	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536541	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
BstUI	chr7 5536543	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	chr7 5536552	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536555	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HpaII, NlaIV	chr7 5536560	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HinP1I	chr7 5536576	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536583	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536588	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	Methylated site frequency		0.00		0.00		0.00

Euploid maternal blood cells

Enzyme	Chromosomal location of CpG site (hg18)	Case 1	Methylation Index	Case 2	Methylation Index	Case 3	Methylation Index
	chr7 5536443	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536449	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HpaII	chr7 5536458	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HinP1I	chr7 5536463	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
BstUI	chr7 5536465	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	chr7 5536472	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HinP1I	chr7 5536476	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536478	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	chr7 5536484	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HinP1I	chr7 5536486	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	chr7 5536492	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	chr7 5536500	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HpaII	chr7 5536520	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536541	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
BstUI	chr7 5536543	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	chr7 5536552	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536555	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HpaII, NlaIV	chr7 5536560	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
HinP1I	chr7 5536576	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536583	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
AciI	chr7 5536588	○○○○○○○○	0.00	○○○○○○○○	0.00	○○○○○○○○	0.00
	Methylated site frequency		0.00		0.00		0.00

Figure 6.11E Bisulfite sequencing data of *β-actin*.

Figure 6.11. Cloning and bisulfite sequencing data

Bisulfite sequencing data of (A) *VAPA-APCDD1 region 1*, (B) *VAPA-APCDD1 region 2*, (C) MAT.18.0071.3, (D) MAT.18.0097.2 and (E) *ß-actin*. For each locus, 2 – 6 pairs of euploid placentas and maternal blood cells from the first trimester were analysed. A total of 8 clones were scored for each sample. The methylated sites are represented by a closed circle, while the unmethylated sites are represented by an opened circle. The MI of each CpG site was given by the number of methylated CpG sites over the total number of CpG sites acress all clones in a sample. The methylated site frequency of each sample was given by the number of methylated CpG sites over the total number of CpG sites across all clones of the whole amplicon in one sample. The positions of the methylation-sensitive enzymes restriction sites are marked above the concerned CpG sites. The genomic locations were mapped according to the Human Genome March 2006 Assembly (hg18).

6.3.4. Detection of VAPA-APCDD1 region 1 by MSRE-mediated qPCR

A quantitative real-time PCR (qPCR) assay was designed to amplify *VAPA-APCDD1*. The region analysed by qPCR spanned altogether five *Hin*P1I and *Hpa*II sites. I aimed to evaluate: (i) the detection rate of digestion-resistant *VAPA-APCDD1* sequences in first- to third- trimester maternal plasma; (ii) the postpartum clearance pattern in maternal plasma, if any, after delivery of the fetus; and (iii) the correlation of the maternal plasma concentrations of digestion-resistant *VAPA-APCDD1* sequences with a fetal genetic marker (Y-chromosome sequences) in pregnancies bearing male fetuses. I evaluated the detection rate of digestion-resistant *VAPA-APCDD1 DNA* sequences with 6 – 10 cases of maternal plasma from the first and second trimesters, as well as 10 pairs of maternal plasma before and after delivery of the fetus.

6.3.4.1 Detection and post-partum clearance of digestion-resistant VAPA-APCDD1 DNA in maternal plasma

<u>*Rate of detection of digestion-resistant VAPA-APCDD1 DNA in maternal plasma*</u>

Digestion-resistant *VAPA-APCDD1* DNA was readily detectable in all the pre-delivery maternal plasma from the first to third trimesters. The median concentrations of digestion-resistant *VAPA-APCDD1* sequences in pre-delivery first, second and third trimester maternal plasma were 39 copies/mL [interquartile range (IQR): 30 to 43], 30 copies/mL (IQR: 24 to 48) and 806 copies/mL (IQR: 398 to 1130), respectively (Figure 6.12). The concentrations of digestion-resistant *VAPA-APCDD1* sequences between the three trimesters of pregnancy were statistically significantly different (Kruskal-Wallis test, $p < 0.001$). Furthermore, digestion-resistant *VAPA-APCDD1* DNA was not detectable in plasma obtained from

non-pregnant individuals (n = 4). The results suggested that digestion-resistant *VAPA-APCDD1* DNA sequences were pregnancy-specific, and the concentrations increased when pregnancy advanced.

As a positive control of DNA extraction, identical aliquots of each maternal plasma samples without restriction enzyme treatment were processed in parallel and the *VAPA-APCDD1* were detectable in all undigested samples. As a control for complete digestion, all digested samples were subjected to the *β-actin* qPCR assay. This assay target a region known to be completely unmethylated in placentas and maternal blood cells and containing the concerned MSRE sites. The results show that all *β-actin* signals were undetectable after enzyme digestion.

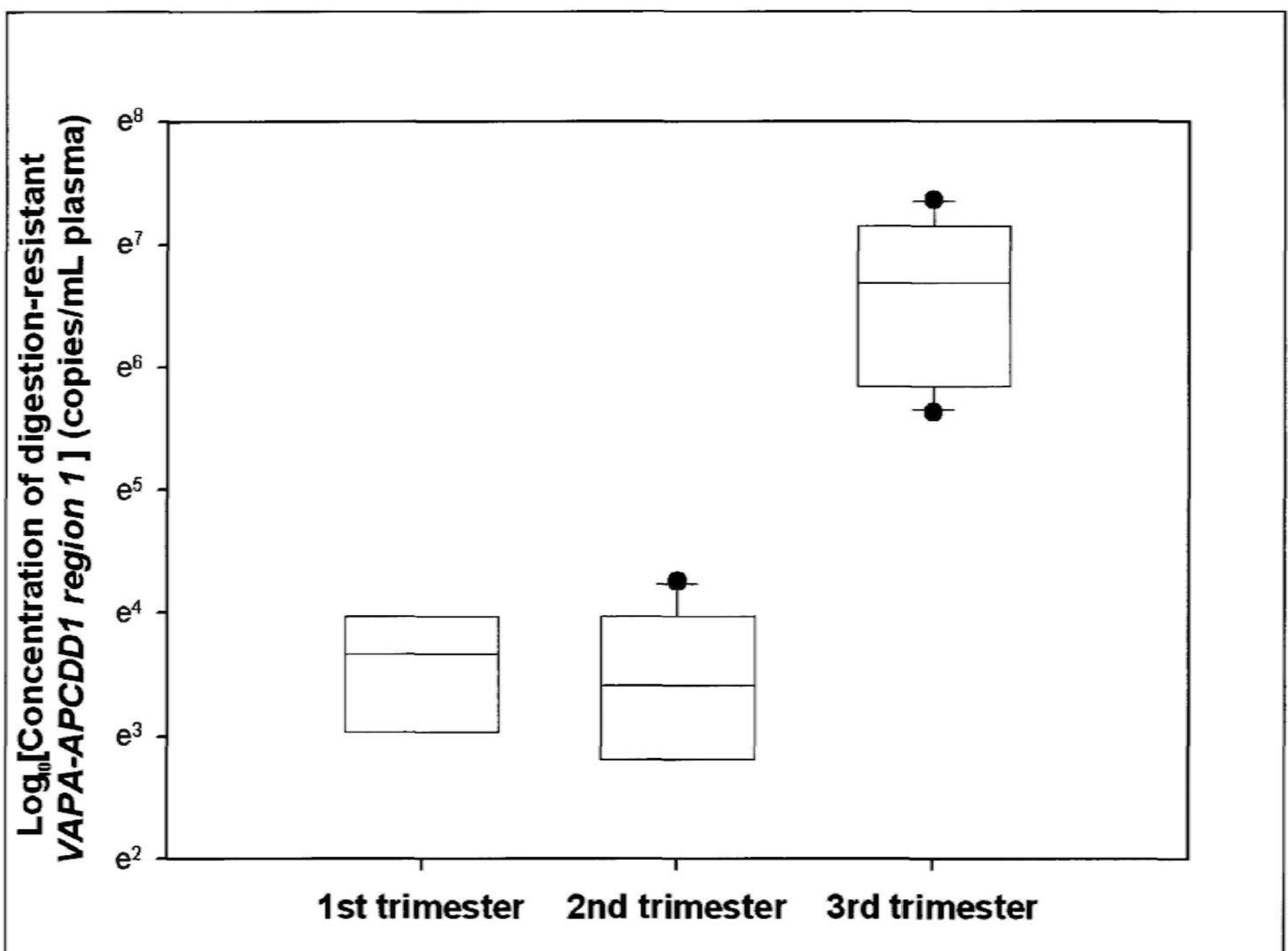
Concentrations of digestion-resistant VAPA-APCDD1 DNA before and after delivery of the fetus The median concentrations of digestion-resistant *VAPA-APCDD1* DNA before delivery of the fetus were 806 copies/mL in 10 third-trimester euploid pregnancies. After delivery, the median concentrations were below the detection limit of the assay (Wilcoxon signed-rank test, P-value=0.002) (Figure 6.13). The results suggested that digestion-resistant *VAPA-APCDD1* sequences were cleared after delivery of the fetus. Both male and female fetuses were involved in the pregnancies tested. The results highlighted that *VAPA-APCDD1* was a gender-independent fetal marker.

6.3.4.2 Correlation of the maternal plasma concentration of digestion-resistant VAPA-APCDD1 sequences with a fetal genetic marker

The source of digestion-resistant *VAPA-APCDD1* DNA in maternal plasma was

further investigated by studying the correlation, if any, of the concentrations of digestion-resistant *VAPA-APCDD1* sequences with the concentrations of *ZFY* (Zinc finger protein, Y-linked), an established genetic marker for detecting fetal DNA in maternal plasma of pregnancies bearing male fetuses (Lun et al. 2008a). Their plasma concentrations correlated significantly ($n = 13$, $r = 0.91$; P-value < 0.00001; Spearman correlation) (Figure 6.14). The results suggested that the digestion-resistant *VAPA-APCDD1* sequences in maternal plasma were derived from the fetus.

Figure 6.12. Plasma concentrations of digestion-resistant *VAPA-APCDD1 region 1* determined by real-time qPCR



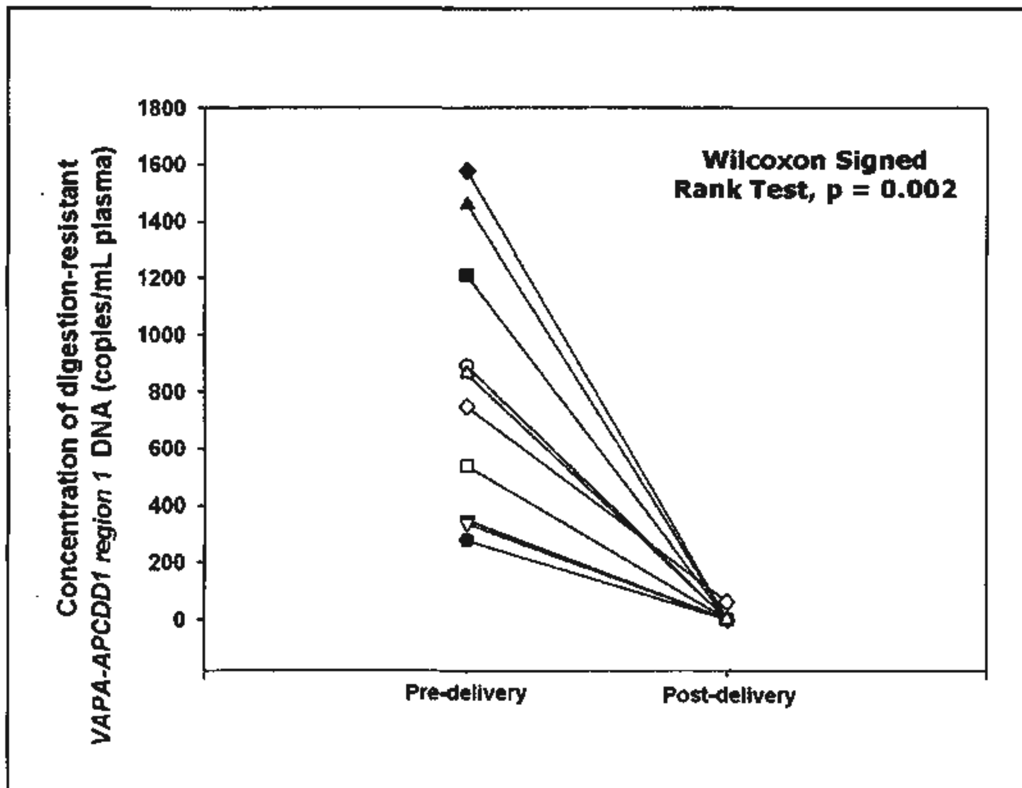


Figure 6.13. Plasma concentrations of digestion-resistant *VAPA-APCDD1 region 1* DNA before and after delivery

Concentrations of digestion-resistant *VAPA-APCDD1 region 1* DNA in 10 paired pre-delivery and post-delivery maternal plasma samples.

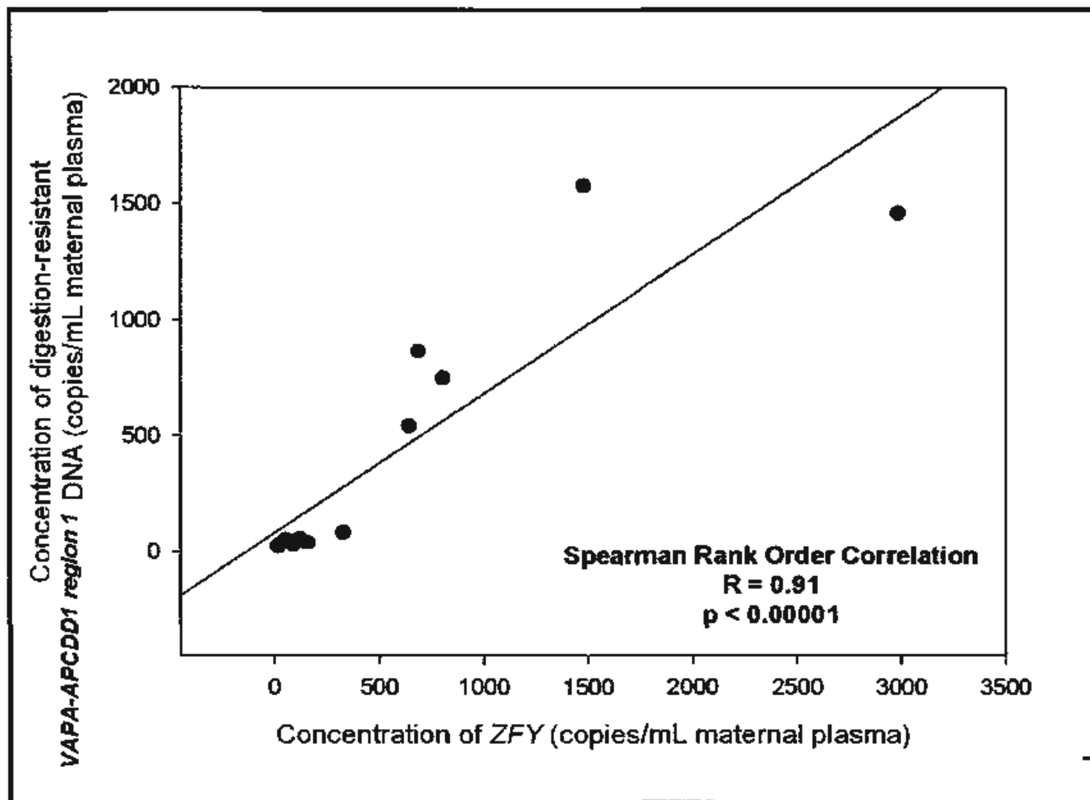


Figure 6.14.

Plasma concentrations of digestion-resistant *VAPA-APCDD1 region 1* and those of an established fetal genetic marker, *ZFY*, in pregnancies bearing male fetuses.

6.4. Discussion

In this study, I have performed a MeDIP-chip analysis to cover essentially all the CpG sites of chromosome 18 to compare the methylation profiles of placenta and maternal blood cells. I have presented a systematic scheme for the evaluation of potential fetal epigenetic markers identified from MeDIP-chip. This scheme included quantitative validation using high-resolution methylation assays, Epityper analysis and bisulfite sequencing, and careful selection using a set of stringent criteria, to identify loci containing clusters of CpG sites consistently methylated across individuals and unmethylated in maternal blood cells. This scheme also included investigation of the detection rate of a potential marker in maternal plasma using quantitative real-time PCR assays. With this series of systematic evaluation, I had successfully identified a panel of novel fetal epigenetic markers on chromosome 18, and illustrated that digestion-resistant *VAPA-APCDD1 region 1* was detectable in maternal plasma and was fetal-derived.

Besides *VAPA-APCDD1 region 1*, the many other genomic loci identified in the current MeDIP-chip dataset as differentially methylated between the fetal (placenta) and maternal (blood cell) DNA can be used to detect and quantify fetal DNA in maternal plasma. These markers offer advantage over fetal genetic markers because they can be applied in pregnancies regardless of the gender or polymorphic status of the fetus (Chim et al. 2005).

As described in Chapter 2.3.1, these identified fetal epigenetic markers can be used to indicate the presence of fetal DNA in maternal plasma when performing fetal rhesus D genotyping, fetal gender determination or the detection of

paternally-inherited polymorphisms (Chan et al. 2006). Combined with a marker for the total (i.e. maternal and fetal) DNA, e.g. the *β-globin* gene, the identified fetal epigenetic markers could also be used to determine the fractional concentration of fetal DNA in a maternal plasma sample.

As described in Chapter 2.3.2 and 2.3.3, these identified fetal epigenetic markers could also be used for non-invasive monitoring of pregnancy-related diseases, such as preeclampsia, preterm labor and hyperemesis gravidarum, which are associated with quantitative aberration of fetal DNA in maternal plasma (Lo et al. 1999b; Sekizawa et al. 2001b; Sugito et al. 2003). The quantitative aberrations, if any, of fetal DNA represented by these markers can be assessed by quantitative real-time PCR (qPCR) (Chapter 4), qMSP (Lo et al. 1999c), or other analytical strategies such as digital MethyLight (Weisenberger et al. 2008) and methyl-BEAMing (Li et al. 2009). These markers also have the potential to be used for detection of fetal trisomy 18, which I would evaluate in the next chapter.

It is worth noting that among the five most promising loci that possess placenta-specific methylation, four of them are located within the gene body. A previous study has reported a pattern of low promoter methylation and high gene-body methylation in highly expressed genes in the human genome (Ball et al. 2009). It would be interesting to further investigate the methylation patterns in different regions of the genes, including the regions close to the transcriptional start site, and to study the expression of those genes in the placenta and maternal blood cells.

In conclusion, I have demonstrated with *VAPA-APCDD1 region 1* that at least some of the loci that were systematically identified in this study could be developed into fetal epigenetic markers that are fetal-specific and detectable in maternal plasma, including those from early-gestation. Since these markers are methylated in the placenta, one could apply the MSRE-mediated detection strategy to specifically distinguish fetal DNA from the co-existing maternal DNA in maternal plasma. Hence, bisulfite treatment, which degrades DNA, could be avoided.

In the next Chapter, I will evaluate the clinical utility of *VAPA-APCDD1 region 1* as an early, non-invasive marker for fetal trisomy 18. Specifically, since digestion-resistant *VAPA-APCDD1 region 1* is fetal-specific in maternal plasma, I will evaluate the feasibility of using it to infer the dosage of the fetal chromosome 18 in maternal plasma by the EGG approach.

Chapter 7 Non-invasive prenatal detection of fetal trisomy 18 by epigenetic-genetic chromosomal dosage analysis

7.1. Introduction

In the previous chapter, I have identified a panel of novel fetal epigenetic markers on chromosome 18 by MeDIP-chip analysis. I have demonstrated that among those novel markers, an intergenic region, namely *VAPA-APCDD1 region 1*, possesses a methylation signature that is specific to the fetus, and further developed it into a fetal epigenetic marker that could be robustly detected in maternal plasma. In this chapter, I aim to evaluate the feasibility of using this fetal epigenetic marker to detect fetal trisomy 18 by the epigenetic-genetic (EGG) chromosome-dosage approach.

