Identification and Characterization of YAP1 as a Functional Oncogene in Gastric Cancer

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Abstract

Gastric cancer is one of the most common malignancies worldwide and is the second most frequent cause of cancer related death. A variety of genetic and epigenetic aberrations underlie development abnormality of gastric cancer.

Array comparative genomic hybridization (array-CGH) was used in this study to analyze the chromosomal aberrations in 9 gastric cancer cell lines. Our results showed good concordance with those of conventional CGH. We correlated the results from array-CGH with expression profiling and found some novel and independent target genes which deserved further confirmation.

The study was focused on the putative oncogene Yes-associated Protein 1 (YAP1) located in 11q22.1. Up-regulation of YAP1 was observed in 92.3% of gastric cancer by immunohistochemistry (IHC) on gastric cancer tissue microarrays. YAP1 nuclear accumulation correlated with cancer specific survival. In addition, multivariate Cox regression showed that YAP1 was an independent predictor of short disease specific survival time for patients with early stage gastric cancer ($P=0.042$) in addition to T stage ($P=0.038$). Knockdown YAP1 in gastric cancer cell lines MKN1 and AGS resulted in a significant reduction in proliferation,

anchorage-dependent colony formation, cell invasion and cell motility. Ectopic YAP1 expression in MKN45 cells promoted anchorage-independent colony formation, induced a more invasive phenotype and accelerated cell growth both *in vitro* and *in vivo*. Microarray analysis highlighted the alteration of MAPK pathway by YAP1. We confirmed a constitutive activation of RAF/MEK/ERK in YAP1-expressing MKN45 cells and further demonstrated that YAP1 enhanced serum/EGF induced c-Fos expression in gastric cancer cells. Furthermore, we demonstrated that ectopic MST1 promoted phosphorylation and cytoplasmic translocation of YAP1 and subsequently quenched the oncogenic function of YAP1 in the nucleus.

Taken together, our findings supported YAP1 is a functional oncogene in gastric cancer. We provided the first evidence that YAP1 exerted the oncogenic function by enhancing the capacity to activate the early response gene pathway. YAP1 could be a prognostic biomarker and potential therapeutic target for gastric cancer.

摘要

胃癌是一種世界性惡性腫瘤同時也是導致死亡率第二位的癌癥，一系列基因方面的改變是胃癌發生的潛在因素。

因此我們首先利用比較基因組雜交技術（array-CGH）分析胃癌細胞株染色體的擴增抑或缺失，這部分結果顯示 array-CGH 與傳統雜交結果相一致。然後我們用基因表達譜（mRNA expression microarray）研究九條胃癌細胞系的基因表達情況，著重分析基因拷貝數與表達水平的關系。通過計算基因拷貝數與基因表達水平之間的 Pearson 相關系數，我們發現了一些值得進一步研究的新的獨立目的基因。

本課題著重對目的基因 YAP1 進行深入研究。YAP1 定位於 11q22.1。通過免疫組化證實 YAP1 在胃癌中高表達（92.3%），同時 YAP1 在細胞核積聚為較差愈後的標誌。同時，多因素回歸分析證實 YAP1 在早期胃癌病人（一期和二期）可以作為一個獨立的預後指標（*P=0.042*）。在 MKN1 和 AGS 中下調 YAP1 可以抑制細胞生長（*P<0.001*）和侵襲能力（*P<0.001*），並可降低集落形成能力（*P<0.001*）。相反，在 MKN45 中重新表達 YAP1 可以促進細胞生長（*P<0.001*），增強癌細胞的侵襲能力（*P<0.001*）和錨著獨立性生長能力（*P<0.001*），在動物實驗中 YAP1 高表達可以提高腫瘤生長速度

（*P<0.001*）。基因表達譜分析揭示 YAP1 可改變 MAPK 信號通路。在穩定表達 YAP1 的 MKN45 克隆中，RAF/MEK/ERK 信號通路持續激活，而且 FBS 或 EGF 刺激可令 c-Fos 蛋白表達上調。我們還證實 YAP1 的上遊基因，抑癌基因 MST1 過表達可以磷酸化 YAP1 並增加 YAP1 在細胞漿中的積聚，達到抑制 YAP1 癌基因的功能。

所有數據表明 YAP1 在胃癌發生過程中具有癌基因功能，我們首次證實 YAP1 可通活激活快速反應基因通路發揮癌基因功能，並揭示該基因的表達具有一定的臨床意義，並可作為治療胃癌的靶基因。

Publications

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Wei Kang, Joanna H.M. Tong, Anthony W.H. Chan, Tin-Lap Lee, Raymond W.M. Lung, Patrick P.S. Leung, Ken K.Y. So, Kaichun Wu, Daiming Fan, Jun Yu, Joseph J.Y. Sung, Ka-Fai To. Yes-associated Protein 1 Exhibits Oncogenic Property in Gastric Cancer and Its Nuclear Accumulation Associates With Poor Prognosis.

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Abbreviations

array-CGH	array based comparative genomic hybridization
bp	base pair
GCA	gastric cancer
CDK	cyclin dependent kinase
CI	confidence interval
CpG	cytosine phosphoguanine dinucleotides
CSS	cancer specific survival
Da	dalton
DNA	deoxyribonucleic aid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediamine tetraacetic acid
FBS	fetal bovine serum
FISH	fluorescence in-situ hybridization
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H. pylori	Helicobacter pylori
H_2O_2	hydrogen peroxidate
IHC	immunohistochemistry
LOH	loss of heterozygosity
microRNA	miRNA
messenger RNA	mRNA
MTT	3- -2, 5-diphenyl tetrazolium bromide
OS	overall survival
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PI	propidium iodide
PVDF	polyvinylidene difluoride
PWH	Prince of Wales Hospital
qRT-PCR	quantitative real-time RT-PCR
RNA	ribonucleic aid
RT-PCR	reverse transcription polymerase chain reaction
siRNA	small interfering RNA
SDS	sodium dodecyl sulfate
SDS-PAGE	sulphate-polyacrylamide gel
TBE	tris borate EDTA
TBS	tris-buffered saline
TBS-T	1 × TBS, 0.1% Tween-20
TMA	tissue microarray
Tris	tris (hydroxymethyl) aminomethane
WHO	world health organization
YAP1	Yes-associated protein 1

Contents

CHAPTER 1. INTRODUCTION AND BACKGROUND

1.1 Gastric cancer

Gastric cancer (GCA) is one of the most common malignancies worldwide and is the second most frequent cause of cancer-related death. The overall prognosis of gastric cancer is poor with a 5-year survival rate below 30% in most countries (Brenner et al., 2009). Primary curative treatment for gastric cancer is surgery. However, gastric cancer is often diagnosed at advanced stage and more than 50% of patients were suffered from unrespectable, locally advanced or metastasis disease. The responses for gastric cancer to conventional chemotherapy treatments remain low. Thus, there is an urgent need to elucidate the underlying biology of gastric carcinogenesis and by which may provide important insights in the development of novel diagnostic, prognostic and therapeutic targets.

The pathogenesis of GCA represents a classic example of gene-environment interactions (Coussens and Werb, 2002; Peek and Blaser, 2002). The suspected several risk factors include high salt diet, smoking, low intake of fruits and vegetables, chronic gastritis with atrophy, intestinal metaplasia and Helicobacter pylori (*H. pylori*) infection (Alm et al., 1999). Over 95% of malignancies of the stomach are adenocarcinomas. Histologically, gastric adenocarcinoma is traditionally classified as intestinal, diffuse or mixed types (Lauren, 1965). Previous studies have documented the importance of genetic alterations affecting known oncogenes, tumor suppressor genes, such as TP53 (Hsieh et al., 1996;

Ochiai et al., 1996; Seruca et al., 1994), and mismatch repair genes in the development of GCA. Several genes, such as c-met, ERBB2, MYC, and MDM2 (Kuniyasu et al., 1992; Tahara, 1995; Yokota et al., 1988), are amplified in some GCA cases, and their amplification is associated with progression and prognosis of gastric cancer patients. A variety of genetic changes could be observed in GCA depending on the histological tumor type and the grade of differentiation, as well as tumor progression.

1.1.1 Epidemiology

GCA is the second commonest cancer in the world and 60% of deaths from GCA occur in developing countries. The highest incidences are in eastern Asia, such as China, Japan and Korea, South America and Eastern Europe. According to the year 2007 statistics published by Hong Kong Cancer registry, gastric cancer is the sixth most common cancer locally with a crude incidence rate of 14.5 per 100,000. The intestinal type is more common in males, while the diffuse type tends to affect younger age groups and is more common in females.

1.1.2 Etiological and risk factors

Both environmental and genetic factors are implicated in gastric carcinogenesis.

H. pylori infection:

H. pylori was first reported by Marshall in 1983 and the evidence has been gathered concerning its role in etiology of GCA. Previous studies strongly

supported that infection with *H. pylori* has association with development of GCA (Cai et al., 2000; Forman, 1991; Gao et al., 2009; Shin et al., 2009). *H. pylori* infection could contribute to the causation of GCA via mechanism that include the development and progression of chronic gastritis (Ohata et al., 2004). There is substantial evidence that genetic differences play roles in clinical outcome of *H. pylori* infection, particularly *H. pylori*-virulence associated genes such as *cag*A, *vac*A, *ice*A and *bab*A (Smith et al., 2006). *H. pylori* could also be a potential activator of NF-*k*B and mitogen-activated protein kinase (MAPK) pathway in gastric epithelial cells. The presence of *H. pylori* leads to the release of mutagenic substances such as metabolites of inducible nitric oxide synthese (iNOS), which is known to promote oncogenesis. *H. pylori* infection is also known to induce the expression of pro-inflammatory cyclooxygenase enzyme (COX-2).

Diet and life habits:

The diet with high intake of foods preserved by smoking, salting or pickling increases risk of stomach cancers (Strumylaite et al., 2006; Tsugane, 2005). It was supposed that the continuous use of high salt would result in early atrophic gastritis, thereby increase the risk of stomach cancer. Foods that contain nitrites and nitrates, such as bacon, ham and processed meats do so. Eating large amounts of red meat, particularly barbecued or well-done, also increases the risk (Gonzalez et al., 2006a). On the other hand, consuming plenty of fruits and vegetables, especially those that are red or deep yellow, such as tomatoes, carrots

and sweet potatoes, helps to protect against GCA (Gonzalez et al., 2006b). Possible protective micronutrients include vitamins C and E, and carotenoids and selenium (Kono and Hirohata, 1996). In addition to that, alcohol consumption (Magalhaes et al., 2008) and long term smocking behavior (Ladeiras-Lopes et al., 2008) also might add the risk of GCA development (Yu et al., 2009).

Genetic factors:

Gastric cancer occurs in some familial diseases with inherited cancer predisposition. Hereditary nonpolyposis colon cancer and familial adenomatous polyposis slightly increase the risk of GCA (Chinnaiyan et al., 2004). Gastric cancers developed in ~11% of HNPCC families and have been shown to occur in families with MSH2, MLH1 or MSH6 germline mutations (Vasen et al., 1996, Aarnio et al., 1997). Li-Fraumeni syndrome with germline mutation of TP53 also increased the risk of GCA (Corso et al., 2009). Hereditary Diffuse Gastric Cancer Syndrome (HDGC) was first reported in a large New Zealand Maori family (Sjodahl et al., 2007). It is a distinctive autosomal dominant inherited gastric cancer susceptibility syndrome and is resulted from germline mutations of E-cadherin (CDH1) gene, which is involved in cellular adhesion (Lynch et al., 2005). Defect in E-cadherin has been linked to the diffuse-type of gastric cancer (Caldas et al., 1999). Affected individuals inherit one copy of the defective gene and somatic mutation or deletion inactivates the other copy in the gastric cancer. In addition, MADH4, CHK2, and caspase-10 germline mutations have also been studied in the HDGC families (Lynch et al., 2005). A silent nucleotide variation

in exon 13 of the CDH1 gene may also contribute to cancer susceptibility, including GCA (Zhu et al., 2004).

Other factors:

Other possible factors include EBV infection, gastric surgery, family cancer syndromes and pernicious anemia. In gastric adenocarcinoma, about 7% cases harbors EBV. EBV is etiologically linked with several human malignancies including nasopharyngeal carcinoma and Burkitt's lymphoma. DNA methylation in EBV-infected stomach cells may be due to overdrive of the cellular defense against foreign DNA, which eventually leads to the development of EBV-associated GCA (Fukayama, 2010). The risk for GCA might increase in people who undergo gastrectomy for benign diseases (Fisher et al., 1993). After gastric surgery, reflux of bile and sometimes pancreatic juices might cause irritation and subsequent chronic gastritis. In addition, the amount of protective gastric acid decreases when nitrite-producing bacterial proliferate. These factors might lead to GCA in some people (Tersmette et al., 1995). It is also reported that pernicious anemia will increase threefold in the risk of GCA (Hsing et al., 1993). It is often associated with atrophic gastritis and develops when stomach is no longer able to make a protein called intrinsic factor that helps the body absorb vitamin B-12. Although pernicious anemia could be treated with B-12 injections, the disease is associated with increase the risk of gastric tumors (Eriksson et al., 1981; Karlson et al., 2000).

1.1.3 Pathology of gastric adenocarcinoma

Approximately 90% of stomach malignancies are adenocarcinomas. Non-Hodgkin's lymphomas and gastrointestinal stromal tumor (GIST) make up most of the remaining 10% (Kelley and Duggan, 2003). Adenosquamous, squamous, and undifferentiated carcinomas also occur but are rare. Several histological classification systems for gastric adenocarcinoma have been described, but the most frequently used are those of the WHO and Lauren (Lauren, 1965). In the World Health Organization (WHO) classification, there are 10 histological types (Brunicardi and Schwartz, 2005). The Lauren classification is commonly employed and makes the distinction between intestinal and diffuse types. The intestinal type consists of cohesive neoplastic cells forming gland-like structures while the diffuse type has lost cell cohesion and resulting in diffuse discohesive cellular infiltration (Kelley and Duggan, 2003). The intestinal type is more common in males and older age groups. Diffuse type carcinomas are relatively more common in younger age groups and have a more equal male-to-female ratio (Lauren, 1965). The gross appearances of advanced GCA are usually classified into four main types: polypoid, fungating, ulcerated and infiltrative. Cancer staging refer to the extend of disease. The most widely used systems for staging of gastric cancer is the tumor-node-metastasis (TNM) staging system. T represents the primary tumor, N represents the degree of spread to regional lymph nodes, and M represents the presence or absence of metastasis. Early gastric cancer is referring to the tumor invasion in mucosa or submucosal while advance gastric cancer referring to tumor invasion to the muscularis

propria or beyond.

The evolution of gastric tumors has been characterized as a multi-step process. For intestinal type adenocarcinoma, it is believed that the process includes gastritis, gastric atrophy, intestinal metaplasia, dysplasia and invasive adenocarcinoma. Intestinal metaplasia is a replacement of gastric epithelium by intestinal-type epithelium and it is believed to be a metaplasia response to chronic inflammation of stomach. Dysplasia is generally considered as a pre-malignant condition that may progress to invasive adenocarcinoma and it is divided into low-grade and high-grade dysplasia.

1.1.4 Biomolecular mechanism of GCA

1.1.4.1 Genetic alterations in GCA

The genetic aberrations include abnormalities of oncogenes, tumor suppressor genes, cell adhesion molecules (E-cadherin, CD44) (Chan, 2006; Hsieh et al., 1999; Lazar et al., 2008; Matsuura et al., 2001; Qian et al., 2008), and cell cycle regulators such as P16, $P27^{Kip1}$ and P53 (Abbaszadegan et al., 2008; De Feo et al., 2009; Galizia et al., 2006; Kim, 2007; Zheng et al., 2005) have been implicated. Genetic instability and alterations in growth factors, such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) (Vidal et al., 2008; Yamada et al., 2007), and cytokines may also contribute to the complex pathways involved in gastric carcinogenesis. Differences exist in the

pathways leading to diffuse and intestinal type gastric carcinoma and these are summarized in some reports (Smith et al., 2006).

Genetic instability and genetic polymorphisms have also involved in gastric tumorigenesis. DNA mismatch repair system dysfunction could lead to microsatellite instability (MSI). MSI could increase accumulation of genetic alterations and might participate in the pathogenesis of sporadic GCAs (Hiyama et al., 2004; Liu et al., 2005; Nakachi et al., 1999). Many reports have demonstrated the relationship between MSI and cancer multiplicity. Gastric cancer with MSI is shown to be associated with favorable survival and less aggressive behavior (dos Santos et al., 1996).

Genetic polymorphism may predispose to increasing risk of GCA (Gonzalez et al., 2002). Cytokines are important mediators that participated in inflammatory response and up-regulation of various inflammatory cytokines, including *IL-1β,* tumor necrosis factor *(TNF-α)* and *INF-γ* is common in *H. pylori* infection. Polymorphisms in human interleukin-*1β (IL-1β)*, interleukin-10 *(IL-10), TNF-A, IFN-G* and *IL-1RN* genes have been reported to influence cytokine expression (Shang and Pena, 2005). *IL-1β* gene promoter polymorphisms and an increased risk of gastric cancer were reported (El-Omar et al., 2000). Polymorphism of *IL-1β* together with *IL-1RN* genes may increase susceptibility of gastric cancer (Shang and Pena, 2005). Polymorphisms in several other cytokines including *IL-10* and *TNF-α* have also shown to be associated with gastric cancer risk

P2/P2 polymorphism of HSP70-2 at position 1267 was associated with a lower risk of gastric cancer in females (Shibata et al., 2009), whereas iNOS Ser (608) Leu allele may be a potential determinant of susceptibility to cigarette-alcohol induced gastric cancer (Shen et al., 2004). Among GCA patients, the single-nucleotide polymorphism (SNP) of E-cadherin, HER-2 and MMP-9 correlated with tumor cell invasion, metastasis and could increase the malignant risk.

1.1.4.2 Epigenetic modification in GCA

The most important epigenetic modifications leading to changed gene expression level are promoter methylation and chromatin remodeling by histone deacetylation, which will cause the downregulation of putative tumor suppressor genes.

The hypermethylation of CpG islands is associated with the downregulation of various tumor suppressor genes and involved in gastric tumorigenesis. Frequently methylated genes were FHIT, E-cadherin, BRCA1 and APC, followed by p14, p16, p15, p73, MGMT and SEMA3B (Bernal et al., 2008). Promoter hypermethylation of Casp8, hMLH1, CDH1 and MDR1 are also described in GCA patients (Poplawski et al., 2008).Other tumor suppressor genes, such as RASSF1A (Ye et al., 2007) , SFRP2 (Yu et al., 2009), CDH4 (Miotto et al., 2004) may also show promoter hypermethylation in GCA samples.

Histone deacetylation is also involved in the transcriptional repression of several tumor suppressor genes, including P21 with hypoacetylation of histone H3 and H4 (Mitani et al., 2005). Transcriptional inactivation of HLTF by aberrant DNA methylation and histone deacetylation may be involved in gastric carcinogenesis through down-regulation of HLTF expression (Hamai et al., 2003). Epigenetic regulation of CHFR may also plays a key role in tumorigenesis in GCA (Satoh et al., 2003). Histone deacetylase inhibitor, trichostatin A (TSA), could suppress proliferation rate, induce apoptosis and alleviate GCA cells invasion ability (Yasui et al., 2003).

1.1.4.3 Oncogenes and tumor suppressor genes in GCA

Some classic oncogenes are activated in gastric carcinoma with variations between different histological subtypes. Mutations of K-ras are often detected in intestinal type gastric adenocarcinomas and the precursor lesions such as intestinal metaplasia and adenomas (Isogaki et al., 1999; Lee et al., 1995; Sano et al., 1991; Song et al., 2009).. Overexpression of COX-2 and K-ras were closely correlated with prognosis of patients with GCA and they may produced synergistic effect in gastric carcinogenesis (Overholtzer et al., 2006; Satiroglu-Tufan et al., 2006). The K-sam (KATOIII cell-derived stomach cancer amplified) oncogene is also frequently activated in gastric carcinomas, and consists of at least four transcriptional variants (Katoh et al., 1992). K-sam is preferentially amplified in 33% of advanced diffuse or scirrhous-type gastric

carcinomas but not in intestinal-type cancers (Hattori et al., 1990). Another oncogene, c-met, encoding a receptor for hepatocyte growth factor / scatter factor is amplified in 19% of intestinal-type and 39% of diffuse-type GCA (Kuniyasu et al., 1992). Expression of the 6.0Kb transcript correlates well with prognostic factors such as tumor staging, depth of tumor invasion and lymph node metastasis (Kuniyasu et al., 1993). Another famous oncogene, c-erbB2, is preferentially amplified in 20% of intestinal type gastric tumors. Also, c-erbB-2 would be involved in the development of relatively early stage gastric carcinogenesis. C-erbB-2 is related with histological type and c-met with lymph node metastasis in gastric carcinomas (Lee et al., 2007; Yokota et al., 1988). Over-expression of c-erbB-2 is also correlated with poorer prognosis and liver metastases (Oda et al., 1990; Yonemura et al., 1991). HER-2 gene amplification and the overexpression of HER-2 protein have been observed in various solid tumors, including gastric carcinomas (Kim et al., 2007).

A key tumor suppressor gene, P53 (Ryu et al., 2007), is frequently inactivated in gastric cancer through loss of heterozygosity (LOH), missense mutation or frame shift deletion (de Moura Gallo et al., 2005). A P53 mutation is one of the most prevalent genetic alternations discovered in human cancers. Some studies also suggested that aberrant P53 expression correlated with GCA cell proliferation rate and P53 mutations may contribute to the increased expression of COX-2 in gastric cancer. Some novel tumor suppressor genes have been reported recently which show downregulation due to promoter hypermethylation, such as ZIC1

(Zhang et al., 2009a), KLF6 (Sangodkar et al., 2009), TSPYL5 (Jung et al., 2008), fibulin 1 (FBLN1) (Yu et al., 2009) and von Hippel-Lindau tumor suppressor gene (VHL) (Cao et al., 2008). In primary human GCA specimens, RUNX3 is frequently inactivated by allele loss or gene silencing due to promoter hypermethylation. The tumorigenicity of human GCA cell lines in nude mice decreased as the level of RUNX3 expression increased, which indicates that RUNX3 is a tumor suppressor of gastric tumors (Bae and Choi, 2004; Vogiatzi et al., 2006).

1.1.4.4 MicroRNA and GCA

MicroRNAs (miRNA) are a class of single-stranded RNA molecules of 21-23 bp in length, which could regulate target genes expression. The miRNAs were first described in 1993 by Lee and colleagues in the Victor Ambros lab (Lee et al., 1993), but this term microRNA was introduced in 2001 in a series of reports (Ruvkun, 2001). MiRNAs are non-coding RNAs. Each primary transcript (pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally further processed into a functional mature miRNA. Mature miRNA are partially complementary to one or more target messenger RNA (mRNA), so their main function is to down-regulate the target gene expression. Animal miRNAs are usually complementary to a site in the 3' UTR of mRNA whereas in plants miRNAs are usually directly complementary to the coding regions of mRNAs (Wang et al., 2004). Perfect or nearly perfect base pairing with the target mRNA promotes cleavage of the target mRNA (Kawasaki and Taira, 2004).

(Maziere and Enright, 2007) MicroRNAs that are partially complementary to the target site can also accelerate deadenylation, causing mRNAs to degrade.

MicroRNAs might function as oncogenes or tumor suppressors in human cancers. In GCA development, some miRNA are downregulated, such as microRNA-143 and -145 (Takagi et al., 2009), miR-141 (Du et al., 2009), miR-31 (Zhang et al., 2009b), miR-106a (Xiao et al., 2009), suggests a function for miRNAs in gastric tumor suppression. The downregulated miRNAs may act as anti-oncomirs and exhibit inhibitory effect on cell proliferation and could be novel diagnostic biomarkers of gastric cancers. Whereas some oncogenetic miRNAs are upregulated in GCA cell lines or primary GCA samples, such as miR-21 (Zhang et al., 2008), whose overexpression may also related in part to *H. pylori* infection. Up-regulation of microRNA-27a (Liu et al., 2009) targeting prohibitin mRNA is also reported in GCA.

1.2 Array-CGH and mRNA expression microarray for screening target genes

1.2.1 Array-CGH in GCA

A microarray-based comparative genomic hybridization (array-CGH), allows high-throughput and quantitative analysis of copy-number changes at high resolution throughout the genome. Array-CGH provides many advantages over conventional CGH and other methods (Albertson and Pinkel, 2003; Cho et al.,

2005; Imoto et al., 2001; Takagi et al., 2009), especially providing a molecular method that enables the high-throughout analysis of each chromosome. Common DNA aberrations include gene amplifications, non-reciprocal translocations and interstitial deletions. Amplifications may be visible cytogenetically as double minutes, chromosomes with homogeneously staining regions or the amplified DNA may be distributed at multiple sites (Albertson and Pinkel, 2003). Breakage of a chromosome or a non-reciprocal translocation event may lead to low level copy number changes whereas homozygous or heterozygous deletions would lead to high level copy number changes and all of them could be detected by array-CGH. In addition to that, array-CGH could provide information of DNA copy number changes involving a DNA segment, not only just the critical genes. There are several factors which could influence the success of array-CGH and the difficulty of array-CGH analysis varies among different application. Array-CGH has been applied to gastric carcinomas to define the novel regions of chromosomal amplifications or deletions (Buffart et al., 2007; Gorringe et al., 2005; Shibata et al., 2009; Takada et al., 2005; Tsukamoto et al., 2008; Varis et al., 2002; Yuan et al., 2008) and they are summarised in Table 1.1 and Table 1.2.

Table 1.1 **Summary of papers about array-CGH in GCA**

Paper	**Main Content**
Chromosomal Imbalances in gastric cancer Correlation With Histologic Subtypes and Tumor Progression. Am J Clin Pathol, 2001;115:828-834	High-level amplifications were found on chromosomes 12, 15, 17, and 20.
Targets of Gene Amplification and Overexpression at 17q in gastric cancer. CANCER RESEARCH, 62, 2625–2629, May 1, 2002	Amplification of 11 known genes (ERBB2, TOP2A, GRB7, ACLY, PIP5K2B, MPRL45, MKP-L, LHX1, MLN51, MLN64, and RPL27) and seven expressed sequence tags (ESTs) that mapped to 17q12-q21 region. Expression analysis showed overexpression of 8 genes (ERBB2, TOP2A, GRB2, AOC3, AP2B1, KRT14, JUP, and ITGA3) and two ESTs.
Analysis of comparative genomic hybridization and loss of heterozygosity in 43 primary gastric carcinomas. Chinese Medical Journal. 116(4), 517-523, 2003	Gains in 3p, 8q, 20, 12q, 13q and losses in 19, 7, 17p, 16, 1p.
DNA copy number changes in young gastric cancer patients with special reference to chromosome 19. British Journal of Cancer (2003) 88, 1914-1919	Analysis of DNA copy number changes revealed frequent DNA copy number increases at chromosomes 17q (52%), 19q (68%) and 20q (64%).
Screening of DNA copy-number aberrations in gastric cancer cell lines by array-based comparative genomic hybridization. Cancer Sci, February 2005, vol.96, no.2, 100–110	Copy-number gains were frequently detected at 1q, 3q, 5p, 7p, 7q, 8q, 11q, 17q, 20p, 20q, Xp and Xq, and losses at 3p, 4p, 4q, 8p, 9p, 18p and 18q. Copy-number gains at 1p, 16p, 20p, 20q and 22q, and losses at 8p, 10p, 10q and 18q were significantly

	frequent in cell lines derived from tumors of the well-differentiated type, whereas copy-number gains at 1q, 7p, 7q, Xp and Xq were frequent in the undifferentiated type.
Genomic loss and epigenetic silencing of very-low-density lipoprotein receptor involved in gastric carcinogenesis. Oncogene (2006) 25, 6554–656	Homozygous deletion at 9p24.2–24.3 using some GCA cell lines including HSC58.
Gastric cancers in young and elderly patients show different genomic profiles. Journal of Pathology, 2007, 211, 45-51	In young patients, amplifications were seen on chromosomes 6p, 6q, 7p, 8q, 11p, 13q, 15q, 16p, 16q, 17q, 18q, and 20q. In old patients, amplifications were seen on 4p, 6p, 6q, 8p, 8q, 11p, 11q, 13q, 16p, and 20q.
DNA copy number profiles of gastric cancer precursor lesions. BMC Genomics, 2007, 8, 345	Gains on chromosomes 8, 9q, 11q and 20, and losses on chromosomes 5q, 6, 10 and 13, likely represent early events in gastric carcinogenesis.
Genome-wide analysis of DNA copy number alterations and gene expression in gastric cancer. Journal of Pathology, 2008, 216, 471-82	Gain at 20q13 was detected in almost all cases (97%). Up-regulation of several candidate genes, such as CDC6, SEC61G, ANP32E, BYSL and FDFT1, was confirmed by immunohistochemistry.
Downregulation of ZIP kinase is associated with tumor invasion, metastasis and poor prognosis in gastric cancer. International Journal of Cancer, 2009, 124, 1587-93	Gains of 16q21, 19q13.1, 5p15.1 and 3q26.31, and losses of 3p21.32, 3p22.2, 19q13.33 and 19p13.3, were frequently detected by array-CGH.