EGG analysis measures the ratio of a fetal-specific epigenetic marker on the chromosome of interest relative to a fetal genetic marker on a reference chromosome. Theoretically, the expected EGG ratio between a fetal epigenetic marker on chromosome 18 to that of a reference chromosome, such as the Y-chromosome, would be 2:1 in the euploid and 3:1 in the trisomy 18 placentas. In the previous study by *Tong* et al. it was shown that as long as the epigenetic marker is specific to the fetus in maternal plasma, such a difference in the ratio in the placenta is readily detectable in maternal plasma (Tong et al. 2010). The relative dosage of fetal chromosome 21 in maternal plasma was elevated in pregnant women carrying trisomy 21 fetuses compared to those carrying euploid fetuses. Using this approach, fetal trisomy 21 can be detected non-invasively even during the first trimester (Tong et al. 2010).

The EGG approach has distinct advantages over other epigenetic approaches, such as the EAR, as it can infer chromosome dosage using a fetal epigenetic marker on the aneuploid chromosome and a fetal genetic marker on any reference chromosome. This reference genetic marker could be located anywhere in the genome as long as it is fetal-specific. For example, for male fetuses, a Y-chromosome marker such as the *ZFY* gene could be used; while for female and male fetuses, any paternally-inherited polymorphic site could be used (Chow et al. 2007). Thus, the EGG approach can be adopted easily to cover the majority of a general population. In contrast, the EAR approach is only applicable to a portion of pregnancies bearing fetuses which are heterozygous for the detected region (fetal epigenetic marker), since allelic ratio assessment is only feasible for these informative cases.

To investigate the feasibility of using the *VAPA-APCDD1 region 1* in the EGG approach for the non-invasive detection of fetal trisomy 18, I have developed a duplex digital PCR assays that would simultaneously amplify the *VAPA-APCDD1 region 1* sequences and *ZFY* after enzyme digestion. The assay would be evaluated with genomic placental DNA and subsequently with maternal plasma.

7.2. Methods

7.2.1. Sample collection and processing

Samples were obtained from women attending the Department of Obstetrics and Gynaecology at the Prince of Wales Hospital, Hong Kong, or the Prenatal Diagnostic and Counselling Department at the Tsan Yuk Hospital, Hong Kong, or the Harris Birthright Research Centre for Fetal Medicine, at the King's College Hospital, London, UK. Plasma from the UK was harvested, kept frozen and sent to Hong

Kong in batches on dry ice. All of the pregnancies were recruited with informed consent among women whose pregnancy was clinically indicated for CVS. The study was approved by the respective institutional review boards. Placental tissues were recruited among women undergoing termination of pregnancy and elective cesarean section. As a control for assay evaluation, blood was taken from non-pregnant individuals.

Maternal peripheral blood samples were processed as describe in Chapter 3. DNA was extracted from peripheral blood cells, CVS, placental tissues and plasma samples as described in Chapter 3.

7.2.2. Epigenetic-genetic (EGG) chromosome dosage analysis

To perform EGG analysis, the samples were first subjected to treatment with methylation-sensitive restriction enzymes as described in Chapter 6.2.5.1. The concentrations of digestion-resistant *VAPA-APCDD1 region 1* DNA sequences were then measured and compared with those of a fetal-specific genetic marker, *ZFY*. As the amplicon region of *ZFY* does not contain MSRE site involved in our digestion protocol, the digestion would not affect the copy number of *ZFY* which would represent the amount of fetal chromosome Y sequences in the tested sample.

If the enzyme digestion has completed, most of the *VAPA-APCDD1 region 1* DNA left in the sample should be derived from the fetus. The amount of digestion resistant *VAPA-APCDD1 region 1* and *ZFY* sequences of the sample would then be determined by a duplex digital PCR assays that targeted the two concerned sequences. Figure 7.1 outlines the workflow of the analysis of maternal plasma

samples with the EGG approach.

7.2.2.1 Digital PCR assay for determination of digestion-resistant VAPA-APCDD1 region 1 to ZFY ratios

The duplex digital PCR assay consisted of two sets of primers and probes: one set targeted *VAPA-APCDD1 region 1* while the other set targeted *ZFY*. Different reporter dyes were used for the probe that targeted the *VAPA-APCDD1 region 1* DNA and the probe that targeted *ZFY*, such that the fluorescent signals from the two targets could be collected simultaneously while distinguishable from each other. The primer and probes sequences were the same as those used for the qPCR assays in Chapter 6. The reactions were set up with 1X TaqMan® Universal PCR Master Mix in a reaction volume of 5 μL for each well, which was then dispended into 384-well reaction plates. The reactions were carried out on the ABI PRISM 7900HT Sequence Detection System. The thermal profile was the same as that of conventional PCR, except the number of cycles was increased from 40 to 50. Collection of all fluorescence data by the "Absolute quantification" application were done with the SDS 2.3.0 software (Applied Biosystems). The primer and probe sequences are listed on Table 7.1.

The numbers of wells with positive signals for each target were directly counted and corrected according to the Poisson-distribution formula (details would be discussed in 7.2.2.3). After digestion with the methylation-sensitive enzymes *HinP*1I and *Hpa*II, the ratio of the digestion-resistant *VAPA-APCDD1 region 1* DNA to *ZFY* of each sample was determined. This ratio would theoretically represent the dosage of the fetal chromosome 18 to chromosome Y in a particular maternal plasma sample.

A duplex assay offered two advantages: First, to determine the relative concentrations between two targets, co-amplifying them in the same reaction chamber could minimise technical variations. Second, co-amplification in the same reaction chamber would halve the amount of maternal plasma required.

To confirm the completeness of the enzyme digestion process, I adopted the *ß-actin* qPCR assay that was described in Chapter 6, but transformed it into a digital PCR assay. Since this assay targeted a region on the *ß-actin* gene that was completely unmethylated in the placenta and maternal blood cells, I expected no amplification signal from the assay if the digestion was completed.

7.2.2.2 Determination of the fractional fetal DNA concentrations

This duplex assay could also be used to determine the fractional fetal DNA concentrations in a particular maternal plasma sample. Without enzyme digestion, the concentrations of *VAPA-APCDD1 region 1* in a particular tested sample would represent the total (i.e. both fetal- and maternal-derived) amount of plasma DNA. The fetal DNA proportion could be calculated based on the following formula:

Fetal DNA proportion = (ZFY) x 2 / (VAPA-APCDD1 region 1) x 100%

Such information would be a piece of useful supplementary information for subsequent maternal plasma analysis. For example, when one failed to amplify any *VAPA-APCDD1 region 1* DNA or *ZFY* sequences after digestion, one could use the undigested portion to check whether the sample was associated with a particularly low fractional fetal DNA concentration. In such case, more DNA might need to be

extracted from a larger volume of maternal plasma to compensate for the low quantity.

The efficiency of this assay for the determination of the fractional fetal DNA concentration would be compared with the digital *ZFY/X* assay that has been well-established for determining fractional fetal DNA concentration in maternal plasma analysis (Lun et al. 2008a).

7.2.2.3 Interpretation of the digital PCR data

The principle of digital PCR has been described in Chapter 3.4.4.2. DNA extracted from plasma with or without digestion was diluted to a concentration such that, on average, one template molecule was present in every two reaction wells. The diluted templates were then dispensed into the reaction chambers of a 384-well plate. Therefore, each reaction well would contain zero, one or more than one template molecule according to the Poisson distribution.

The Poisson equation is given as:

$$P(n) = \frac{m^n e^{-m}}{n!}$$

where n is the number of template molecules per well, $P(n)$ is the probability of n template molecules in a particular well, and m is the average number of template molecules in a particular digital PCR experiment.

The proportion of wells with no template molecule was given by e^{-m}. For example, at an average concentration of one template molecular per two wells, the proportion of

wells with no template molecule was estimated to be $e^{-0.5}$, which is 60.7% (0.607). The proportion of wells containing one or more template molecules would therefore be 39.4% (100% – 60.7%).

After each digital PCR run, the number of positive wells was counted to calculate the total number of molecules present in the reaction wells according to the following formula (Poisson correction):

The total no. of molecules = - Ln [(no. of negative wells) / no. of total reactions] X no. of total reactions

The *m* value was calculated by dividing the total number of molecules after Poisson correction by the total number of reactions performed for each run. In the duplex *VAPA-APCDD1 region 1/ZFY* assay, the numbers of positive wells for each fluorescent dye were counted, and the respective *m* value for each marker was calculated separately.

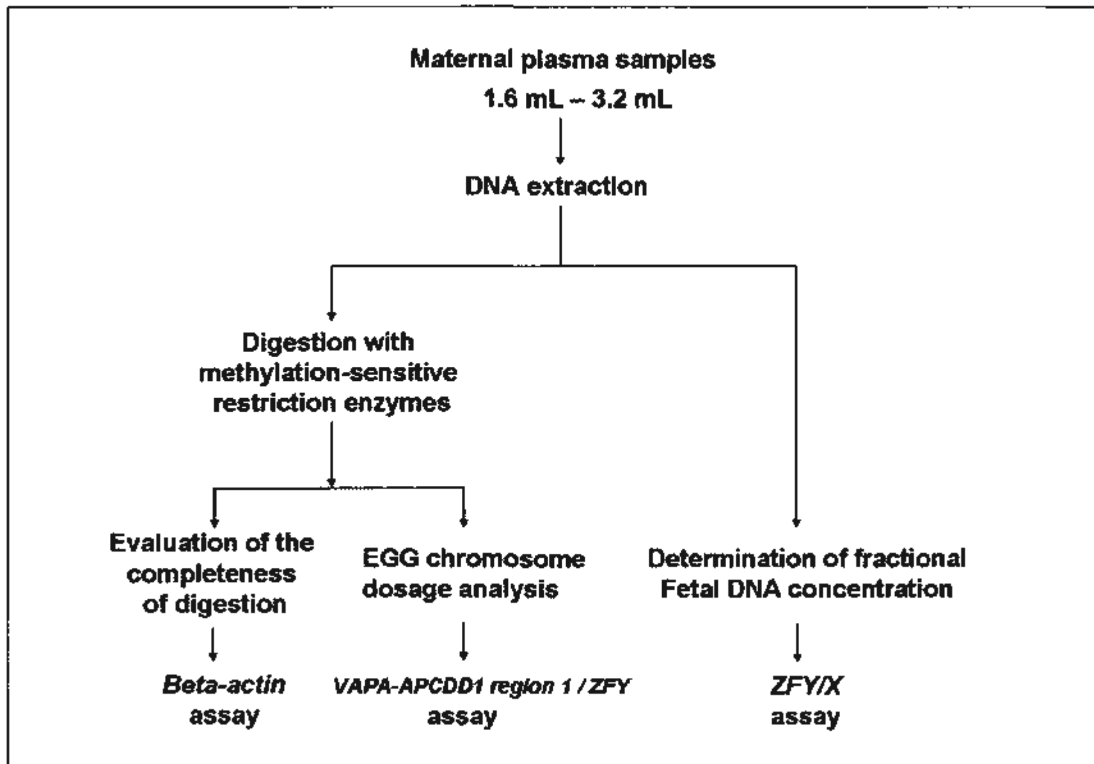


Figure 7.1. Workflow of the EGG chromosome dosage analysis of maternal plasma.

Maternal plasma was collected and extracted for cell-free DNA. A portion was digested with methylation-sensitive restriction enzymes, while another portion was sampled for assessment of the fractional fetal DNA concentration by the *ZFY/X* digital PCR assay. Among the digested samples, a portion would be tested with the *β-actin* digital PCR assay to ensure complete digestion, while another portion would be tested with the *VAPA-APCDD1 region 1/ZFY* duplex assay to determine the EGG chromosome dosage ratio.

Table 7.1. Specifications of the digital PCR assays for *VAPA-APCDD1 region 1/ZFY* (duplex), *β-actin* and *ZFX/Y* (duplex).

Digital PCR

VAPA-APCDD1 region 1 / ZFY (Duplex)

Total no of reactions = 384

		Final concentration			
	Water	variable	50℃	2 min	
	2X TaqMan Universal master mix	1X	95℃	10 min	50 cycles
VAPA-APCDD1 region 1	Forward primer	1500nM	95℃	15s	
	Reverse primer	1500nM	60℃	1 min	
	Probe (FAM)	250nM			
ZFY	Forward primer	300nM			
	Reverse primer	300nM			
	Probe (VIC)	100nM			
	DNA	variable			
	Total	5			

The fluorescent TaqMan probes contained VIC as reporter and BHQ1 (MGBNFQ) as quencher

β-actin

Total no of reactions = 96

	Final concentration			
Water	variable	50℃	2 min	
2X TaqMan Universal master mix	1X	95℃	10 min	50 cycles
Forward primer	900nM	95℃	15s	
Reverse primer	900nM	60℃	1 min	
Probe (VIC)	250nM			
DNA	variable			
Total	5			

ZFXY

Total no of reactions = 192

		Final concentration			
	Water	variable	50℃	2 min	
	2X TaqMan Universal master mix	1X	95℃	10 min	45 cycles
ZFXY	Forward primer	900nM	95℃	15s	
	Reverse primer	900nM	60℃	1 min	
	ZFY-probe (FAM)	125nM			
	ZFX-probe (VIC)	125nM			
	DNA	variable			
	Total	5			

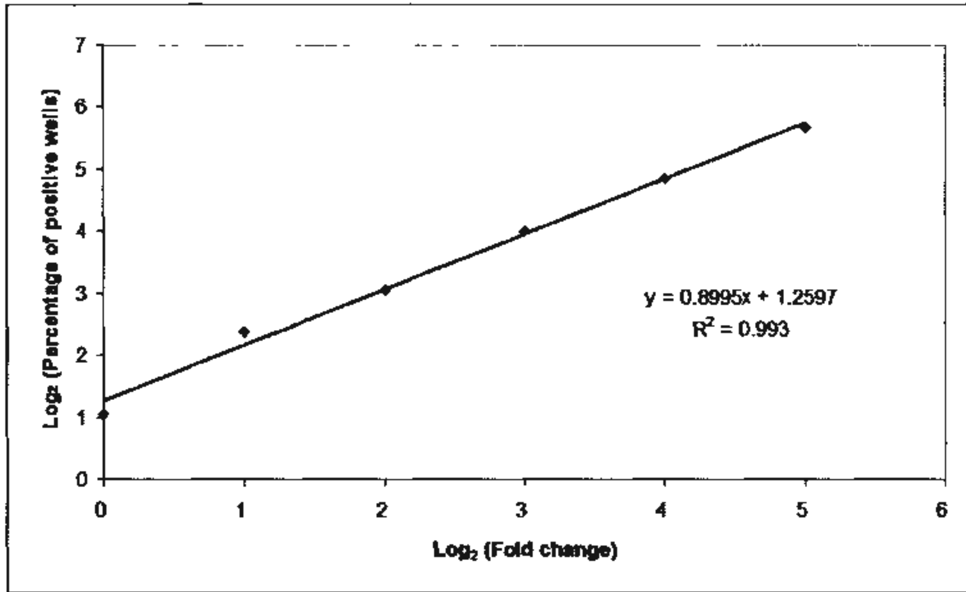
The fluorescent TaqMan probes contained FAM as reporter and BHQ1 (Black-hole quencher 1) as quencher
The fluorescent TaqMan probes contained VIC as reporter and BHQ1 ((MGBNFQ) as quencher

7.3. Results

7.3.1 Evaluation of PCR efficiencies

Single-molecule detection test The lower detection limitation of the assay was evaluated with the following procedures: Different template concentrations were prepared by 2-fold serial dilutions of a particular DNA sample. The lowest dilution should aim at achieving around 1% of positive wells, which was around 3 wells in a 384-well setting. The dilution series was subjected to digital PCR reaction and the log_2-transformed numbers of positive wells were plotted against the log_2-transformed fold-increase at each concentration. Our group have established a model and determined that, if the detection limit is down to single molecule level, theoretically, for every 2-fold increase in template concentration, there would be a 2-fold increase of positive wells, and thus would give a slope of around 0.96. If the lower detection limit was 2, according to the model, the slope would deviate from 0.96 to approximately 1.5. More details on this single molecule detection test are described in Appendix II. The detection limit test for *VAPA-APCDD1 region 1/ZFY* assay was shown in Figure 7.2.

(A)



(B)

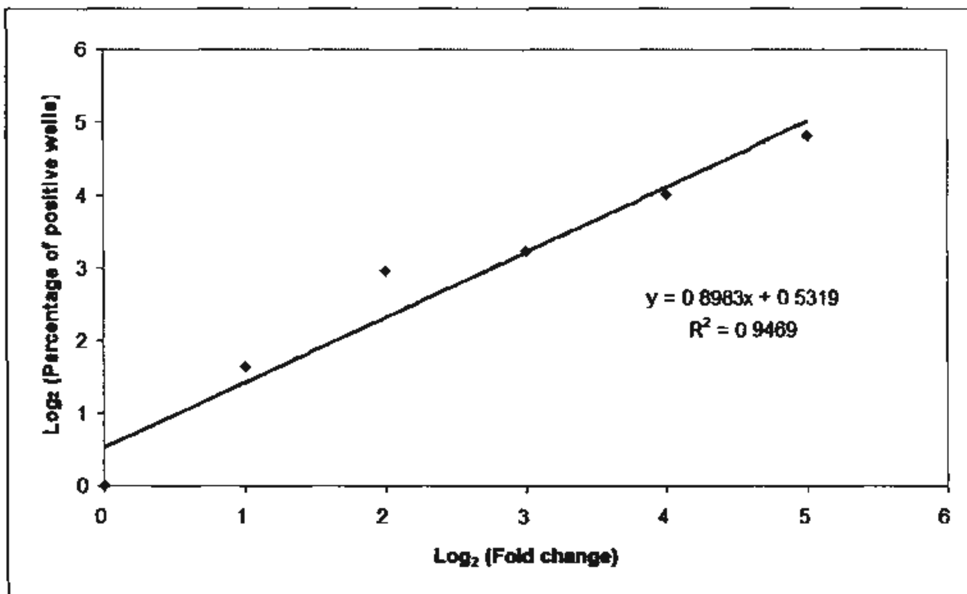


Figure 7.2. Detection limit test of (A) the *VAPA-APCDD1 region 1* assay and (B) the *ZFY* duplex digital PCR assay. The results suggest that both assays are approaching single molecule detection. Details of the testing procedures are described in Appendix II.

Determination of fractional fetal DNA concentration: I analysed 9 maternal plasma samples with the *VAPA-APCDD1 region 1 /ZFY* assay before enzyme digestion and calculated the fractional fetal DNA concentration. It was found that the results were comparable to those obtained using the *ZFX/Y* duplex assay. The data are shown in Figure 7.3 ($r = 0.83$; P-value < 0.02; Spearman correlation).

Non-pregnant female controls: The specificity of the *VAPA-APCDD1 region 1* assay was checked with non-pregnant female controls ($n = 4$) (Table 7.2A). The data showed that there were virtually no detectable signals of digestion-resistant *VAPA-APCDD1 region 1*. Thus, the assay detected pregnancy-specific *VAPA-APCDD1 region 1* DNA in plasma. The completeness of digestion of all plasma samples were confirmed by the absence of signals in the *ß-actin* digital PCR assay.

Feasibility of the EGG analysis demonstrated using placental tissues I investigated if this approach was feasible to differentiate trisomy 18 from euploid with placentas obtained from the first trimester (Figure 7.4). I tested five pairs of placenta DNA samples from the first trimester pregnancies with male fetuses with the *VAPA-APCDD1 region 1 / ZFY* duplex assays. The ratios of the Poisson-corrected counts of *VAPA-APCDD1 region 1* and *ZFY* in euploid and T18 samples were significantly different (Mann-Whitney rank-sum test, $p = 0.029$). A reference interval, defined as the **mean ratio of *VAPA-APCDD1 region 1* to *ZFY* ± 1.96SD**, for the euploid samples as 1.20 – 1.66. All of the T18 cases had a ratio greater than the upper reference limit (Table 7.2B and Figure 7.4).

Table 7.2 Evaluation of the duplex *VAPA-APCDD1 region 1/ZFY* duplex digital PCR assays with (A) non-pregnant control and (B) placental tissues obtained from T18 and normal pregnancies.