Table 1.2 **Summary of amplification region and possible oncogenes reported in GCA array-CGH**

Chromosomal region	**Possible oncogenic targets**	**References**
1q21.1-q21.3	ARNT and S100A families,SELENBP1, JTB	Atiye et al., 2005; Wong et al., 2003; Koon et al., 2004
3p22.1	Beta-catenin	Suriano et al., 2005
3q26.33-q27.2	SOX2, EPHB3	Schwartz et al., 2003
4q13.3	ADAMTS3, ANKRD17, CXCL family, DCK	Handsley and Edwards, 2005
7p15.3	HOXA family	Rossi et al., 2005
8p23.1	TNKS, GATA4, MFHAS1,DEFA4, DLC1	Weiss et al., 2003
8q24.22	SLA, CGI-72, KIAA0143, WISP1	Douglas et al., 2004; Heidenblad et al., 2005
11q13.3	CCND1	Douglas et al., 2004
12p12.1	K-RAS	Sakakura et al., 1999; Heidenblad et al., 2005
13q22	KLF12	Nakamura et al., 2009
14q21	SDCCAG1, POLE2, MAP4K5, CDKL1	Pimhkakhoan et al., 2000
17q12	PPP1R1B, ERBB2,GRB7, STARD3	Varis et al., 2004; Marx et al., 2009; Tapia et al., 2007
17q21.2	KRT family	Koon et al., 2004
17q23.3	TRAP240, DKFZP564	Monni et al., 2001; Heidenblad et al., 2004
18q11.2	MIC1, NPC1, LAMA3, CABYR	Hermsen et al., 2001; Heidenblad et al., 2004
19q13.32	SAE1, BBC3, DHX34	Heidenblad et al., 2004
—	p42.3 gene	Xu X et al; 2007

1.2.2 mRNA expression microarray profiling in GCA

DNA copy number change could influence expression level of mRNA. Association between gene amplification and increased expression level has been demonstrated for oncogenes such as MYC, ERBB2, and gene deletion and decreased mRNA level have been shown for tumor suppressor gene TP53. Large scale evaluation of gene expression level became imperative. With advances and facilities in mRNA expression microarray chips, it is easy to check several thousands expressed genes and even the entire human transcriptome. High-density mRNA microarrays have been used to perform genome-wide screening studies for different types of human cancers (Berchuck, 2009; Dumur et al., 2008; Goto et al., 2006; Katoh et al., 1992; Lacroix et al., 2008; Onda et al., 2004; Tsai et al., 2007). mRNA expression level is regulated not only by DNA copy number changes, but also by many other possible mechanisms, such as transcription factors, epigenetic modifications, point mutations and polymorphisms (Albertson et al., 2003). In GCA, comprehensive examination of both DNA copy number and mRNA expression patterns has been reported in several papers (Fernandez et al., 2009; Fu et al., 2008; Kim et al., 2007; Myllykangas et al., 2008).

In the mRNA expression microarray analysis reports (Dong et al., 2007; Fu et al., 2008; Myllykangas et al., 2008; Overholtzer et al., 2006; Trautmann et al., 2005), it is found that genes with the highest statistical significance included ERBB2, MUC1, GRB7, PPP1R1B and PPARBP with concomitant changes in copy number and expression (Myllykangas et al., 2008). Several other target genes

were detected in mRNA expression microarray of primary GCA samples. Genes, including OPCML, RNASE1, YES1 and ACK1 may play a role in the tumorigenesis and metastasis of GCA (Fu et al., 2008). Secernin 1 could be a novel immunotherapy target for GCA screened out from the expression profiles of cDNA microarray (Imoto et al., 2001). Overholtzer et al reported that a total of 153 genes up-regulated and 204 down-regulated in diffuse-type gastric cancer by the expression microarray profiling (Overholtzer et al., 2006). Gene expression profiling by cDNA microarray analysis provides futher insights in understanding of biological properties of cancer and may help discovering novel clinical diagnostic markers and therapeutic targets (Overholtzer et al., 2006).

CHAPTER 2. STUDY OBJECTIVES

Gastric cancer is one of the most common malignancies and leading cause of cancer-related death in China including Hong Kong with overall 5-year survival rate is only 10-20%. There is a compelling need to explore the molecular mechanisms and therefore identify the novel targets that contribute to gastric carcinogenesis for early detection, prognosis prediction and effective treatment.

The objectives of the current study are:

1. To investigate genetic changes and to identify potential oncogenes and tumor suppressor genes in gastric cancer by comprehensive genome and transcriptome profiling.
2. To characterize the functional roles of putative oncogene YAP1 in gastric cancer development.

The general plan of study has been as follows:

Objective 1:

1. The chromosomal aberrations on 9 gastric cancer cell lines are investigated by high-resolution Agilent 44K Human Genome Array-CGH analysis.
2. The expression profiles of 9 gastric cancer cell lines are compared to normal gastric mucosa by using Agilent 4x44K Whole Human Genome Oligo Microarray system.

3. Differentially expressed genes with high correlation between DNA copy number changes and expression level variations are identify for further study.

Objective 2:

1. YAP1 expression in GCA is evaluated by immunohistochemistry and Western blot and correlated with clinicopathologic parameters.
2. The biological functions of YAP1 in GCA are determined by proliferation assay, colony formation, cell invasion, flow cytometry analysis through knocking down or ectopic expressing YAP1 in gastric cancer cell lines coupled with *in vivo* study.
3. The possible downstream effectors of YAP1 are identified by expression microarray using GeneChip HuGene U133A & B probe array sets (Affymetrix, Inc).
4. The upstream regulation of YAP1 by Hippo pathway will be investigated.

CHAPTER 3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Nine GCA cell lines

There are at least 40 GCA cell lines cultured in biology lab in the world and 9 of them were selected, MKN45, MKN28, KATO-III, AGS, N87, SNU1, SNU16, MKN1, MKN7, as our research material. Each cell line has its biological characteristics, including histology, metastasis status and level of differentiation (Table 3.1). These cell lines are grown in RPMI 1640 (GIBCO, Invitrogen) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen), 100U/ml penicillin and 10μg/ml streptomycin and maintained in log phase growth in a humidified atmosphere of 5% CO_2 at 37℃.

3.1.2 RNA sample from a commercially available normal gastric tissue is perused from Ambion for the mRNA expression study.

3.1.3 GCA patient samples and normal gastric tissue specimens

A total of 130 patients with confirmed gastric adenocarcinoma were examined. All of the patients were recruited from the Prince of Wales Hospital (PWH) , Shatin, Hong Kong and were subjected to standard management protocol. All conventional clinical pathological data were evaluated. The median age of the 130

patients in the tissue array at the time of diagnosis was 69.0 years (range, 35~88 years). This included 83 men and 47 women (male / female = 1.77 : 1). Tumor type, site, histology, grade and differentiation were assessed and recorded (Type: intestinal / diffuse / mixed = 4.50 : 1.72 : 1; HP status: absence / presence = 0.59 : 1; Grade: I / II / III = 0.08 : 0.75 : 1; Stage: I / II / III / IV = 0.39 : 0.39 : 1.04 : 1). Tumor was staged according to the TNM (AJCC 7th edition) staging system. All histological assessment was made by an experienced pathologist. All cancer patients were treated according to a standard protocol. The median and mean follow-up duration since the time of diagnosis were 17.2 months and 37.5 months respectively (range from 0.3 to 143.4 months). All cases were diagnosed between 1998 and 2002 and collected by K.F.T. and J.H.M.T. The tissue microarray and immunohistochemistry staining was prepared by S.C.Y.S. The study protocol was approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong.

Also, 16 paired GCA biopsy specimens from primary tumor and paired non-tumor sites were obtained from GCA patients during surgery according to a standard protocol, before any therapeutic intervention. The biopsy specimens were frozen in –80℃ for molecular analyses. All cases were diagnosed between 1998 and 2002 in PWH, Shatin, Hong Kong. 10 of them were prepared for Western blotting and 6 of them for IHC. In addition to that, 10 morphologically normal gastric mucosal biopsy samples and 13 morphologically normal gastric mucosa from excision specimens were also included. Dr. Yu Jun and Dr. Alfred Cheng from

Institute Digestive Disease (IDD) also provided 30 paired GCA / paired non-tumoral gastric tissue cDNAs for YAP1 qRT-PCR analysis.

3.1 **Biological features of nine GCA cell lines**

	Cell Line	Gender	Histology	P53	Source of tumor	Growth Property
1	MKN45	Female	Poorly differentiated adenocarcinoma	Wild type	Liver metastasis	Adherent
2	MKN28	Female	Well-differentiated tubular adenocarcinoma	Mutant	Lymph node	Adherent
3	KATO-III	Male	Signet-ring cell carcinoma	Deleted	Pleural fluid	Mixed
4	AGS	Female	Moderate / poorly differentiated	Wild type	Stomach	Adherent
5	N87	Male	Well-differentiated adenocarcinoma	Mutant	Liver metastasis	Adherent
6	SNU1	Male	Poorly differentiated	Wild type	Stomach, ascites	Suspension
7	SNU16	Female	Poorly differentiated	Mutant	Stomach, ascites	Suspension
8	MKN1	Male	Well differentiated, adenosquamous	Mutant	Lymph node	Adherent
9	MKN7	Male	Well-differentiated tubular adenocarcinoma	Mutant	Lymph node	Adherent

3.2 Methods

3.2.1 DNA extraction and array-CGH

3.2.1.1 DNA extraction (Wizard® SV Genomic DNA Purification System, Promega, USA)

Wash the cells once with 1 × PBS. Add 150μl of Lysis Buffer to the washed cells in the tissue culture plate. Mix lysate by pipetting. Transfer sample lysate from the tissue culture plate to a Minicolumn assembly. Then Place the Minicolumn / Collection Tube assembly containing the sample lysate into a microcentrifuge and spin at 13,000 × g for 3 minutes. Remove the Minicolumn from the Minicolumn / Collection Tube assembly and discard the liquid in the Collection Tube. Replace the Minicolumn into the Collection Tube. Add 650μl of Wash Solution to each Minicolumn / Collection Tube assembly. Centrifuge at 13,000 × g for 1 minute. Discard the liquid in the Collection Tube and replace the Minicolumn into the empty Collection Tube. Repeat Steps three times for a total of four washes of the Minicolumn. After the last wash, empty the Collection Tube and reassemble the Minicolumn / Collection Tube assembly. Centrifuge for 13,000 × g for 2 minutes to dry the binding matrix. Remove the Minicolumn and place in a new, labeled 1.5ml microcentrifuge tube. Add 250μl 4℃ nuclease-free water to Minicolumn. Incubate for 2 minutes at room temperature. Place the Minicolumn / elution tube assembly into the centrifuge and spin at 13,000 × g for 1 minute. Cap the elution tube containing purified genomic DNA and store at -20

℃.

3.2.1.2 Array-CGH (Agilent Technologies, USA)

DNA concentration was measured by NanoDrop 1000 (Thermo Fisher Scientific). Array-CGH was accomplished according to protocol (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis) and the result was analyzed by Agilent G4175AA CGH Analytics 3.4 (Agilent Technologies, USA).

3.2.2 RNA extraction and mRNA expression microarray profiling

3.2.2.1 Total RNA extraction (RNeasy® Mini Handbook, QIAGEN)

Disrupt cells or tissues in 350µl Buffer RLT. Homogenize by passing lysate at least 5 times through a 20-gauge needle fitted to an RNase-free syringe. Add 1 volume of 70% ethanol to the cleared lysate, and mix by pipetting. Apply up to 700µl of the sample, including any precipitate that may have formed, to an RNeasy mini column placed in a 2ml collection tube. Close the tube gently, and centrifuge for 1 minute 13,000rpm. Discard the flowthrough. Add 700µl Buffer RW1 to the RNeasy column. Close the tube gently, and centrifuge for 1 minute 13,000rpm. Discard the flow-through. Add another 500µl Buffer RPE to the RNeasy column. Close the tube gently, and centrifuge for 1 minute at 13,000rpm. Discard the flow-through. Repeat this step to dry the RNeasy silica-gel membrane. To elute, transfer the RNeasy column to a new 1.5ml collection tube. Pipet 30µl RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube

gently, and centrifuge for 1 minute 13,000rpm. Repeat this step as described with a second volume of RNase-free water. Elute into the same collection tube. Store the Total RNA in -80℃.

3.2.2.2 mRNA expression microarray profiling

RNA concentration was measured by NanoDrop 1000 (Thermo Fisher Scientific).

For the 9 GCA cell lines, the mRNA expression microarray was completed according to protocol (Two-Color Microarray-Based Gene Expression Analysis, Agilent Technologies) and the data was summarized and analyzed by GeneSpring GX Software (Agilent Technologies, USA).

For the later expression profiling such as knocking down YAP1 in AGS or overexpression YAP1 in MKN45, Human genome U133 Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA) was applied to identify the transcript expression profiles. It provides comprehensive genome wide expression analysis over 47,000 transcripts and variants. The raw data were quantile normalized by robust multiarray average (RMA) algorithm and analyzed in Partek Genomics Suite 6.4 (Partek, St. Charles, MO). Differential gene expression was evaluated using one-way ANOVA. A fold change cutoff of 1.5 and false discovery rates less than 5% were set to identify differentially expressed gene between YAP1-expressing MKN45 cells to vector controls, or siYAP1 knockdown AGS cells to the scramble controls. The integrated gene network analysis on the gene set with significant

expression changes were generated by Ingenuity Pathways Analysis (IPA, Ingenuity® Systems, www.ingenuity.com). Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity. Fischer's exact test was used to calculate a *P-value* determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. A *P-value* of less than 0.05 was considered significant. A *P-value* of less than 0.001 was considered highly significant.

3.2.3 RT-PCR and qRT-PCR

1) First-Strand cDNA Synthesis (Invitrogen): The following 20μl reaction volume can be used for 2μg of total RNA.

Add the following components to a nuclease-free microcentrifuge tube: 1μl random primers (300ng/μl); 2μg of total RNA; 1μl 10mM dNTP Mix; Sterile, distilled water to 14μl. Heat mixture to 65℃ for 5 minutes and incubate on ice for at least 1 minute. Collect the contents of the tube by brief centrifugation and add: 4μl 5 × First-Strand Buffer; 1μl 0.1M DTT; 1μl of SuperScript™ III RT (200units/μl). Mix by pipetting gently up and down, incubate tube at 25℃ for 5 minutes. Incubate at 50℃ for 60 minutes. Inactivate the reaction by heating at 70

℃ for 15 minutes and keep in -20℃. The cDNA can now be used as a template for amplification in PCR.

2) PCR

Add the following to a PCR reaction tube:

Component	**Volume**
10 × PCR Buffer	2.0μl
$MgCl_2$, 50 mM	1.5μl
dNTP mix (10mM)	1.5μl
Sense primer (10μM)	0.5μl
Antisense primer (10μM)	0.5μl
Taq DNA polymerase (5U/μl)	0.2μl
cDNA (from first strand reaction)	1.0μl
Autoclaved distilled water	to 25μl

Heat reaction to 94℃ for 2 minutes to denature. Perform 35 cycles of PCR. Annealing and extension conditions are primer and template dependent and must be determined empirically.

3) qRT-PCR (quantitative real-time RT-PCR)

qRT-PCR was used to quantitative differences in mRNA expression among the liver tissues of groups. We used SYBR Green Master Mix (Applied Biosystems) with later dissociation curve analysis for YAP1 (Forward:

CAGCAACTGCAGATGGAGAA; Reverse: ACATCCCGGGAGAAGACACT), whereas for GAPDH we used Taqman mix (Applied Biosystems). The primers were designed with Primer3, and diluted at 10μM in nuclease-free water.

2μl of each cDNA product at 100 × dilution was prepared for experiment reaction and Sybrgreen was used in the procedure of the assay according to the manufacturer's instructions. A no-template-control and standard samples were prepared for each pair of primers. The contents of the reactions were transferred to a MicroAmp optical 96-well reaction plate which then was sealed with a MicroAmp optical adhesive cover (Applied Biosystems).

The PCR reactions were performed in the 7500 Fast Real-Time PCR system (Applied Biosystems). Each Ct value was normalized by housekeeping gene GAPDH and calculated using the 2^ (-Delta Delta Ct) method..

Experiment reactions

Component	**Volume**
2 × SYBR Green Master Mix	10.0μl
Forward Primer	0.25μl
Reverse Primer	0.25μl
Template (diluted cDNA)	2.0μl
Nuclease-Free water	7.5μl
Total volume	20μl

3.2.4 Protein expression analysis

3.2.4.1 Protein extraction, concentration detection and antibodies used

A) Protein extraction

Put the stored samples on ice and add RIPA lysis buffer depending on the size of the cell pallet. Lyse the cells by pipetting up and down several times and leave on ice for at least 10 minutes. Spin at 13000rpm for 10 minutes at 4℃. Transfer the supernatant to a screw-cap eppendorf tube and the residual lysate will be used for protein quantification. Store the samples at -80℃. Special note: Solid tissues (such as primary GCA samples or mouse samples from in vivo study) are first broken down mechanically using a homogenizer or by sonication. Protease inhibitors are often added to prevent the digestion of the sample by its own enzymes. Cells may also be broken open by one of the above mechanical methods. The nuclear and cytoplasmic fraction was extracted using NE-PER Nuclear and cytoplasmic extraction reagents (Thermo scientific).

B) Protein concentration quantification (BCA^{TM} Protein Assay Kit, Pierce, IL)

Pipette 12.5µl of each standard or unknown protein sample duplicate into a 96-well microplate plate. Add 200µl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds. Cover plate and incubate at 37℃ for 30 minutes and cool at RT. Measure the absorbance at 570nm on plate reader

(Victor3, Perken Elmer).

C) Table 3.2 **Chemical reagents used for protein manipulation**

Reagents	**Manufacturers**
Bovine serum albumin (BSA)	Sigma, St. Louis, MO
Bromophenol blue	BIO-RAD, Hercules, CA
EDTA Disodium Salt, Dihydrate	USB Corporation, Cleveland, Ohio
Glycerol	USB Corporation, Cleveland, Ohio
Glycine	BIO-RAD Laboratories, CA
Methanol	Lab-scan, Patumwan, Bangkok
Millipore Immobilon Western	Millipore Corporation, Billerica,
NaCl	USB Corporation, Cleveland, Ohio
Nonidet P-40	USB Corporation, Cleveland, Ohio
Non-fat milk	Nestle, Australia
PD98059 (MEK1 Inhibitor)	Cell Signaling, #9900
Polyvinylidene difluoride (PVDF) membrane	GE Healthcare, Amersham
Phosphate Buffered Saline Tablets	Sigma, St. Louis, MO
protease inhibitor	Promega, Madison, WI
Sodium Dodecyl Sulfate	USB Corporation, Cleveland, Ohio
20% SDS Solution (Sodium dodecyle sulfate)	USB Corporation, Cleveland, Ohio
Tris	USB Corporation, Cleveland, Ohio
Tris-HCL	USB Corporation, Cleveland, Ohio

Tween-20	USB Corporation, Cleveland, Ohio
X-ray film	FUJIFILM Corporation, Tokyo
Diaminobenzidine (DBA) substrate kit	Dako Cytomation, California

D) Table 3.3 **Antibodies for Western blotting and immunohistochemistry**

Antibodies	**Manufactures**	**Cat. No.**	**Diluted**
Anti-AKT	Cell Signaling	#9272	1:1000
Anti-beta-catenin	BD Transduction	610154	1:1000
Anti-cyclin D1	Cell Signaling	#2926	1:1000
Anti-cyclin D3	Cell Signaling	#2936	1:2000
Anti-CDK4	Cell Signaling	#2906	1:2000
Anti-CDK6	Cell Signaling	#3136	1:2000
Anti-c-Fos	Cell Signaling	#2250	1:1000
Anti-c-Raf	Cell Signaling	#9422	1:1000
Anti-Caspase-8 (1C12)	Cell Signaling	#9746	1:1000
Anti-Cleaved Caspase-3	Cell Signaling	#9661	1:1000
Anti-Cleaved PARP (Asp214)	Cell Signaling	#9541	1:1000
Anti-MEK1/2	Cell Signaling	#4694	1:1000
Anti-c-Myc	Cell Signaling	#9402	1:1000
Anti-Mouse IgG-HRP	Dako	00049039	1:30,000
Anti-MST1	Abcam	Ab51134	1:1000
Anti-MST2	Cell Signaling	#3952	1:1000

Anti-Notch3	Cell Signaling	#2889	1:1000
Anti-Notch4 (L5C5)	Cell Signaling	#2423	1:1000
Anti-Numb (C29G11)	Cell Signaling	#2756	1:1000
Anti-Phospho-YAP1 (S127)	Cell Signaling	#4911	1:1000
Anti-Phospho-P44/42 MAPK	Cell Signaling	#9106	1:2000
Anti-P44/42 MAP Kinase	Cell Signaling	#9102	1:1000
Anti-Phospho-AKT (S473)	Cell Signaling	#9271	1:1000
Anti-Phospho-Rb (Ser807/811)	Cell Signaling	#9308	1:1000
Anti-P21	Cell Signaling	#2946	1:2000
Anti-PCNA	Lab Vision	#MS-106-P	1:1000
Anti-PI3 Kinase p110 Alfa	Cell Signaling	#4255	1:1000
Anti-Phospho-P38 MAPK	Cell Signaling	#4511	1:1000
Anti-Phospho-Stat3 (T705)	Cell Signaling	#9145	1:2000
Anti-p-Stat5a (Ser780)	Santa Cruz	SC-101805	1:1000
Anti-P73	Abcam	Ab14430	1:1000
Anti-Phospho-c-Raf (Ser338)	Cell Signaling	#9427	1:1000
Anti-Phospho-MEK1/2	Cell Signaling	#9154	1:1000
Anti-RASSF1A	Santa Cruz		1:1000
Anti-Rabbit IgG-HRP	Dako	00028856	1:10,000
Anti-Stat3	Cell Signaling	#9139	1:1000
Anti-YAP1	Abcam	ab52771	1:10000

3.2.4.2 Western blotting

A) Gel electrophoresis:

30μg protein samples were mixed with 2 × SDS loading buffer (Tris, pH 6.8, Bromophenol blue, 4% w/v SDS, 10% w/v beta-mercaptoethanal, 20% glycerol). The protein was denatured by heating at 95℃ for 5 minutes and cooled on ice. The protein sample per lane was resolved on a 12% sulphate-polyacrylamide gel (SDS-PAGE). Broad range protein markers (H-018, Houbio Life Technologies) were loaded to determine molecular weights. Electrophoresis was carried out at room temperature at 120V for one and a half hour.

B) Wet transfer of proteins:

After electrophoresis, the gel was removed from the tank and immersed in pre-chilled transfer buffer. Then the proteins were electrotransfer to PVDF membrane using a Bio-Rad Trans-Blot cell. The transfer sandwich was assembled with sponge on the bottom, followed by PVDF membrane, stacking gel and sponge. The transfer was performed for 60 minutes at 120V in cold transfer buffer with stirring.

C) Immunoblotting:

After transfer, the PVDF membrane was blocked with non-fat milk (5% non-fat milk, 1 × TBS, 0.1% Tween-20) for one hour at room temperature. After blocking, the membrane was washed three times with 5 minute respectively in TBS-T. Dilute primary antibody with 5% non-fat milk or 3% BSA in TBS-T according to

recommended dilution factor. Approximately 5ml for each membrane. Then the membranes were incubated with diluted primary antibody under gentle shake at room temperature for 1.5 hours or overnight at 4℃. Wash the membrane 3 times with TBS-T, each with 5 minutes. Then the membrane was incubated with HRP-conjugated secondary antibody under gentle shake for one hour at room temperature. Following three times TBS-T washing for 5 minutes each.

D) Signal detection:

Mix HRP substrate solution according to 1:1 ratio (1ml for 2 membranes). Incubate the membrane upside down for 1 minute in the substrate solution. Remove excess solution on the membrane and fix it on the cassette with scotch tape. In dark, place a sheet of film on the membrane and expose for certain time, and then develop the film. Align the film with the membrane and mark the protein marker, determine the size of the bands to the marker. Finally record the exposure time and date on the film.

3.2.4.3 IHC (immunohistochemistry) staining

A) Solutions and reagents needed

Xylene; 1 × Tris buffered saline (TBS); 3% hydrogen peroxidate (H_2O_2) solution; 1 × Citrate buffer; DAB Substrate Kit; Harris Hematoxylin; Graded alcohols; Blocking solution: 10% nonimmunized goat serum in TBS.

B) Deparaffinization

Sections were immersed in three washes of xylene for 5 minutes each. Sections were immersed in two washes of 100% ethanol for 5 minutes each. Sections were immersed in two washes of 75% ethanol for 5 minutes each. Sections were immersed in distillated water for 5 minutes.

C) Staining

Sections were incubated in 3% H_2O_2 solution for 25 minutes to block endogenous Peroxidase activities. Transfer to one distilled water for 1 minute and rinse briefly with TBS. Microwave 2 minutes at P-10 followed by 10 minutes at P-2 in Citrate Buffer to make antigen retrieval. Incubate section in 5% normal Goat serum in TBS for 10 minutes. Primary antibody was added with dilution in blocking solution. Incubated for 2 hours at room temperature or overnight at 4℃. Slides were rinsed 3 times with TBS, 5 minutes each time. The HRP labeled secondary antibody diluted in blocking solution were applied to the tissue sections on the slide and incubated for 30 minutes at room temperature. Removed secondary antibody solution and washed section three times with TBS for 5 minutes each. DAB substrate solution was prepared by adding 1 drop of DAB chromagen to every 1 ml of DAB buffer (finally 1:50). Drain PBS from slides and apply the DAB substrate solution. Allow slides to incubate for at most 5 minutes until the desired color intensity is reached. Immerse the sections in water as soon as the sections were developed.

D) Counterstain slides:

Slides were dipped for 1 minute in Harris Hematoxylin and followed slides were rinsed thoroughly in water. Slides were dipped in to Acid Alcohol for 1 second and followed slides were rinsed thoroughly in water. Dehydrated through 4 washes of alcohol (75%, 75%, 100% and 100%). Clear the slides in 3 washes of xylene and air-dry the slides. Each 5 minutes. Coverslip using mounting solution with cover slides.

E) Scoring: In IHC scoring, tumor heterogeneity in expression was assessed by two pathologists (K.F.T. and A.W.H.C.) using a semi-quantitative method. As total YAP and p-YAP1 (S127) could be located both in the nucleus and cytoplasm, we scored YAP1 separately according to its localization.

The nuclear expression of YAP1 was scored by estimating proportion of tumor cells with positive nuclear staining (0, none; 1, <=10%; 2, 10 to <=50%; 3, >50%). The cytoplasmic expression of YAP1 was assessed by assigning a proportion score and an intensity score. The proportion score was according to proportion of tumor cells with positive cytoplasmic staining (0, none; 1, <=10%; 2, 10 to <=25%; 3, >25 to 50%; 4, >50%). The intensity score was assigned for the average intensity of positive tumor cells (0, none; 1, weak; 2, intermediate; 3, strong). The cytoplasmic score of YAP1 was the product of proportion and intensity scores, ranging from 0 to 12. The cytoplasmic expression was categorized into low (score 0 to 3), intermediate (score 4-6), and high (score 7-12).