Table 7.2A Post-digestoin Digital PCR data of non-pregnant controls

Digital PCR data after enzyme digestion (copies)

Sample	Wells	VAPA-APCDD1 region 1	ZFY	Wells	*ß-actin*
Control 21	192	6	0	96	0
Control 23	192	6	0	96	0
Control 24	192	6	0	96	0
Control 25	192	3	0	96	0

Table 7.2B Correlation of the fetal concentration determined by the *ZFY/X* assay and the *VAPA-APCDD1 / ZFY* assay

Digital PCR data before enzyme digestion (copies)

		*VAPA-APCDD1 region 1 / ZFY**						*ZFY/X***					
Sample	Gestational age (week)	Wells	VAPA-APCDD1 region 1	m	ZFY	m	Fetal %	Wells	ZFX	m	ZFY	m	Fetal %
TY0055	13.4	192	116	0.60	12	0.06	21.4%	192	133	0.89	10	0.05	14.3%
TY0067	12.6	192	154	0.80	5	0.03	6.6%	192	152	0.79	8	0.04	10.0%
TY0085	12.1	192	116	0.60	19	0.10	32.6%	192	114	0.59	10	0.05	16.5%
TY0096	12.9	192	145	0.76	10	0.05	14.1%	192	44	0.23	3	0.02	12.8%
TY0018	12.9	192	159	0.83	7	0.04	9.0%	192	191	0.99	11	0.06	10.9%
M5421	38.0	192	154	0.80	17	0.09	21.7%	192	186	0.97	19	0.10	18.5%
M5427	38.3	192	148	0.77	9	0.05	12.5%	192	163	0.85	13	0.07	15.2%
M5496	38.1	192	135	0.70	8	0.04	12.1%	192	183	0.95	16	0.08	15.7%
M5479	38.3	192	95	0.50	19	0.10	39.7%	192	102	0.53	23	0.12	37.3%

Fetal DNA concentration = % of fetal-derived copies of DNA in maternal plasma
* Fetal % = ZFY x 2/ VAPA-APCDD1 region 1
**Fetal % = (ZFY x 2)/(ZFX + ZFY)

Spearman Rank Order Correlation	
Correlation Coefficient	0.83
P-value	0.02

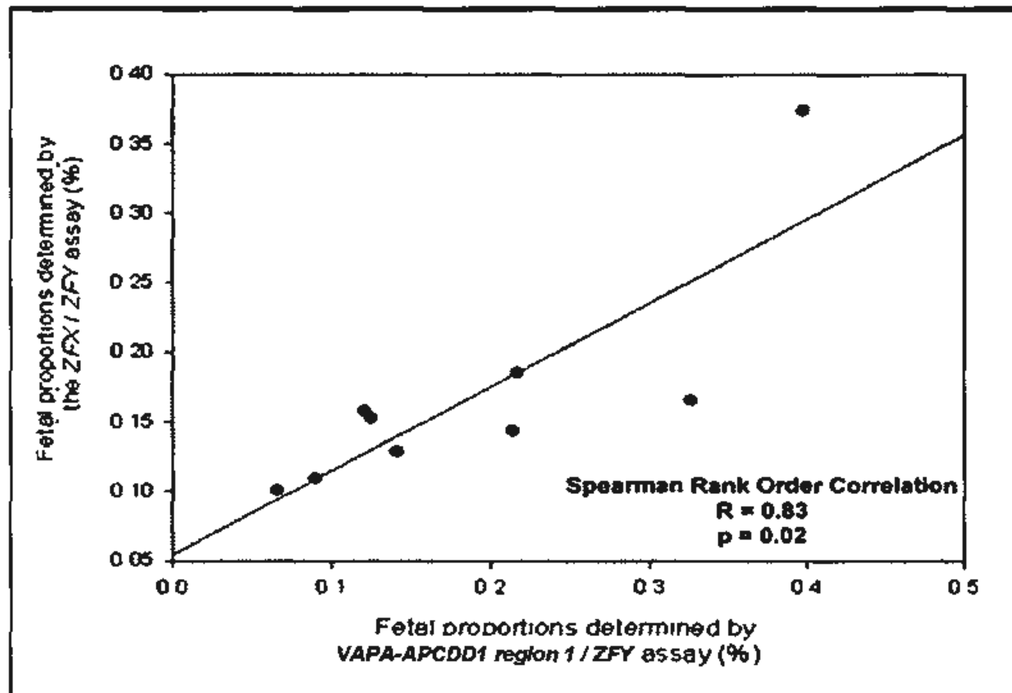


Figure 7.3.

Fractional fetal DNA concentration determined by *VAPA-APCDD1 region 1 / ZFY* assay and that by *ZFY*/X assay. ($n = 6$, $r = 0.83$; $p < 0.02$; Spearman correlation).

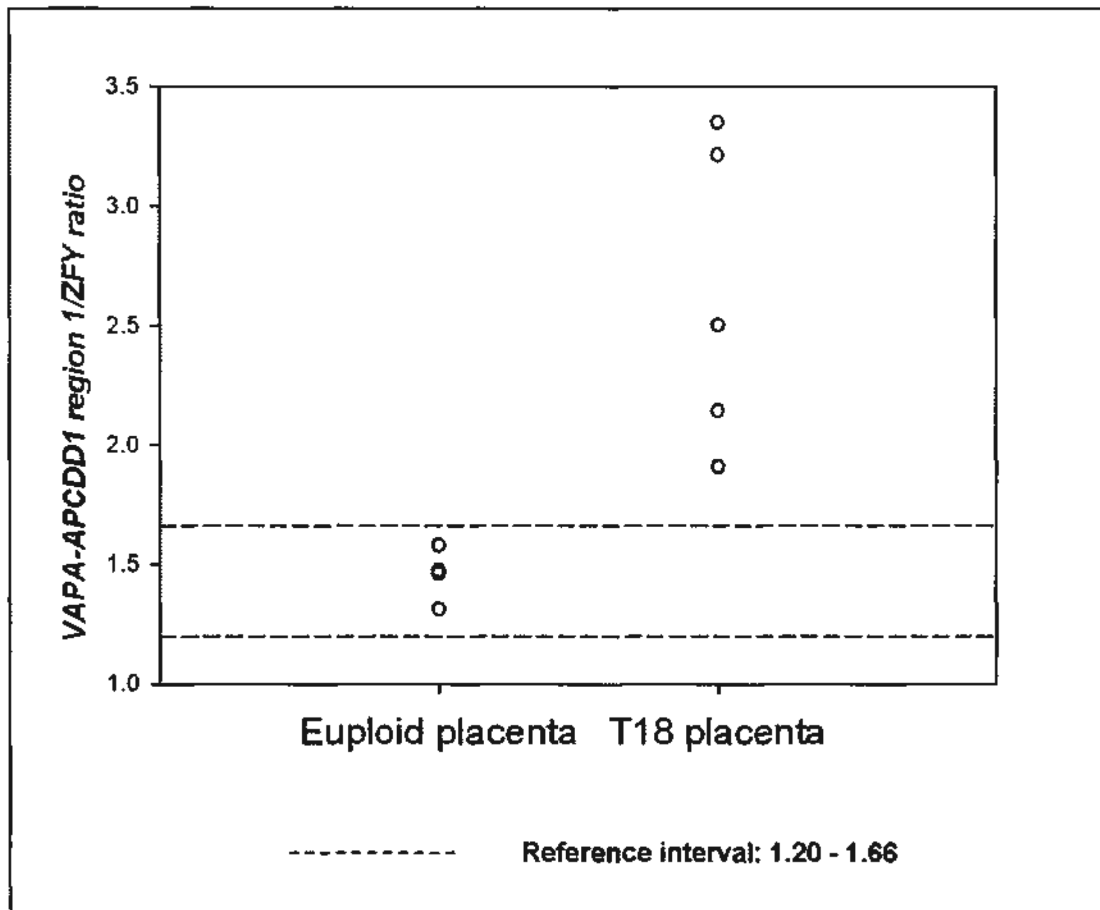


Figure 7.4. Epigenetic-genetic chromosome dosage analysis of placental tissues

First-trimester placental tissues from 5 euploid cases were analysed and compared with 5 other T18 cases. The ratio of *VAPA-APCDD1 region 1/ZFY* was determined for each case. A normal reference interval was determined as 1.20 – 1.66 (dotted line).

7.3.2 Non-invasive prenatal detection of fetal trisomy 18 by the EGG approach

As mentioned in Chapter 3.3.3, the high precision of digital PCR is attributed to the direct counting of individual template molecules through multiple analyses on highly diluted nucleic acids (Vogelstein et al. 1999). However, the accuracy of digital PCR analysis would decrease when a high proportion of positive wells contain more than one molecule (Lo et al. 2007a; Vogelstein et al. 1999). For example, if one was doing the relative chromosome dosage analysis, the average template concentration (m) should be maintained as 0.5 per reaction well (Lo et al. 2007a).

In practice, the ratio between the two targets would vary depending on multiple factors, such as whether all the unmethylated sequences had been completely removed by MSRE and the average template concentration of each target in a particular plasma sample. Therefore, in the current study I had ensured that the two groups of tested samples had a comparable average number of *ZFY* (reference) molecules (median = 0.08 for euploid and 0.05 for T18) for fair comparison.

I analysed 27 maternal plasma samples obtained from pregnancies with euploid male fetuses and 9 from pregnancies with T18 male fetuses. The median gestational ages of the two groups were matched (*Table 7.2*). The samples were subjected to the EGG analysis as outlined in Figure 7.1.

The results showed that the relative plasma concentrations of digestion-resistant *VAPA-APCDD1 region 1* DNA relative to *ZFY* are significantly higher in T18 cases compared with euploid cases (Mann-Whitney rank-sum test, P-value < 0.001) (*Figure 7.5*). A reference interval, defined as the mean ratio of digestion-resistant

VAPA-APCDD1 region 1 to *ZFY* ±1.96SD was established as 0.34 – 3.04. Based on this interval, 26 out of 27 euploid cases and 8 out of 9 T18 were correctly classified, giving a sensitivity of 88.9% and a specificity of 96.3% (*Figure 7.5*). All of the digested maternal plasma samples showed no detectable *ß-actin* signals implying the completeness of the digestion process. Figure 7.6 showed some of the cases as an illustration.

In my study, one euploid and one trisomy 18 case were misclassified as false positive and false negative, respectively. One possible explanation would be the presence of a low percentage of placental mosaicism, in which there is a discrepancy among the chromosome numbers of individual cells in the placenta (Kalousek et al. 1989). However, as placental mosaicism occurs in only approximately 1 – 2 % of viable pregnancies at 9 - 11 weeks of gestation (Harrison et al. 1993), it is not likely to be crucial to the reliability of the EGG analysis. To validate the results, I performed the EGG analysis on the respective placental villus samples of the false negative and false positive cases. Indeed, I found that the ratio of *VAPA-APCDD1 region 1 / ZFY* for the euploid case (false positive) was 1.31 while that for the trisomy 18 case (false negative) was 2.50, which were consistent with the karyotyping results. Therefore, it was unlikely that the misclassification was due to placental mosaicism.

One of the potential factors that affect the ratio of *VAPA-APCDD1 region 1 / ZFY* of individual cases would be the respective average template concentration of *ZFY* and digestion-resistant *VAPA-APCDD1 region 1* DNA. Thus, it would be optimal to determine a specific set of normal reference interval at each average concentration of the reference molecule. Given with the limited sample size, the current study serves

as a demonstration of the feasibility of the EGG approach. The diagnostic accuracy of the test at individual template concentration would need to be further evaluated in a cohort with more samples.

Table 7.3 Characteristics of the maternal plasma samples used for testing the EGG approach

	Number of cases		Gestational age / week [median (IQR)]	
	Euploid	Trisomy 18	Euploid	Trisomy 18
First trimester	18	9	14.1 (12.9 – 16.6)	13.3 (12.9 – 14.5)
Second trimester	4	-		
Third trimester	5	-		
Total	27	9		

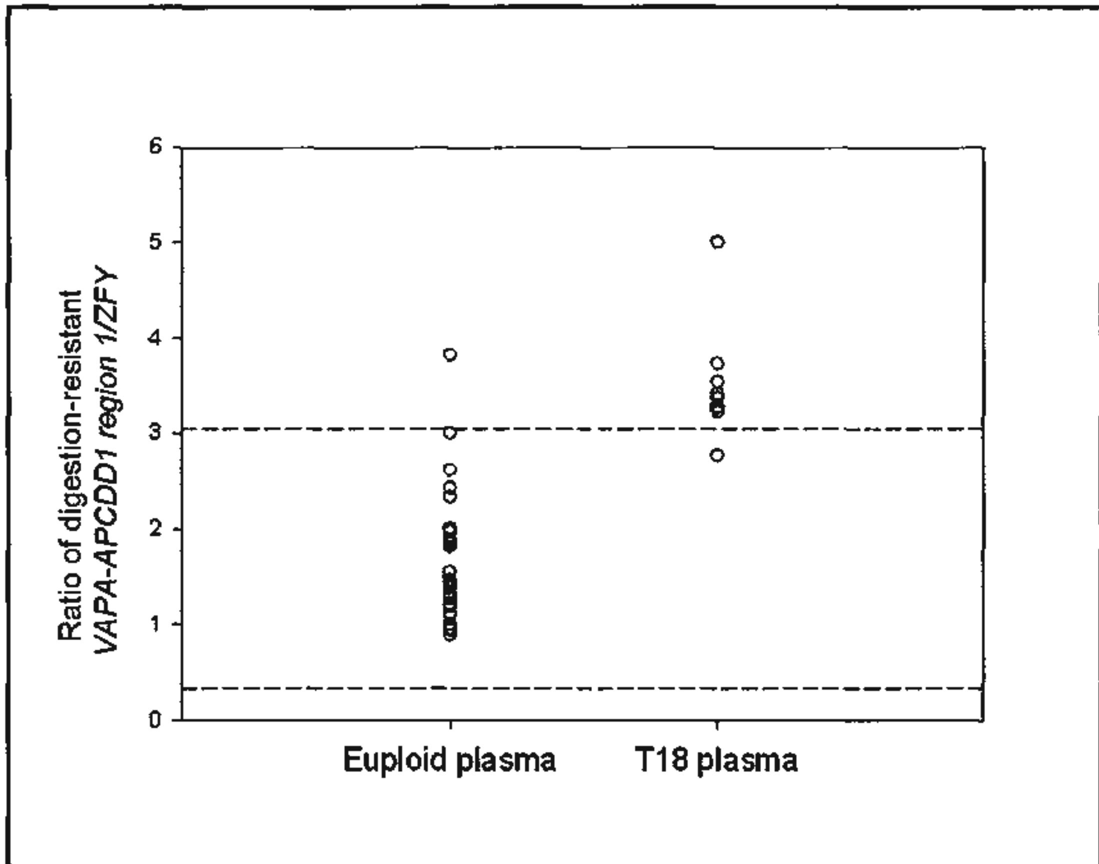


Figure 7.5. Epigenetic-genetic chromosome dosage analysis of maternal plasma samples

Maternal plasma from 27 euploid cases were analysed and compared with 9 T18 cases. The ratio of digestion-resistant *VAPA-APCDD1 region 1/ZFY* was determined for each case. The two groups of ratios were significantly different from each other (Mann-Whitney rank sum test, P-value < 0.001). A normal reference interval was determined as 0.34 –3.04 (dotted line). Eight out of nine T18 cases had a ratio higher than the upper limit of the reference. The sensitivity and specificity of the assay are 88.9% and 96.3 %, respectively.

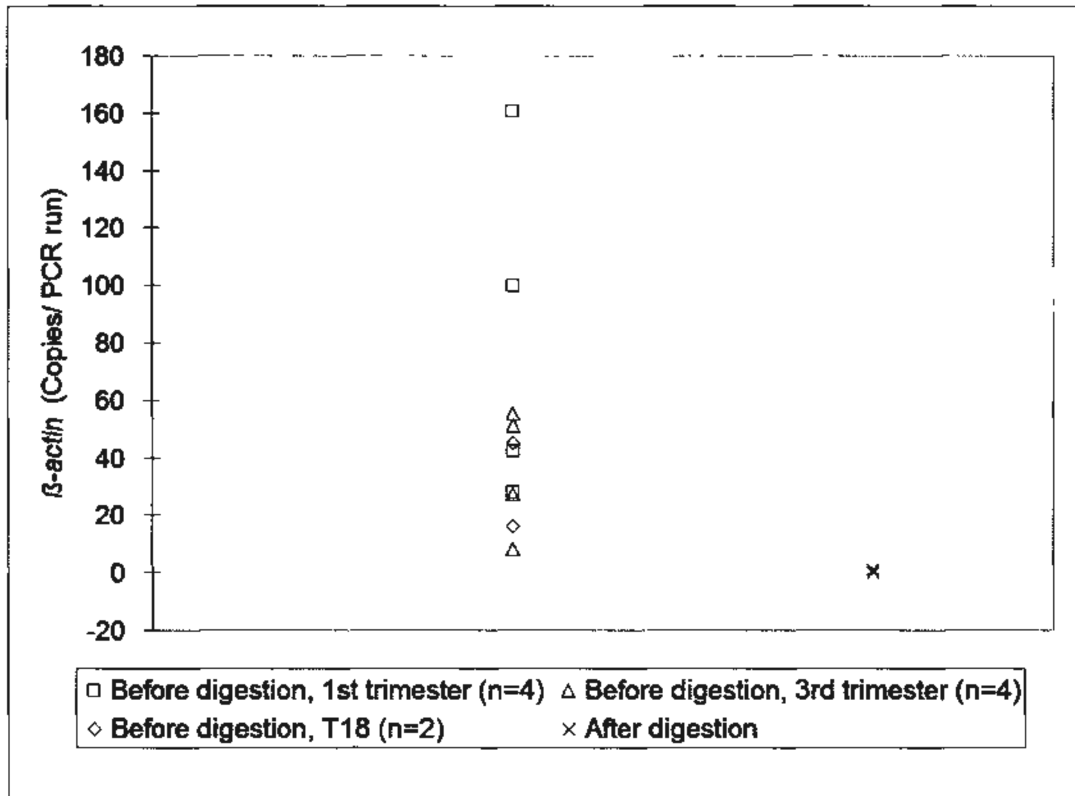


Figure 7.6. Plasma concentration of the *ß-actin* control before and after enzyme digestion.

The *ß-actin* assay was specifically designed to check the completeness of the enzyme digestion. Every plasma samples involved in the EGG test were checked with this assay, and in all case, the *ß-actin* sequences were undetectable after enzyme digestion. This figure only shows a few cases as an illustration. For all the other cases, only the enzyme-digested samples were tested to confirm the completeness of digestion.

7.4. Discussion

In this Chapter, I have demonstrated the feasibility of using *VAPA-APCDD1 region 1* to detect fetal trisomy 18 non-invasively. It provides further evidence to demonstrate the clinical application of the markers that were identified in Chapter 6.

Besides the Y-chromosome fetal genetic markers, Tong and co-workers had also used another fetal epigenetic marker, namely *RASSF1A* on chromosome 3, as the reference for relative chromosome dosage analysis (Tong et al. 2010). However, the authors found that the accuracy in detecting fetal trisomy 21 was adversely affected, by the individual variation in DNA methylation level of this fetal epigenetic marker (Tong et al. 2010). Thus, using a fetal genetic marker as the reference for dosage comparison would give higher accuracy in detecting fetal trisomy. Hence, I have used a fetal genetic marker as the reference.

Before this study, the non-invasive detection of trisomy 18 by fetal nucleic acids analysis was technically challenging. For example, the methylation-based EAR approach requires the presence of a polymorphic site within a placenta-specific methylation marker, and could only be applied to fetus that is heterozygous to that particular polymorphism (Tong et al. 2006). To extend the EAR approach to cover the majority of a general population, one would need to identify more EAR markers with fetal-specific epigenetic signatures overlapping with a polymorphic site with high heterozygosity. Moreover, if the fetal epigenetic marker is unmethylated in the placenta, the use of methylation-sensitive restriction enzymes to remove maternal background is no longer applicable. The use of bisulfite would degrade substantial amount of DNA. Consequently, a large volume of maternal plasma would be needed.

Besides, a SNP on a fetal-specific mRNA, namely the *SERPINB2* [*serpin peptidase inhibitor, clade B (ovalbumin), member 2*] mRNA transcribed from chromosome 18 was adopted to detect the allelic imbalance of fetal chromosome 18 in maternal plasma (Tsui et al. 2009). However, the low detection rate of the *SERPINB2* mRNA in maternal plasma has hindered the robustness of that approach. More recently, our group have demonstrated the potential of the use of massively parallel genomic sequencing for detecting fetal chromosomal aneuploidies from maternal plasma (Chiu et al. 2008; Chiu et al. 2010). However, its throughput is currently limited by the turnaround time required for sample and library preparation.