The scoring was independently assessed by two pathologists (K.F.T. and A.W.H.C.)

3.2.5 YAP1 Knocking down assays

3.2.5.1 SiRNA transfection

Transfection was performed using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) and the final siRNA (validated siRNA from Qiagen) concentration was 20nM according to the recommendation of Qiagen.

3.2.5.2 MTT assays for transient transfection

We used 96-well plates for 6 days MTT analysis (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega), 5 wells for YAP1 siRNA, 5 wells for scramble siRNA control and 5 wells for mock control (about 2000 cells/well). The value of 570nm wavelength light absorption (Victor3, Perken Elmer, Waltham, MA) was measured and documented.

3.2.5.3 Monolayer colony formation assays

For colony formation assays in monolayer cultures, we used 6-well plates, 3 for YAP1 siRNA and 3 for scramble siRNA. The time point was 14 days after transfection. Colonies were fixed with 70% ethanol for 15 minutes and stained with 2% crystal violet. Colonies with cell numbers of more than 50 cells per colony were counted. The experiments were performed in triplicate wells in 3

independent experiments.

3.2.5.4 Cell invasion assays

For invasion assays, the assays were performed using a 24-well invasion chamber (BD Biocoat Matrigel Invasion Chamber, BD Biosciences). Cells were harvested from culture dishes 24 hours after transfection, washed three times with culture medium containing 1% FBS, and resuspended in medium without FBS. Then 300ul of the cell suspension (5×10^4 cells) was added into the transwells, with 500ul of culture medium containing 10% FBS in the lower chamber. After 24 hours incubation at 37℃, cells that invaded through the matrigel membrane were fixed with 100% methanol for 2 minutes and stained with 1% Toluidine blue for another 2 minutes. We counted the cell number under microscope and took photograph. For statistics analysis, we counted the cell number in 5 view fields of each chamber randomly and took the average cell number.

3.2.5.5 Cell migration assays

For wound healing assays, after transfection with siRNA, the cell layers were carefully wounded using sterile tips and washed twice with fresh medium. Cell migration pictures were photographed and documented under microscope at the beginning and 48 hours after wound formation.

3.2.5.6 Flow cytometry analysis for cell cycle arrest and early apoptosis

For cell cycle analysis, MKN1 and AGS cell collecting time point was 24 hours

after transfection in 6-well plates. Cells were harvested using cold PBS and fixed in 70% cold ethanol for at least 0.5 hour in 4℃ and treated with 1ng/mL RNase A for 10 minutes at 37℃. Cellular DNA was stained with 15ng/ml propidium iodide (PI) for 30 minutes at 37℃ in the dark. The cells then were sorted by FACS Calibur Flow Cytometer (Becton Dickinson, CA) and cell-cycle profiles were determined using the ModFitLT software (Becton Dickinson, San Diego, CA). For early apoptosis analysis, Annexin V-FITC Apoptosis Detection Kit (BioVision, CA) was used. 5×10^5 cells were suspended in 500μl 1 × Binding Buffer. After adding 5μl Annexin V-FITC and 5μl PI, the cells were incubated at room temperature for 5 minutes in the dark. Finally, cells were sorted by FITC signal detector (Becton Dickinson, CA).

3.2.6 YAP1 overexpression assays

3.2.6.1 Plasmid construction and transfection

YAP1 (commercially from Addgene, Cambridge, MA, the full-length YAP1 was sequence verified and subcloned into pcDNA3.1+) and pcDNA3.1+ empty vector concentration was 2ug/well (6-well plate) using GeneJuice transfection reagent (Novagen). We also selected several stable clones of pcDNA3.1+ empty vector and stable YAP1 expression clones using G418 (Merck, Darmstadt, Germany). Restoration of YAP1 expression was confirmed by Western blot analysis.

3.2.6.2 MTT assays of stable clones

We also applied 6-day MTT analysis (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega) in 96-well plates, 5 wells for empty vector control, 5 wells for stable expressing YAP1 cells under the condition of 10% FBS (high serum condition) and 1% FBS (low serum condition) separately. The value of 570nm wavelength light absorption (Victor3, Perkin Elmer) was measured and documented.

3.2.6.3 Cell invasion assays of stable clones

For invasion assays, the assays were also performed using a 24-well invasion chamber (BD Biocoat Matrigel Invasion Chamber, BD Biosciences) as mentioned before. MKN45 cells with pcDNA3.1+ empty vector or stable-YAP1-expressing constructs were placed in the chambers for 24 hours in the culture incubator before examination.

3.2.6.4 Anchorage independent soft agar colony formation assays

For anchorage-independent growth assays, cells were plated in soft agar to evaluate the ability of cells to grow in an anchorage-independent manner. The bottom layer was prepared using 2ml of the RPMI-1640 containing 0.7% agar, together with 10% FBS. It was placed in 6-well plates, and allowed to solidify in 4℃ for 10 minutes. MKN45 cells (5000/well) transfected with empty vector or stable-YAP1-expressing constructs were suspended and seeded on 6-well plates containing 0.35% agar in RPMI-1640 medium, separately. After 2 weeks culture in 37℃ incubator, cells were stained with 0.005% crystal violet and examined.

Colonies could be seen by naked eye were counted. The experiments were performed in triplicate wells.

3.2.6.5 Flow cytometry analysis of stable clones for early apoptosis

For cell cycle analysis, several stable clones of empty vector and stable-expression YAP construct were subjected flow cytometry analysis. In addition to that, the cells were treated with Doxorubicin (1μM), Cisplatin (100μM) for 24 hours then the cells were harvested and fixed in 70% cold ethanol for at least 0.5 hour in 4℃ and treated with 1ng/ml RNase A for 10 minutes at 37℃. Cellular DNA was stained with 15ng/ml PI for 30 minutes at 37℃ in the dark. The cells then were sorted by FACS Calibur Flow Cytometer (Becton Dickinson, CA) and cell-cycle profiles were determined using the ModFitLT software (Becton Dickinson, San Diego, CA).

3.2.6.6 Starvation / stimulation assays

For starvation / stimulation assays, the cells, including AGS and MKN1 with knocking down YAP1, MKN45 / pcDNA3.1+ or YAP1, were starved for 24hrs, then the cells underwent stimulation of 10% FBS or EGF (50ng/ml). The cells were collected according to the time point 0min, 1min, 5mins, 15mins, 30mins, 60mins and 90mins and waiting for Western blotting analysis. PD98059 (20uM, Cell signaling, #9900) was also used to inhibit MEK1 in order to investigate whether c-Fos change was due to ERK change in stimulation.

3.2.6.7 In vivo study

For in vivo tumorigenicity study, MKN45 cells (1×10^6cells suspended in 0.1ml PBS) with stable YAP1 expression or empty vector clones were injected subcutaneously into the dorsal flank of 4-week-old male Balb/c nude mice (empty vector in left and stable YAP1 in right) separately. Tumor diameter was measured and documented every 3 days until the end of week 3. Tumor volume (mm^3) was estimated by measuring the longest and shortest diameter of the tumor and calculating as follows: volume = $(\text{shortest diameter})^2 \times (\text{longest diameter}) \times 0.5$, as described by Yu (Yu et al., 2009). The animal handling and all experimental procedures were approved by the Department of Health of Hong Kong. This experiment was also repeated three times using different clones and different passages.

3.2.7 Statistics

Correlation coefficients studies were assessed by Pearson correlation. Pearson correlation coefficients between DNA copy number changes and mRNA expression levels for each gene were calculated in Microsoft Excel by Thomas Lo. Gene screening was performed using a custom script in R (http://www.r-project.org).

Correlations between YAP1 nuclear/cytoplasmic stain and clinicopathologic parameters were assessed by the non-parametric Spearman's rho rank test. The TMA IHC was analyzed according to overall survival (OS) and cancer specific

survival (CSS). Overall survival is the percentage of people in a study who have survived for a certain period of time, usually reported as time since diagnosis or treatment. It denotes the percentage of individuals in the group who are likely to be alive after a particular duration of time. For CSS only deaths from GCA are counted. OS and CSS as a function of the markers studied were estimated by the Kaplan-Meier method and the log-rank test was used to test for differences. For those variables being statistically significant found in the univariate survival analysis ($P<0.05$), the Cox proportional hazards model with the likelihood ratio statistics was employed to further evaluate them for multivariate survival analysis. The strength of the associations between the marker expression levels and patient and tumor characteristics were tested with the Mann–Whitney U-test or the Kruskal–Wallis H-test.

The Mann–Whitney U test was used to compare the difference in biological behavior between YAP1-expressing MKN45 cells and empty vector-transfected MKN45, or siYAP1-knockdown AGS cells and scramble siRNA-transfected AGS cells.

All statistical analyses were carried out by using statistical program SPSS version 16.0. A two-tailed *P-value* of <0.05 was regarded as statistically significant. A *P-value* of less than 0.001 was considered highly significant.

CHAPTER 4. RESULTS

4.1 DNA copy number changes in GCA cell lines

The DNA copy number changes in 9 gastric cancer cell lines were determined by Array-CGH, a technique with powerful potential for high-throughout identification of genetic aberrations. In array-CGH, the reference and test DNA samples were differentially labeled with fluorescent tags (Cy5 and Cy3, respectively), and hybridized to genomic arrays. After hybridization, the fluorescence ratio (Cy3:Cy5) was determined, which revealed copy-number differences between the two DNA samples.

The data-value thresholds in the experiment were shown in Table 4.1. The thresholds for the log2 ratio of gains and losses were set at log2 ratios of +0.58 and −0.58, respectively, because the values within this range were observed in a normal to normal hybridization experiment that they were considered as signal noise (Kim et al., 2007). Copy number gain was defined as the log2 ratio greater than 0.58 and amplification was defined as greater than 2. The heterozygous and homozygous deletions were defined with value thresholds of -1 and -2, respectively. For a specific gene or a specific chromosomal fragment, the high-frequency amplification was defined when 5 or more cell lines showed amplification in this region. Whereas low-frequency amplification indicated that no more than 4 cell lines demonstrated amplification in this region.

The genome-wide frequency plot of copy number alterations was shown in Figure 4.1, in which red represented copy-number gains and green represented losses. Clones were ordered as the position of each gene and alterations were defined by log2 ratio thresholds of -0.58 and 0.58 for copy number gains and losses, respectively. Chromosomal gains were more frequently detected than losses in our array-CGH analysis of GCA. Using the threshold values mentioned above, a total of 7439 genes showed copy number gain in any of the GCA cell line, which encompassed 1099 amplification genes. The copy number loss was observed in 4584 genes (Table 4.2). Regions of high-frequency amplification (5 or more cell lines) involved chromosomes 1p36.32-36.12, 5p, 7p, 8q22.22, 11q12.1-13.4, 12q13.12-13.2, 19p, 19q13.2-13.32, 20p, 20q, and X, and recurrent loss (5 or more cell lines) localized at chromosomes 3p14.1, 4p, 4q, 5q14.1-23.3, 6q, 8p, 9p24.2-21.3, and 10p (Table 4.3).

Table 4.1 **Thresholds of data-value in array-CGH experiment**

Log2 ratio		**Number of Cell Lines (total 9)**	
Normal	-0.58~0.58	High-frequency amplification	≥5
Copy number gain	>0.58	Low-frequency amplification	≤4
Amplification	≥2	High-frequency deletion	≥5
Copy number loss	<-0.58	Low-frequency deletion	≤4
Homozygous deletion	≤-2		

Table 4.2 **Gene number with different thresholds**

Content	**Definition**	**Gene number**
Copy number gain	Log2 ratio>0.58 in any GCA cell line	7439
Amplification	Log 2 ratio≥2 in any GCA cell line	1099
Copy number loss	Log 2 ratio<-0.58 in any GCA cell line	4584
Heterozygous deletion	-2<Log 2 ratio≤-1 in any GCA cell line	2571
Homozygous deletion	Log 2 ratio≤-2 in any GCA cell line	139

Table 4.3 **Recurrent DNA copy number change regions in 9 GCA cell lines**

Alteration		**Region**
Amplification	Low-level	1q22, 10q34.12-34.2, 14q, 16p, 17q12-21.32.
	High-level	1P36.32-36.12, 5p, 7p, 8q22.22, 11q12.1-13.4.
Deletion	Low-level	1q31.3, 2q24.3-32.3, 7q21.11-21.13, 12p, 16p, 18q.
	High-level	3p14.1, 4p, 4q, 5q14.1-23.3, 6q, 8p, 9p24.2-21.3, 10p.

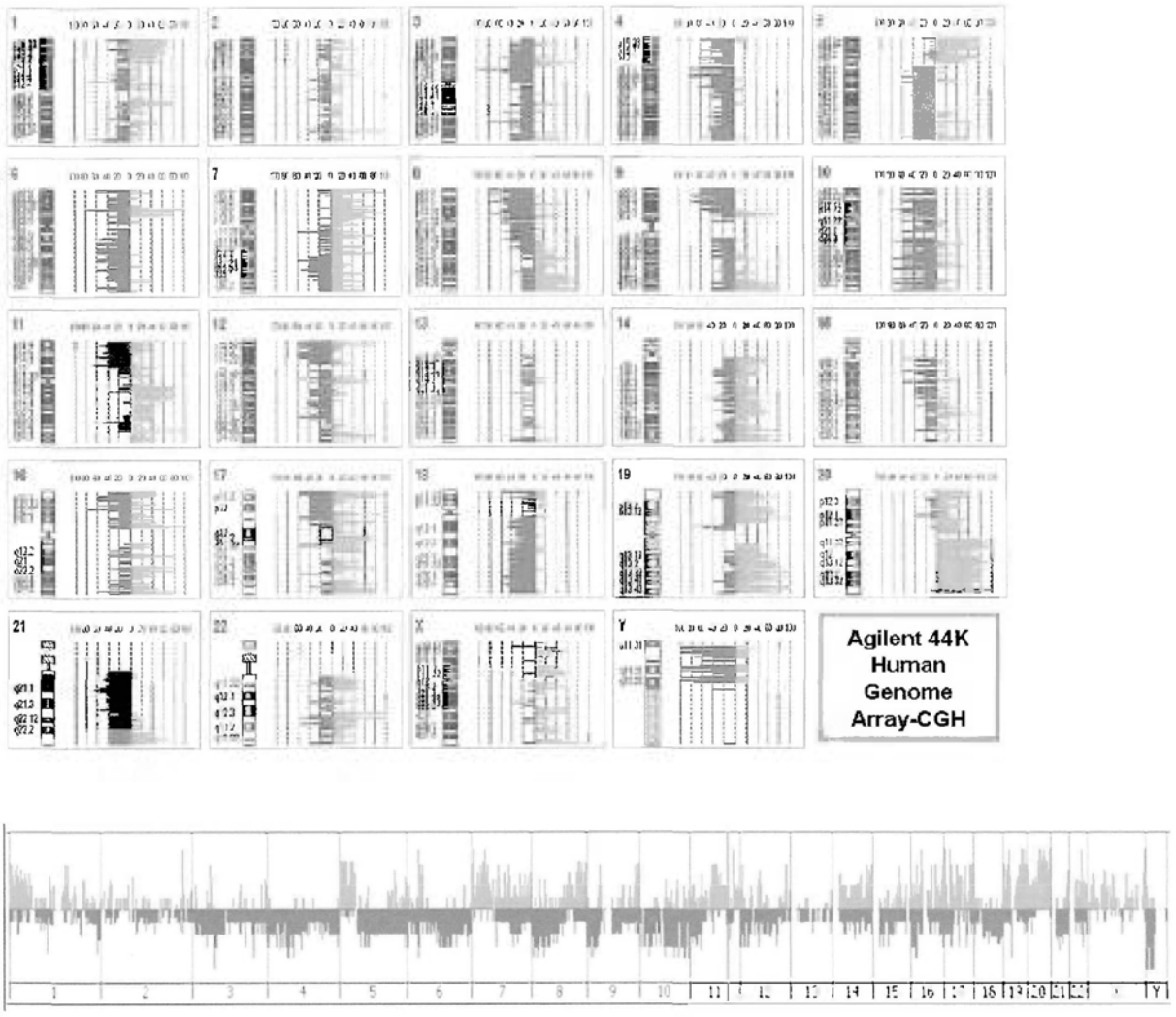


Figure 4.1 **Genome-wide frequency plots of copy number alterations of each chromosome.** Red denoted copy-number gains and green represented losses. Clones were ordered as the position of each gene and alterations were defined by log2 ratio thresholds of -0.58 and 0.58 for copy number gains and losses respectively. The lower panel was the horizontal figure of copy number change frequency of these 9 GCA cell lines, taken all chromosomes together.

Array-CGH could provide DNA copy number changes with high resolution. A representative high-level amplification of 1p36.12 in MKN45 cells was shown in Figure 4.2. In this aberration summary, a large gain region was found in the p arm of chromosome 1. A total of 9 candidate genes (CaMKIIN alpha, ECE1, ALPL, RAP1GA1, ELA3B, ELA3A, EPHA8, EPHB2, and E2F2) showed high-level amplification (log2 ratio>2) at 1p36.12 in MKN45 cells. Figure 4.3 demonstrated another representative aberration summary for chromosome 7 of MKN45 cells. A high-level amplification region (log2 ratio>2) on 7q31.31 which encompassing 8 candidate genes (KCND2, TM4SF12, ING3, WNT16, FAM3C, PTPRZ1, AASS, and CADPS2) was observed.

DNA copy number changes in each GCA cell line were summarized in Table 4.4. For example, in MKN45, the gain regions were mainly located in 1p, 1q23.2, 5p15.32, 7p, 7q31.31,10q, 11q13.2, 16p, 17q, 20q, and Xp, and loss regions were located in 1q42.2, 2p22.2-22.3, 4q, 6q, 8p, 9p, 12q21.31, 13q, 18q, and Y. Table 4.5 listed some "special genes" that showed both high-level amplification and homozygous deletion in different GCA cell lines (e.g. MGAT, GNL3 and SLC25A13), and/or amplification and homozygous deletion on different positions of the gene as demonstrated by different probe sites (e.g. CACNA2D)

However, these findings will be future verified by FISH or genomic DNA copy number qPCR.

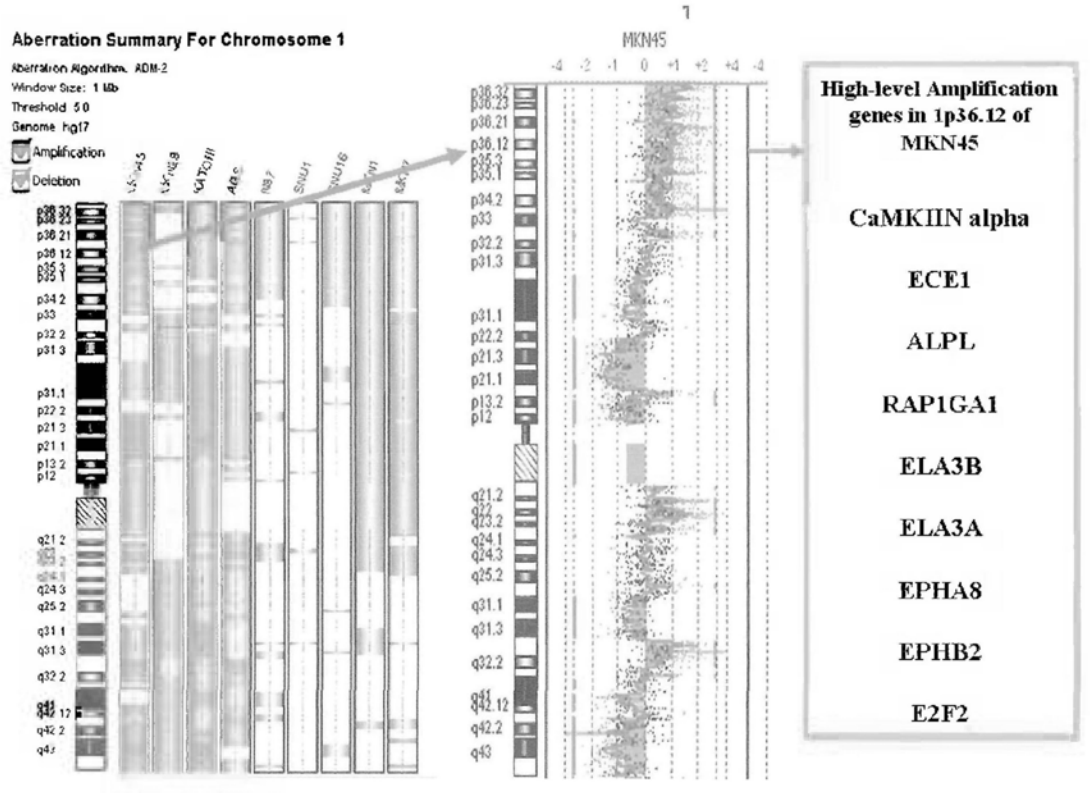


Figure 4.2 **Genes with high-level amplification in 1p36.12 of MKN45.** The genes in this region with high-level amplification include CaMKIIN alpha, ECE1, ALPL, RAP1GA1, ELA3B, ELA3A, EPHA8, EPHB2 and E2F2.

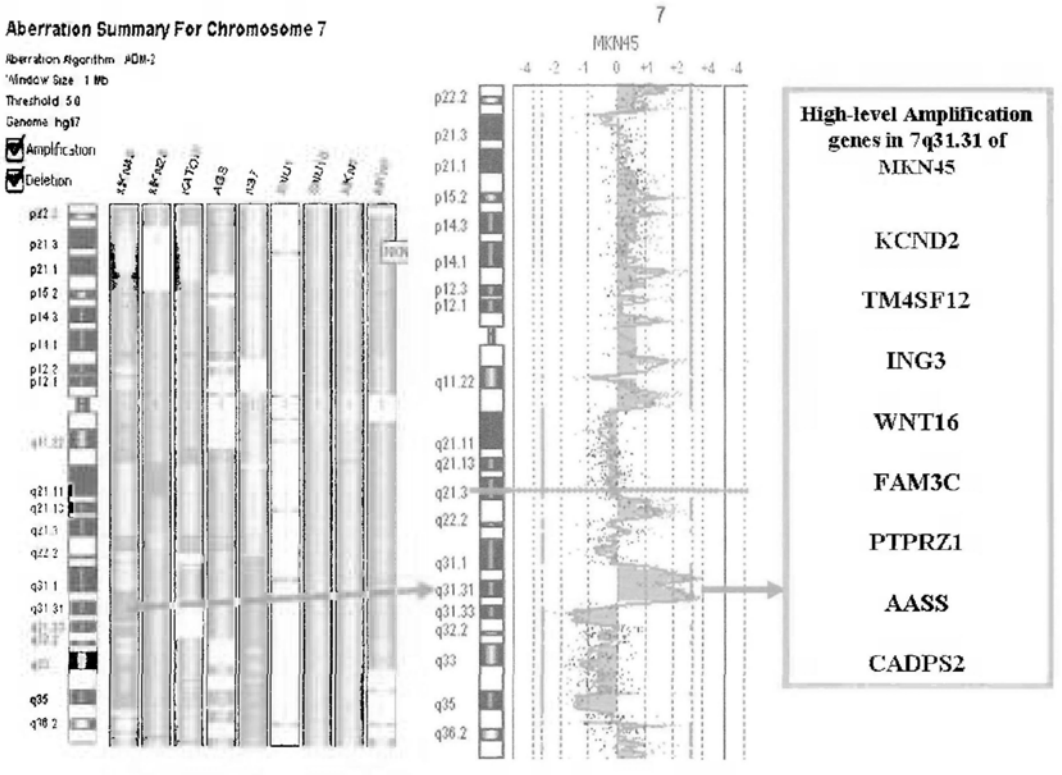


Figure 4.3 **Genes with high-level amplification in 7q31.31 of MKN45.** The genes in this region with high-level amplification include KCND2, TM4SF12, ING3, WNT16, FAM3C, PTPRZ1, AASS and CADPS2.

Table 4.4 **Gene copy number aberration regions in each GCA cell line**

Cell Line	Gains	Losses
MKN45	1p, 1q23.2, 5p15.32, 7p, 7q31.31,10q, 11q13.2, 16p, 17q, 20q, Xp.	1q42.2, 2p22.2-22.3, 4q, 6q, 8p, 9p, 12q21.31, 13q, 18q, Y.
MKN28	7p22.2, 11q13.2, 15q15.2, 16p, 20q11.22.	1q, 3p, 4p, 4q, 5p, 8p, 13q, 18q, Y.
KATO-III	3p25.2, 7p, 9q34.12-34.2, 11q13.2, 14q22.32, 15p, 16p, 17q, 19q,	1q, 2q, 4q, 4q, 5p, 6q, 8q, 10p, 12q, 13q, 18q, Y.
AGS	1q, 11q13.2-23.4, 13q, 14p, 17q, 19q, 20p, 20q, 22q, X.	2q, 4q, 5q, 6q, 7q, 8p, 18q, Y.
N87	5p, 8q21.11-24.23, 20q.	1p36.22-35.3, 3p, 5q, 6p, 6q, 7q22.2-32.2, 8p, 10p, 11p,
SNU1	20p, 20q.	4q32.3-34.3.
SNU16	1p36.32-34.2, 7p, 7q, 14q, 19p, 20p, 20q, X.	3p, 3q, 4p, 4q, 5q, 6p, 6q, 8p, 9p, 9q, 10p, 13q, 16p, 17p,
MKN1	1p, 4p16.2, 5p, 5q, 7p, 7q, 9q, 11q12.3-13.4, 12p12.1, 20q, 21q, X.	8p, 10p, 10q, 14q12-24.2, 15q, 16q, 18p, 22q, Y.
MKN7	1p36.32-35.1, 2p, 5p, 6p, 7p, 11p, 11q, 14q, 17q, 18p, 20p, 20q, X.	1p31.1-12, 4p, 7q, 8q, 10p, 10q, 12p, Y.

Table 4.5 **Special genes which showed both high-level amplification and homozygous deletion in different GCA cell lines**

Gene	Region	Cell line (Log2 ratio)
MGAT5	2q21.2	MKN28(2.087)/KatoIII(-2.352)
FLJ10375	3p22.1	MKN28(-2.006)/ N87(4.939)
GNL3	3p22.1	MKN1(2.810)/MKN7(2.295)/MKN28(-2.040)
CACNA2D	3p22.1	MKN28(-2.467/2.760)
ADAMTS6	5q12.3	MKN28(-2.304)/KatoIII(-2.066)/N87(2.248)
RFXDC1	6q22.1	MKN28(-2.031)/KatoIII(2.316)
TPST1	7q11.21	MKN45(2.184)/MKN28(-2.183)
SLC25A13	7q21.3	MKN28(-2.286)/KatoIII(2.311)
FBXO25	8p23.3	MKN28(-2.857)/MKN1(2.671)/MKN7(2.260)
GATA4	8p23.1	MKN28(-2.517)/KatoIII(2.116)/MKN7(-2.132)
NKX3-1	8p21.2	MKN28(-2.034)/KatoIII(2.034)
RNF38	9p13.2	N87(-2.055)/MKN1(2.278)
GRK5	10q26.11	KatoIII(2.865)/MKN1(-2.112)
ATE1	10q26.13	KatoIII(-2.153)/SNU16(5.783)
TMEM16C	11p14.2	MKN28(2.060)/KatoIII(-2.342)
SLC5A12	11p14.2	MKN45(2.123)/KatoIII(-2.119)
LGR4	11p14.1	MKN45(2.514)/KatoIII(-2.155)
PDHX	11p13	KatoIII(-2.075)/SNU16(4.596)
GRIA4	11q22.3	MKN45(-2.004)/MKN7(2.118)
VWF	12p13.31	MKN45(2.629)/MKN28(-2.067)
KRAS2	12p12.1	MKN28(-2.243)/MKN1(2.799)
PTHLH	12p11.22	MKN28(-2.055)/MKN1(2.066)

UBL3	13q12.3	MKN28(-2.224)/KatoIII(2.461)
EML1	14q32.2	MKN28(-2.160)/KatoIII(2.096)
AKT1	14q32.33	MKN45(2.521)/MKN28(-2.385)/KatoIII(3.088)
SPG7	16q24.3	MKN28(-2.035)/KatoIII(2.542)
RPH3AL	17p13.3	KatoIII(2.396)/N87(-2.033)
ABR	17p13.3	KatoIII(2.483)/N87(-2.163)/MKN7(-2.087)
ATP1B2	17p13.1	MKN45(2.221)/MKN28(2.001)/KatoIII(-2.589)
DNAH9	17p12	MKN28(-2.134)/KatoIII(2.220)
CDK3	17q25.1	MKN45(2.000)/MKN1(-2.075)
LAMA3	18q11.2	MKN28(-2.006)/MKN7(2.268)
NFATC1	18q23	MKN45(2.128)/MKN28(-2.014)
CLPTM1	19q13.31	MKN45(2.778)/KatoIII(2.813)/AGS(3.461)/MKN1(-2.3)
NF2	22q12.2	KatoIII(2.310)/MKN1(-2.003)
APOBEC3A	22q13.1	KatoIII(2.138)/MKN1(-5.409)
MTMR1	Xq28	KatoIII(2.467)/MKN1(-3.780)

4.2 Correlation of DNA copy number changes with mRNA expression profile

One of the biological implications of a copy number change of a gene would be the accompanying changes in the amount of mRNA synthesized from that gene (Kim et al., 2007). DNA copy number changes usually occurred repeatedly in tumors and were important to the carcinogenesis, especially when loss of gene copies on tumor suppressor genes with reduced expression level or gain of gene copies on oncogenes with increased expression level. To investigate the correlation between DNA copy number changes and gene expression in GCA, mRNA expression microarray profiling was performed on the 9 GCA cell lines.