Thus, the currently proposed EGG approach has strategic advantages to overcome these technical difficulties. Moreover, the cost of instrument and operation of this methylation-based approach is relatively low, especially when compared with the massively parallel sequencing platform. Thus it may be more easily implemented into routine clinical usage. To extend this approach to female fetuses, one could use a fetal genetic marker based on paternally-inherited polymorphism as the reference instead of the Y-specific marker. Such a polymorphic site do not need to overlap with the methylation markers. Thus this EGG approach will provide more flexibility in assay design than other methylation-based approach such as the EAR.

In conclusion, the non-invasive detection of fetal trisomy 18 can be achieved by the EGG approach. This potential clinical application is facilitated by the fetal epigenetic markers identified in Chapter 6. Given the robustness of the EGG approach and its simple and low-cost operational procedures, it has a high potential to be applied in the routine clinical setting. However further evaluation of the reliability requires

study in a cohort of more pregnant women.

My work descried in this thesis has also demonstrated that the systematic search in Chapter 6 is able to identify fetal epigenetic markers that are detectable in maternal plasma and are clinically useful. It is envisioned that this systematic search could be further applied to identify more fetal epigenetic markers on other aneuploid chromosome, such as chromosome 13. The EGG approach could also be extended to the non-invasive prenatal detection of fetal trisomy 13, which is the third commonest fetal trisomy worldwide.

Section V Concluding remarks

This section concludes the significance of the work described in this thesis. Remarks on future prospects would be given.

Chapter 8 Conclusion and future prospects

8.1 The progress of non-invasive prenatal diagnosis through epigenetics

The discovery of cell-free fetal DNA in the maternal circulation has accelerated the development of non-invasive prenatal diagnostic methods. The extensive research efforts in the past 15 years have demonstrated the high potential of cell-free fetal DNA analysis in clinical diagnostic usage (Chapter 1.3). The development of circulating fetal DNA markers has also evolved from paternally-inherited genetic markers, which are only applicable to a subset of population, to universally applicable epigenetic and gene expression markers. These developments have extended the potential application of cell-free fetal nucleic acids to essentially all pregnancies, regardless of the gender or the polymorphic status of the fetus (Chapter 2). Furthermore, sophisticated analytical platforms, such as digital PCR, allows precise measurement of the quantitative changes of fetal chromosome dosage in maternal plasma of aneuploid pregnancies (Chapter 3). All of these advancements have led to the development of a variety of non-invasive prenatal diagnostic strategies.

8.2 Fetal epigenetic markers for quantitative assessment of fetal DNA in pre-eclampsia

Previous studies using male-specific markers have demonstrated quantitative aberrations of fetal DNA in the plasma of pregnant women who suffered from pre-eclampsia (Lo et al. 1999b). The study described in Chapter 4 demonstrated that

there is a similar elevation for a fetal epigenetic marker, hypermethylated *RASSF1A*. The clinical significance of such finding is four-fold: **First**, in terms of coverage, the detection of fetal DNA using hypermethylated *RASSF1A* has advantages over male-specific fetal genetic markers, as it can be used in pregnancies bearing both male and female fetuses. In particular, as *RASSF1A* is a placenta-specific methylation signature (Chiu et al. 2007), its detection do not require any prior assessment of the polymorphic status of the fetus and thus can be applied to essentially all pregnancies. **Second**, in terms of the rate of detection, fetal-derived hypermethylated *RASSF1A* is detected using a strategy based on the selective removal of background unmethylated DNA by methylation-sensitive restriction enzymes (Chan et al. 2006). Such a detection strategy does not require bisulfite treatment, which degrades DNA, and is expected to be particularly useful in clinical samples where the amounts of fetal DNA molecules are low, such as early-gestation maternal plasma. **Third**, previous studies have suggested that the plasma concentrations of male-specific fetal marker are elevated before the onset of clinical symptoms of pre-eclampsia (Leung et al. 2001b; Levine et al. 2004), and its potential to predict pre-eclampsia in low-risk population has also been evaluated. (Farina et al. 2004b). It would be interesting to investigate whether hypermethylated *RASSF1A* could be used as a gender-independent predictive marker of pre-eclampsia. Previous studies have showed that the elevation of cell-free fetal DNA in pre-eclampsia began at the gestational age of 17 weeks (Levine et al. 2004). Thus, the predictive value of the hypermethylated *RASSF1A* would need to be further evaluated in mid-trimester maternal plasma. **Fourth**, apart from pre-eclampsia, quantitative aberrations of male-specific cell-free fetal DNA were also described in preterm labor (Farina et al. 2005; Leung et al. 1998), and other pregnancy-associated disorders (Sekizawa et al.

2002; Sekizawa et al. 2001b; Sugito et al. 2003). It would be interesting to investigate whether hypermethylated *RASSF1A* demonstrate a similar extent of aberrant changes in maternal plasma of pregnancies associated with these disorders.

8.3 The search for new DNA methylation markers for non-invasive prenatal diagnosis

The study described in Chapter 5 outlines a candidate gene approach to search for pre-eclampsia-associated DNA methylation signatures. To further extent the coverage of the study, one possible strategy is to use microarray to systematically identify genes that are aberrantly expressed in pre-eclamptic pregnancies (Enquobahrie et al. 2008; Founds et al. 2009), and to then study their DNA methylation patterns. Another strategy would be to utilize quantitative methylation profiling technique to compare the global methylation patterns between pre-eclamptic and uncomplicated samples. For example, genome-wide detection of methylation profiles might be done by enrichment of methylated DNA with an antibody (MeDIP) (Weber et al. 2005), differentiation cleavage by methylation-sensitive restriction enzymes (Hatada et al. 2006; Khulan et al. 2006) or methylation-specific amplification of bisulfite-converted DNA (Gitan et al. 2002), and then followed by hybridization to high-resolution microarray. Furthermore, quantitative analytical platforms such as the mass spectrometry-based Epityper system allows rapid and high-throughput analysis of DNA methylation at a resolution close to single-CpG site (Ehrich et al. 2005; Ragoussis et al. 2006). In the past, such high-resolution data are mainly obtained via cloning and bisulfilte sequence, which is unfortunately tedious and of low throughput. The combined usage

of a high-coverage profiling tool and a high-throughput validation platform would increase the efficiency of the search for new DNA methylation markers. Using MeDIP-chip and the Epityper platform, the work described in Chapter 6 has identified a panel of novel fetal epigenetic markers on chromosome 18. These markers are located throughout the whole chromosome 18 with more than half of them located within the gene body (Chapter 6). Such high-coverage screening would be much more difficult if it was performed without a global methylation profiling tool. Furthermore, the inclusion of a systematic evaluation scheme is also important for the efficient validation of the potential candidates identified. As demonstrated in Chapter 6, such a scheme would be useful for the identification of fetal epigenetic markers that could be detected in maternal plasma even from the early gestation.

DNA methylation profiling by MeDIP-chip has also been adopted in studies of cancer (Cheung et al. 2010; Tomazou et al. 2008; Weng et al. 2009). The MeDIP procedures might be followed by massively parallel sequencing to further increase the resolution of the analysis (Li et al. 2010); (Gu et al.). It is envisioned that these strategies would further extend the search for disease-associated DNA methylation signatures.

8.4 Digital EGG analysis permits non-invasive prenatal detection of fetal trisomy 18 in as early as the first-trimester

Non-invasive assessment of fetal aneuploidy status is an important goal in the field of prenatal diagnosis. Since the discovery of cell-free fetal DNA in maternal plasma, many strategies have been proposed to prenatally assess the aneuploidy status of the

fetus. These approaches include the assessment of allelic imbalance of heterozygous polymorphic site on fetal-specific transcripts (e.g. *PLAC4*, the RNA-SNP approach) (Lo et al. 2007b; Lo et al. 2007c) or fetal-specific epigenetic signatures (e.g. *SERPINB5*, the EAR approach) (Tong et al. 2006). However, the application of these approaches would require the presence of a SNP within the fetal-specific sequences. Prior assessment of the polymorphic status of the fetus on the specific allele involved would be needed. In order to extend the diagnosis to cover a large population of pregnancies, the EGG approach was developed to infer the relative dosage of fetal epigenetic markers on the aneuploid chromosome with respect to a fetal genetic marker on a reference chromosome (Tong et al. 2010). It is useful for the non-invasive prenatal detection of trisomy 21 (Tong et al. 2010) and trisomy 18 (Chapter 7) in maternal plasma even in the first trimester. Although a male-specific genetic marker was used in these examples, the coverage of the EGG approach could be further increased by using one or more than one paternally-inherited SNP(s) located on any chromosome that is not involved in the aneuploidy. On the other hand, the high analytical precision of digital PCR has further enhanced the diagnostic accuracy of the EGG approach (Chapter 7) (Tong et al. 2010). The experimental procedures could be simplified by using a 384-well reaction system with careful management of the average template concentrations of the reference molecules (Chapter 7), or by implementation of a microfluidic system (Tong et al. 2010). The demonstrated robustness of the EGG approach suggested that it has a high potential to be applied into clinical usage.

8.5 Future prospects

The past few years have been a fruitful period for the development of noninvasive

prenatal diagnostic tests. The emergence of sophisticated analytical platforms, such as digital PCR and massively parallel sequencing, has catalyzed the development of noninvasive prenatal diagnosis (Chiu et al. 2009; Chiu et al. 2010; Lo 2009; Lo et al. 2007b; Lun et al. 2008b). In particular, the sequencing-based methods allow one to detect the small quantitative changes of genomic distribution of the chromosome directly from maternal plasma in a locus-independent manner (Chiu et al. 2008; Chiu et al. 2010; Fan et al. 2008). However, the high instrumental and operational costs of the sequencing-based methods might hinder their rapid implementation into the clinical setting. Before their costs further decline, which is expected to happen in the near future, diagnostic tests that are based on fetal epigenetic markers or other fetal-specific targets are relatively more economical to be implemented clinically. The demonstrated improved robustness of combining epigenetic approaches with microfluidic digital PCR system for the detection of trisomy 18 is only one among many other successful examples (Lo et al. 2007b; Lo et al. 2007c; Tong et al. 2010). The analytical power of these tests would need to be evaluated with large-scale trials before these approaches could be put into routine usage.

8.6 Final remarks

The identification and development of fetal epigenetic markers are indispensable for the progress of non-invasive prenatal diagnosis. It is envisioned that, in the near future, more fetal epigenetic markers will be discovered and adopted in the development of non-invasive prenatal diagnostic tests. With the rapid advances in analytical power, it is expected that the diagnostic accuracy associated with these tests would be further improved. After systematic evaluation of their reliability, these approaches may eventually be applied in the clinical setting as an economical choice

for pregnant women who opt for safer prenatal diagnosis.

Appendix

Appendix I Summary of the Epityper data for the validation of MeDIP-chip analysis

Table 1 Comparison of the methylation data of the potential loci between T18 and euploid placentas

Table 2 Variations of the methylation data of the potential loci in placentas obtained from 10 euploid pregnancies

Appendix I Table 1 Comparison of the methylation data of the potential loci between T18 and euploid placentas

Assay ID	CpG unit	chromosomal location	MI in T18 placenta #1	MI in T18 placenta #2	MI in T18 placenta #3	MI in T18 placenta #4	MI in T18 placenta #5	MI in T18 placenta #6	MI in Euploid placenta #1	MI in Euploid placenta #2	MI in Euploid placenta #3	MI in Euploid placenta #4	MI in Euploid placenta #5	MI in Euploid placenta #6	Mann-Whitney P-value
MAT 18 0094 1	1	10022553	0.25	0.36	0.71	0.56	0.55	0.62	0.56	0.38	0.41	0.46	0.54	0.69	0.937
	2	10022563	0.33	0.65	0.77	0.68	0.68	0.78	0.75	0.66	0.67	0.68	0.70	0.71	0.937
	3	10022575	0.33	0.38	0.63	0.44	0.50	0.64	0.50	0.37	0.36	0.47	0.43	0.59	0.589
	4	10022590	0.31	0.52	0.75	0.64	0.67	0.74	0.73	0.52	0.48	0.57	0.63	0.69	0.589
	5	10022605	0.31	0.48	0.72	0.54	0.63	0.68	0.63	0.52	0.46	0.52	0.57	0.56	0.589
	6	10022618	0.21	0.47	0.62	0.49	0.54	0.66	0.52	0.40	0.42	0.44	0.44	0.51	0.240
	7	10022629	0.31	0.62	0.76	0.70	0.71	0.76	0.75	0.65	0.63	0.67	0.67	0.70	0.589
	8	10022645-52	0.39	0.73	0.80	0.75	0.75	0.79	0.72	0.72	0.71	0.73	0.75	0.77	0.394
	9	10022666	0.39	0.73	0.80	0.75	0.75	0.79	0.72	0.72	0.71	0.73	0.75	0.77	0.394
	10	10022692	0.33	0.65	0.77	0.68	0.68	0.78	0.75	0.66	0.67	0.68	0.70	0.71	0.937
MAT 18 0094 2	1	10022765	0.29	0.88	0.82	0.92	0.91	0.88	0.56	0.65	0.99	0.88	0.90	1.00	0.589
	2	10022778	0.15	0.87	0.82	0.88	0.92	0.82	0.75	0.85	1.00	0.88	0.75	1.00	0.699
	3	10022805-9	NA	NA	0.82	0.68	NA	1.00	0.70	1.00	NA	NA	0.91	1.00	0.629
	4	10022827	0.05	0.89	0.73	0.74	0.79	0.70	0.71	0.77	0.96	0.61	0.69	0.40	0.699
	5	10022834	0.15	0.94	0.78	0.91	0.88	0.98	0.99	0.77	1.00	0.87	0.70	1.00	0.589
	6	10022842	0.36	0.81	0.58	0.61	0.66	0.50	0.75	0.41	0.73	0.59	0.53	0.61	0.818
	7	10022854	0.11	0.64	0.55	0.66	0.47	0.70	0.64	0.48	0.21	0.47	0.51	0.38	0.240
	8	10022863	0.09	0.82	0.70	0.84	0.78	0.92	0.69	0.25	0.96	0.82	0.82	1.00	0.699
	9	10022873-80	0.29	0.88	0.82	0.92	0.91	0.88	0.56	0.65	0.99	0.88	0.90	1.00	0.589
	10	10022902-6	0.16	0.93	0.82	0.92	0.95	0.98	0.73	0.46	1.00	0.93	0.88	0.97	0.937
	11	10022927	0.11	0.90	0.78	0.86	0.87	0.91	0.69	0.42	0.94	0.74	0.78	0.37	0.310
	12	10022966-70	0.13	0.95	0.84	0.93	0.92	0.78	0.31	0.84	0.81	1.00	0.92	1.00	0.589
	13	10022983	0.16	0.79	0.67	0.82	0.82	0.84	0.64	0.41	0.91	0.70	0.73	0.36	0.485
MAT 18 0096 1	1	72292169-77	0.11	0.73	0.59	0.54	0.44	0.35	0.76	0.43	0.66	0.80	0.52	0.36	0.394
	2	72292197	0.03	0.47	0.51	0.39	0.18	0.30	0.47	0.22	0.30	0.52	0.26	0.13	1.000
	3	72292200	0.07	0.29	0.33	0.37	0.18	0.29	0.39	0.23	0.37	0.49	0.36	0.28	0.180
	4	72292249-55	0.16	0.68	0.83	0.60	0.41	0.46	0.62	0.50	0.66	0.86	0.47	NA	0.429
	5	72292278	0.06	0.47	0.30	0.48	0.37	0.24	0.42	0.39	0.16	0.41	0.06	0.04	0.589
	6	72292291-3	0.08	0.55	0.45	0.33	0.16	0.33	0.22	0.30	0.33	0.28	0.30	0.31	0.485
	7	72292315-23	0.13	0.57	0.43	0.36	0.19	0.29	0.48	0.45	0.45	0.54	0.26	0.22	0.394
	8	72292333	0.11	0.43	0.41	0.28	0.24	0.23	0.52	0.24	0.55	0.34	0.24	0.21	0.589
MAT 18 0097 2	1	75542913	NA	NA	0.64	0.66	0.62	0.48	0.45	NA	0.61	0.68	NA	NA	0.857
	2	75542944	0.17	0.37	0.46	0.47	0.42	0.34	0.21	0.19	0.40	0.53	0.20	0.38	0.699
	3	75542966	0.26	0.42	0.51	0.82	0.78	0.44	0.35	NA	0.78	0.96	0.30	0.75	0.792
	4	75542972	0.42	0.49	0.62	0.71	0.50	0.46	0.50	0.61	0.62	0.43	0.31	0.43	0.485
	5	75542983	NA	0.50	0.59	0.67	0.65	0.41	0.55	NA	0.88	0.81	0.46	0.85	0.310
	6	75543066	0.35	0.21	0.31	0.42	0.37	0.35	0.18	0.04	0.36	0.44	0.21	0.32	0.394
	7	75543093	0.54	0.71	0.53	0.55	0.52	0.42	0.45	NA	0.56	0.61	0.43	0.46	0.792
	8	75543118	0.81	0.73	0.80	0.70	0.79	0.77	0.53	0.76	0.70	0.79	0.72	0.84	0.485
	9	75543155-65	0.97	1.00	1.00	0.89	0.90	0.92	1.00	1.00	0.79	1.00	0.79	1.00	0.818
	10	75543216	0.81	0.73	0.80	0.70	0.79	0.77	0.53	0.76	0.70	0.79	0.72	0.84	0.485

Appendix I Table 1 Comparison of the methylation data of the potential loci between T18 and euploid placentas (continued)

Assay ID	CpG unit	chromosomal location	MI in T18 placenta #1	MI in T18 placenta #2	MI in T18 placenta #3	MI in T18 placenta #4	MI in T18 placenta #5	MI in T18 placenta #6	MI in Euploid placenta #1	MI in Euploid placenta #2	MI in Euploid placenta #3	MI in Euploid placenta #4	MI in Euploid placenta #5	MI in Euploid placenta #6	Mann-Whitney P-value
MAT.18.0098.1	1	54075710	0.65	0.84	0.80	0.77	0.77	0.79	0.78	0.80	0.68	0.73	0.72	0.75	0.310
	2	54075727-32	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.000
	3	54075768	0.70	0.65	0.57	0.58	0.63	0.57	0.54	0.60	0.53	0.59	0.47	0.51	0.065
	4	54075824	0.65	0.84	0.80	0.77	0.77	0.79	0.78	0.80	0.68	0.73	0.72	0.75	0.310
	5	54075868	0.52	0.81	0.76	0.73	0.74	0.72	0.83	0.72	0.70	0.65	0.67	0.70	0.310
	6	54075909	0.65	0.84	0.80	0.77	0.77	0.79	0.78	0.80	0.68	0.73	0.72	0.75	0.310
	7	54075947	0.64	0.83	0.81	0.83	0.81	0.77	0.81	0.79	0.73	0.81	0.75	0.86	0.699
MAT.18.0071.3	1	27486381	0.50	0.88	0.91	0.86	0.89	0.89	1.00	0.99	0.89	0.93	0.81	NA	0.126
	2	27486441	0.48	0.67	0.67	0.66	0.68	0.64	0.35	0.77	0.64	0.70	0.60	NA	1.000
	3	27486461	0.61	0.76	0.75	0.74	0.75	0.76	0.83	0.81	0.71	0.74	0.75	NA	0.662
	4	27486523	0.59	0.87	0.84	0.53	0.88	0.85	0.94	0.95	0.84	0.86	0.83	NA	0.429
	5	27486619-22	0.59	0.79	0.83	0.81	0.82	0.78	0.83	0.89	0.81	0.83	0.76	NA	0.247
TAS 18 0841.3	1	27486860	0.94	0.72	0.85	0.46	0.76	0.66	0.59	NA	0.88	NA	0.83	0.94	0.476
	2	27486863	0.97	0.99	0.98	0.97	0.97	0.97	0.60	NA	0.99	NA	0.94	0.96	0.171
	3	27486897	0.59	0.85	0.83	0.78	0.83	0.86	0.64	NA	0.91	NA	0.88	0.73	0.762
	4	27486956	0.54	0.78	0.88	0.76	0.76	0.88	0.73	NA	0.89	NA	0.85	0.57	0.762
TAS.18.1887.2	1	72227952	0.27	0.68	0.56	0.68	0.61	NA	0.62	0.70	0.67	0.70	0.45	NA	0.421
	2	72227903	0.25	0.38	0.33	0.31	0.41	NA	0.41	0.36	0.46	0.38	0.29	NA	0.421
	3	72227851	0.27	0.68	0.56	0.68	0.61	NA	0.62	0.70	0.67	0.70	0.45	NA	0.421
	4	72227809	0.21	0.62	0.50	0.62	0.46	NA	0.64	0.58	0.53	0.60	0.48	NA	0.548
	5	72227578	0.25	0.38	0.33	0.31	0.41	NA	0.41	0.36	0.46	0.38	0.29	NA	0.421
TAS.18.0675.1	1	'19035903	0.54	0.46	0.54	0.56	0.56	0.57	0.57	0.59	0.38	0.56	NA	0.46	0.792
	2	'19035909	0.63	0.61	0.65	0.81	0.66	0.83	0.99	1.00	0.53	0.66	0.98	0.58	0.589
	3	'19035937	0.69	0.73	0.72	0.79	0.77	0.82	0.53	0.56	0.41	0.50	0.54	0.83	0.065
	4	'19035945	0.90	0.75	0.77	0.88	0.82	0.83	0.66	0.82	0.42	0.73	0.87	0.68	0.065
	5	'19035954	0.79	0.81	0.83	0.82	0.80	0.81	0.70	0.74	0.44	0.72	0.72	0.96	0.065
	6	'19035985	0.36	0.78	0.80	0.85	0.78	0.90	0.79	0.80	0.40	0.80	0.79	0.93	0.699
	7	'19036029	0.78	0.79	0.70	0.92	0.76	0.88	0.72	0.79	0.44	0.91	0.91	0.82	0.937
	8	'19036061	0.35	0.50	0.61	0.60	0.69	0.56	0.46	0.77	0.29	0.67	0.82	0.40	0.937
	9	'19036069	0.48	0.70	0.77	0.78	0.73	0.71	0.74	0.75	0.32	0.65	0.71	0.83	0.937
	10	'19036133	0.90	0.97	0.94	0.97	0.98	0.87	0.97	0.98	1.00	0.98	0.95	0.93	0.240
	11	'19036184	0.89	0.98	1.00	1.00	1.00	1.00	0.99	1.00	0.92	0.98	1.00	1.00	0.818