For the 1099 genes with log2 ratio>2 in any of the 9 cell lines (Table 4.2), Pearson correlation coefficients were calculated between the DNA copy number changes and the mRNA expression levels (Figure 4.4). A total of 134 genes had Pearson correlation coefficients greater than 0.6, and 8 genes showed high correlation coefficient greater than 0.9. Four representative genes (PPP2R2B, ERBB2, PERLD1 and FGFR1OP2) demonstrating high correlation of DNA copy number changes and mRNA expression levels were showed in Figure 4.5.

For the 2571 genes with log2 ratio<-1 in any of the 9 gastric cancer cell lines (Table 3), Pearson correlation coefficients were also calculated between the DNA copy number changes and the mRNA expression levels. A group of 475 genes had Pearson correlation coefficients greater than 0.6 and 21 genes showed

high correlation coefficient greater than 0.9 (Figure 4.6). Figure 4.7 showed 4 representative genes in this group (MPDU1, UHRF2 BAP1 and ARIH2).

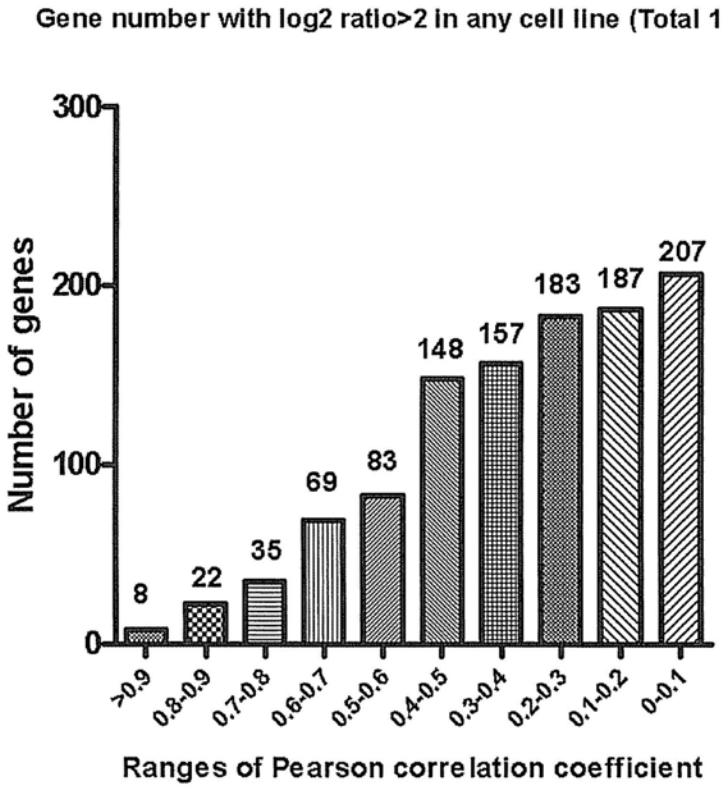


Figure 4.4 **Number of genes according to the range of Pearson correlation coefficient (Genes with array-CGH log2 ratio>2 in any cell line).** Total 1099 genes were subjected to Pearson correlation calculation. A group of 134 genes had Pearson correlation coefficients greater than 0.6 and 8 genes showed high correlation coefficient larger than 0.9.

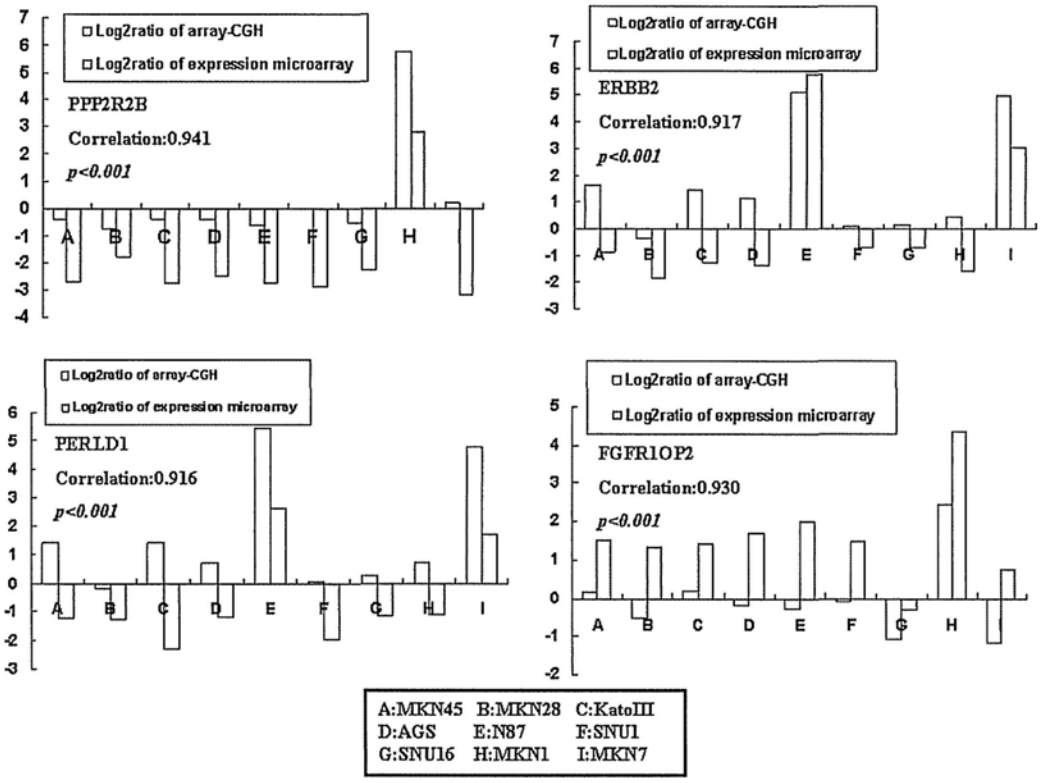


Figure 4.5 **Four representative genes from Figure 4.4 showing high correlation between DNA copy number change and mRNA expression level.** These four genes have Pearson correlation coefficients greater than 0.9, indicating that DNA copy number change correlated with the gene expression level.

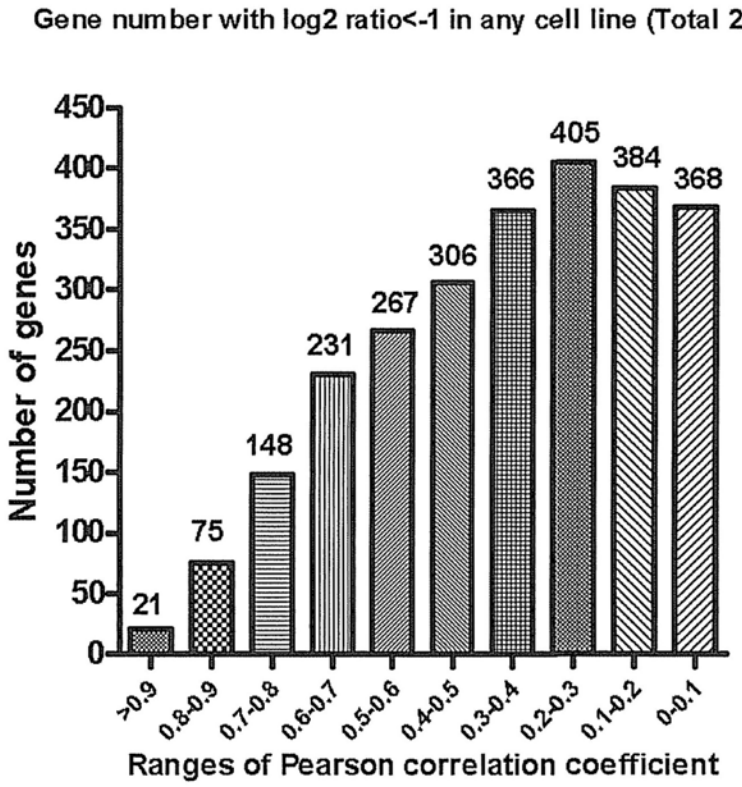


Figure 4.6 **Number of genes according to the range of Pearson correlation coefficient (Genes with array-CGH log2 ratio<-1 in any GCA cell line)** A total of 2571 genes were subjected to Pearson correlation calculation. A group of 475 genes had Pearson correlation coefficients greater than 0.6 and 21 genes showed high correlation coefficient greater than 0.9.

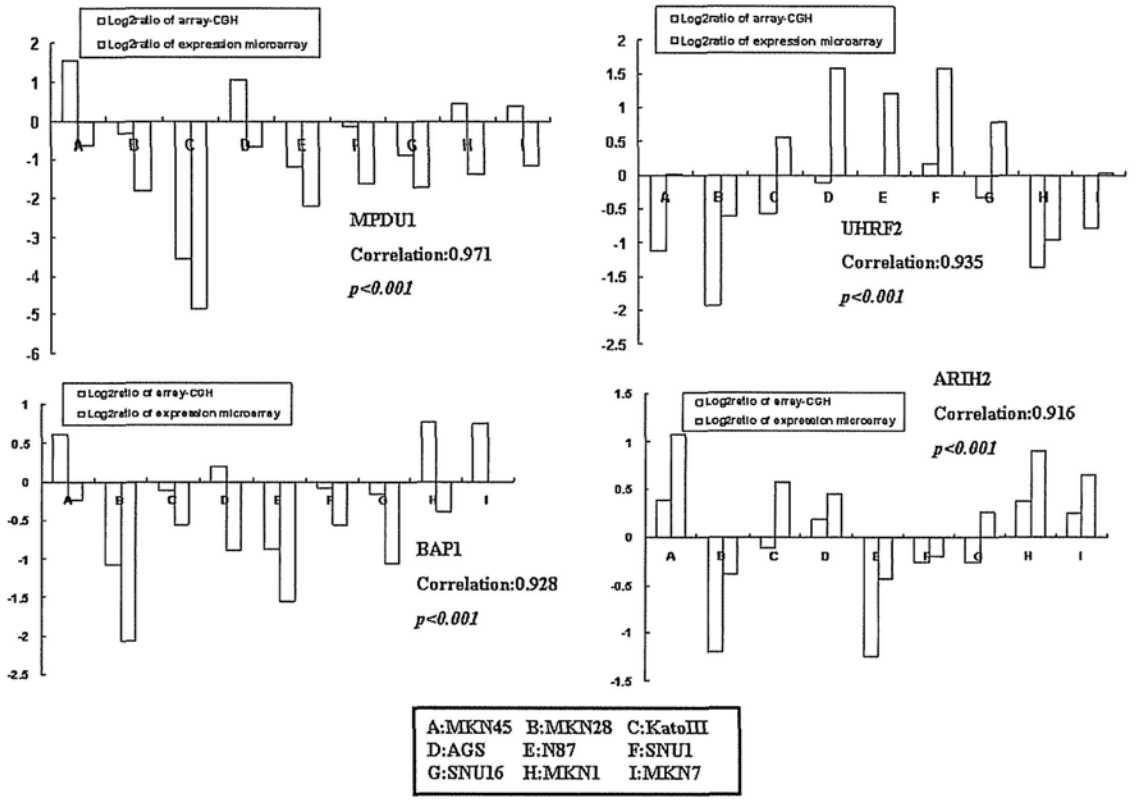


Figure 4.7 **Four representative genes in Figure 4.6 showing high correlation between DNA copy number change and mRNA expression level.** These four genes have Pearson correlation coefficient greater than 0.9.

On the other hand, some genes showed negative correlation between copy-number changes and mRNA expression levels. Four representative genes including ASB2, TSPYL2, MFRP, ABTB1 were shown in Figure 4.8. Further studies were required to uncover the underlying mechanisms for the discrepancies between DNA copy number change and mRNA expression level. For example, a rapid turnover, epigenetic modification or post-transcriptional regulation might contribute to the reverse relationship.

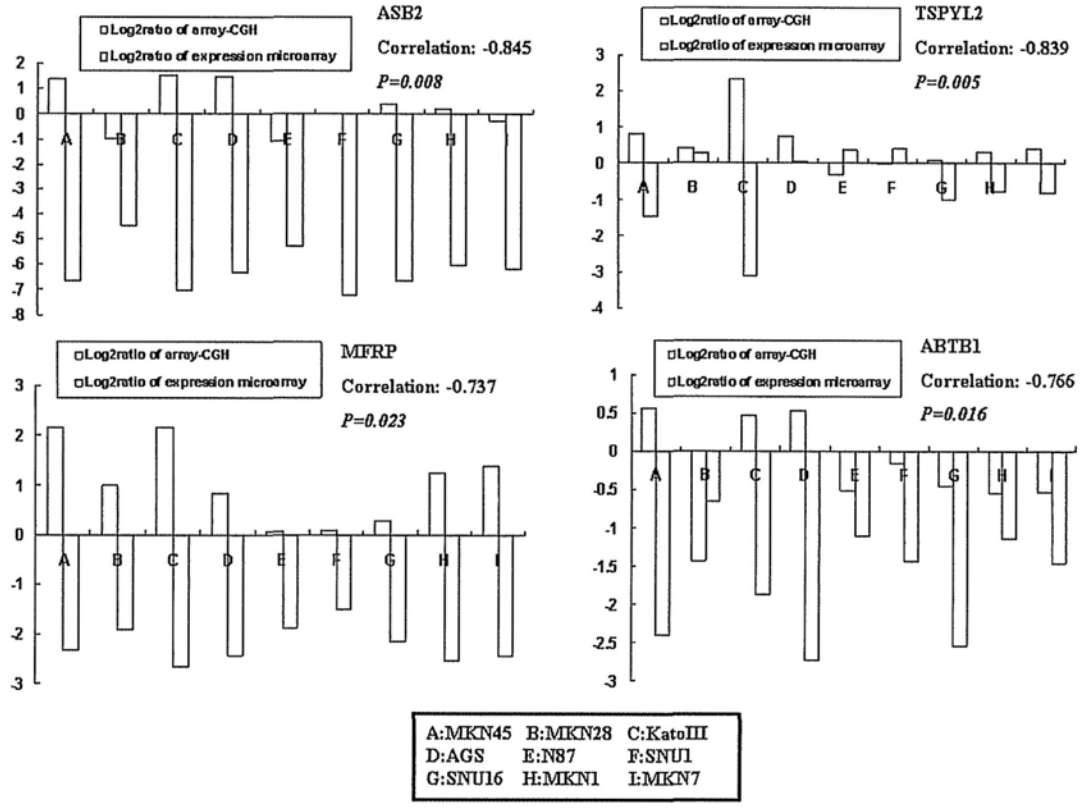


Figure 4.8 **Representative genes showing high negative correlation coefficient between DNA copy number change and mRNA expression level.**

4.3 Identification and characterization of Yes-Associated protein 1 (YAP1) as functional oncogene in gastric cancer

4.3.1 Change of DNA copy number of *YAP1* in GCA cell lines

Yes-associated protein 1 (YAP1) is a 65 KDa proline-rich phosphoprotein located on chromosome 11q22.1. Gain of *YAP1* DNA copy number was found in gastric cancer cell lines MKN7, MKN1 and N87 by array-CGH analysis. There was no obvious copy number change of *YAP1* in cell lines AGS, MKN28, KATOIII, SNU1 and SNU16. Interestingly, array-CGH showed a homozygous deletion of *YAP1* gene in MKN45 cells involving 2 out of 4 consecutive intragenic probes (Table 4.6, Figure 4.9). PCR amplifications using exonic primers confirmed the homozygous deletion of exons 1-4 of *YAP1* gene while exons 5-7 were retained (Figure 4.9). RT-PCR was performed using primers flanking different exons as indicated in Table 4.8. The result was consistent with deletion of exons 1-4 (Figure 4.10). Intriguiningly, we were able to detect exons 5 to 7 of the *YAP1* transcript from MKN45 cells, suggesting a deletion/rearrangement might occur. However, we failed to amplify the 5' sequence of this transcript using Rapid Amplification of 5' complementary DNA ends (5' RACE). Further investigation is required to confirm the identity of the potential fusion partner and the biological significance of the potential chimerical transcript.

Table 4.6 **Array-CGH data (log 2 ratio) of *YAP1*.**

Probe	MKN28	KatoIII	MKN45	SNU16	SUN1	MKN7	MKN1	N87	AGS
1	-0.91	0.4138	-3.613	0.06	-0.0307	1.2226	1.159	1.195	-0.592
2	0.226	0.7247	-4.364	0.137	0.1090	0.4392	0.261	1.167	-0.1
3	-0.15	0.1015	-0.625	0.02	-0.0667	0.9622	0.827	0.955	-0.451
4	0.445	0.7188	-1.051	0.007	-0.1027	0.9723	0.945	1.016	-0.322

Table 4.7 **Primers set for PCR of *YAP1* gene**

Exon	Forward primer	Reverse primer	size (bp)
1	5'-CCCGACTCCTTCTTCAAGC	5'-ACGACTCCAGTTCCACTTCG	203
2	5'-GCAGTTGGGAGCTGTTTCTC	5'-TGTCTTTGCCATCTCCCAAC	165
3	5'-GGAGCAGTGAGATGCTGTGA	5'-GCCATGTTGTTGTCTGATCG	159
4	5'-CCATGAACCAGAGAATCAGT	5'-CCGAAGCAGTTCTTGCTGTT	179
5	5'-TCTGCACGGTTACTCTGATGA	5'-ACATCCCGGGAGAAGACACT	176
6	5'-TTCAGACATTGCAGGACAGG	5'-GGTTCGAGGGACACTGTAGC	207
7	5'-GGAACCTAGGCAAATGACCA	5'-ACTGGAAAGCAGGGAAACCT	225

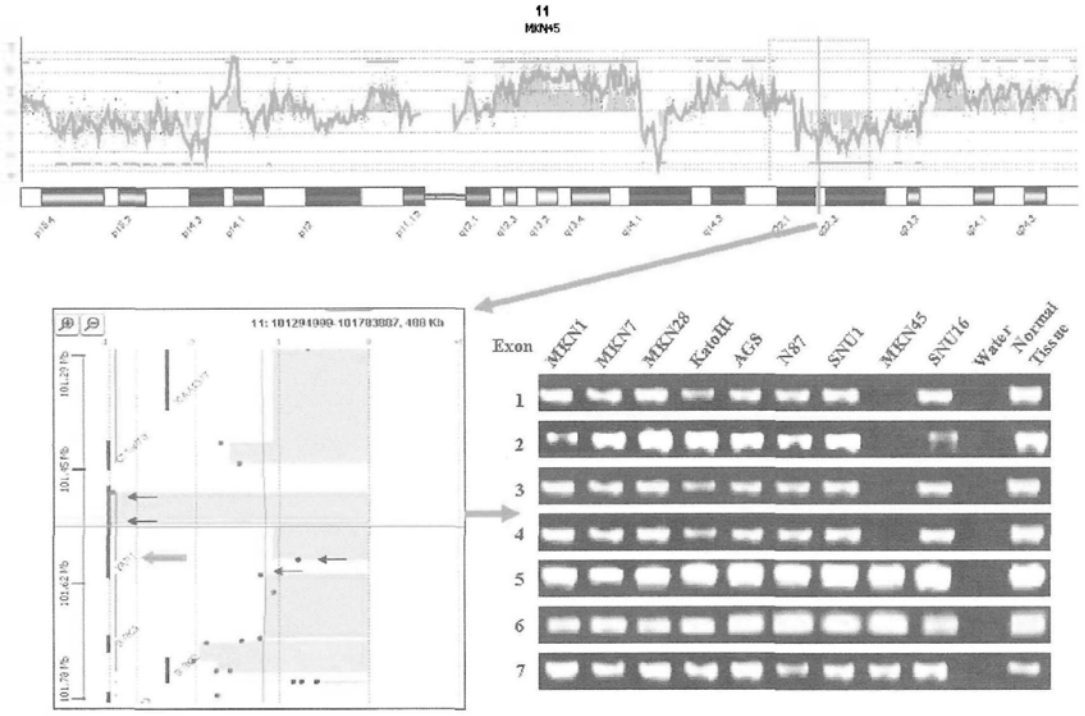


Figure 4.9 **Homozygous intragenic deletion of *YAP1* in MKN45.** The upper panel showed a whole-chromosome view of data from chromosome 11. The boxed region at 11q22.1 containing *YAP1* was covered by 4 oligonucleotide probes. Homozygous deletion was detected with 2 consecutive probes with log2-ratio less than -2. PCR amplification of *YAP1* DNA from 9 GCA cell lines confirmed the deletion of exons 1-4 in MKN45 and exons 5-7 of *YAP1* were retained.

Table 4.8 **Primers set for RT-PCR of *YAP1***

Exon	Forward primer	Reverse primer	size (bp)
1F/7R	5'-CCCGACTCCTTCTTCAAGC	5'-GCAGCCAAAACAGACTCCAT	1050
2F/7R	5'-GCAGTTGGGAGCTGTTTCTC	5'-GCAGCCAAAACAGACTCCAT	928
3F/7R	5'-TCCACCAGTGCAGCAGAATA	5'-GCAGCCAAAACAGACTCCAT	673
4F/7R	5'-CAGCAACTGCAGATGGAGAA	5'-GCAGCCAAAACAGACTCCAT	514
5F/7R	5'-ACAGGAGTTAGCCCTGCGTA	5'-GCAGCCAAAACAGACTCCAT	445
6F/7R	5'-GCAGCCAAAACAGACTCCAT	5'-GCAGCCAAAACAGACTCCAT	241

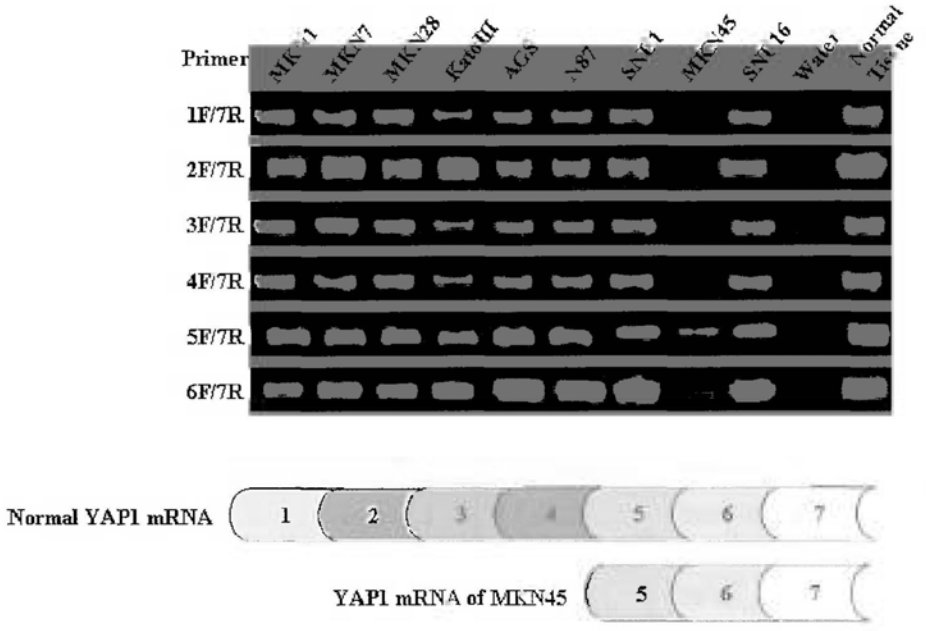


Figure 4.10 **Schematics of *YAP1* transcript in MKN45 cells.** RT-PCR using primers flanking different exons successfully amplified exons 5-7 of *YAP1* mRNA but not exons 1-4. The result was consistent with the loss of exons 1-4 in MKN45 cells.

4.3.2 YAP1 is upregulated in GCA cell lines

Expression microarray analysis revealed the up-regulation of *YAP1* mRNA in 7/9 gastric cancer cell lines compared to normal gastric tissue. The finding was validated by qRT-PCR (Figure 4.11). Western blotting and immunohistochemistry (IHC) were performed to investigate the protein expression level of YAP1 in GCA cell lines. As showed Figure 4.12, most cell lines showed strong or moderate YAP1 expression except MKN45. In addition, the 5 morphologically normal gastric mucosal samples showed negative YAP1 expression. In concordance with the results from Western blotting, 6 cell lines (MKN28, AGS, NCI-N87, SNU1, MKN1 and MKN7) showed strong *YAP1* immunoreactivity with both cytoplasmic and nuclear stain by IHC (Figure 4.13).

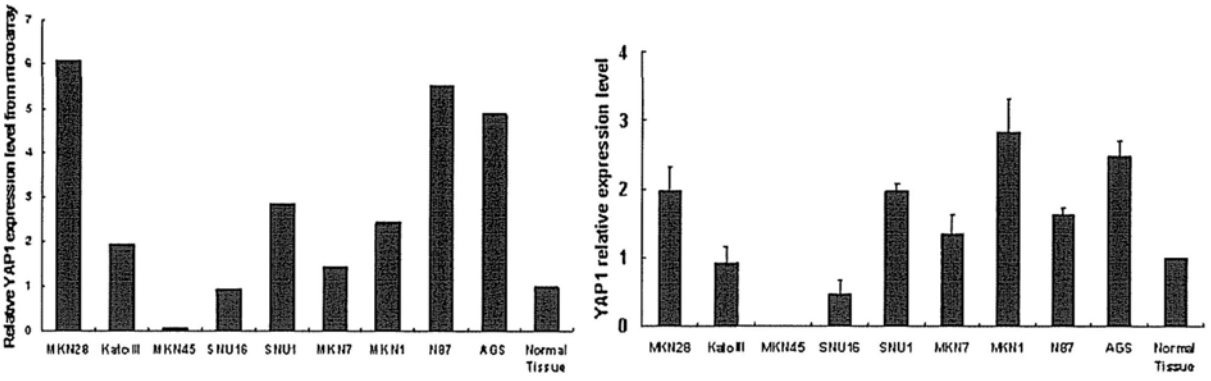


Figure 4.11 ***YAP1*** **mRNA level was upregulated in GCA cell lines.** Upregulation of *YAP1* mRNA was found in GCA cell lines compared with normal gastric tissue by expression microarray analysis and confirmed by qRT-PCR.

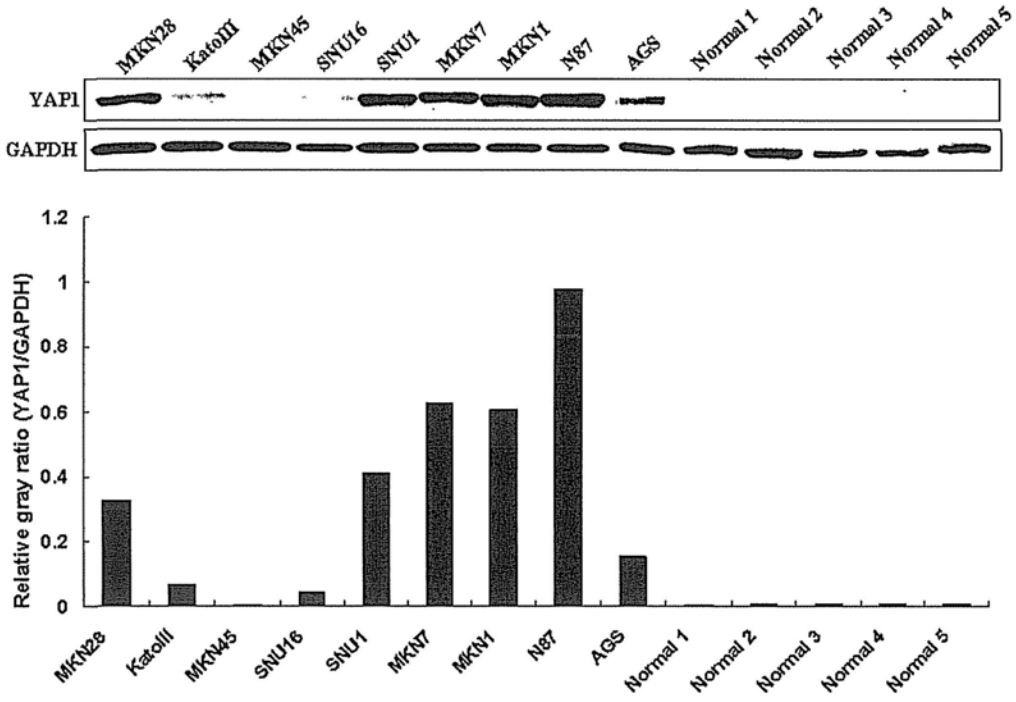


Figure 4.12 **YAP1 protein level was upregulated in GCA cell lines.** All cell lines showed strong or moderate YAP1 expression except MKN45 which showed negative YAP1 expression, whereas no YAP1 protein was detected in 5 normal gastric mucosal tissues.

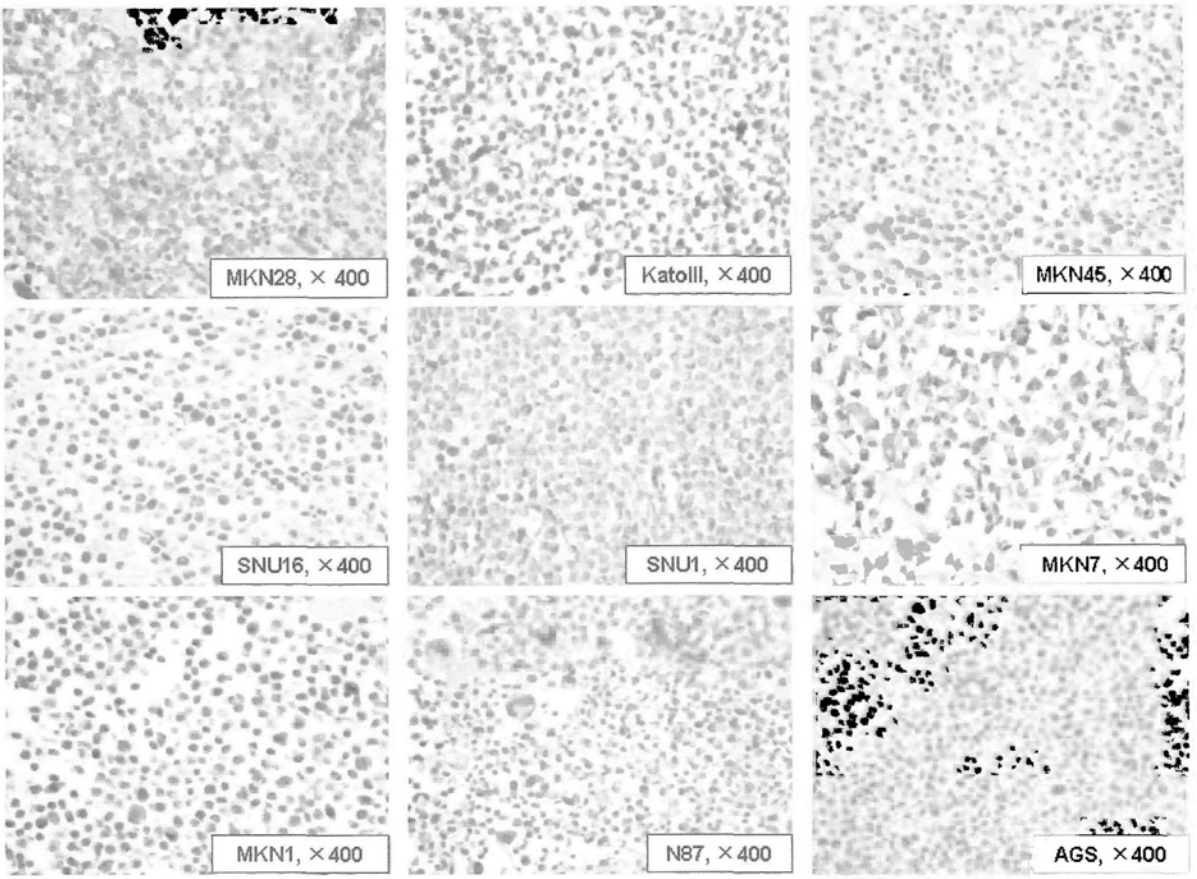


Figure 4.13 **Total YAP1 IHC in GCA cell lines.** Strong expression of total YAP1 was observed in most of the GCA cell lines which can be found in both cytoplasm and nucleus. MKN45 cells were negative for YAP1 expression.

YAP1 could be phosphorylated at S127 by several proteins, such as Lats1 (Zhao et al.), AKT and Merlin. It has been postulated that the extent of p-YAP1(S127) might represent AKT activity (Basu et al., 2003). We analyzed the level of p-YAP1(S127) in GCA cell lines by Western blotting and IHC. P-YAP1(S127) was detected in 7 out of 9 lines (Figure 4.14). IHC demonstrated that p-YAP1(S127) was mainly located in the cytoplasm (Figure 4.15).

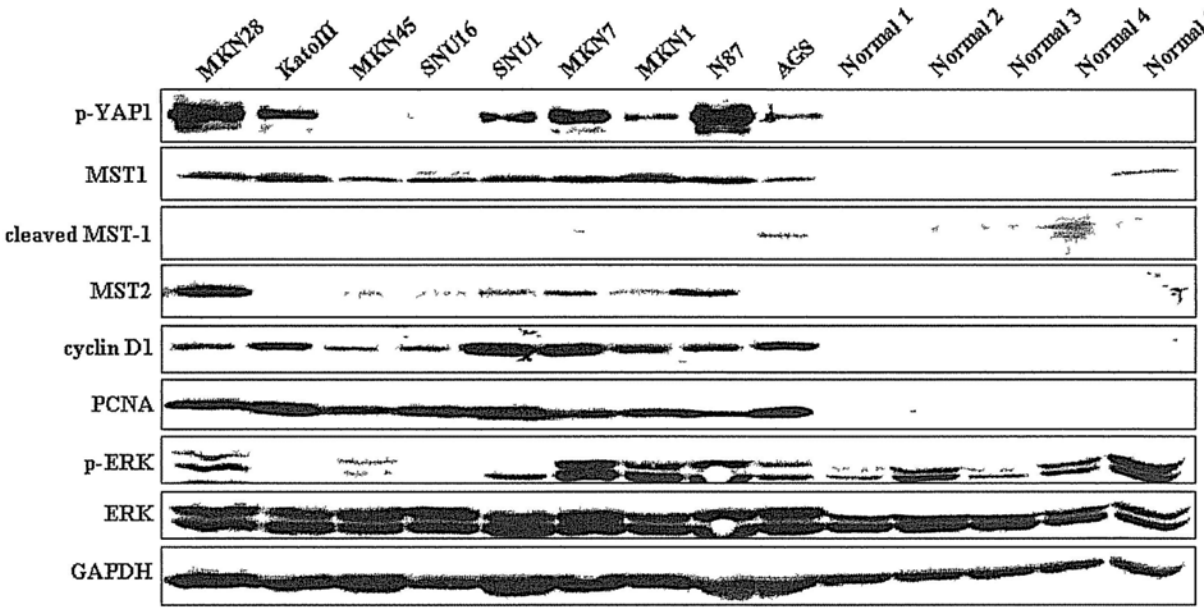


Figure 4.14 **Western blotting of p-YAP1 (S127) and other related proteins in GCA cell lines.** YAP1 could be phosphorylated in S127 and some possible downstream proteins were also upregulated in GCA cell lines. P-YAP1 (S127), cyclin D1 and PCNA were activated in GCA cell lines compared with normal gastric epithelium tissue.

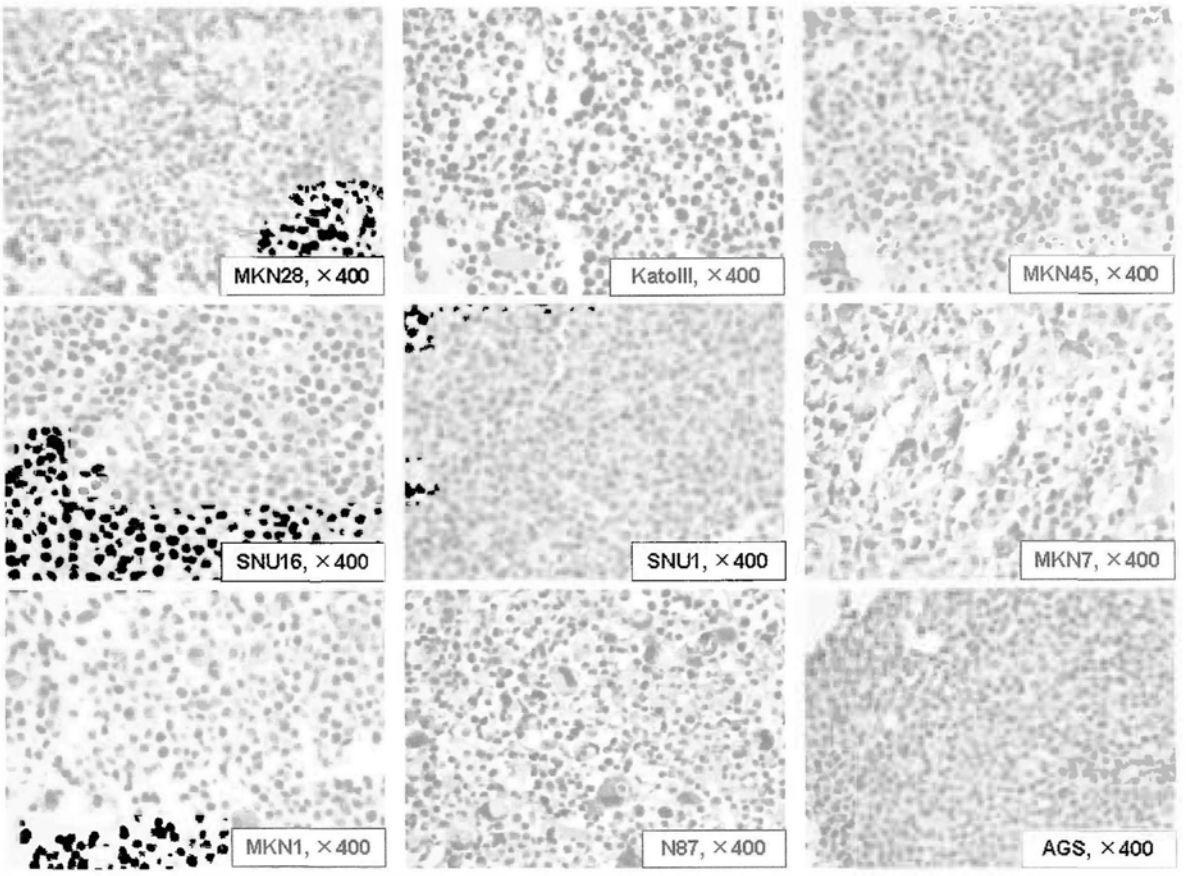


Figure 4.15 **P-YAP1 (S127) IHC of GCA cell lines.** P-YAP1 (S127) was mainly located in the cytoplasm but not in the nucleus.

4.3.3 YAP1 is upregulated in primary GCA

To investigate the YAP1 expression level in primary gastric cancer tissues, western blotting was performed in 10 paired GCA / non-cancerous tissues. Nine out of 10 cases (90%) showed upregulated YAP1 expression in tumor tissues compared to the non-cancerous tissues. Case 6 showed negative YAP1 expression both in the tumor and non-cancerous tissues (Figure 4.16). IHC of another 6 paired GCA cases demonstrated upregulation o f YAP1 in all 6 tumors compared to the non-cancerous tissues. In gastric cancer tissues, strong and diffuse YAP1 immunoreactivity was frequently observed in both nucleus and cytoplasm (Figure 4.17). Whereas in non-cancerous gastric mucosa, YAP1 expression was infrequently expressed in the glandular epithelial cells and the YAP1 immunoreactivity mainly located at the proliferative zone of the gastric glands.

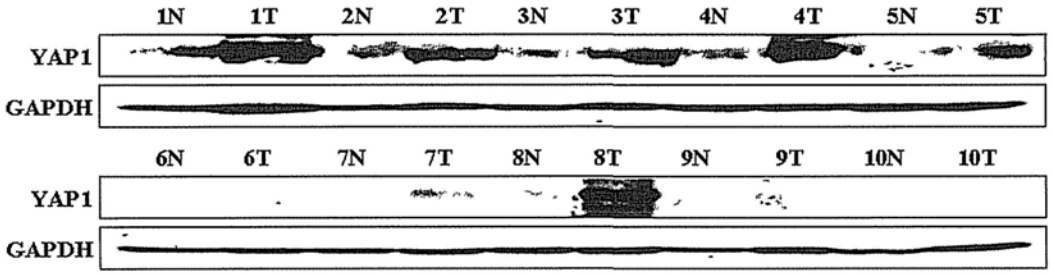


Figure 4.16 **YAP1 showed high expression level in paired GCA samples by Western blotting.** Western Blotting of 10 paired primary GCA samples showed upregulation of total YAP1. N represented non-cancerous gastric mucosal tissue and T denoted tumor tissue. Nine of 10 cases (90%) showed stronger expression of YAP1 in GCA than the normal counterparts. Only in case 6, no YAP1 expression was detected in both tumor and normal tissues.

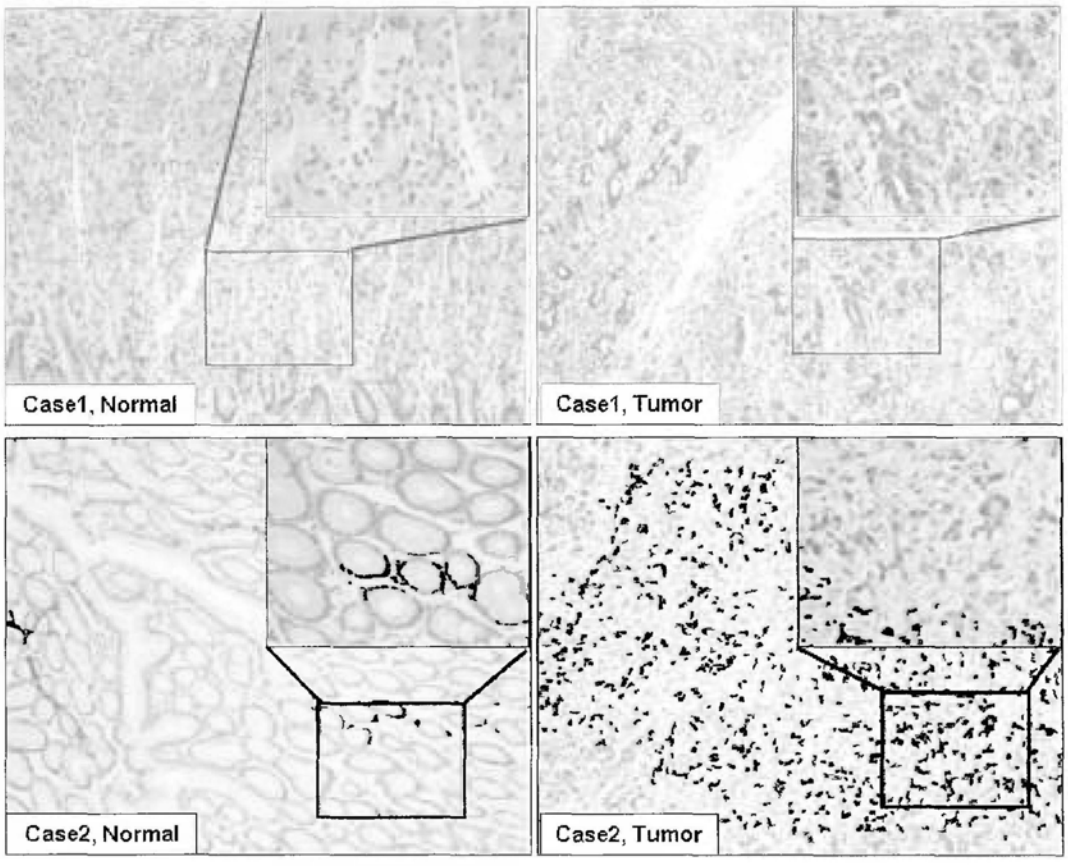


Figure 4.17 **YAP1 showed upregulated expression level by IHC of paired GCA samples.** YAP1 was upregulated in GCA compared with non-cancerous gastric tissue. Strong and diffuse YAP1 immunoreactivity was observed in GCA. Total YAP1 could be located both in the nucleus and cytoplasm of the gastric tumor cells. In non-cancerous gastric mucosa, YAP1 expression was infrequently expressed in the glandular epithelial cells.

4.3.4 YAP1 nuclear accumulation predicted poor prognosis in GCA

To investigate the clinical significance of YAP1 in GCA and its prognostic value, we evaluate the YAP1 protein expression in a cohort of 129 GCA by IHC. The formalin-fixed paraffin-embedded GCA tissues diagnosed between 1998 and 2002 in the Prince of Wales Hospital, Hong Kong was retrieved. The median age of the patients was 69 years (range 38-88 years) and male to female ratio was 1.8:1. The median follow-up time was 17.2 months (range 0.3-143.4 months). The study protocol was approved by the Joint CUHK-NTE Clinical Research Ethics Committee, Hong Kong.

Gastric carcinoma cells often exhibited cytoplasmic and nuclear expression of YAP1 (Figure 4.18). Since the subcellular localization is critical in determining the functions of YAP1, the cytoplasmic and nuclear staining were scored separately. Marked nuclear immunoreactivity was seen in 42% (54/129) of the GACs. Intermediate and low nuclear stain were noted in 31% (40/129) and 19% (25/129), respectively. Ten GAC samples (8%) showed negative nuclear stain of YAP1. For the cytoplasmic localization of YAP1, strong and moderate immunoreactivity were seen in 32% (41/129) and 42% (54/129) of GACs, respectively. Weak and negative YAP1 cytoplasmic immunoreactivity accounted for 26% (34/129) of the tumor samples. IHC was also performed in 23 non-cancerous gastric mucosal tissues. A small proportion of epithelial cells on the proliferative zone or at the neck region of gastric gland exhibited YAP1 immunoreactivity. Expression of YAP1 was significantly higher in GCA than

non-cancerous gastric mucosa ($P<0.001$, Table 4.9).

The expression of p-YAP1 (S127) was also evaluated on the same cohort of GCA using IHC. Half of cases showed moderate to strong p-YAP1 (S127) staining and p-YAP1 (S127) mainly localized in the cytoplasm of the tumor cells (Figure 4.19).

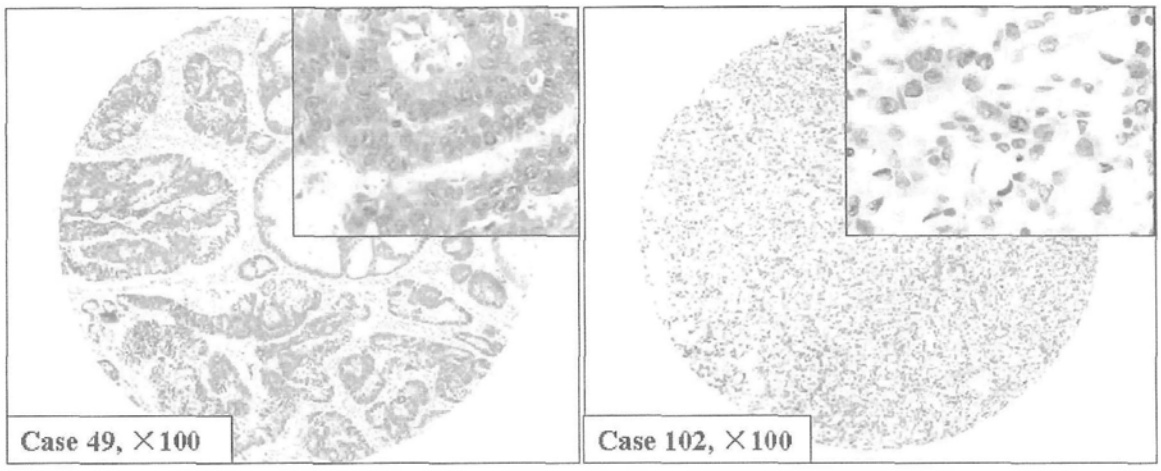


Figure 4.18 **Representative figures of total YAP1 expression in gastric cancers.** Strong cytoplasmic and nuclear YAP1 stain was detected in tumor cells from an intestinal type (left panel) and a diffuse type (right panel) GCA.

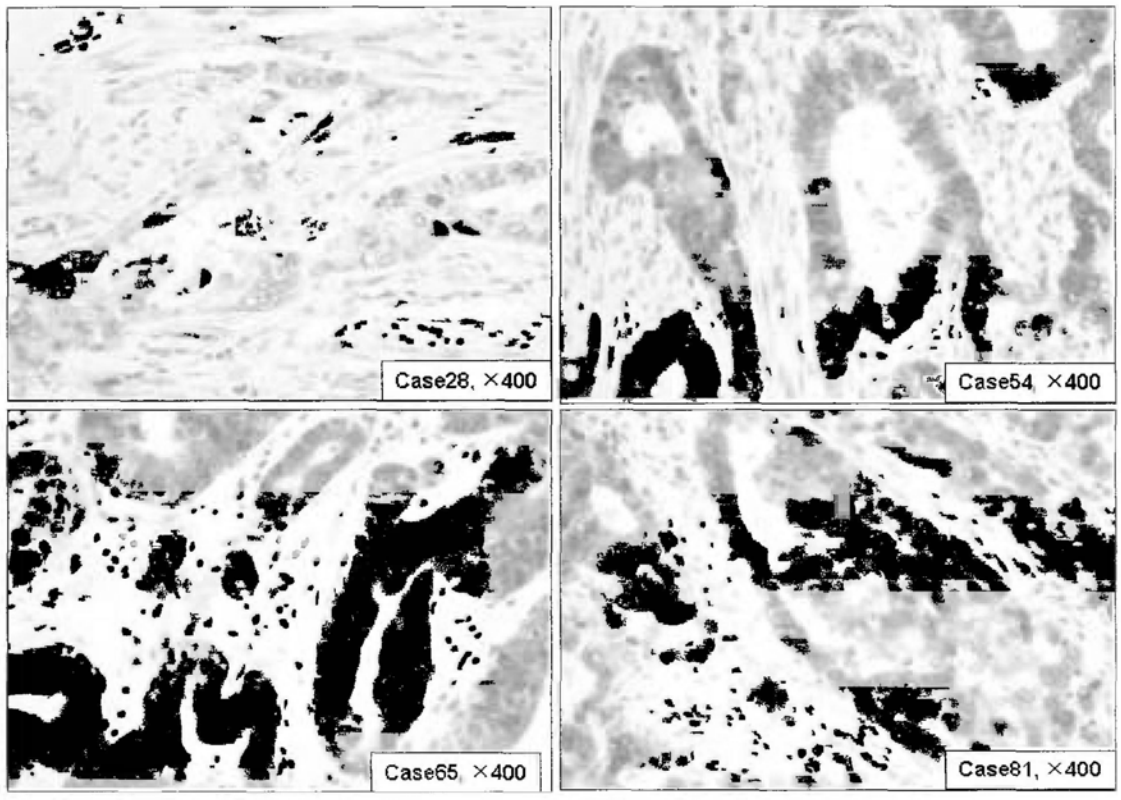


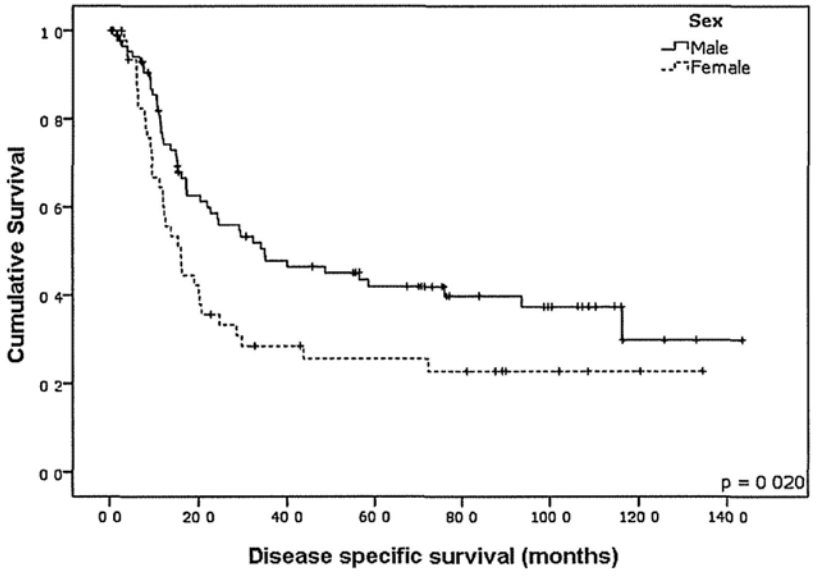
Figure 4.19 **Representative pictures of p-YAP1 (S127) expression in GCA samples.** P-YAP1 (S127) was mainly located in the cytoplasm.

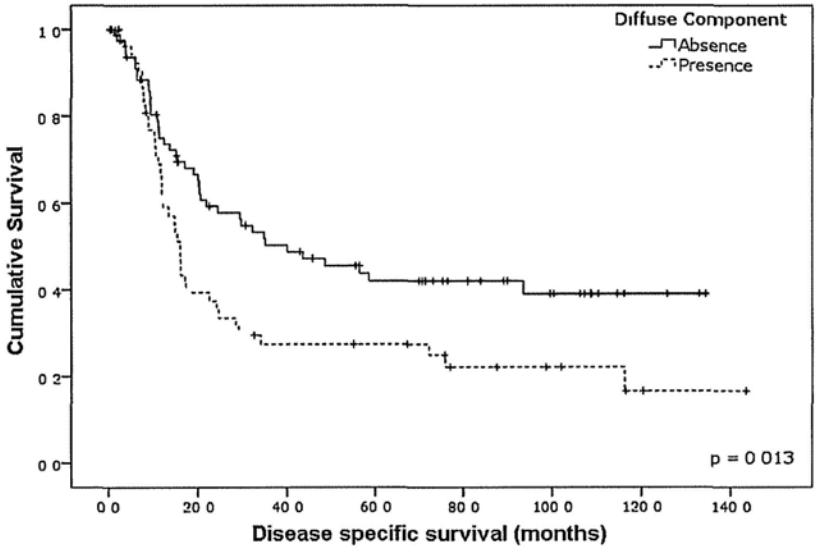
Univariate analysis indicated that other factors including female gender (*P=0.027*), histology with diffuse component (*P=0.021*), higher tumor grade (*P=0.037*) and advance stage (*P<0.001*) also correlated with poor survival (Figure 4.20). Up-regulation of YAP1 nuclear expression in gastric cancer associated with a poorer disease specific survival (gastric cancer specific survival in this project, which means the patients died from gastric cancer was counted, otherwise excluded) by univariate analysis (*P=0.021*, Figure 4.21 upper panel). Especially in patients with early stage diseases (stage I & II), a strong association for higher nuclear YAP1 expression (score 2 or above) with shorter disease specific survival (*P<0.001*, Figure 4.21 lower panel) was demonstrated. By multivariate Cox proportional hazards regression analysis, only stage was independently associated with disease specific survival (*P<0.0001*). Nuclear expression of YAP1 did not associate with age, gender, histological type, grading, staging, or the presence of H. pylori (Table 4.10). Cytoplasmic expression of YAP1 did not associate with survival or other clinicopathologic parameters.