Appendix I Table 2 Variations of the methylation data of the potential loci in placentas obtained from 10 euploid pregnancies

Locus ID	Assay ID	CpG unit	chromosome	chromosomal location	MI in placenta #1	MI in placenta #2	MI in placenta #3	MI in placenta #4	MI in placenta #5	MI in placenta #6	MI in placenta #7	MI in placenta #8	MI in placenta #9	MI in placenta #10	Median MI in placenta	Coefficient of variation (%) of the placental MI (obtained from placental DNA of 10 individuals)
MAT 18 0094	MAT 18 0094 1	1	18	10022553	0.52	0.49	0.49	0.57	0.49	0.56	0.38	0.41	0.46	0.54	0.49	13%
		2	18	10022563	0.65	0.61	0.65	0.67	0.66	0.75	0.66	0.67	0.68	0.70	0.67	5%
		3	18	10022575	0.41	0.38	0.44	0.49	0.45	0.50	0.37	0.36	0.47	0.43	0.43	11%
		4	18	10022590	0.63	0.55	0.61	0.68	0.54	0.73	0.52	0.48	0.57	0.63	0.59	13%
		5	18	10022605	0.54	0.50	0.54	0.63	0.50	0.63	0.52	0.46	0.52	0.57	0.53	10%
		6	18	10022618	0.43	0.34	0.49	0.49	0.43	0.52	0.40	0.42	0.44	0.44	0.44	12%
		7	18	10022629	0.67	0.63	0.71	0.68	0.72	0.75	0.65	0.63	0.67	0.67	0.67	6%
		8	18	10022645-52	0.68	0.68	0.73	0.73	0.72	0.72	0.72	0.71	0.73	0.75	0.72	3%
		9	18	10022666	0.68	0.68	0.73	0.73	0.72	0.72	0.72	0.71	0.73	0.75	0.72	3%
		10	18	10022692	0.65	0.61	0.65	0.67	0.66	0.75	0.66	0.67	0.68	0.70	0.67	5%
MAT 18 0094	MAT 18 0094 2	1	18	10022765	0.93	0.65	0.73	0.92	0.81	0.56	0.65	0.99	0.88	0.90	0.85	18%
		2	18	10022778	0.81	0.59	0.63	0.75	0.71	0.75	0.85	1.00	0.88	0.75	0.75	16%
		3	18	10022805-9	0.99	0.72	0.62		0.81	0.70	1.00			0.91	0.81	18%
		4	18	10022827	0.83	0.66	0.69	0.83	0.82	0.71	0.77	0.96	0.61	0.69	0.74	14%
		5	18	10022834	0.82	0.70	0.74	0.94	0.83	0.99	0.77	1.00	0.87	0.70	0.83	13%
		6	18	10022842	0.66	0.48	0.55	0.71	0.35	0.75	0.41	0.73	0.59	0.53	0.57	24%
		7	18	10022854	0.22	0.39	0.40	0.66	0.15	0.64	0.48	0.21	0.47	0.51	0.43	43%
		8	18	10022863	0.77	0.64	0.54	0.87	0.81	0.69	0.25	0.96	0.82	0.82	0.79	29%
		9	18	10022873-80	0.93	0.65	0.73	0.92	0.81	0.56	0.65	0.99	0.88	0.90	0.85	18%
		10	18	10022902-6	0.98	0.77	0.65	1.00	0.85	0.73	0.46	1.00	0.93	0.88	0.86	21%
		11	18	10022927	0.80	0.57	0.85	0.92	0.80	0.69	0.42	0.94	0.74	0.78	0.79	21%
		12	18	10022966-70	0.87	0.59	0.64	0.90	0.29	0.31	0.84	0.81	1.00	0.92	0.83	35%
		13	18	10022983	0.60	0.58	0.55	0.71	0.67	0.64	0.41	0.91	0.70	0.73	0.66	20%
MAT 18 0071	MAT 18 0071 3	1	18	27486381	0.82	0.78	0.81	0.78	0.81	1.00	0.99	0.89	0.93	0.81	0.82	10%
		2	18	27486441	0.86	0.83	0.83	0.83	0.87	0.35	0.77	0.64	0.70	0.60	0.80	22%
		3	18	27486461	0.75	0.84	0.93	0.81	1.00	0.83	0.81	0.71	0.74	0.75	0.81	11%
		4	18	27486523	0.77	0.72	0.79	0.65	0.84	0.94	0.95	0.84	0.86	0.83	0.83	11%
		5	18	27486619-22	1.00	0.92	0.82	0.97	0.93	0.83	0.89	0.81	0.83	0.76	0.86	9%

Appendix I Table 2 Variations of the methylation data of the potential loci in placentas obtained from 10 euploid pregnancies (continued)

Locus ID	Assay ID	CpG unit	chromosome	chromosomal location	MI in placenta #1	MI in placenta #2	MI in placenta #3	MI in placenta #4	MI in placenta #5	MI in placenta #6	MI in placenta #7	MI in placenta #8	MI in placenta #9	MI in placenta #10	Median MI in placenta	Coefficienct of variation (%) of the placental MI (obtained from placental DNA of 10 individuals)
TAS.18.1887	TAS.18.1887.2	1	18	72227952	0.65	0.56	0.45	0.57	0.89	0.62	0.70	0.67	0.70	0.45	0.64	21%
		2	18	72227903	0.31	0.29	0.29	0.32	0.91	0.41	0.36	0.46	0.38	0.29	0.34	47%
		3	18	72227861	0.66	0.56	0.45	0.57	0.89	0.62	0.70	0.67	0.70	0.45	0.64	21%
		4	18	72227809	0.70	0.65	0.52	0.60	0.00	0.64	0.58	0.53	0.60	0.48	0.59	37%
		5	18	72227578	0.31	0.29	0.29	0.32	0.91	0.41	0.36	0.46	0.38	0.29	0.34	47%
MAT.18.0096	MAT.18.0096.1	1	18	72292169-77	0.45	0.56	0.56	0.81	0.50	0.76	0.43	0.66	0.80	0.52	0.56	24%
		2	18	72292197	0.26	0.49	0.36	0.59	0.29	0.47	0.22	0.30	0.52	0.26	0.33	35%
		3	18	72292200	0.06	0.35	0.13	0.36	0.38	0.39	0.23	0.37	0.49	0.36	0.36	42%
		4	18	72292249-55	0.49	0.70	0.61	0.73	0.45	0.62	0.50	0.66	0.86	0.47	0.62	22%
		5	18	72292278	0.08	0.30	0.20	0.64	0.05	0.42	0.39	0.16	0.41	0.06	0.25	72%
		6	18	72292291-3	0.24	0.39	0.38	0.71	0.17	0.22	0.30	0.33	0.28	0.30	0.30	45%
		7	18	72292315-23	0.23	0.44	0.49	0.75	0.30	0.48	0.45	0.45	0.54	0.26	0.45	34%
		8	18	72292333	0.22	0.36	0.38	0.61	0.17	0.52	0.24	0.56	0.34	0.24	0.35	42%
MAT.18.0097	MAT.18.0097.2	1	18	75542913	0.64	0.73	0.57	0.65	0.83	0.45	0.61	0.68	0.64	0.66	0.65	15%
		2	18	75542944	0.50	0.77	0.59	0.77	0.73	0.21	0.40	0.53	0.63	0.61	0.61	32%
		3	18	75542956	0.77	0.78	0.75	0.95	0.98	0.35	0.78	0.96	0.97	0.96	0.87	24%
		4	18	75542972	0.53	0.61	0.58	0.55	0.86	0.50	0.62	0.43	0.40	0.58	0.57	23%
		5	18	75542983	0.68	0.77	0.76	0.79	0.93	0.56	0.68	0.81	0.66	0.72	0.74	14%
		6	18	75543066	0.40	0.49	0.39	0.48		0.18	0.36	0.44	0.40	0.49	0.40	24%
		7	18	75543093	0.47	0.58	0.65	0.79	0.76	0.45	0.56	0.61	0.42	0.56	0.57	21%
		8	18	75543118	0.63	0.76	0.78	0.91	0.86	0.53	0.70	0.79	0.69	0.76	0.76	15%
		9	18	75543155-65	0.89	1.00	0.92	1.00	1.00	1.00	0.79	1.00	0.99	0.99	0.99	8%
		10	18	75543216	0.63	0.76	0.78	0.91	0.85	0.53	0.70	0.79	0.69	0.76	0.76	15%

Appendix II Detection limit verification for digital PCR assays

The detection limit of every newly developed digital PCR assays were tested with the following procedures.

First, different template concentrations of DNA / RNA samples were prepared by serial 2-fold dilution. The lowest dilution (1X) was aimed at achieving approximately 3 – 6 copies of DNA, and those with higher concentrations theoretically should contain 2X, 4X, 8X and 16X amount of DNA. Such a series of different template concentrations of DNA was subjected to digital PCR anaylsis. The number of positive wells were counted separately for each target. The number of positive wells and the fold change in template concentrations was then $\log_2$ transformed and plotted against each other.

If the detection limit of the system is down to single molecule level, the slope of the plot would be approximately 0.97. Therefore, if the detection limit is approaching 2 molecules, the slope of the plot would be approximately 1.5 (Figure i).

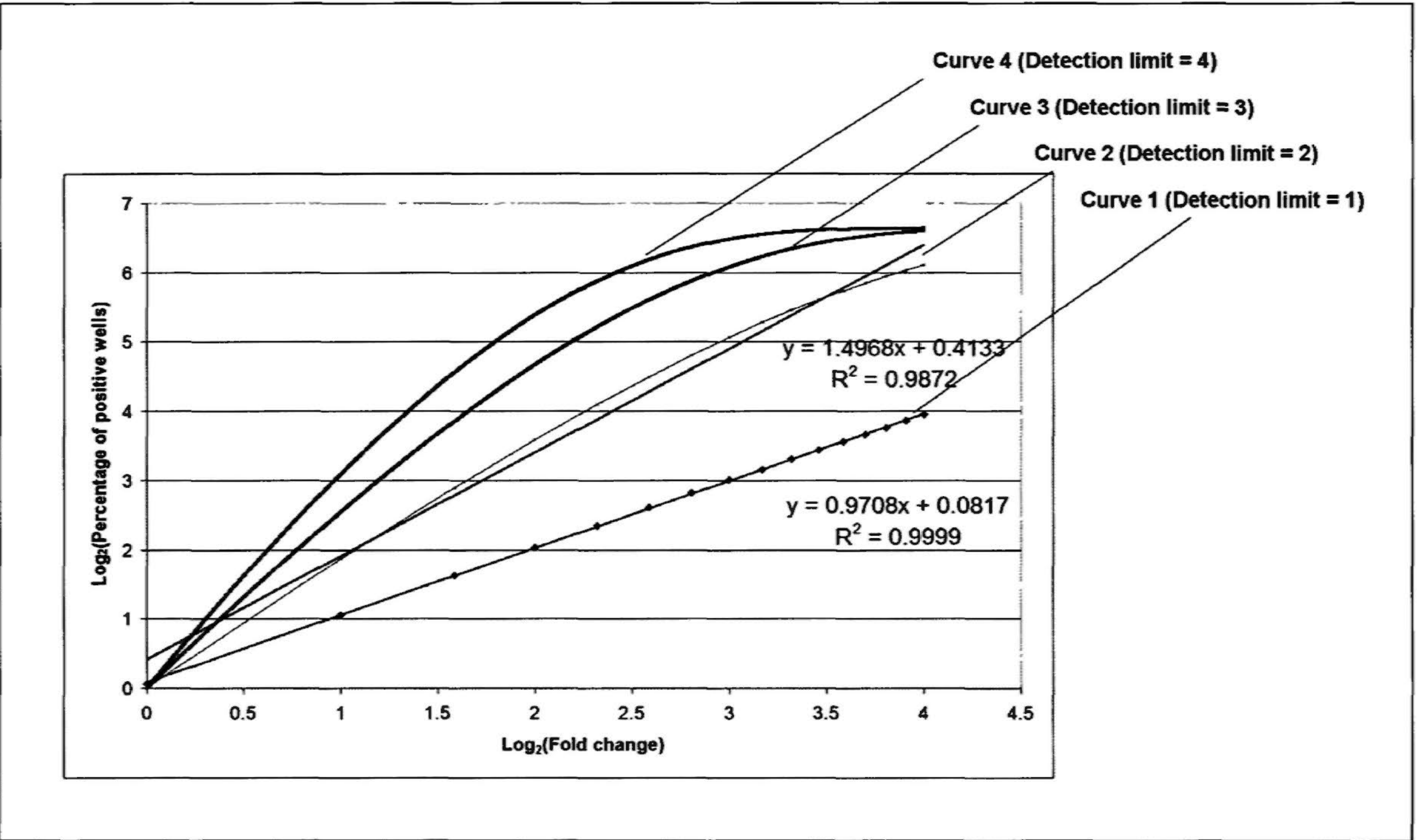


Figure i.

The theoretical relationship of $\log_2$ (percentage of positive wells) against $\log_2$ (template concentration) at different detection limits

REFERENCES

Adekunle, O., Gopee, A., el-Sayed, M., and Thilaganathan, B. 1999. Increased first trimester nuchal translucency: pregnancy and infant outcomes after routine screening for Down's syndrome in an unselected antenatal population. *Br J Radiol* **72:** 457-460.

Agathanggelou, A., Cooper, W.N., and Latif, F. 2005. Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. *Cancer Res* **65:** 3497-3508.

Aitken, D.A., Wallace, E.M., Crossley, J.A., Swanston, I.A., van Pareren, Y., van Maarle, M., Groome, N.P., Macri, J.N., and Connor, J.M. 1996. Dimeric inhibin A as a marker for Down's syndrome in early pregnancy. *N Engl J Med* **334:** 1231-1236.

Akesson, H.O. and Forssman, H. 1966. A study of maternal age in Down's syndrome. *Ann Hum Genet* **29:** 271-276.

Alberry, M., Maddocks, D., Jones, M., Abdel Hadi, M., Abdel-Fattah, S., Avent, N., and Soothill, P.W. 2007. Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. *Prenat Diagn* **27:** 415-418.

Alfirevic, Z., Sundberg, K., and Brigham, S. 2003. Amniocentesis and chorionic villus sampling for prenatal diagnosis. *Cochrane Database Syst Rev*: CD003252.

Amicucci, P., Gennarelli, M., Novelli, G., and Dallapiccola, B. 2000. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. *Clin Chem* **46:** 301-302.

Angert, R.M., LeShane, E.S., Lo, Y.M.D., Chan, L.Y., Delli-Bovi, L.C., and Bianchi, D.W. 2003. Fetal cell-free plasma DNA concentrations in maternal blood are stable 24 hours after collection: analysis of first- and third-trimester samples. *Clin Chem* **49:** 195-198.

Aoki, E., Uchida, T., Ohashi, H., Nagai, H., Murase, T., Ichikawa, A., Yamao, K., Hotta, T., Kinoshita, T., Saito, H. et al. 2000. Methylation status of the p15INK4B gene in hematopoietic progenitors and peripheral blood cells in myelodysplastic syndromes. *Leukemia* **14:** 586-593.

Ball, M.P., Li, J.B., Gao, Y., Lee, J.H., LeProust, E.M., Park, I.H., Xie, B., Daley, G.Q., and Church, G.M. 2009. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nat Biotechnol* **27:** 361-368.

Barton, S.C., Surani, M.A., and Norris, M.L. 1984. Role of paternal and maternal genomes in mouse development. *Nature* **311:** 374-376.

Baylin, S.B., Belinsky, S.A., and Herman, J.G. 2000. Aberrant methylation of gene promoters in cancer---concepts, misconcepts, and promise. *J Natl Cancer Inst* **92:** 1460-1461.

Beck, S., Olek, A., and Walter, J. 1999. From genomics to epigenomics: a loftier view of life. *Nat Biotechnol* **17:** 1144.

Beck, S. and Rakyan, V.K. 2008. The methylome: approaches for global DNA methylation profiling. *Trends Genet* **24:** 231-237.

Benachi, A., Steffann, J., Gautier, E., Ernault, P., Olivi, M., Dumez, Y., and Costa, J.M. 2003. Fetal DNA in maternal serum: does it persist after pregnancy? *Hum Genet* **113:** 76-79.

Benn, P.A., Fang, M., Egan, J.F., Horne, D., and Collins, R. 2003. Incorporation of inhibin-A in second-trimester screening for Down syndrome. *Obstet Gynecol* **101:** 451-454.

Benn, P.A., Kaminsky, L.M., Ying, J., Borgida, A.F., and Egan, J.F. 2002. Combined second-trimester biochemical and ultrasound screening for Down syndrome. *Obstet Gynecol* **100:** 1168-1176.

Beroud, C., Karliova, M., Bonnefont, J.P., Benachi, A., Munnich, A., Dumez, Y., Lacour, B., and Paterlini-Brechot, P. 2003. Prenatal diagnosis of spinal muscular atrophy by genetic analysis of circulating fetal cells. *Lancet* **361:** 1013-1014.

Bianchi, D.W. 1998. Fetal DNA in maternal plasma: the plot thickens and the placental barrier thins. *Am J Hum Genet* **62:** 763-764.

Bianchi, D.W. 2000. Fetal cells in the mother: from genetic diagnosis to diseases associated with fetal cell microchimerism. *Eur J Obstet Gynecol Reprod Biol* **92:** 103-108.

Bianchi, D.W. 2004. Circulating fetal DNA: its origin and diagnostic potential-a review. *Placenta* **25 Suppl A:** S93-S101.

Bianchi, D.W., Flint, A.F., Pizzimenti, M.F., Knoll, J.H., and Latt, S.A. 1990. Isolation of fetal DNA from nucleated erythrocytes in maternal blood. *Proc Natl Acad Sci U S A* **87:** 3279-3283.

Bianchi, D.W., Simpson, J.L., Jackson, L.G., Elias, S., Holzgreve, W., Evans, M.I., Dukes, K.A., Sullivan, L.M., Klinger, K.W., Bischoff, F.Z. et al. 2002. Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data. National Institute of Child Health and Development Fetal Cell Isolation Study. *Prenat Diagn* **22:** 609-615.