For the p-YAP1 (S127) IHC in this cohort of GCA patients, both the nuclear and cytoplasmic staining of p-YAP1 (S127) had no correlation with age, sex, site, histological type, and stage or disease free survival.

To further verify the clinical significance of YAP1 as a predictive marker for disease specific survival in early stage gastric cancer, we recruited another 65

cases of stage I and II gastric cancers for further analysis. In a cohort of total 101 early stage gastric cancers, the YAP1-negative subgroup (n=23) had a significantly better prognosis than YAP1-positive subgroup (n=78) by Kaplan-Meier analysis (*P=0.040*, Figure 4.22). In addition, multivariate Cox regression also showed that YAP1 was an independent predictor of short disease specific survival time for patients with early stage gastric cancer (*P=0.042*) in addition to T stage (*P=0.038*).


Sex
Male
Female
Cumulative Survival
1 0
0 8
0 6
0 4
0 2
0 0
p = 0 020
0 0
20 0
40 0
60 0
80 0
100 0
120 0
140 0
Disease specific survival (months)


Diffuse Component
Absence
Presence
Cumulative Survival
1 0
0 8
0 6
0 4
0 2
0 0
p = 0 013
0 0
20 0
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100 0
120 0
140 0
Disease specific survival (months)

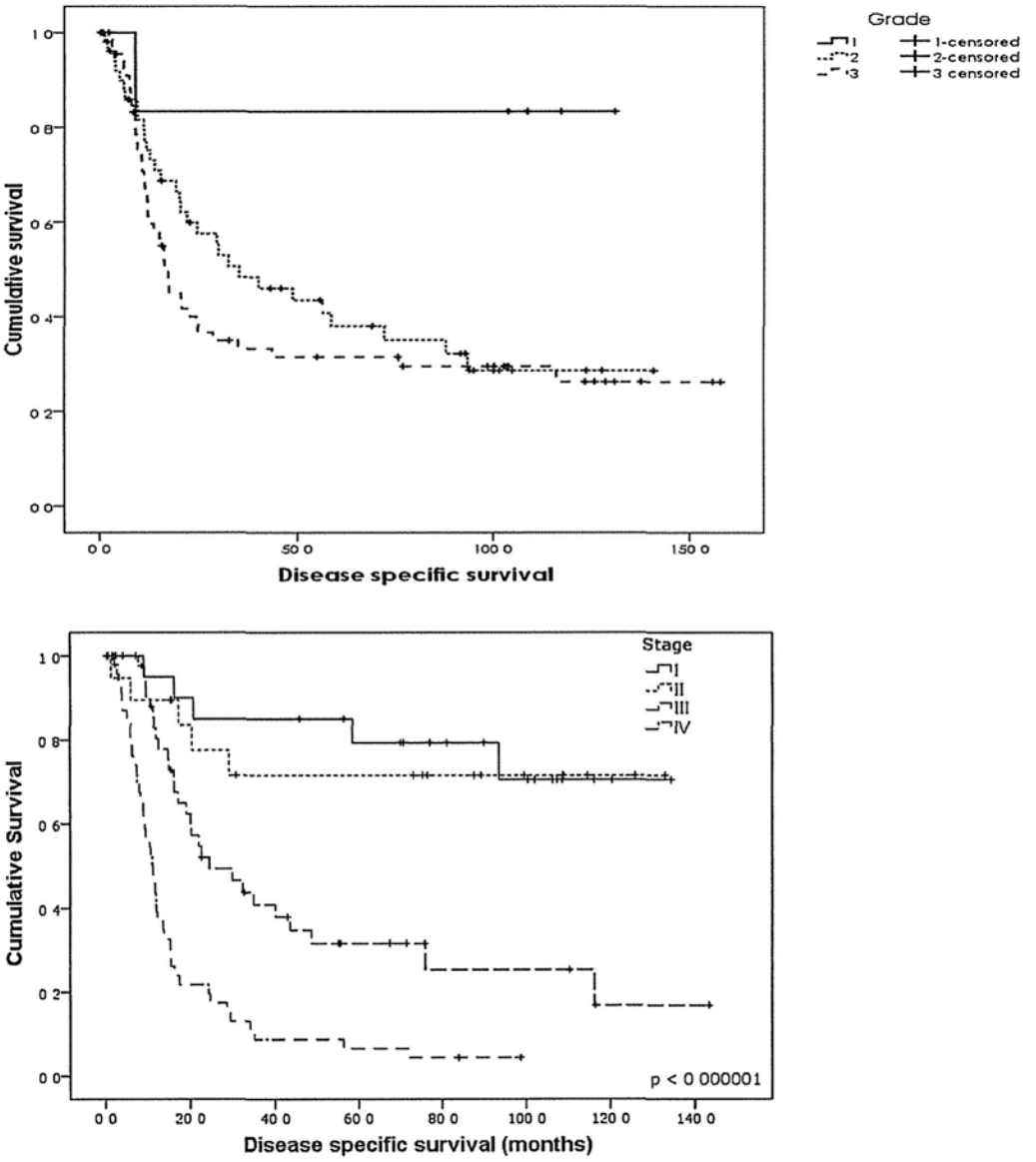


Figure 4.20 **Clinicopathologic parameters with prognostic significance in this cohort of GCA patients.** By univariate Cox regression analysis, sex, TNM stage, tumor grade and diffused histological component were associated with disease specific survival.

Table 4.9 **Total YAP1 expression was significantly higher in GCA than non-cancerous gastric mucosa.**

Characteristic	**Normal Samples (n=23)**	**GCA cases (n=129)**	***P-value***
Total YAP1 IHC staining			*<0.001*
Negative	17	10	
Positive	6	119	

Table 4.10 **Correlation of YAP1 nuclear expression with clinicopathologic features.** *P-value* is given for comparing the distribution of characteristics between patient clinicopathologic characteristics and YAP1 nuclear staining.

		YAP1 Nuclear Staining								
YAP1 Score		**0**		**1**		**2**		**3**		***P-value***
Sex	M	9	(10.84%)	14	(16.87%)	24	(28.92%)	36	(43.37%)	*0.267*
	F	1	(2.13%)	11	(23.40%)	16	(34.04%)	19	(40.43%)	
Age		63.8	+/-8.1	61.2	+/-12.9	71	+/-10.9	66.6	+/-12.2	*0.120*
Type	Intestinal	7	(8.64%)	14	(17.28%)	25	(30.86%)	35	(43.21%)	*0.953*
	Diffuse	2	(6.45%)	6	(19.35%)	9	(29.03%)	14	(45.16%)	
	Mixed	1	(5.56%)	5	(27.78%)	6	(33.33%)	6	(33.33%)	
Diffuse Component	Absence	7	(8.64%)	14	(17.28%)	25	(30.86%)	35	(43.21%)	*0.869*
	Presence	3	(6.12%)	11	(22.45%)	15	(30.61%)	20	(40.82%)	
Grade	1	2	(33.33%)	0	(0.00%)	3	(50.00%)	1	(16.67%)	*0.941*

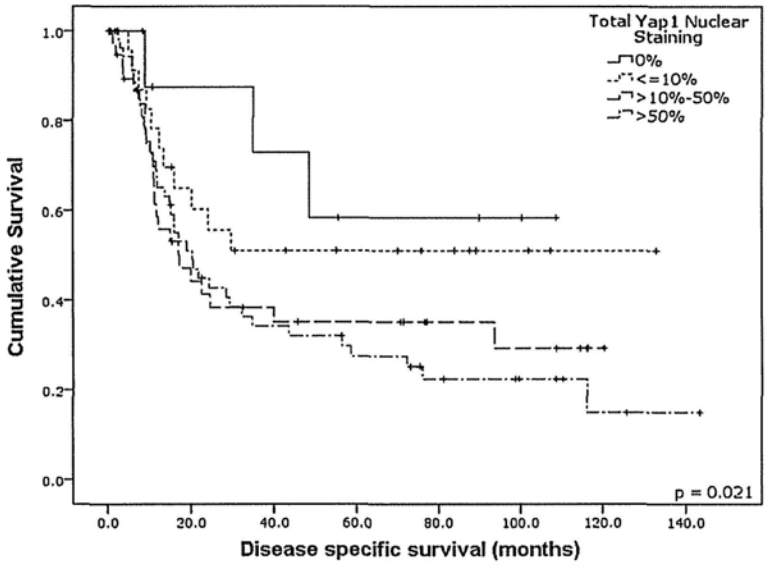
	2	3	(5.66%)	11	(20.75%)	14	(26.42%)	25	(47.17%)	
	3	5	(7.04%)	14	(19.72%)	23	(32.39%)	29	(40.85%)	
Stage	I	2	(11.11%)	3	(16.67%)	7	(38.89%)	6	(33.33%)	*0.316*
	II	1	(5.56%)	4	(22.22%)	5	(27.78%)	8	(44.44%)	
	III	4	(8.33%)	10	(20.83%)	16	(33.33%)	18	(37.50%)	
	IV	3	(6.52%)	8	(17.39%)	12	(26.09%)	23	(50.00%)	
Stage (T)	1	0	(0.00%)	1	(12.50%)	3	(37.50%)	4	(50.00%)	*0.993*
	2	5	(13.89%)	5	(13.89%)	11	(30.56%)	15	(41.67%)	
	3	4	(5.13%)	19	(24.36%)	23	(29.49%)	32	(41.03%)	
	4	1	(12.50%)	0	(0.00%)	3	(37.50%)	4	(50.00%)	
Stage (N)	0	2	(10.00%)	6	(30.00%)	8	(40.00%)	4	(20.00%)	*0.319*
	1	1	(2.78%)	5	(13.89%)	12	(33.33%)	18	(50.00%)	
	2	5	(11.63%)	9	(20.93%)	11	(25.58%)	18	(41.86%)	

	3	2	(6.45%)	5	(16.13%)	9	(29.03%)	15	(48.39%)	
Stage (M)	0	9	(8.11%)	20	(18.02%)	35	(31.53%)	47	(42.34%)	*0.827*
	1	1	(5.26%)	5	(26.32%)	5	(26.32%)	8	(42.11%)	
Helicobacter pylori	Absence	5	(11.36%)	10	(22.73%)	13	(29.55%)	16	(36.36%)	*0.760*
	Presence	5	(6.76%)	14	(18.92%)	24	(32.43%)	31	(41.89%)	

Table 4.11 **Univariate analysis of the association between clinicopathologic features and disease specific survival (DSS) in patients with gastric adenocarcinoma.**

Clinicopathologic features (No. of patients)	Median survival time (months)	Univariate analysis (Kaplan-Meier) ***P-value***
Sex		
Male (83)	34.8	
Female (46)	16.1	***0.027***
Diffuse Component		
Absence (80)	35.1	
Presence (49)	15.0	***0.021***
Grade		
1 (6)	>100	
2 (52)	32.3	
3 (71)	16.2	***0.037***
Stage		
I (18)	>100	
II (18)	>100	
III (48)	24.5	
IV (45)	11.1	***<0.0001***
Stage (T)		
1 (8)	>100	
2 (36)	>100	
3 (77)	15.2	
4 (8)	8.9	***<0.0001***
Stage (N)		

0 (20)	>100	
1 (36)	24.7	
2 (43)	20.3	
3 (30)	11.7	***<0.0001***
Stage (M)		
0 (111)	32.3	
1 (18)	8.3	***<0.0001***
Helicobacter pylori		
Absence (50)	17.4	
Presence (79)	29.8	*0.188*
YAP1 Nucleus		
0 (10)	>100	
1 (25)	>100	
2 (40)	17.4	
3 (54)	20.4	***0.021***



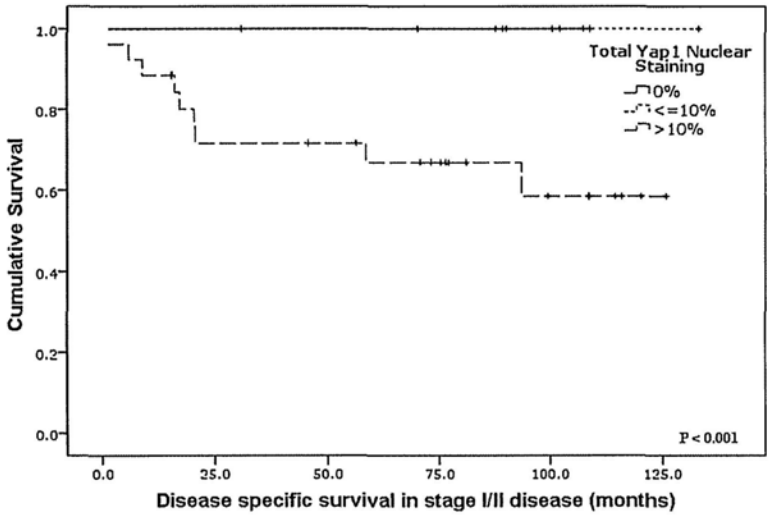


Figure 4.21 **YAP1 nuclear staining could predict poor prognosis in a cohort of GCA patients.** Kaplan-Meier survival curves according to nuclear scoring of total YAP1 IHC. Nuclear YAP1accumulation indicated a poor survival result in this cohort of patients with GCA (*P=0.021*). Nuclear YAP1accumulation (score 2 & 3) correlated with poor prognosis in early stage (stage I & II) GCA patients with much more significance (*P<0.001*).

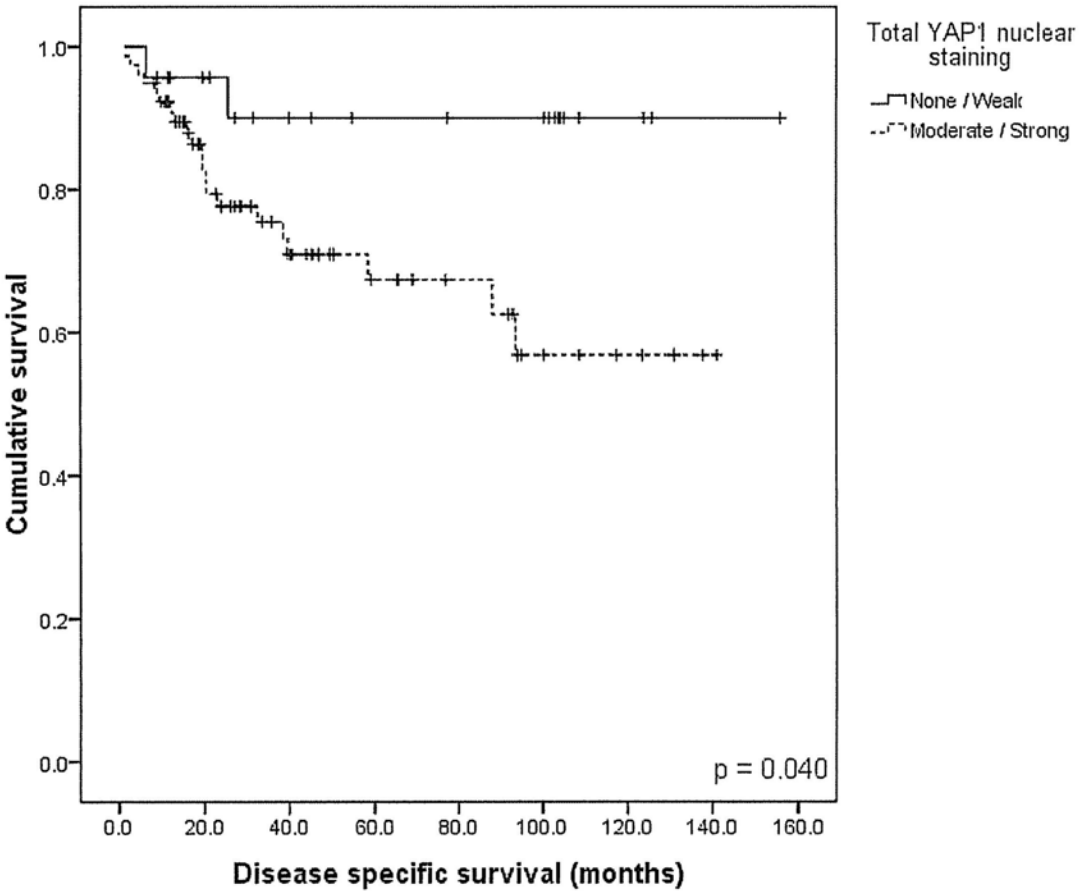


Figure 4.22 **Kaplan-Meier plots of disease free survival according to YAP1 nuclear expression status in a cohort of 101 early stage (I/II) gastric cancers.**

4.3.5 Effect of YAP1 knockdown in GCA cell lines

Frequent upregulation of YAP1 in GCA suggested a potential oncogenetic role of this gene. In order to investigate the functional role of YAP1 in gastric tumorigenesis, we knocked down the endogenous YAP1 expression in MKN1 and AGS cells with YAP1 siRNA. Successful knockdown of YAP1 in both mRNA and protein level were confirmed by qRT-PCR and Western blotting (Figure 4.23).

siYAP1 inhibited cell growth and colony formation in GCA cell lines

A significantly decreased proliferation rate ($P<0.001$, Figure 4.24) was observed in these two cell lines upon YAP1 knockdown compared with scramble siRNA and mock control groups. Monolayer colony formation assays were performed to assess the effect of YAP1 knockdown on anchorage dependent growth of MKN1 and AGS cells. YAP1 siRNA significantly reduced monolayer colony formation in both cell lines under normal culture condition ($P<0.001$, Figure 4.25).

siYAP1 inhibited GCA cell migration and invasion

Migration and invasion ability are essential for tumor progression as well as metastasis. To evaluate the effect of YAP1 in cancer cell migration and invasiveness, we first performed monolayer wound healing assay in MKN1 and AGS cells. The ability for cells to migrate and close the wound gaps were assessed 48 hours after transfection. Suppression of wound healing ability by YAP1 siRNA was observed in both MKN1 and AGS cells, suggesting YAP1

siRNA inhibited the migration of GCA cells (Figure 4.26).

To assess the ability of YAP1 in modulating cancer cells invasion, invasion assays using Matrigel model was performed. A significant reduction in the number of invasive cells through the Matrigel-coated Boyden chamber was noted on YAP1 knockdown ($P<0.001$, Figure 4.27), indicating that knocking down YAP1 inhibited the invasiveness of GCA cells.

siYAP1 induced G1 arrest in GCA cells

Since a growth inhibitory effect was observed in siYAP1 transfected cells, we analyzed the transfectants for cell cycle parameters using flow cytometry. Twenty-four hours after transfection, accumulation of G1 cells increased in siYAP1 transfectant compared to the scramble siRNA controls (Figure 4.28). Cells in the G1 phase were increased from 55% to 72% in MKN1, and 37% to 49% in AGS cells. This cycle arrest might be induced by ERK (P44/42) phosphorylation, and P21 showed upregulation after transfection as downstream protein of p-ERK, indicating that knocking down YAP1 could influence cell cycle progression through cell cycle arrest. This finally resulted in the hypophosphorylation of p-Rb (S807/811). At the mean time, some associated protein, such as p-AKT (S473) and p-Stat3 (T705) showed inactivation accompanied with the decreasing expression of YAP1 (Figure 4.29).

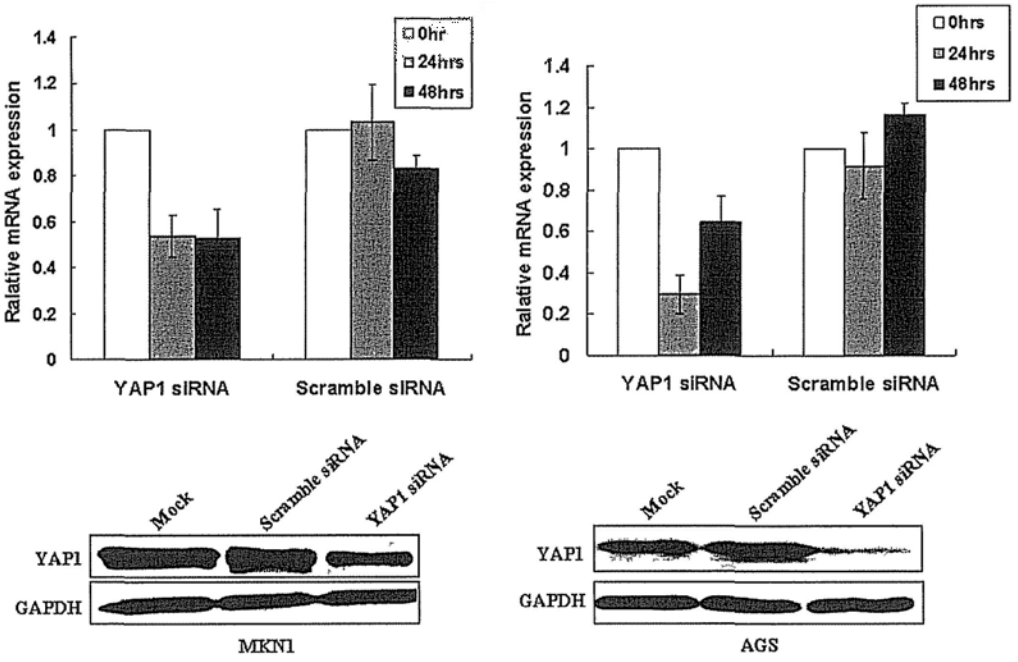


Figure 4.23 **YAP1 expression was confirmed by qRT-PCR (upper panels) and Western blotting (lower panels) in MKN1 and AGS 24 hours after transfection with YAP1 siRNA.**

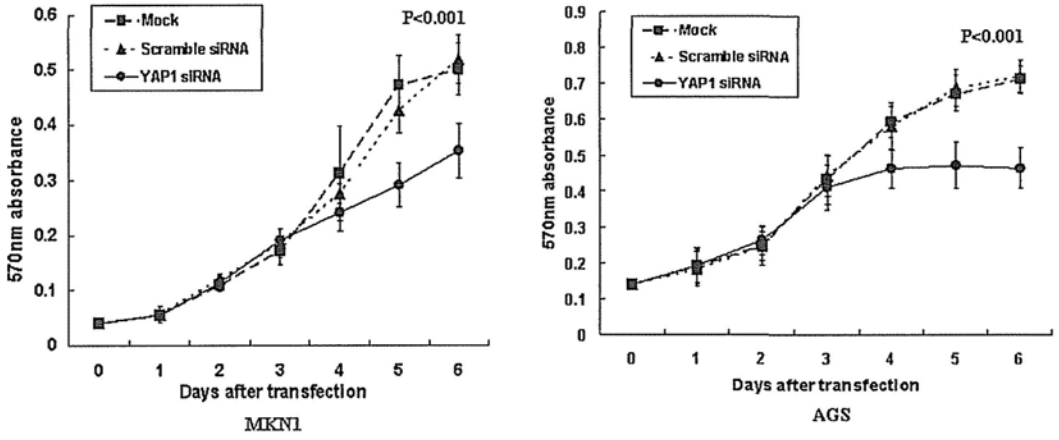


Figure 4.24 **MTT assays after transfection with siYAP1.** MTT assays suggested that knockdown YAP1 by siRNA significantly suppressed proliferation in MKN1 and AGS cells (*P<0.001*). The mean and SD obtained from seven experiments were plotted.

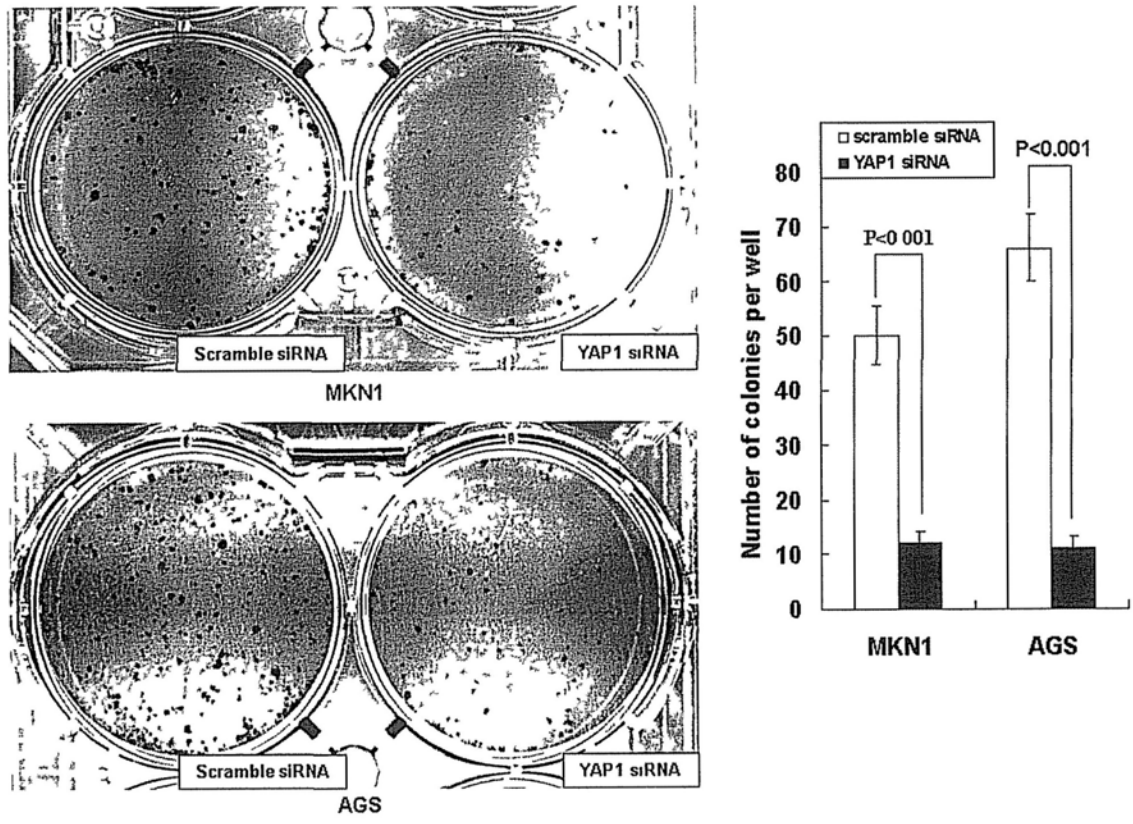


Figure 4.25 **Monolayer colony formation assays after transfection with YAP1 siRNA.** Monolayer colony formation assay showed that YAP1 knockdown reduced anchorage-dependent colony formation. The experiment was done in triplicate and the error bars represented SDs (*P<0 001*).

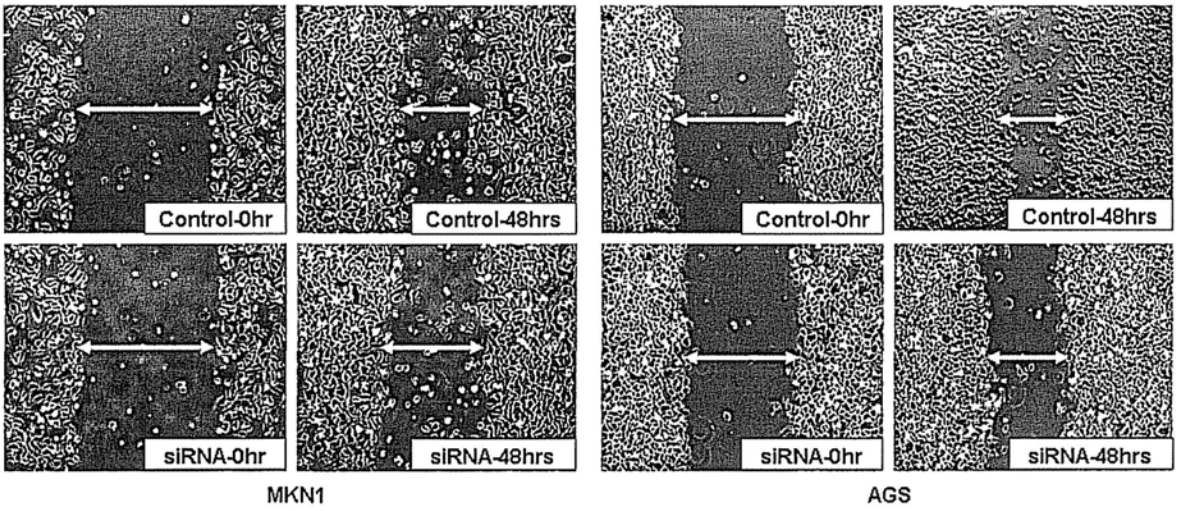


Figure 4.26 **Cell migration assays after transfection with YAP1 siRNA and negative control.** Suppression of wound healing ability by YAP1 siRNA in MKN1 and AGS cells was noted.

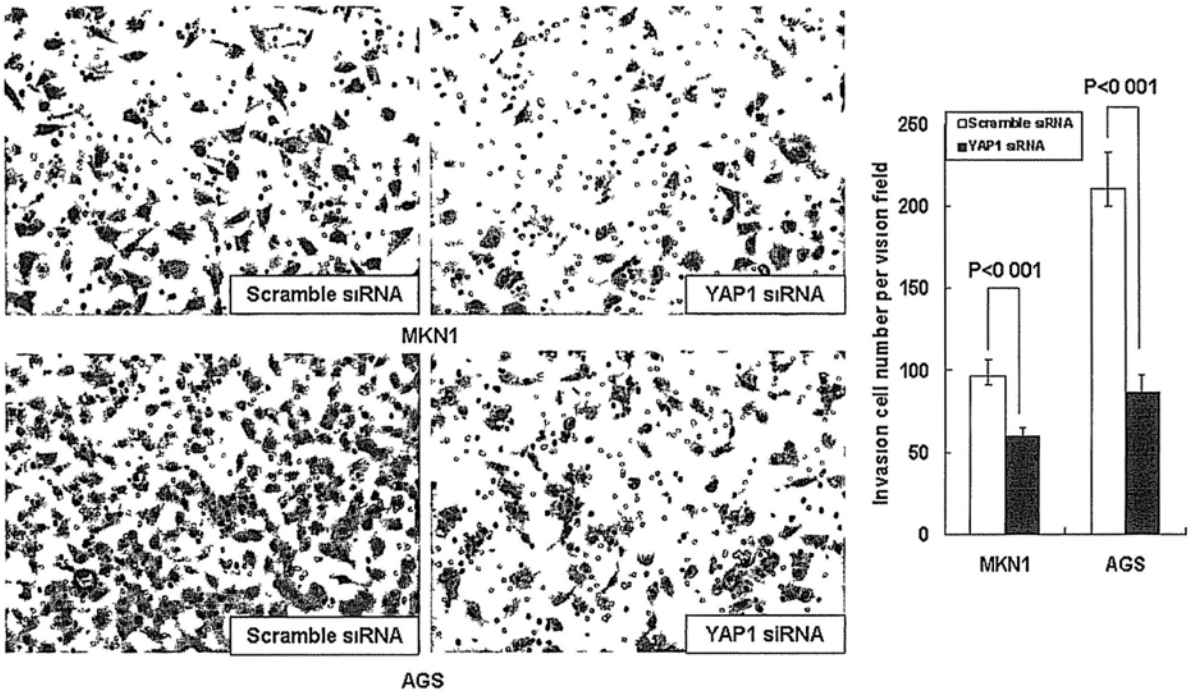
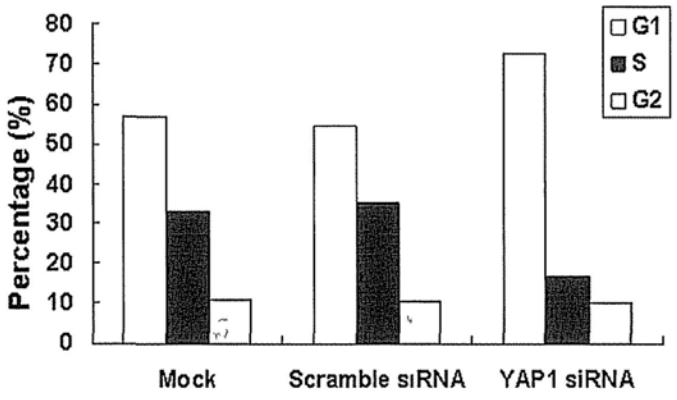
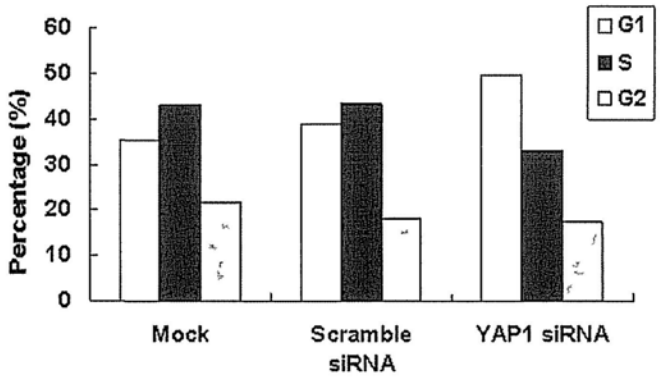


Figure 4.27 **Cell invasion assays using Matrigel model.** A significant reduction in the invasive ability was demonstrated on YAP1 knockdown compared with scramble siRNA treated cells. Representative images of cells invaded through the Matrigel-coated membrane to the underside of micropores were shown (*P<0 001*).



MKN1



AGS

Figure 4.28 **Cell cycle analysis by flow cytometry after transfection with YAP1 siRNA.** Flow cytometric analysis suggested the accumulation of cells in G1 phase 24 hours after siYAP1 treatment. Representative data of three independent experiments was shown. Cells in the G1 phase were increased from 55% to 72% in MKN1 and 37% to 49% in AGS cells.

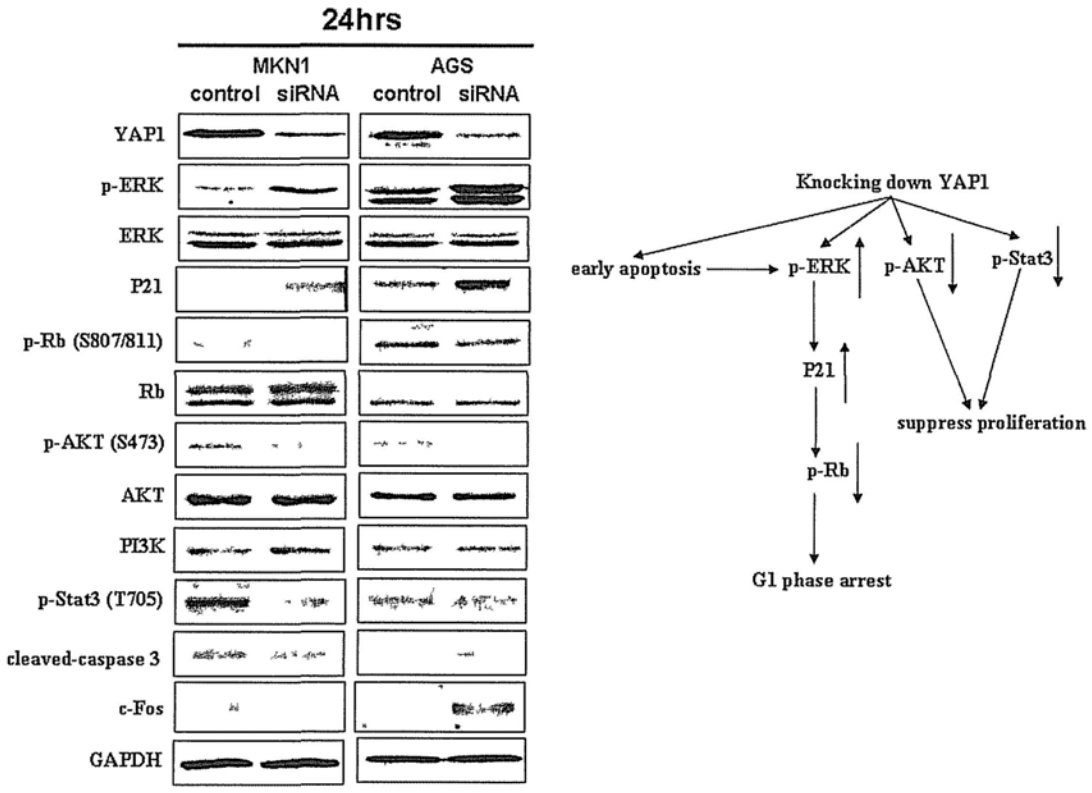


Figure 4.29 **Downstream protein analysis in MKN1 and AGS after YAP1 knockdown.** The cell cycle arrest might be induced by ERK (P44/42) phosphorylation, and P21 showed upregulation after siYAP1 transfection as downstream protein of p-ERK, which finally resulted in Rb hypophosphorylation. This result demonstrated that YAP1 knockdown could influence cell cycle progression through cell cycle arrest. At the mean time, some associated protein, such as p-AKT (S473) and p-Stat3 (T705) showed decreased level accompanied with the decreasing expression of YAP1. The right panel is a schematic figure describing the left protein assays.

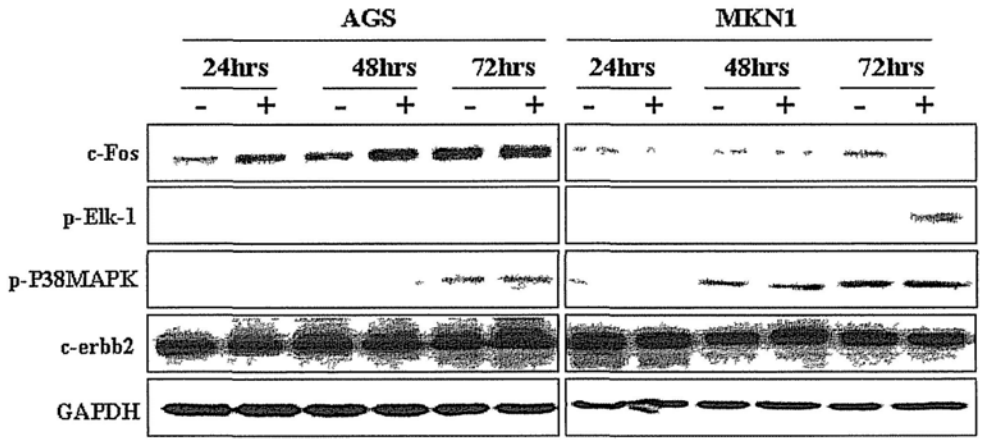
Interestingly, we found that c-Fos and p-Elk-1 showed a reverse expression pattern upon YAP1 knockdown in AGS and MKN1 cells. In AGS, siYAP1 up regulated c-Fos and down regulated p-Elk-1. On the contrary, it down regulated c-Fos and up regulated p-Elk-1 in MKN1 cells. However, siYAP1 knockdown in both cell lines did not influence the expression level of p-P38MAPK and c-erbB2 (Figure 4.30).

siYAP1 induced apoptosis was cell context-dependent

To examine the role of YAP1 in regulating apoptosis, we measured apoptosis in siYAP1 transfected MKN1 and AGS cells and control cells 24 hours after transfection. The percentage of sub-G1 cells was increased from 0.56% to 17% in AGS cells. However, siYAP1 did not change the percentage of sub-G1 cells in MKN1 (Figure 4.31). Similar finding was observed by Annexin V assay. Thus, the apoptotic response to siYAP1 appeared to be cell context-dependent. This might be related to cell line-specific endogenous genetic program and mechanisms such as p53 status. MKN1 cells harbor the TP53 mutation at the DNA-binding (core) domain (V143A), whereas AGS cells carry the wild type TP53 (Figure 4.32). Further studies will instigate whether the different TP53 status might contribute to the different apoptotic response to YAP1 knockdown between the two cell lines.

Taken together, siRNA knockdown of YAP1 in MKN1 and AGS cells resulted in a significant reduction in proliferation, anchorage-dependent colony formation

and cell invasion. G1 arrest was observed in both cell lines upon YAP1 knockdown yet the apoptotic response appeared to be cell context-dependent.



"-" means scramble siRNA and "+" means YAP1 siRNA.

Figure 4.30 **c-Fos and p-Elk-1 showed a reverse expression pattern in AGS and MKN1 after YAP1 knockdown.** In AGS cells, siYAP1 up-regulated c-Fos for 3 consecutive days, whereas down regulated p-Elk-1. On the contrary, it down regulated c-Fos and up-regulated p-Elk-1 in MKN1 cells. SiYAP1 did not alter the expression level of p-P38MAPK and c-erbB2 in both cell lines.

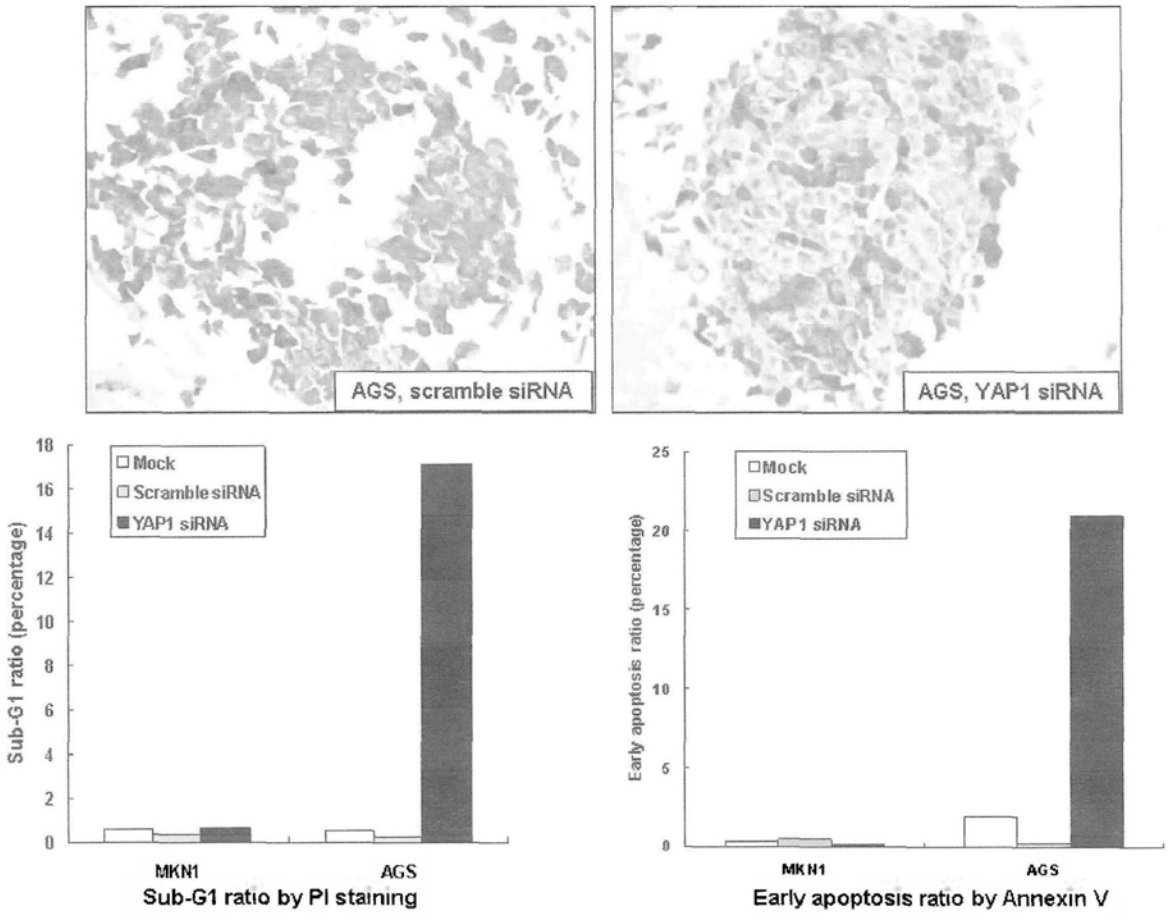


Figure 4.31 **siYAP1 induced apoptosis in AGS but not MKN1 cells.** Upper panel: YAP1 IHC confirmed successful knockdown of YAP1 in AGS cells. Apoptosis was measured by sub-G1 phase (lower left panel) and Annexin V assay (lower right panel).

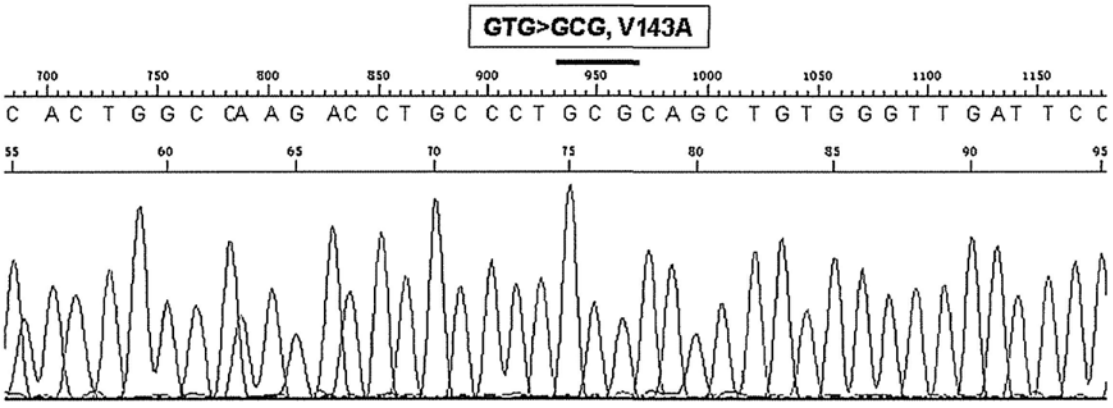


Figure 4.32 **MKN1 cells carried a mutation (V143A, GTG>GCG) at exon 5 of TP53 gene.**

4.3.6 The effect of YAP1 overexpression in GCA cells

The expression of YAP1 was completely lost in gastric cancer cell line MKN45 due to an intragenic homozygous deletion. It therefore provided an in vitro model to study the YAP1 ectopic expression. We transfected pcDNA3.1(+)/YAP1 or empty vector into MKN45 cells. Re-expression of YAP1 was confirmed by Western blotting (Figure 4.33). Transient transfection of YAP1 expression vector into MKN45 cells did not change the proliferation rate in high and low serum conditions. This was because the low transfection efficiency of MKN45 (lower than 15% efficiency). But transient YAP1 expression could induce upregulation of p-ERK, cyclin D1 and CDK4 (Figure 4.33). Re-expression of YAP1 was confirmed by immunohistochemistry and Western blot.

Several clones that stably expressing YAP1 and the corresponding empty vector-transfected cells were selected by G418. The YAP1 expressing MKN45 cells and the empty vector transfected MMKN45 cells were designated YAP1-MKN45 and vector-MKN45, respectively. Re-expression of YAP1 in MKN45 cells was confirmed by Western blotting and IHC. YAP1 could induce morphology change in the early passage of stable clones, e.g. swollen, cell scattering and cell-cell detachments (Figure 4.34). Reason of this phenomenon might lie in YAP1 induces epithelium mesenchymal transition (EMT) in MKN45, but this need to be confirmed by western blot of EMT markers.

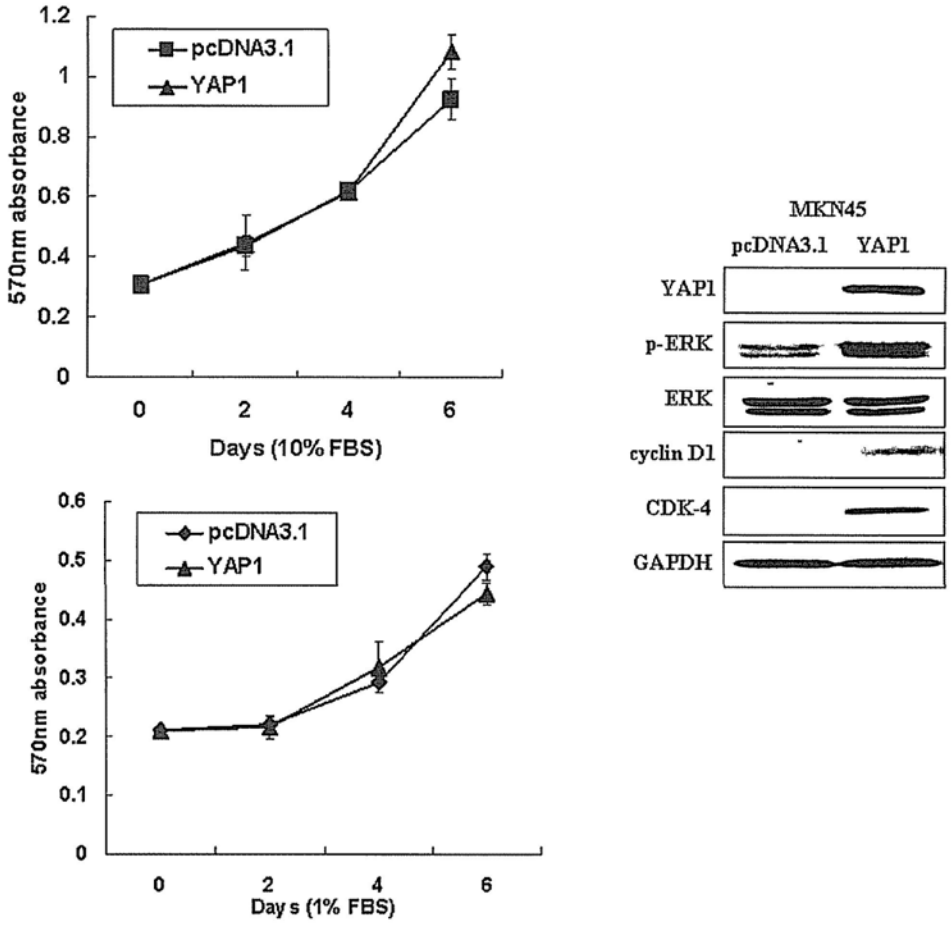


Figure 4.33 **Transient transfection of YAP1 to MKN45 cells.** Left panel: YAP1 did not change cell proliferation of transiently transfected MKN45 cells by MTT assay. Right panel: Western blotting showed upregulation of p-ERK, Cyclin D1 and CDK4 in YAP1 expressing MKN45 cells.

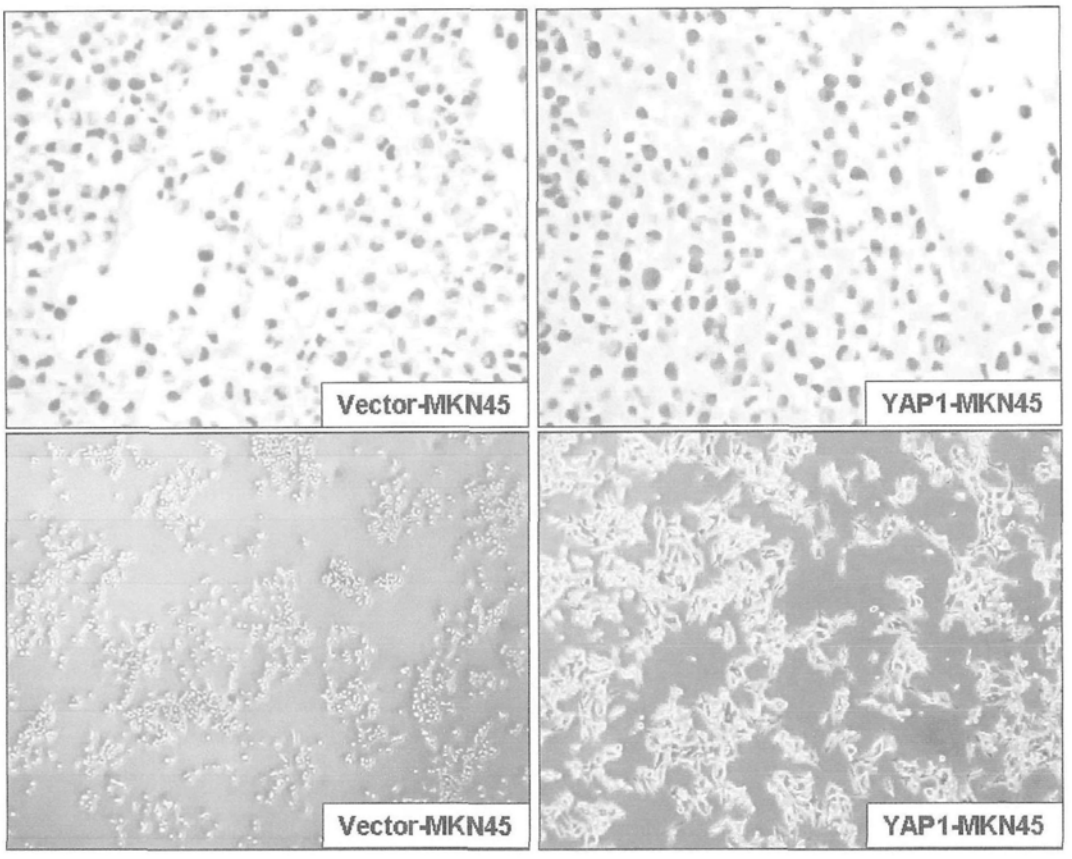


Figure 4.34 **MKN45 cells stably expressing YAP1.** Upper panel: re-expression of YAP1 in MKN45 cells was confirmed by IHC. Lower panel: YAP1 could induce morphology changes in the early passage of stable clones, such as swollen, cell scattering and detachment from each other.

YAP1 overexpression promoted the growth of GCA cells

Increased cell proliferation was noted in YAP1-MKN45 both in high (10% FBS) or low (1% FBS) serum conditions compared with vector-MKN45 cells. To investigate the potential mechanisms by which YAP1 modulates cell growth, we examined the possible alterations in several canonical pathways. Constitutive activation of MAPK, AKT, beta-Catenin and P38MAPK was noted in MKN45 cells stably expressing YAP1, suggesting a possible involvement of YAP1 in these pathways. On the other hand, YAP1 re-expression did not change the level of p-Stat3, PCNA, Cyclin D1 and CDK4 in MKN45 cells (Figure 4.35).

YAP1 overexpression increased GCA cell invasion

The cell invasive ability was assessed by in vitro Matrigel cell invasion assay, which tested the ability of tumor cells to permeate through a reconstituted basement membrane barrier (Matrigel). A significant increase in the number of invading cells through the Matrigel coated membrane in YAP1-MKN45 cells was demonstrated when compared with the vector-MKN45 cells ($P<0.001$, Figure 4.36). The results indicated that YAP1 could strengthen the invasion ability of GCA cells and might be involved in cancer cell metastasis.

YAP1 promoted anchorage-independent growth of GCA cells

We further examined the effect of YAP1 on the ability to form colonies in soft agar, a more stringent parameter of oncogenic property. YAP1-expressing MKN45 cells formed larger colonies in soft agar by 14 days and the number of

colonies was significantly increased by 44 folds ($P<0.001$) compared to the vector controls (Figure 4.37), suggesting that YAP1 promoted anchorage-independent growth.