Bianchi, D.W., Williams, J.M., Sullivan, L.M., Hanson, F.W., Klinger, K.W., and Shuber, A.P. 1997. PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. *Am J Hum Genet* **61:** 822-829.

Bianchi, D.W., Zickwolf, G.K., Weil, G.J., Sylvester, S., and DeMaria, M.A. 1996. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci U S A* **93:** 705-708.

Bischof, P., Meisser, A., and Campana, A. 2001. Biochemistry and molecular biology of trophoblast invasion. *Ann N Y Acad Sci* **943:** 157-162.

Bogic, L.V., Brace, R.A., and Cheung, C.Y. 2001. Developmental expression of vascular endothelial growth factor (VEGF) receptors and VEGF binding in ovine placenta and fetal membranes. *Placenta* **22:** 265-275.

Borrell, A., Casals, E., Fortuny, A., Farre, M.T., Gonce, A., Sanchez, A., Soler, A., Cararach, V., and Vanrell, J.A. 2004. First-trimester screening for trisomy 21 combining biochemistry and ultrasound at individually optimal gestational ages. An interventional study. *Prenat Diagn* **24:** 541-545.

Borzychowski, A.M., Sargent, I.L., and Redman, C.W. 2006. Inflammation and pre-eclampsia. *Semin Fetal Neonatal Med* **11:** 309-316.

Brenner, S., Johnson, M., Bridgham, J., Golda, G., Lloyd, D.H., Johnson, D., Luo, S., McCurdy, S., Foy, M., Ewan, M. et al. 2000. Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat Biotechnol* **18:** 630-634.

Brown, M.A., Hague, W.M., Higgins, J., Lowe, S., McCowan, L., Oats, J., Peek, M.J., Rowan, J.A., and Walters, B.N. 2000. The detection, investigation and management of hypertension in pregnancy: full consensus statement. *Aust N Z J Obstet Gynaecol* **40:** 139-155.

Callinan, P.A. and Feinberg, A.P. 2006. The emerging science of epigenomics. *Hum Mol Genet* **15 Spec No 1:** R95-101.

Causevic, M. and Mohaupt, M. 2007. 11beta-Hydroxysteroid dehydrogenase type 2 in pregnancy and preeclampsia. *Mol Aspects Med* **28:** 220-226.

Cawley, S., Bekiranov, S., Ng, H.H., Kapranov, P., Sekinger, E.A., Kampa, D., Piccolboni, A., Sementchenko, V., Cheng, J., Williams, A.J. et al. 2004. Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. *Cell* **116:** 499-509.

Chan, K.C.A., Ding, C., Gerovassili, A., Yeung, S.W., Chiu, R.W.K., Leung, T.N., Lau, T.K., Chim, S.S.C., Chung, G.T., Nicolaides, K.H. et al. 2006. Hypermethylated RASSF1A in maternal plasma: A universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. *Clin Chem* **52:** 2211-2218.

Chan, K.C.A., Zhang, J., Hui, A.B., Wong, N., Lau, T.K., Leung, T.N., Lo, K.W., Huang, D.W., and Lo, Y.M.D. 2004. Size distributions of maternal and fetal DNA in maternal plasma. *Clin Chem* **50:** 88-92.

Chan, L.Y., Leung, T.N., Chan, K.C.A., Tai, H.L., Lau, T.K., Wong, E.M., and Lo, Y.M.D. 2003. Serial analysis of fetal DNA concentrations in maternal plasma in late pregnancy. *Clin Chem* **49:** 678-680.

Chang, H.W., Lee, S.M., Goodman, S.N., Singer, G., Cho, S.K., Sokoll, L.J., Montz, F.J., Roden, R., Zhang, Z., Chan, D.W. et al. 2002. Assessment of plasma DNA levels, allelic imbalance, and CA 125 as diagnostic tests for cancer. *J Natl Cancer Inst* **94:** 1697-1703.

Chang, H.W. and Shih Ie, M. 2005. Digital single-nucleotide polymorphism analysis for allelic imbalance. *Methods Mol Med* **103:** 137-141.

Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Nguyen, J.T., Barbisin, M., Xu, N.L., Mahuvakar, V.R., Andersen, M.R. et al. 2005. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* **33:** e179.

Chen, X.Q., Stroun, M., Magnenat, J.L., Nicod, L.P., Kurt, A.M., Lyautey, J., Lederrey, C., and Anker, P. 1996. Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat Med* **2:** 1033-1035.

Cheung, H.H., Lee, T.L., Davis, A.J., Taft, D.H., Rennert, O.M., and Chan, W.Y. 2010. Genome-wide DNA methylation profiling reveals novel epigenetically regulated genes and non-coding RNAs in human testicular cancer. *Br J Cancer* **102:** 419-427.

Cheung, M.C., Goldberg, J.D., and Kan, Y.W. 1996. Prenatal diagnosis of sickle cell anaemia and thalassaemia by analysis of fetal cells in maternal blood. *Nat Genet* **14:** 264-268.

Chim, S.S.C., Jin, S., Lee, T.Y., Lun, F.M.F., Lee, W.S., Chan, L.Y., Jin, Y., Yang, N., Tong, Y.K., Leung, T.Y. et al. 2008. Systematic search for placental DNA-methylation markers on chromosome 21: toward a maternal plasma-based epigenetic test for fetal trisomy 21. *Clin Chem* **54:** 500-511.

Chim, S.S.C., Tong, Y.K., Chiu, R.W.K., Lau, T.K., Leung, T.N., Chan, L.Y., Oudejans, C.B., Ding, C., and Lo, Y.M.D. 2005. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. *Proc Natl Acad Sci U S A* **102:** 14753-14758.

Chiu, R.W.K., Cantor, C.R., and Lo, Y.M.D. 2009. Non-invasive prenatal diagnosis by single molecule counting technologies. *Trends Genet* **25:** 324-331.

Chiu, R.W.K., Chan, K.C.A., Gao, Y., Lau, V.Y., Zheng, W., Leung, T.Y., Foo, C.H., Xie, B., Tsui, N.B.Y., Lun, F.M.F. et al. 2008. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. *Proc Natl Acad Sci U S A* **105:** 20458-20463.

Chiu, R.W.K., Chim, S.S.C., Wong, I.H., Wong, C.S., Lee, W.S., To, K.F., Tong, J.H., Yuen, R.K., Shum, A.S., Chan, J.K. et al. 2007. Hypermethylation of RASSF1A in human and rhesus placentas. *Am J Pathol* **170:** 941-950.

Chiu, R.W.K., Lau, T.K., Cheung, P.T., Gong, Z.Q., Leung, T.N., and Lo, Y.M.D. 2002a. Noninvasive prenatal exclusion of congenital adrenal hyperplasia by maternal plasma analysis: a feasibility study. *Clin Chem* **48:** 778-780.

Chiu, R.W.K., Lau, T.K., Leung, T.N., Chow, K.C.K., Chui, D.H., and Lo, Y.M.D. 2002b. Prenatal exclusion of beta thalassaemia major by examination of maternal plasma. *Lancet* **360:** 998-1000.

Chiu, R.W.K., Poon, L.L.M., Lau, T.K., Leung, T.N., Wong, E.M., and Lo, Y.M.D. 2001. Effects of blood-processing protocols on fetal and total DNA quantification in maternal plasma. *Clin Chem* **47:** 1607-1613.

Chiu, R.W.K., Sun, H., Akolekar, R., Clouser, C., Lee, C., McKernan, K., Zhou, D., Nicolaides, K.H., and Lo, Y.M.D. 2010. Maternal plasma DNA analysis with massively parallel sequencing by ligation for noninvasive prenatal diagnosis of trisomy 21. *Clin Chem* **56:** 459-463.

Chow, K.C.K., Chiu, R.W.K., Tsui, N.B.Y., Ding, C., Lau, T.K., Leung, T.N., and Lo, Y.M.D. 2007. Mass spectrometric detection of an SNP panel as an internal positive control for fetal DNA analysis in maternal plasma. *Clin Chem* **53:** 141-142.

Christiansen, M. and Norgaard-Pedersen, B. 2005. Inhibin A is a maternal serum marker for Down's syndrome early in the first trimester. *Clin Genet* **68:** 35-39.

Chu, T., Burke, B., Bunce, K., Surti, U., Allen Hogge, W., and Peters, D.G. 2009. A microarray-based approach for the identification of epigenetic biomarkers for the noninvasive diagnosis of fetal disease. *Prenat Diagn* **29:** 1020-1030.

Chua, S., Wilkins, T., Sargent, I., and Redman, C. 1991. Trophoblast deportation in pre-eclamptic pregnancy. *Br J Obstet Gynaecol* **98:** 973-979.

Cirpan, T., Akercan, F., Terek, M.C., Kazandi, M., Ozcakir, H.T., Giray, G., and Sagol, S. 2007. Evaluation of VEGF in placental bed biopsies from preeclamptic women by immunohistochemistry. *Clin Exp Obstet Gynecol* **34:** 228-231.

Coolen, M.W., Statham, A.L., Gardiner-Garden, M., and Clark, S.J. 2007. Genomic profiling of CpG methylation and allelic specificity using quantitative high-throughput mass spectrometry: critical evaluation and improvements. *Nucleic Acids Res* **35:** e119.

Cooper, J.C., Sharkey, A.M., Charnock-Jones, D.S., Palmer, C.R., and Smith, S.K. 1996. VEGF mRNA levels in placentae from pregnancies complicated by pre-eclampsia. *Br J Obstet Gynaecol* **103:** 1191-1196.

Costa, J.M., Benachi, A., and Gautier, E. 2002. New strategy for prenatal diagnosis of X-linked disorders. *N Engl J Med* **346:** 1502.

Costa, J.M., Benachi, A., Gautier, E., Jouannic, J.M., Ernault, P., and Dumez, Y. 2001. First-trimester fetal sex determination in maternal serum using real-time PCR. *Prenat Diagn* **21:** 1070-1074.

Diebold, J., Arnholdt, H., Lai, M.D., and Lohrs, U. 1991. C-myc expression in early human placenta--a critical evaluation of its localization. *Virchows Arch B Cell Pathol Incl Mol Pathol* **61:** 65-73.

Diehl, F., Li, M., Dressman, D., He, Y., Shen, D., Szabo, S., Diaz, L.A., Jr., Goodman, S.N., David, K.A., Juhl, H. et al. 2005. Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc Natl Acad Sci U S A* **102:** 16368-16373.

DiFederico, E., Genbacev, O., and Fisher, S.J. 1999. Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. *Am J Pathol* **155:** 293-301.

Ding, C., Chiu, R.W., Lau, T.K., Leung, T.N., Chan, L.C., Chan, A.Y., Charoenkwan, P., Ng, I.S., Law, H.Y., Ma, E.S. et al. 2004a. MS analysis of single-nucleotide differences in circulating nucleic acids: Application to noninvasive prenatal diagnosis. *Proc Natl Acad Sci U S A* **101:** 10762-10767.

Ding, C., Chiu, R.W.K., Lau, T.K., Leung, T.N., Chan, L.C., Chan, A.Y., Charoenkwan, P., Ng, I.S., Law, H.Y., Ma, E.S. et al. 2004b. MS analysis of single-nucleotide differences in circulating nucleic acids: Application to noninvasive prenatal diagnosis. *Proc Natl Acad Sci U S A* **101:** 10762-10767.

Driscoll, D.A. and Gross, S. 2009. Clinical practice. Prenatal screening for aneuploidy. *N Engl J Med* **360:** 2556-2562.

Driscoll, D.J., Waters, M.F., Williams, C.A., Zori, R.T., Glenn, C.C., Avidano, K.M., and Nicholls, R.D. 1992. A DNA methylation imprint, determined by the sex of the parent, distinguishes the Angelman and Prader-Willi syndromes. *Genomics* **13:** 917-924.

Dungy, L.J., Siddiqi, T.A., and Khan, S. 1991. C-jun and jun-B oncogene expression during placental development. *Am J Obstet Gynecol* **165:** 1853-1856.

Ehrich, M., Nelson, M.R., Stanssens, P., Zabeau, M., Liloglou, T., Xinarianos, G., Cantor, C.R., Field, J.K., and van den Boom, D. 2005. Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc Natl Acad Sci U S A* **102:** 15785-15790.

Ehrlich, M. 2002. DNA methylation in cancer: too much, but also too little. *Oncogene* **21:** 5400-5413.

Emlen, W. and Mannik, M. 1984. Effect of DNA size and strandedness on the in vivo clearance and organ localization of DNA. *Clin Exp Immunol* **56:** 185-192.

Enquobahrie, D.A., Meller, M., Rice, K., Psaty, B.M., Siscovick, D.S., and Williams, M.A. 2008. Differential placental gene expression in preeclampsia. *Am J Obstet Gynecol* **199:** 566 e561-511.

Escudero, C. and Sobrevia, L. 2008. A hypothesis for preeclampsia: adenosine and inducible nitric oxide synthase in human placental microvascular endothelium. *Placenta* **29:** 469-483.

Evans, P.C., Lambert, N., Maloney, S., Furst, D.E., Moore, J.M., and Nelson, J.L. 1999. Long-term fetal microchimerism in peripheral blood mononuclear cell subsets in healthy women and women with scleroderma. *Blood* **93:** 2033-2037.

Faas, B.H., Beuling, E.A., Christiaens, G.C., von dem Borne, A.E., and van der Schoot, C.E. 1998. Detection of fetal RHD-specific sequences in maternal plasma. *Lancet* **352:** 1196.

Fan, H.C., Blumenfeld, Y.J., Chitkara, U., Hudgins, L., and Quake, S.R. 2008. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc Natl Acad Sci U S A* **105:** 16266-16271.

Farina, A., LeShane, E.S., Lambert-Messerlian, G.M., Canick, J.A., Lee, T., Neveux, L.M., Palomaki, G.E., and Bianchi, D.W. 2003. Evaluation of cell-free fetal DNA as a second-trimester maternal serum marker of Down syndrome pregnancy. *Clin Chem* **49:** 239-242.

Farina, A., LeShane, E.S., Romero, R., Gomez, R., Chaiworapongsa, T., Rizzo, N., and Bianchi, D.W. 2005. High levels of fetal cell-free DNA in maternal serum: a risk factor for spontaneous preterm delivery. *Am J Obstet Gynecol* **193:** 421-425.

Farina, A., Sekizawa, A., Iwasaki, M., Matsuoka, R., Ichizuka, K., and Okai, T. 2004a. Total cell-free DNA (beta-globin gene) distribution in maternal plasma at the second trimester: a new prospective for preeclampsia screening. *Prenat Diagn* **24:** 722-726.

Farina, A., Sekizawa, A., Sugito, Y., Iwasaki, M., Jimbo, M., Saito, H., and Okai, T. 2004b. Fetal DNA in maternal plasma as a screening variable for preeclampsia. A preliminary nonparametric analysis of detection rate in low-risk nonsymptomatic patients. *Prenat Diagn* **24:** 83-86.

Fasco, M.J., Treanor, C.P., Spivack, S., Figge, H.L., and Kaminsky, L.S. 1995. Quantitative RNA-polymerase chain reaction-DNA analysis by capillary electrophoresis and laser-induced fluorescence. *Anal Biochem* **224:** 140-147.

Fatouros, I.G., Destouni, A., Margonis, K., Jamurtas, A.Z., Vrettou, C., Kouretas, D., Mastorakos, G., Mitrakou, A., Taxildaris, K., Kanavakis, E. et al. 2006. Cell-free plasma DNA as a novel marker of aseptic inflammation severity related to exercise overtraining. *Clin Chem* **52:** 1820-1824.

Faxen, M., Nisell, H., and Kublickiene, K.R. 2001. Altered mRNA expression of ecNOS and iNOS in myometrium and placenta from women with preeclampsia. *Arch Gynecol Obstet* **265:** 45-50.

Fazzari, M.J. and Greally, J.M. 2004. Epigenomics: beyond CpG islands. *Nat Rev Genet* **5:** 446-455.

Ferguson-Smith, A.C., Moore, T., Detmar, J., Lewis, A., Hemberger, M., Jammes, H., Kelsey, G., Roberts, C.T., Jones, H., and Constancia, M. 2006. Epigenetics and imprinting of the trophoblast -- a workshop report. *Placenta* **27 Suppl A:** S122-126.

Finning, K., Martin, P., Summers, J., Massey, E., Poole, G., and Daniels, G. 2008. Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study. *BMJ* **336:** 816-818.

Finning, K.M., Martin, P.G., Soothill, P.W., and Avent, N.D. 2002. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion* **42:** 1079-1085.

Flori, E., Doray, B., Gautier, E., Kohler, M., Ernault, P., Flori, J., and Costa, J.M. 2004. Circulating cell-free fetal DNA in maternal serum appears to originate from cyto- and syncytio-trophoblastic cells. Case report. *Hum Reprod* **19:** 723-724.

Founds, S.A., Conley, Y.P., Lyons-Weiler, J.F., Jeyabalan, A., Hogge, W.A., and Conrad, K.P. 2009. Altered global gene expression in first trimester placentas of women destined to develop preeclampsia. *Placenta* **30:** 15-24.

Fournie, G.J., Courtin, J.P., Laval, F., Chale, J.J., Pourrat, J.P., Pujazon, M.C., Lauque, D., and Carles, P. 1995. Plasma DNA as a marker of cancerous cell death. Investigations in patients suffering from lung cancer and in nude mice bearing human tumours. *Cancer Lett* **91:** 221-227.

Fournie, G.J., Martres, F., Pourrat, J.P., Alary, C., and Rumeau, M. 1993. Plasma DNA as cell death marker in elderly patients. *Gerontology* **39:** 215-221.

Frazier, M.L., Xi, L., Zong, J., Viscofsky, N., Rashid, A., Wu, E.F., Lynch, P.M., Amos, C.I., and Issa, J.P. 2003. Association of the CpG island methylator phenotype with family history of cancer in patients with colorectal cancer. *Cancer Res* **63:** 4805-4808.

Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L., and Paul, C.L. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A* **89:** 1827-1831.

Fuke, C., Shimabukuro, M., Petronis, A., Sugimoto, J., Oda, T., Miura, K., Miyazaki, T., Ogura, C., Okazaki, Y., and Jinno, Y. 2004. Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study. *Ann Hum Genet* **68:** 196-204.

Gama-Sosa, M.A., Midgett, R.M., Slagel, V.A., Githens, S., Kuo, K.C., Gehrke, C.W., and Ehrlich, M. 1983. Tissue-specific differences in DNA methylation in various mammals. *Biochim Biophys Acta* **740:** 212-219.

Gannage, M., Amoura, Z., Lantz, O., Piette, J.C., and Caillat-Zucman, S. 2002. Feto-maternal microchimerism in connective tissue diseases. *Eur J Immunol* **32:** 3405-3413.

Germain, S.J., Sacks, G.P., Sooranna, S.R., Sargent, I.L., and Redman, C.W. 2007. Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles. *J Immunol* **178:** 5949-5956.

Gheorghe, C.P., Goyal, R., Mittal, A., and Longo, L.D. 2010. Gene expression in the placenta: maternal stress and epigenetic responses. *Int J Dev Biol* **54:** 507-523.

Gitan, R.S., Shi, H., Chen, C.M., Yan, P.S., and Huang, T.H. 2002. Methylation-specific oligonucleotide microarray: a new potential for high-throughput methylation analysis. *Genome Res* **12:** 158-164.

Glish, G.L. and Vachet, R.W. 2003. The basics of mass spectrometry in the twenty-first century. *Nat Rev Drug Discov* **2:** 140-150.

Grunau, C., Clark, S.J., and Rosenthal, A. 2001. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucleic Acids Res* **29:** E65-65.

Gu, H., Bock, C., Mikkelsen, T.S., Jager, N., Smith, Z.D., Tomazou, E., Gnirke, A., Lander, E.S., and Meissner, A. Genome-scale DNA methylation mapping of clinical samples at single-nucleotide resolution. *Nat Methods* **7:** 133-136.

Gutierrez, M.I., Siraj, A.K., Khaled, H., Koon, N., El-Rifai, W., and Bhatia, K. 2004. CpG island methylation in Schistosoma- and non-Schistosoma-associated bladder cancer. *Mod Pathol* **17:** 1268-1274.

Hamai, Y., Fujii, T., Yamashita, T., Kozuma, S., Okai, T., and Taketani, Y. 1998. Evidence for basic fibroblast growth factor as a crucial angiogenic growth factor, released from human trophoblasts during early gestation. *Placenta* **19:** 149-155.

Han, J.Y., Kim, Y.S., Cho, G.J., Roh, G.S., Kim, H.J., Choi, W.J., Paik, W.Y., Rho, G.J., Kang, S.S., and Choi, W.S. 2006. Altered gene expression of caspase-10, death receptor-3 and IGFBP-3 in preeclamptic placentas. *Mol Cells* **22:** 168-174.

Hanson, M.A. and Gluckman, P.D. 2005. Developmental processes and the induction of cardiovascular function: conceptual aspects. *J Physiol* **565:** 27-34.

Hardy, D.B. and Yang, K. 2002. The expression of 11 beta-hydroxysteroid dehydrogenase type 2 is induced during trophoblast differentiation: effects of hypoxia. *J Clin Endocrinol Metab* **87:** 3696-3701.

Harrison, K.J., Barrett, I.J., Lomax, B.L., Kuchinka, B.D., and Kalousek, D.K. 1993. Detection of confined placental mosaicism in trisomy 18 conceptions using interphase cytogenetic analysis. *Hum Genet* **92:** 353-358.

Hatada, I., Fukasawa, M., Kimura, M., Morita, S., Yamada, K., Yoshikawa, T., Yamanaka, S., Endo, C., Sakurada, A., Sato, M. et al. 2006. Genome-wide profiling of promoter methylation in human. *Oncogene* **25:** 3059-3064.

Heid, C.A., Stevens, J., Livak, K.J., and Williams, P.M. 1996. Real time quantitative PCR. *Genome Res* **6:** 986-994.

Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D., and Baylin, S.B. 1996. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* **93:** 9821-9826.

Herzenberg, L.A., Bianchi, D.W., Schroder, J., Cann, H.M., and Iverson, G.M. 1979. Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence-activated cell sorting. *Proc Natl Acad Sci U S A* **76:** 1453-1455.

Hesson, L.B., Cooper, W.N., and Latif, F. 2007. The role of RASSF1A methylation in cancer. *Dis Markers* **23:** 73-87.

Higuchi, R., Fockler, C., Dollinger, G., and Watson, R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y)* **11:** 1026-1030.

Holland, P.M., Abramson, R.D., Watson, R., and Gelfand, D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci U S A* **88:** 7276-7280.

Holliday, R. and Grigg, G.W. 1993. DNA methylation and mutation. *Mutat Res* **285:** 61-67.

Holzgreve, W., Ghezzi, F., Di Naro, E., Ganshirt, D., Maymon, E., and Hahn, S. 1998. Disturbed feto-maternal cell traffic in preeclampsia. *Obstet Gynecol* **91:** 669-672.

Honda, H., Miharu, N., Ohashi, Y., Samura, O., Kinutani, M., Hara, T., and Ohama, K. 2002. Fetal gender determination in early pregnancy through qualitative and quantitative analysis of fetal DNA in maternal serum. *Hum Genet* **110:** 75-79.

Houfflin-Debarge, V., O'Donnell, H., Overton, T., Bennett, P.R., and Fisk, N.M. 2000. High sensitivity of fetal DNA in plasma compared to serum and nucleated cells using unnested PCR in maternal blood. *Fetal Diagn Ther* **15:** 102-107.

Huppertz, B., Frank, H.G., Kingdom, J.C., Reister, F., and Kaufmann, P. 1998. Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. *Histochem Cell Biol* **110:** 495-508.

Iafrate, A.J., Feuk, L., Rivera, M.N., Listewnik, M.L., Donahoe, P.K., Qi, Y., Scherer, S.W., and Lee, C. 2004. Detection of large-scale variation in the human genome. *Nat Genet* **36:** 949-951.

Imudia, A.N., Kilburn, B.A., Petkova, A., Edwin, S.S., Romero, R., and Armant, D.R. 2008. Expression of heparin-binding EGF-like growth factor in term chorionic villous explants and its role in trophoblast survival. *Placenta* **29:** 784-789.

Invernizzi, P., Biondi, M.L., Battezzati, P.M., Perego, F., Selmi, C., Cecchini, F., Podda, M., and Simoni, G. 2002. Presence of fetal DNA in maternal plasma decades after pregnancy. *Hum Genet* **110:** 587-591.

Ishkanian, A.S., Malloff, C.A., Watson, S.K., DeLeeuw, R.J., Chi, B., Coe, B.P., Snijders, A., Albertson, D.G., Pinkel, D., Marra, M.A. et al. 2004. A tiling resolution DNA microarray with complete coverage of the human genome. *Nat Genet* **36:** 299-303.

Janatpour, M.J., Utset, M.F., Cross, J.C., Rossant, J., Dong, J., Israel, M.A., and Fisher, S.J. 1999. A repertoire of differentially expressed transcription factors that offers insight into mechanisms of human cytotrophoblast differentiation. *Dev Genet* **25:** 146-157.

Janneau, J.L., Maldonado-Estrada, J., Tachdjian, G., Miran, I., Motte, N., Saulnier, P., Sabourin, J.C., Cote, J.F., Simon, B., Frydman, R. et al. 2002. Transcriptional expression of genes involved in cell invasion and migration by normal and tumoral trophoblast cells. *J Clin Endocrinol Metab* **87:** 5336-5339.

Johnson-Hopson, C.N. and Artlett, C.M. 2002. Evidence against the long-term persistence of fetal DNA in maternal plasma after pregnancy. *Hum Genet* **111:** 575.

Johnson, W.E., Li, W., Meyer, C.A., Gottardo, R., Carroll, J.S., Brown, M., and Liu, X.S. 2006. Model-based analysis of tiling-arrays for ChIP-chip. *Proc Natl Acad Sci U S A* **103:** 12457-12462.

Jokhi, P.P., King, A., and Loke, Y.W. 1994. Reciprocal expression of epidermal growth factor receptor (EGF-R) and c-erbB2 by non-invasive and invasive human trophoblast populations. *Cytokine* **6:** 433-442.

Jones, P.A. 2002. DNA methylation and cancer. *Oncogene* **21:** 5358-5360.

Jones, P.A. and Baylin, S.B. 2002. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* **3:** 415-428.

Jones, P.A. and Baylin, S.B. 2007. The epigenomics of cancer. *Cell* **128:** 683-692.

Jones, P.A. and Takai, D. 2001. The role of DNA methylation in mammalian epigenetics. *Science* **293:** 1068-1070.

Kalinina, O., Lebedeva, I., Brown, J., and Silver, J. 1997. Nanoliter scale PCR with TaqMan detection. *Nucleic Acids Res* **25:** 1999-2004.

Kalousek, D.K., Barrett, I.J., and McGillivray, B.C. 1989. Placental mosaicism and intrauterine survival of trisomies 13 and 18. *Am J Hum Genet* **44:** 338-343.

Kellogg, D.E., Sninsky, J.J., and Kwok, S. 1990. Quantitation of HIV-1 proviral DNA relative to cellular DNA by the polymerase chain reaction. *Anal Biochem* **189:** 202-208.

Khraiwesh, B., Arif, M.A., Seumel, G.I., Ossowski, S., Weigel, D., Reski, R., and Frank, W. Transcriptional control of gene expression by microRNAs. *Cell* **140:** 111-122.

Khulan, B., Thompson, R.F., Ye, K., Fazzari, M.J., Suzuki, M., Stasiek, E., Figueroa, M.E., Glass, J.L., Chen, Q., Montagna, C. et al. 2006. Comparative isoschizomer profiling of cytosine methylation: the HELP assay. *Genome Res* **16:** 1046-1055.

Klump, B., Hsieh, C.J., Nehls, O., Dette, S., Holzmann, K., Kiesslich, R., Jung, M., Sinn, U., Ortner, M., Porschen, R. et al. 2003. Methylation status of p14ARF and p16INK4a as detected in pancreatic secretions. *Br J Cancer* **88:** 217-222.

Koffler, D., Agnello, V., Winchester, R., and Kunkel, H.G. 1973. The occurrence of single-stranded DNA in the serum of patients with systemic lupus erythematosus and other diseases. *J Clin Invest* **52:** 198-204.

Laird, P.W. 2005. Cancer epigenetics. *Hum Mol Genet* **14 Spec No 1:** R65-76.

Lambert, N. and Nelson, J.L. 2003. Microchimerism in autoimmune disease: more questions than answers? *Autoimmun Rev* **2:** 133-139.

Lau, T.W., Leung, T.N., Chan, L.Y., Lau, T.K., Chan, K.C.A., Tam, W.H., and Lo, Y.M.D. 2002. Fetal DNA clearance from maternal plasma is impaired in preeclampsia. *Clin Chem* **48:** 2141-2146.

Lazar, L., Rigo, J., Jr., Nagy, B., Balogh, K., Mako, V., Cervenak, L., Mezes, M., Prohaszka, Z., and Molvarec, A. 2009. Relationship of circulating cell-free DNA levels to cell-free fetal DNA levels, clinical characteristics and laboratory parameters in preeclampsia. *BMC Med Genet* **10:** 120.

Leach, R.E., Khalifa, R., Ramirez, N.D., Das, S.K., Wang, J., Dey, S.K., Romero, R., and Armant, D.R. 1999. Multiple roles for heparin-binding epidermal growth factor-like growth factor are suggested by its cell-specific expression during the human endometrial cycle and early placentation. *J Clin Endocrinol Metab* **84:** 3355-3363.

Leach, R.E., Romero, R., Kim, Y.M., Chaiworapongsa, T., Kilburn, B., Das, S.K., Dey, S.K., Johnson, A., Qureshi, F., Jacques, S. et al. 2002. Pre-eclampsia and expression of heparin-binding EGF-like growth factor. *Lancet* **360:** 1215-1219.

Lee, T., LeShane, E.S., Messerlian, G.M., Canick, J.A., Farina, A., Heber, W.W., and Bianchi, D.W. 2002. Down syndrome and cell-free fetal DNA in archived maternal serum. *Am J Obstet Gynecol* **187:** 1217-1221.

Leon, S.A., Shapiro, B., Sklaroff, D.M., and Yaros, M.J. 1977. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* **37:** 646-650.

Leung, D.N., Smith, S.C., To, K.F., Sahota, D.S., and Baker, P.N. 2001a. Increased placental apoptosis in pregnancies complicated by preeclampsia. *Am J Obstet Gynecol* **184:** 1249-1250.

Leung, T.N., Zhang, J., Lau, T.K., Chan, L.Y., and Lo, Y.M.D. 2001b. Increased maternal plasma fetal DNA concentrations in women who eventually develop preeclampsia. *Clin Chem* **47:** 137-139.

Leung, T.N., Zhang, J., Lau, T.K., Hjelm, N.M., and Lo, Y.M.D. 1998. Maternal plasma fetal DNA as a marker for preterm labour. *Lancet* **352:** 1904-1905.

Levine, R.J., Qian, C., Leshane, E.S., Yu, K.F., England, L.J., Schisterman, E.F., Wataganara, T., Romero, R., and Bianchi, D.W. 2004. Two-stage elevation of cell-free fetal DNA in maternal sera before onset of preeclampsia. *Am J Obstet Gynecol* **190:** 707-713.

Li, E., Beard, C., and Jaenisch, R. 1993. Role for DNA methylation in genomic imprinting. *Nature* **366:** 362-365.

Li, L.C. and Dahiya, R. 2002. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* **18:** 1427-1431.

Li, M., Chen, W.D., Papadopoulos, N., Goodman, S.N., Bjerregaard, N.C., Laurberg, S., Levin, B., Juhl, H., Arber, N., Moinova, H. et al. 2009. Sensitive digital quantification of DNA methylation in clinical samples. *Nat Biotechnol* **27:** 858-863.

Li, M., Diehl, F., Dressman, D., Vogelstein, B., and Kinzler, K.W. 2006. BEAMing up for detection and quantification of rare sequence variants. *Nat Methods* **3:** 95-97.

Li, N., Ye, M., Li, Y., Yan, Z., Butcher, L.M., Sun, J., Han, X., Chen, Q., Zhang, X., and Wang, J. 2010. Whole genome DNA methylation analysis based on high throughput sequencing technology. *Methods*.

Li, Y., Di Naro, E., Vitucci, A., Zimmermann, B., Holzgreve, W., and Hahn, S. 2005. Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. *JAMA* **293:** 843-849.

Lichtenstein, A.V., Melkonyan, H.S., Tomei, L.D., and Umansky, S.R. 2001. Circulating nucleic acids and apoptosis. *Ann N Y Acad Sci* **945:** 239-249.

Lindheimer, M.D. and Umans, J.G. 2006. Explaining and predicting preeclampsia. *N Engl J Med* **355:** 1056-1058.

Lippman, Z., Gendrel, A.V., Colot, V., and Martienssen, R. 2005. Profiling DNA methylation patterns using genomic tiling microarrays. *Nat Methods* **2:** 219-224.

Lister, R., Pelizzola, M., Dowen, R.H., Hawkins, R.D., Hon, G., Tonti-Filippini, J., Nery, J.R., Lee, L., Ye, Z., Ngo, Q.M. et al. 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* **462:** 315-322.

Lo, Y.M.D. 2009. Noninvasive prenatal detection of fetal chromosomal aneuploidies by maternal plasma nucleic acid analysis: a review of the current state of the art. *BJOG* **116:** 152-157.

Lo, Y.M.D. and Chan, K.C.A. 2006. Setting up a polymerase chain reaction laboratory. *Methods Mol Biol* **336:** 11-18.

Lo, Y.M.D., Corbetta, N., Chamberlain, P.F., Rai, V., Sargent, I.L., Redman, C.W., and Wainscoat, J.S. 1997. Presence of fetal DNA in maternal plasma and serum. *Lancet* **350:** 485-487.

Lo, Y.M.D., Hjelm, N.M., Fidler, C., Sargent, I.L., Murphy, M.F., Chamberlain, P.F., Poon, P.M., Redman, C.W., and Wainscoat, J.S. 1998a. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* **339:** 1734-1738.

Lo, Y.M.D., Lau, T.K., Zhang, J., Leung, T.N., Chang, A.M., Hjelm, N.M., Elmes, R.S., and Bianchi, D.W. 1999a. Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. *Clin Chem* **45:** 1747-1751.

Lo, Y.M.D., Leung, T.N., Tein, M.S., Sargent, I.L., Zhang, J., Lau, T.K., Haines, C.J., and Redman, C.W. 1999b. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. *Clin Chem* **45:** 184-188.

Lo, Y.M.D., Lo, E.S., Watson, N., Noakes, L., Sargent, I.L., Thilaganathan, B., and Wainscoat, J.S. 1996. Two-way cell traffic between mother and fetus: biologic and clinical implications. *Blood* **88:** 4390-4395.

Lo, Y.M.D., Lun, F.M., Chan, K.C.A., Tsui, N.B.Y., Chong, K.C., Lau, T.K., Leung, T.Y., Zee, B.C., Cantor, C.R., and Chiu, R.W. 2007a. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. *Proc Natl Acad Sci U S A* **104:** 13116-13121.

Lo, Y.M.D., Lun, F.M.F., Chan, K.C.A., Tsui, N.B.Y., Chong, K.C., Lau, T.K., Leung, T.Y., Zee, B.C., Cantor, C.R., and Chiu, R.W. 2007b. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. *Proc Natl Acad Sci U S A* **104:** 13116-13121.

Lo, Y.M.D., Tein, M.S., Lau, T.K., Haines, C.J., Leung, T.N., Poon, P.M., Wainscoat, J.S., Johnson, P.J., Chang, A.M., and Hjelm, N.M. 1998b. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* **62:** 768-775.

Lo, Y.M.D., Tsui, N.B.Y., Chiu, R.W.K., Lau, T.K., Leung, T.N., Heung, M.M., Gerovassili, A., Jin, Y., Nicolaides, K.H., Cantor, C.R. et al. 2007c. Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. *Nat Med* **13:** 218-223.

Lo, Y.M.D., Wong, I.H., Zhang, J., Tein, M.S., Ng, M.H., and Hjelm, N.M. 1999c. Quantitative analysis of aberrant p16 methylation using real-time quantitative methylation-specific polymerase chain reaction. *Cancer Res* **59:** 3899-3903.

Lo, Y.M.D., Zhang, J., Leung, T.N., Lau, T.K., Chang, A.M., and Hjelm, N.M. 1999d. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* **64:** 218-224.

Loncar, J., Barnabei, V.M., and Larsen, J.W., Jr. 1995. Advent of maternal serum markers for Down syndrome screening. *Obstet Gynecol Surv* **50:** 316-320.

Longo, M.C., Berninger, M.S., and Hartley, J.L. 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* **93:** 125-128.

Lui, Y.Y., Chik, K.W., Chiu, R.W.K., Ho, C.Y., Lam, C.W., and Lo, Y.M.D. 2002a. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. *Clin Chem* **48:** 421-427.

Lui, Y.Y. and Dennis, Y.M. 2002b. Circulating DNA in plasma and serum: biology, preanalytical issues and diagnostic applications. *Clin Chem Lab Med* **40:** 962-968.

Lun, F.M.F., Chiu, R.W.K., Allen Chan, K.C.A., Yeung Leung, T., Kin Lau, T., and Dennis Lo, Y.M.D. 2008a. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. *Clin Chem* **54:** 1664-1672.

Lun, F.M.F., Tsui, N.B.Y., Chan, K.C.A., Leung, T.Y., Lau, T.K., Charoenkwan, P., Chow, K.C.K., Lo, W.Y., Wanapirak, C., Sanguansermsri, T. et al. 2008b. Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. *Proc Natl Acad Sci U S A* **105:** 19920-19925.

Maccani, M.A. and Marsit, C.J. 2009. Epigenetics in the placenta. *Am J Reprod Immunol* **62:** 78-89.

Malone, F.D., Canick, J.A., Ball, R.H., Nyberg, D.A., Comstock, C.H., Bukowski, R., Berkowitz, R.L., Gross, S.J., Dugoff, L., Craigo, S.D. et al. 2005. First-trimester or second-trimester screening, or both, for Down's syndrome. *N Engl J Med* **353:** 2001-2011.

Maloney, S., Smith, A., Furst, D.E., Myerson, D., Rupert, K., Evans, P.C., and Nelson, J.L. 1999. Microchimerism of maternal origin persists into adult life. *J Clin Invest* **104:** 41-47.

Martin, D.C., Fowlkes, J.L., Babic, B., and Khokha, R. 1999. Insulin-like growth factor II signaling in neoplastic proliferation is blocked by transgenic expression of the metalloproteinase inhibitor TIMP-1. *J Cell Biol* **146:** 881-892.

Masuzaki, H., Miura, K., Yoshiura, K.I., Yoshimura, S., Niikawa, N., and Ishimaru, T. 2004. Detection of cell free placental DNA in maternal plasma: direct evidence from three cases of confined placental mosaicism. *J Med Genet* **41:** 289-292.

McClelland, M., Nelson, M., and Raschke, E. 1994. Effect of site-specific modification on restriction endonucleases and DNA modification methyltransferases. *Nucleic Acids Res* **22:** 3640-3659.

Mendel, P. and Metais P. 1948. Les acides nucleiques du plasma sanguine chez l'homme. *C. R. Acad. Sci. Paris* **142**: 241–243.

Michell, N.P., Langman, M.J., and Eggo, M.C. 1997. Insulin-like growth factors and their binding proteins in human colonocytes: preferential degradation of insulin-like growth factor binding protein 2 in colonic cancers. *Br J Cancer* **76:** 60-66.

Mockler, T.C., Chan, S., Sundaresan, A., Chen, H., Jacobsen, S.E., and Ecker, J.R. 2005. Applications of DNA tiling arrays for whole-genome analysis. *Genomics* **85:** 1-15.

Mohn, F., Weber, M., Schubeler, D., and Roloff, T.C. 2009. Methylated DNA immunoprecipitation (MeDIP). *Methods Mol Biol* **507:** 55-64.

Mulder, J., McKinney, N., Christopherson, C., Sninsky, J., Greenfield, L., and Kwok, S. 1994. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. *J Clin Microbiol* **32:** 292-300.

Murata, M., Kodama, H., Goto, K., Hirano, H., and Tanaka, T. 1996. Decreased very-low-density lipoprotein and low-density lipoprotein receptor messenger ribonucleic acid expression in placentas from preeclamptic pregnancies. *Am J Obstet Gynecol* **175:** 1551-1556.

Nawroz, H., Koch, W., Anker, P., Stroun, M., and Sidransky, D. 1996. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med* **2:** 1035-1037.

Nelson, J.L. 1999. Microchimerism: implications for autoimmune disease. *Lupus* **8:** 370-374.

Ng, E.K., Tsui, N.B.Y., Lau, T.K., Leung, T.N., Chiu, R.W.K., Panesar, N.S., Lit, L.C., Chan, K.W., and Lo, Y.M.D. 2003. mRNA of placental origin is readily detectable in maternal plasma. *Proc Natl Acad Sci U S A* **100:** 4748-4753.

Novakovic, B., Rakyan, V., Ng, H.K., Manuelpillai, U., Dewi, C., Wong, N.C., Morley, R., Down, T., Beck, S., Craig, J.M. et al. 2008. Specific tumour-associated methylation in normal human term placenta and first-trimester cytotrophoblasts. *Mol Hum Reprod* **14:** 547-554.

Novakovic, B., Sibson, M., Ng, H.K., Manuelpillai, U., Rakyan, V., Down, T., Beck, S., Fournier, T., Evain-Brion, D., Dimitriadis, E. et al. 2009. Placenta-specific methylation of the vitamin D 24-hydroxylase gene: implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface. *J Biol Chem* **284:** 14838-14848.

Novakovic, B., Wong, N.C., Sibson, M., Ng, H.K., Morley, R., Manuelpillai, U., Down, T., Rakyan, V.K., Beck, S., Hiendleder, S. et al. 2010. DNA methylation-mediated down-regulation of DNA methyltransferase-1 (DNMT1) is coincident with, but not essential for, global hypomethylation in human placenta. *J Biol Chem* **285:** 9583-9593.

Ohlsson, R. 1989. Growth factors, protooncogenes and human placental development. *Cell Differ Dev* **28:** 1-15.

Old, R.W., Crea, F., Puszyk, W., and Hulten, M.A. 2007. Candidate epigenetic biomarkers for non-invasive prenatal diagnosis of Down syndrome. *Reprod Biomed Online* **15:** 227-235.

Papageorgiou, E.A., Fiegler, H., Rakyan, V., Beck, S., Hulten, M., Lamnissou, K., Carter, N.P., and Patsalis, P.C. 2009. Sites of differential DNA methylation between placenta and peripheral blood: molecular markers for noninvasive prenatal diagnosis of aneuploidies. *Am J Pathol* **174:** 1609-1618.

Park, J.S., Namkoong, S.E., Lee, H.Y., Kim, S.J., Hong, K.J., Kim, I.S., Kim, K.U., and Shim, B.S. 1992. Expression and amplification of cellular oncogenes in human developing placenta and neoplastic trophoblastic tissue. *Asia Oceania J Obstet Gynaecol* **18:** 57-64.

Poon, L.L.M., Leung, T.N., Lau, T.K., Chow, K.C.K., and Lo, Y.M.D. 2002. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. *Clin Chem* **48:** 35-41.

Prat, M., Narsimhan, R.P., Crepaldi, T., Nicotra, M.R., Natali, P.G., and Comoglio, P.M. 1991. The receptor encoded by the human c-MET oncogene is expressed in hepatocytes, epithelial cells and solid tumors. *Int J Cancer* **49:** 323-328.

Ragoussis, J., Elvidge, G.P., Kaur, K., and Colella, S. 2006. Matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry in genomics research. *PLoS Genet* **2:** e100.

Redman, C.W., Sacks, G.P., and Sargent, I.L. 1999. Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol* **180:** 499-506.

Redman, C.W. and Sargent, I.L. 2003. Pre-eclampsia, the placenta and the maternal systemic inflammatory response--a review. *Placenta* **24 Suppl A:** S21-27.

Redman, C.W. and Sargent, I.L. 2005. Latest advances in understanding preeclampsia. *Science* **308:** 1592-1594.

Roberts, J.M. and Cooper, D.W. 2001. Pathogenesis and genetics of pre-eclampsia. *Lancet* **357:** 53-56.

Roberts, J.M. and Gammill, H.S. 2005. Preeclampsia: recent insights. *Hypertension* **46:** 1243-1249.

Roncalli, M., Bulfamante, G., Viale, G., Springall, D.R., Alfano, R., Comi, A., Maggioni, M., Pollak, J.M., and Coggi, G. 1994. C-myc and tumour suppressor gene product expression in developing and term human trophoblast. *Placenta* **15:** 399-409.

Saito, H., Sekizawa, A., Morimoto, T., Suzuki, M., and Yanaihara, T. 2000. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. *Lancet* **356:** 1170.

Saito, Y. and Jones, P.A. 2006. Epigenetic activation of tumor suppressor microRNAs in human cancer cells. *Cell Cycle* **5:** 2220-2222.

Sane, D.C., Anton, L., and Brosnihan, K.B. 2004. Angiogenic growth factors and hypertension. *Angiogenesis* **7:** 193-201.

Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74:** 5463-5467.

Schneid, H., Seurin, D., Vazquez, M.P., Gourmelen, M., Cabrol, S., and Le Bouc, Y. 1993. Parental allele specific methylation of the human insulin-like growth factor II gene and Beckwith-Wiedemann syndrome. *J Med Genet* **30:** 353-362.

Schuster, S.C. 2008. Next-generation sequencing transforms today's biology. *Nat Methods* **5:** 16-18.

Sekizawa, A., Jimbo, M., Saito, H., Iwasaki, M., Sugito, Y., Yukimoto, Y., Otsuka, J., and Okai, T. 2002. Increased cell-free fetal DNA in plasma of two women with invasive placenta. *Clin Chem* **48:** 353-354.

Sekizawa, A. and Saito, H. 2001a. Prenatal screening of single-gene disorders from maternal blood. *Am J Pharmacogenomics* **1:** 111-117.

Sekizawa, A., Sugito, Y., Iwasaki, M., Watanabe, A., Jimbo, M., Hoshi, S., Saito, H., and Okai, T. 2001b. Cell-free fetal DNA is increased in plasma of women with hyperemesis gravidarum. *Clin Chem* **47:** 2164-2165.

Selvaggi, L., Ribatti, D., Loverro, G., Sorino, S., Greco, P., and Roncali, L. 1995. Angiogenesis in pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol* **59:** 83-89.

Sharkey, A.M., Jokhi, P.P., King, A., Loke, Y.W., Brown, K.D., and Smith, S.K. 1994. Expression of c-kit and kit ligand at the human maternofetal interface. *Cytokine* **6:** 195-205.

Sibai, B., Dekker, G., and Kupferminc, M. 2005. Pre-eclampsia. *Lancet* **365:** 785-799.

Sibai, B.M. 2003. Diagnosis and management of gestational hypertension and preeclampsia. *Obstet Gynecol* **102:** 181-192.

Sirchia, S.M., Ferguson, A.T., Sironi, E., Subramanyan, S., Orlandi, R., Sukumar, S., and Sacchi, N. 2000. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. *Oncogene* **19:** 1556-1563.

Smid, M., Galbiati, S., Vassallo, A., Gambini, D., Ferrari, A., Viora, E., Pagliano, M., Restagno, G., Ferrari, M., and Cremonesi, L. 2003. No evidence of fetal DNA persistence in maternal plasma after pregnancy. *Hum Genet* **112:** 617-618.

Smid, M., Lagona, F., de Benassuti, L., Ferrari, A., Ferrari, M., and Cremonesi, L. 1999. Evaluation of different approaches for fetal DNA analysis from maternal plasma and nucleated blood cells. *Clin Chem* **45:** 1570-1572.

Soleymanlou, N., Jurisica, I., Nevo, O., Ietta, F., Zhang, X., Zamudio, S., Post, M., and Caniggia, I. 2005. Molecular evidence of placental hypoxia in preeclampsia. *J Clin Endocrinol Metab* **90:** 4299-4308.

Somerset, D.A., Kilby, M.D., Strain, A.J., and Afford, S. 1998. Differential expression of HGF and Met in human placenta. *J Clin Endocrinol Metab* **83:** 1400-1401.

Sorenson, G.D., Pribish, D.M., Valone, F.H., Memoli, V.A., Bzik, D.J., and Yao, S.L. 1994. Soluble normal and mutated DNA sequences from single-copy genes in human blood. *Cancer Epidemiol Biomarkers Prev* **3:** 67-71.

Soundararajan, R. and Rao, A.J. 2004. Trophoblast 'pseudo-tumorigenesis': significance and contributory factors. *Reprod Biol Endocrinol* **2:** 15.

Spencer, K. 1999. Second trimester prenatal screening for Down's syndrome using alpha-fetoprotein and free beta hCG: a seven year review. *Br J Obstet Gynaecol* **106:** 1287-1293.

Spugnardi, M., Tommasi, S., Dammann, R., Pfeifer, G.P., and Hoon, D.S. 2003. Epigenetic inactivation of RAS association domain family protein 1 (RASSF1A) in malignant cutaneous melanoma. *Cancer Res* **63:** 1639-1643.

Stepan, H., Faber, R., Dornhofer, N., Huppertz, B., Robitzki, A., and Walther, T. 2006. New insights into the biology of preeclampsia. *Biol Reprod* **74:** 772-776.

Stewart, F.J., Panne, D., Bickle, T.A., and Raleigh, E.A. 2000. Methyl-specific DNA binding by McrBC, a modification-dependent restriction enzyme. *J Mol Biol* **298:** 611-622.

Strickland, S. and Richards, W.G. 1992. Invasion of the trophoblasts. *Cell* **71:** 355-357.

Stroun, M., Anker, P., Maurice, P., and Gahan, P.B. 1977. Circulating nucleic acids in higher organisms. *Int Rev Cytol* **51:** 1-48.

Stroun, M., Anker, P., Maurice, P., Lyautey, J., Lederrey, C., and Beljanski, M. 1989. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* **46:** 318-322.

Sugito, Y., Sekizawa, A., Farina, A., Yukimoto, Y., Saito, H., Iwasaki, M., Rizzo, N., and Okai, T. 2003. Relationship between severity of hyperemesis gravidarum and fetal DNA concentration in maternal plasma. *Clin Chem* **49:** 1667-1669.

Swinkels, D.W., de Kok, J.B., Hendriks, J.C., Wiegerinck, E., Zusterzeel, P.L., and Steegers, E.A. 2002. Hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome as a complication of preeclampsia in pregnant women increases the amount of cell-free fetal and maternal DNA in maternal plasma and serum. *Clin Chem* **48:** 650-653.

Sykes, P.J., Neoh, S.H., Brisco, M.J., Hughes, E., Condon, J., and Morley, A.A. 1992. Quantitation of targets for PCR by use of limiting dilution. *Biotechniques* **13:** 444-449.

Tan, E.M., Schur, P.H., Carr, R.I., and Kunkel, H.G. 1966. Deoxybonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J Clin Invest* **45:** 1732-1740.

Tang, K., Fu, D.J., Julien, D., Braun, A., Cantor, C.R., and Koster, H. 1999a. Chip-based genotyping by mass spectrometry. *Proc Natl Acad Sci U S A* **96:** 10016-10020.

Tang, N.L., Leung, T.N., Zhang, J., Lau, T.K., and Lo, Y.M.D. 1999b. Detection of fetal-derived paternally inherited X-chromosome polymorphisms in maternal plasma. *Clin Chem* **45:** 2033-2035.

Thu, K.L., Vucic, E.A., Kennett, J.Y., Heryet, C., Brown, C.J., Lam, W.L., and Wilson, I.M. 2009. Methylated DNA immunoprecipitation. *J Vis Exp*.

Tomazou, E.M., Rakyan, V.K., Lefebvre, G., Andrews, R., Ellis, P., Jackson, D.K., Langford, C., Francis, M.D., Backdahl, L., Miretti, M. et al. 2008. Generation of a genomic tiling array of the human major histocompatibility complex (MHC) and its application for DNA methylation analysis. *BMC Med Genomics* **1:** 19.

Tong, Y.K., Chiu, R.W.K., Leung, T.Y., Ding, C., Lau, T.K., Leung, T.N., and Lo, Y.M.D. 2007. Detection of restriction enzyme-digested target DNA by PCR amplification using a stem-loop primer: application to the detection of hypomethylated fetal DNA in maternal plasma. *Clin Chem* **53:** 1906-1914.

Tong, Y.K., Ding, C., Chiu, R.W.K., Gerovassili, A., Chim, S.S.C., Leung, T.Y., Leung, T.N., Lau, T.K., Nicolaides, K.H., and Lo, Y.M.D. 2006. Noninvasive prenatal detection of fetal trisomy 18 by epigenetic allelic ratio analysis in maternal plasma: Theoretical and empirical considerations. *Clin Chem* **52:** 2194-2202.

Tong, Y.K., Jin, S., Chiu, R.W.K., Ding, C., Chan, K.C.A., Leung, T.Y., Yu, L., Lau, T.K., and Lo, Y.M.D. 2010. Noninvasive prenatal detection of trisomy 21 by an epigenetic-genetic chromosome-dosage approach. *Clin Chem* **56:** 90-98.

Tost, J. and Gut, I.G. 2002. Genotyping single nucleotide polymorphisms by mass spectrometry. *Mass Spectrom Rev* **21:** 388-418.

Tsui, N.B.Y., Akolekar, R., Chiu, R.W.K., Chow, K.C.K., Leung, T.Y., Lau, T.K., Nicolaides, K.H., and Lo, Y.M.D. 2010. Synergy of total PLAC4 RNA concentration and measurement of the RNA single-nucleotide polymorphism allelic ratio for the noninvasive prenatal detection of trisomy 21. *Clin Chem* **56:** 73-81.

Tsui, N.B.Y., Wong, B.C., Leung, T.Y., Lau, T.K., Chiu, R.W.K., and Lo, Y.M.D. 2009. Non-invasive prenatal detection of fetal trisomy 18 by RNA-SNP allelic ratio analysis using maternal plasma SERPINB2 mRNA: a feasibility study. *Prenat Diagn* **29:** 1031-1037.

Tsumita, T. and Iwanaga, M. 1963. Fate of injected deoxyribonucleic acid in mice. *Nature* **198:** 1088-1089.

Tusnady, G.E., Simon, I., Varadi, A., and Aranyi, T. 2005. BiSearch: primer-design and search tool for PCR on bisulfite-treated genomes. *Nucleic Acids Res* **33:** e9.

van Rooij, E., Sutherland, L.B., Qi, X., Richardson, J.A., Hill, J., and Olson, E.N. 2007. Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **316:** 575-579.

Vogelstein, B. and Kinzler, K.W. 1999. Digital PCR. *Proc Natl Acad Sci U S A* **96:** 9236-9241.

Vucic, E.A., Wilson, I.M., Campbell, J.M., and Lam, W.L. 2009. Methylation analysis by DNA immunoprecipitation (MeDIP). *Methods Mol Biol* **556:** 141-153.

Wald, N.J., Cuckle, H.S., Densem, J.W., Nanchahal, K., Royston, P., Chard, T., Haddow, J.E., Knight, G.J., Palomaki, G.E., and Canick, J.A. 1988. Maternal serum screening for Down's syndrome in early pregnancy. *BMJ* **297:** 883-887.

Wald, N.J., Kennard, A., and Hackshaw, A.K. 1995. First trimester serum screening for Down's syndrome. *Prenat Diagn* **15:** 1227-1240.

Wald, N.J., Rish, S., and Hackshaw, A.K. 2003. Combining nuchal translucency and serum markers in prenatal screening for Down syndrome in twin pregnancies. *Prenat Diagn* **23:** 588-592.

Walknowska, J., Conte, F.A., and Grumbach, M.M. 1969. Practical and theoretical implications of fetal-maternal lymphocyte transfer. *Lancet* **1:** 1119-1122.

Wang, R.Y., Gehrke, C.W., and Ehrlich, M. 1980. Comparison of bisulfite modification of 5-methyldeoxycytidine and deoxycytidine residues. *Nucleic Acids Res* **8:** 4777-4790.

Wataganara, T., LeShane, E.S., Farina, A., Messerlian, G.M., Lee, T., Canick, J.A., and Bianchi, D.W. 2003. Maternal serum cell-free fetal DNA levels are increased in cases of trisomy 13 but not trisomy 18. *Hum Genet* **112:** 204-208.

Weatherall, D.J. 1997. The thalassaemias. *BMJ* **314:** 1675-1678.

Weber, M., Davies, J.J., Wittig, D., Oakeley, E.J., Haase, M., Lam, W.L., and Schubeler, D. 2005. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet* **37:** 853-862.

Weisenberger, D.J., Trinh, B.N., Campan, M., Sharma, S., Long, T.I., Ananthnarayan, S., Liang, G., Esteva, F.J., Hortobagyi, G.N., McCormick, F. et al. 2008. DNA methylation analysis by digital bisulfite genomic sequencing and digital MethyLight. *Nucleic Acids Res* **36:** 4689-4698.

Weng, Y.I., Huang, T.H., and Yan, P.S. 2009. Methylated DNA immunoprecipitation and microarray-based analysis: detection of DNA methylation in breast cancer cell lines. *Methods Mol Biol* **590:** 165-176.

Witters, I. and Fryns, J.R. 2007. Fetal nuchal translucency thickness. *Genet Couns* **18:** 1-7.

Wittmaack, F.M., Gafvels, M.E., Bronner, M., Matsuo, H., McCrae, K.R., Tomaszewski, J.E., Robinson, S.L., Strickland, D.K., and Strauss, J.F., 3rd. 1995. Localization and regulation of the human very low density lipoprotein/apolipoprotein-E receptor: trophoblast expression predicts a role for the receptor in placental lipid transport. *Endocrinology* **136:** 340-348.

Wong, I.H., Lo, Y.M.D., Zhang, J., Liew, C.T., Ng, M.H., Wong, N., Lai, P.B., Lau, W.Y., Hjelm, N.M., and Johnson, P.J. 1999. Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. *Cancer Res* **59:** 71-73.

Xue, W.C., Chan, K.Y., Feng, H.C., Chiu, P.M., Ngan, H.Y., Tsao, S.W., and Cheung, A.N. 2004. Promoter hypermethylation of multiple genes in hydatidiform mole and choriocarcinoma. *J Mol Diagn* **6:** 326-334.

Yung, T.K., Chan, K.C.A., Mok, T.S., Tong, J., To, K.F., and Lo, Y.M.D. 2009. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. *Clin Cancer Res* **15:** 2076-2084.

Zandi-Nejad, K., Luyckx, V.A., and Brenner, B.M. 2006. Adult hypertension and kidney disease: the role of fetal programming. *Hypertension* **47:** 502-508.

Zhang, Y., Rohde, C., Tierling, S., Jurkowski, T.P., Bock, C., Santacruz, D., Ragozin, S., Reinhardt, R., Groth, M., Walter, J. et al. 2009. DNA methylation analysis of chromosome 21 gene promoters at single base pair and single allele resolution. *PLoS Genet* **5:** e1000438.

Zhong, X.Y., Holzgreve, W., and Hahn, S. 2002. Cell-free fetal DNA in the maternal circulation does not stem from the transplacental passage of fetal erythroblasts. *Mol Hum Reprod* **8:** 864-870.

Zhong, X.Y., Laivuori, H., Livingston, J.C., Ylikorkala, O., Sibai, B.M., Holzgreve, W., and Hahn, S. 2001. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. *Am J Obstet Gynecol* **184:** 414-419.

Zhou, W., Galizia, G., Lieto, E., Goodman, S.N., Romans, K.E., Kinzler, K.W., Vogelstein, B., Choti, M.A., and Montgomery, E.A. 2001. Counting alleles reveals a connection between chromosome 18q loss and vascular invasion. *Nat Biotechnol* **19:** 78-81.

Zhou, Y., Genbacev, O., and Fisher, S.J. 2003. The human placenta remodels the uterus by using a combination of molecules that govern vasculogenesis or leukocyte extravasation. *Ann N Y Acad Sci* **995:** 73-83.

Zimmermann, B.G., Grill, S., Holzgreve, W., Zhong, X.Y., Jackson, L.G., and Hahn, S. 2008. Digital PCR: a powerful new tool for noninvasive prenatal diagnosis? *Prenat Diagn* **28:** 1087-1093.