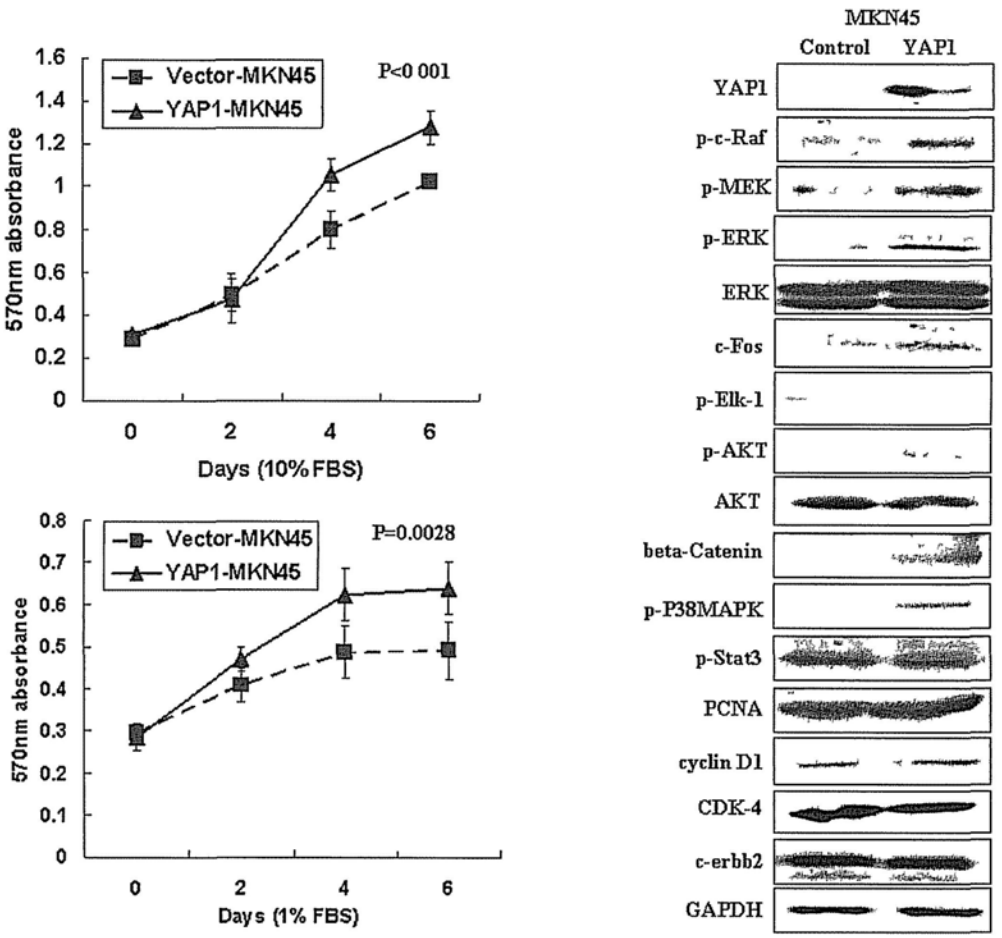


Figure 4.35 **YAP1 promoted the growth of MKN45 in both high and low serum condition** (*P<0.001*). Left panels: MTT assay, the mean and SD obtained from three experiments were plotted. Right panel: The expression levels of growth-related proteins were evaluated by Western blotting in YAP1-MKN45 and vector-MKN45 cells.

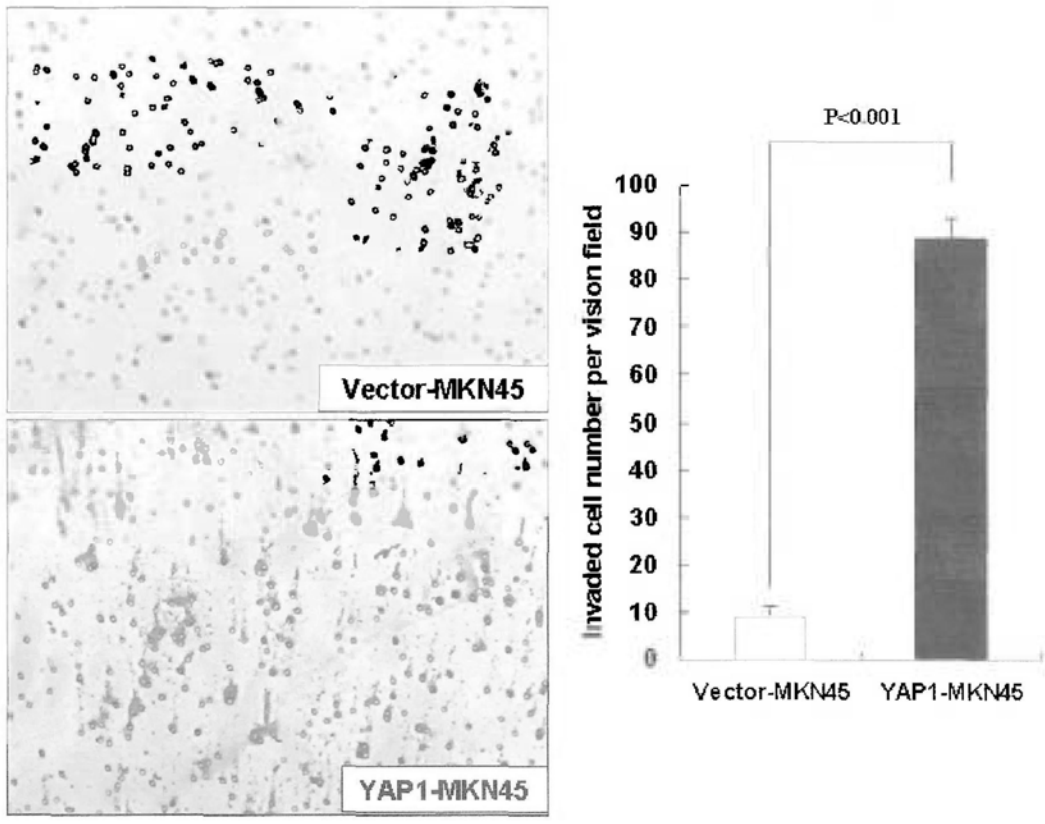


Figure 4.36 **YAP1 increased the invasiveness of MKN45 cells**. A significant increase of invasion ability of MKN45 cells through the Matrigel-coated membrane was observed ($P<0.001$) in YAP1 transfected cells compared with vector controls. The experiment was done in triplicate and the error bars represented SDs.

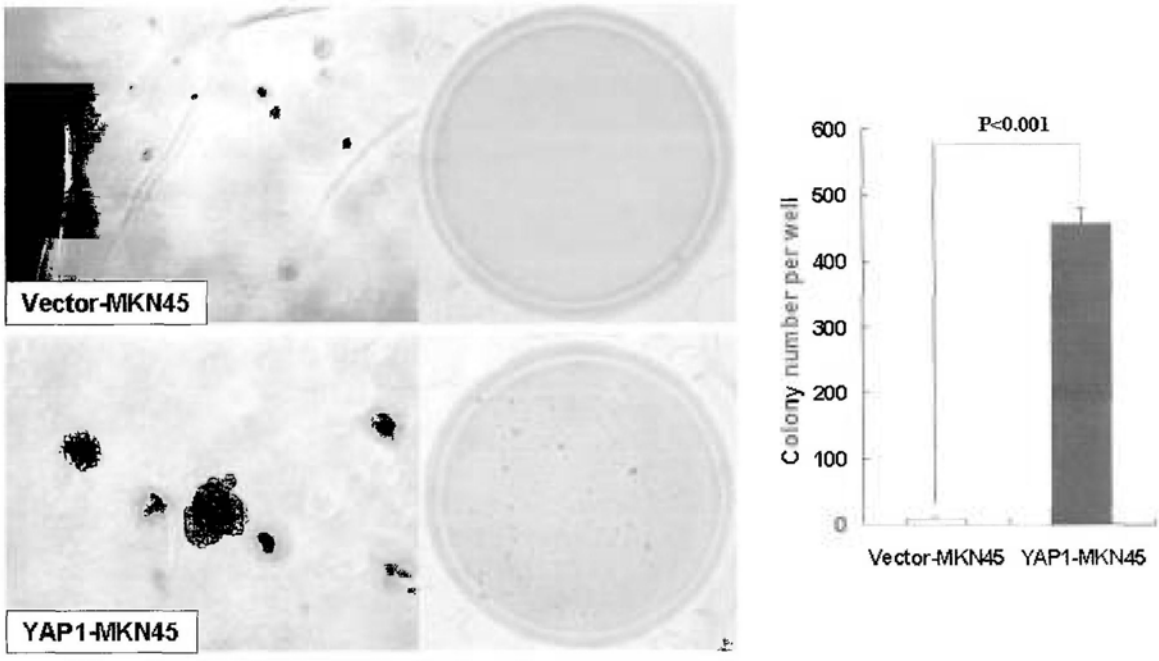


Figure 4.37 **YAP1 promoted anchorage-independent growth of MKN45 cells.** YAP1-expressing cells formed larger colonies in soft agar by 14 days and the number of colonies was significantly increased compared to the empty vector controls (*P<0.001*). The experiment was done in triplicate and the error bars represented SDs.

YAP1 facilitated in vivo growth of MKN45 cells

The effect of YAP1 expression on *in vivo* growth of tumor was studied by subcutaneous injection of YAP1-expressing MKN45 clones into nude mice. The tumor growth in YAP1-expressing clones was significantly enhanced compared with the vector control clones. A photo of the tumor-bearing mice and the *in vivo* growth curve of YAP1-MKN45 and vector-MKN45 cells were shown in Figure 4.38. YAP1-MKN45 cells and vector-transfected were injected subcutaneously to the right and left dorsal flank of nude mice, respectively. YAP1-MKN45 formed larger tumors on the right dorsal flank than vector-MKN45 on the left dorsal flank 3 weeks after injection ($P<0.001$, Figure 4.38). The expression of YAP1 in xenografts was confirmed by immunohistochemistry. Both cytoplasmic and nuclear localization were noted in xenografts of YAP1-MKN45. We examined the expression level of several growth-related proteins in YAP1-MKN45 xenografts. Upregulation of cyclin D3, CDK4 and CDK6 were noted in YAP1-MKN45 xenografts, suggesting that YAP1 might promote cell proliferation by modulating cell cycle progression (Figure 4.39).

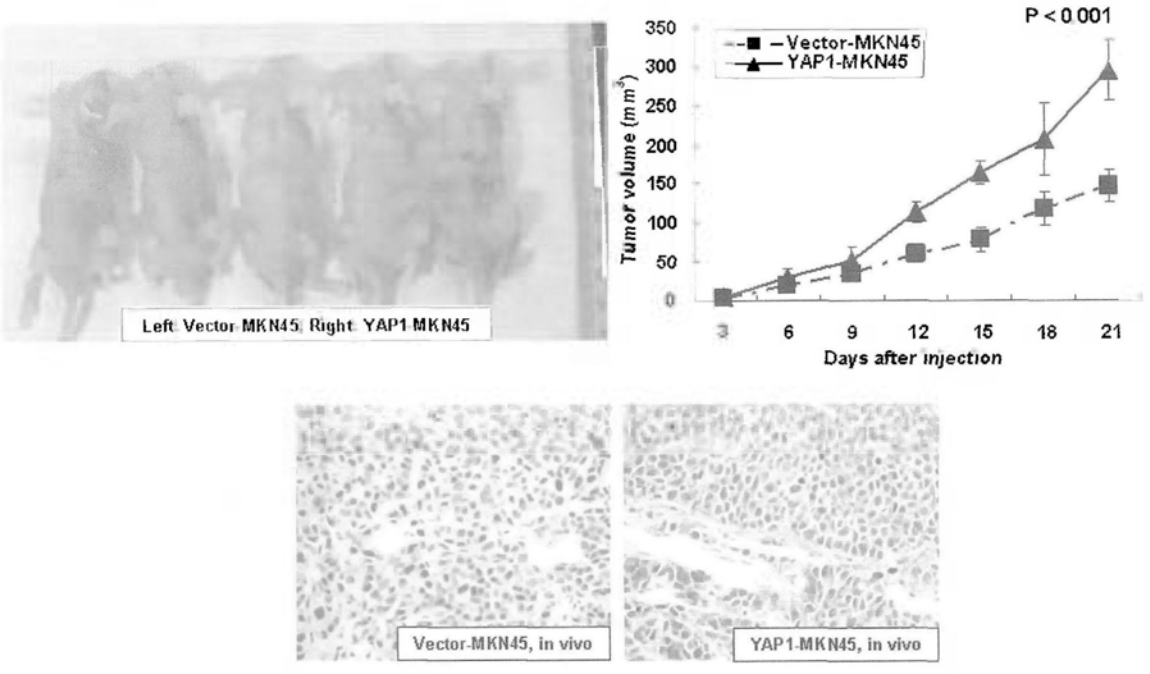


Figure 4.38 **YAP1 promoted tumor growth *in vivo*.** YAP1-MKN45 cells and vector-MKN45 cells were injected subcutaneously to the right and left dorsal flank of nude mice, respectively. Three weeks after injection, the tumor size of YAP1-MKN45 was significantly larger than vector-transfected controls. The mean and SD obtained from five mice were plotted. Representative results from 3 independent experiments were shown. The YAP1 expression in xenograft tumors was confirmed by immunohistochemistry in the lower panel.

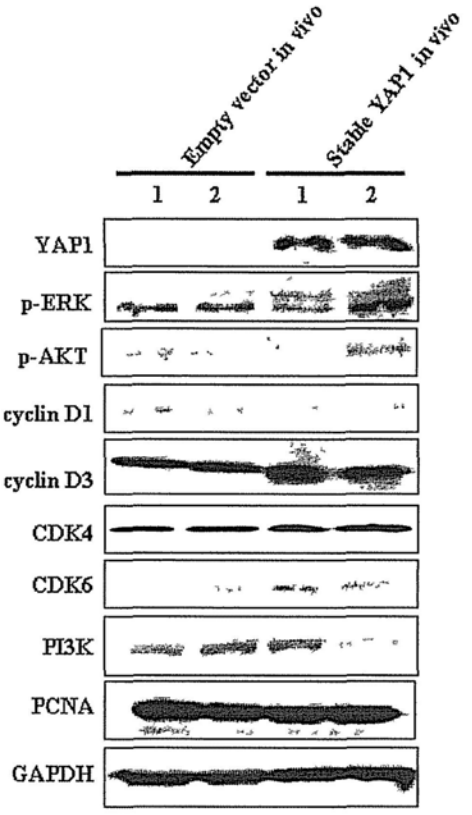


Figure 4.39 **Expression of growth-related proteins in xenografts.** Western blotting was performed to evaluate the levels of growth-related proteins in YAP1-MKN45 and vector-MKN45 xenograft tumors.

4.3.7 Expression profiling for downstream targets of YAP1

To gain insight into the mechanisms by which YAP1 exerts the oncogenic function, we compared gene expression profiles from YAP1-expressing MKN45 cells (YAP1-MKN45) versus vector controls (vector-MKN45), and YAP1-knockdown AGS cells (siYAP1-AGS) versus scramble controls (scramble-AGS). Differential gene expression was evaluated using one-way ANOVA. Genes that had greater than 1.5-fold differences in expression levels over the controls with false discovery rates (FDR) <5% were selected for Ingenuity pathway analysis. Selected up and down regulated genes were validated by qRT-PCR (Table 4.12 and Table 4.13).

To enhance the specificity of identified potential targets, we specifically screened for genes that demonstrated opposite expression in YAP1-expressing and YAP1-knockdown cells. A total of 1021 differentially expressed genes were overlapped between YAP1-expressing (*in vivo*) and YAP1-knockdown cells. About 94% (962/1021) of the overlapped gene population were opposite in expression trend, suggesting they were highly related to YAP1 regulation. Among them 494 genes were up-regulated in siYAP1-AGS and down-regulated in YAP1-MKN45 while 468 genes were up-regulated in YAP1-MKN45 and down-regulated in siYAP1-AGS (Figure 4.40).

We then used the Ingenuity Pathways Analysis to identify network of interacting genes and the biological functions that were most significant to the data set. Of

the 962 genes, 464 were eligible for network analysis. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity. A total of 27 partially overlapped networks with IPA score >11 were linked to YAP1 regulation. All networks involved more than 11 focus genes. Top functions of these genes were related to cancer, cell cycle, digestive system development and function, small molecule biochemistry and lipid metabolism. The ten networks with the highest rank are showed in Table 4.14.

Close examination of the networks identified that mitogen-activated kinases (MAPK) family genes were enriched in several networks. For example, the network associated with "cancer, cellular movement and neurological disease", incorporating 23 focus genes, was centered on ERK (Left panel of Figure 4.41). Another network built on 22 focus genes was mostly centered on MAPK, and associated with "lipid metabolism, molecular transport and small molecule biochemistry" (Right panel of Figure 4.41). MAPK pathway is involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. The pathway is also a key target of cell transformation in tumor development. We compared the expression of MAPK family genes and other growth related genes in YAP1-MKN45 cells to vector-MKN45. Activation of MAPK pathway by YAP1 as indicated by elevated phosphorylated-c-Raf/MEK1/2/ERK1/2 and c-Fos was observed in Figure 4.35.

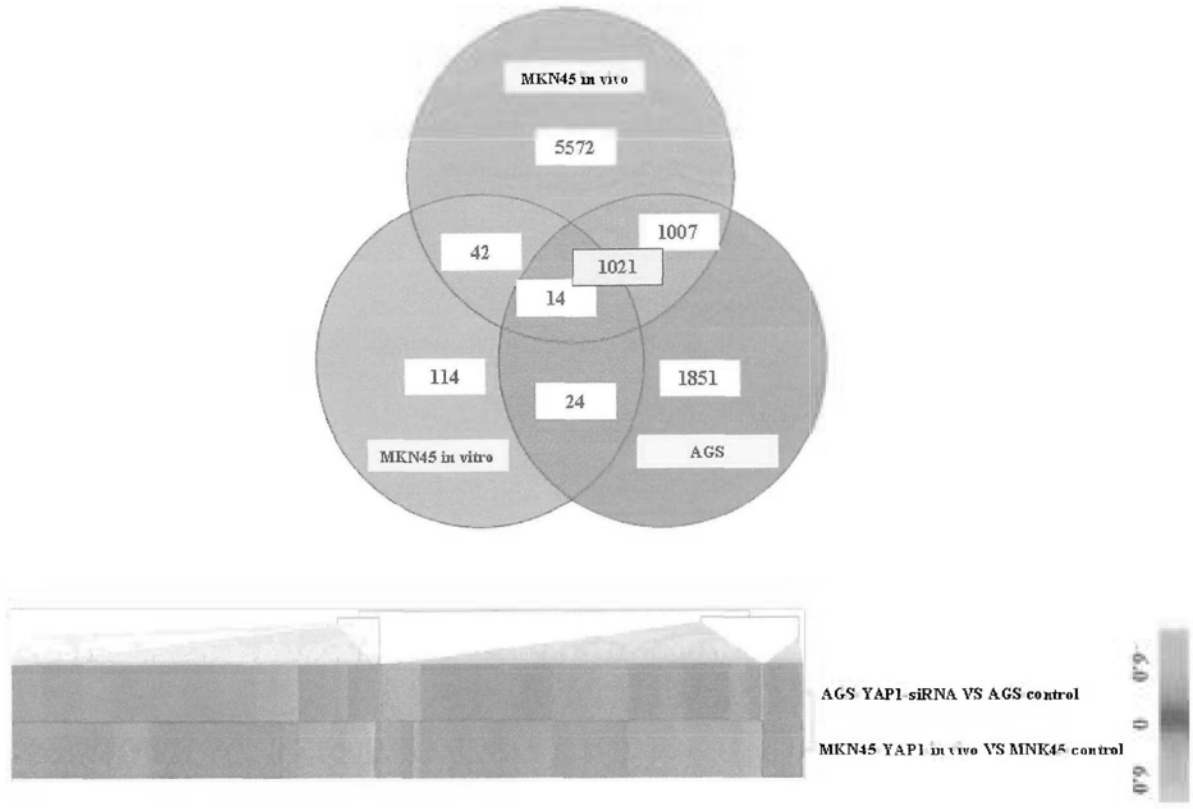


Figure 4.40 **Venn diagram and clustering analysis in expression profiling.** A total of 1021 differentially expressed genes were overlapped between YAP1-MKN45 *in vivo* and YAP1-knockdown AGS cells. About 94% (962 in 1021) of the overlapped gene population were opposite in expression trend. Clustering analysis in the lower panel revealed that most intersect genes which exhibited significance (fold change>2 in YAP1-MKN45 *in vivo* group and fold change >1.5 in siYAP1-AGS group) showed reverse expression level.

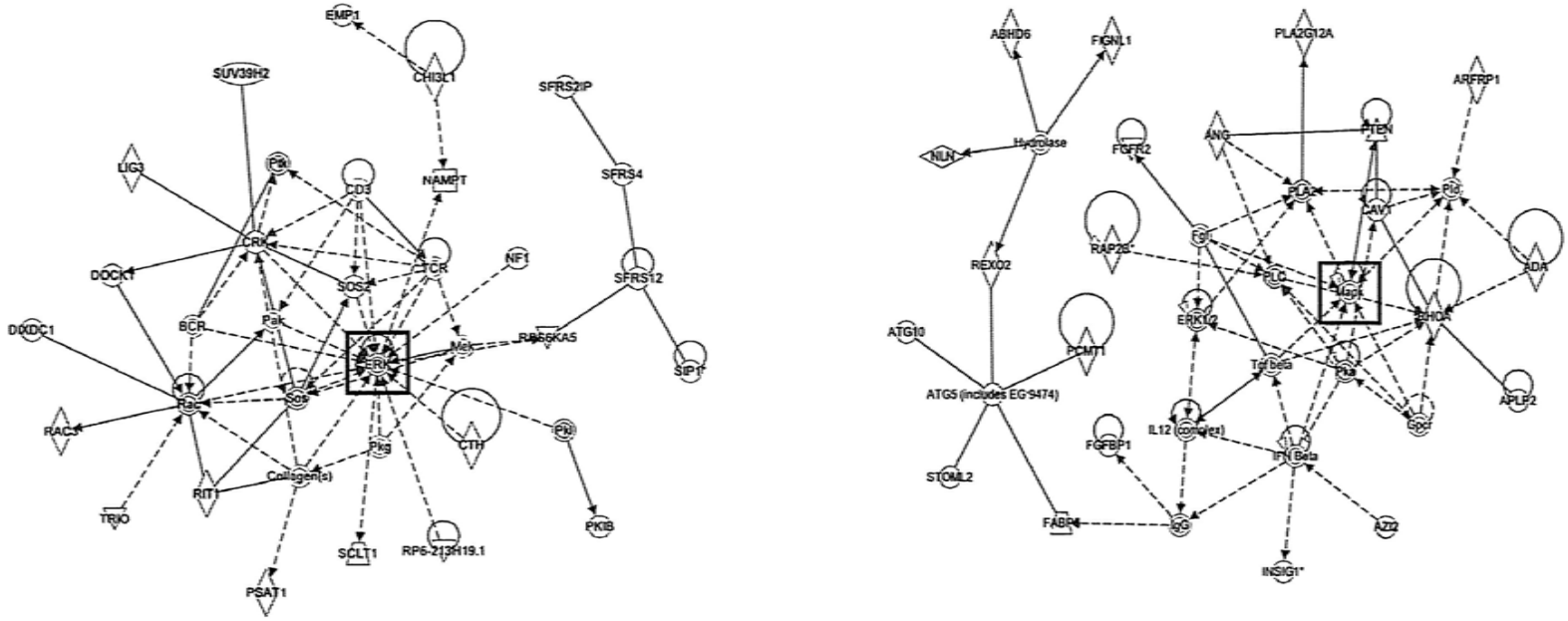


Figure 4.41 **Enrichment of MAPK family genes in networks as determined by Ingenuity pathway analysis.** Genes depicted in gray were identified by microarray results as differentially expressed and demonstrated an opposite expression pattern between YAP1-MKN45 and siYAP1-AGS. Left, Network associated with cancer, cellular movement and neurological disease. Right, Network associated with lipid metabolism, molecular transport and small molecule biochemistry.

Table 4.12 **PCR primers used in the current study.**

Gene Name	Forward	Reverse	Size	Note
YAP1	CAGCAACTGCAGATGGAG	ACATCCCGGGAGAAGAC	150	Exon4/5
RYBP	TGCACCTTCAGAAACAGT	TGGCATACTGTTGTGCCA	127	Exon1/2
FOXP1	TTCAGGGGTAAGACGTGA	AAGACCGCCGCACTCTAG	174	Exon4/6
LRRFIP1	GAACGGGAATGCTTATTGG	GTCTGTTGGCATCCCTTT	157	Exon22/24
KLF10	GTGCCTCTCTCCAGCAGA	TCCAACTGCAGCTCATTG	144	Exon1/2
EXT1	TGCTGGTATTCAAGGGGA	TGTGCTTTTGCCAGTCTT	135	Exon1/2
IFI16	CAACCAAAGAAAAGGCTG	GGTGGAGCTGACAATGA	136	Exon4/5
SASH1	GTGGACGTGCTCAGTGAA	GAACAGGAAAGTGGGCA	141	Exon42/43
CUL3	CCAGGGCTTATTGGATCTG	CCCTTTGACTCCCTTTTTC	182	Exon4/5
SMAD3	CTGTGTGAGTTCGCCTTCA	TTAGTGTTTTCGGGGATG	173	Exon7/10
PGF	GGGGAAGAGGAGGAGAG	AGCAGGGAAACAGTTGG	189	Exon3/4
KITLG	GGATGGATGTTTTGCCAAG	TCTTTCACGCACTCCACA	172	Exon19/20

TCF4	CGAATCACATGGGACAGA	CTTGCGTCTGCGATTCAT	171	Exon6/9
CDC25C	GGGGAGATAACTGCCACT	AAGCTGTGCTGGGCTACA	141	Exon3/6
JAK1	CGCTCTGGGAAATCTGCTA	AGGTCAGCCAGCTCCTTA	124	Exon17/18
FH	TCGATTTTTGGGTTCTGGT	CCATGGTCATTGCTTCAC	122	Exon7/8
MAP3K2	AGTCCTGAGACCAGCAAG	GGATCCCTCAAACAGCCA	107	Exon12/13
SKAP2	CGGTGGTGTGCTCTCAGTA	TCTTTGGGAGAAGCTGCT	200	Exon6/8
PIK3C2A	TGGGACATGCACAGATGTT	CTGACAGCAGAGGTCCA	137	Exon23/24
RFC3	CAAAGGCTCCTTGAAGTT	ACCCAGCTGTAGACGATG	171	Exon7/9
PLAU	ACCCATGGTCTTCCATTTG	GTTGCACCAGTGAATGTT	109	Exon2b/4
CDK8	TATCAGCGTTCCAATCCAC	AGATTCCGATGCAGCTCA	147	Exon12/13
RASAL2	TCCTCACAGACAGCCAGT	GTGGGTACTCGGGAAACT	150	Exon2/3
FGFR2	GTCCCATCTGACAAGGGA	TCTACGTCTCCTCCGACC	161	Exon6/7

Table 4.13 **Validation of microarray results by quantitative RT-PCR.** Possible downstream genes selected for validation from expression microarray after knocking down YAP1 in AGS and MKN45 / YAP1 *in vivo*. Some associated genes were isolated from clustering analysis for validation by qRT-PCR and the results were concordant with the expression microarray results.

Gene Name	Description	Function	Fold change	
			Microarray	qRT-PCR
siYAP1-AGS vs. scramble-AGS				
RYBP	RING1 and YY1 binding protein	apoptosis	2.557	1.454
FOXP1	Forkhead box P1	tumor suppressor	2.646	1.424
LRRFIP1	Leucine rich repeat (in FLII) interacting protein 1	transcriptional repressor	2.379	1.357
KLF10	Kruppel-like factor 10	inhibits cell growth	2.615	1.812
EXT1	Exostoses (multiple) 1	putative tumor suppressor	2.315	1.468
IFI16	interferon, gamma-inducible protein 16	inhibits cell growth	2.316	8.554
SASH1	SAM and SH3 domain containing 1	tumor suppressor	2.765	2.119

CUL3	Cullin 3	ubiquitination of cyclin E and D1	2.477	1.641
SMAD3	SMAD family member 3	tumor suppressor	4.200	1.066
PGF	placental growth factor	proliferation and migration	-2.045	-1.201
KITLG	KIT ligand	cell migration	-1.907	-1.319
TCF4	Transcription factor 4	transcription	-2.315	-1.386
CDC25C	cell division cycle 25 homolog C	suppress p53-induced growth arrest	-1.988	-1.348
		YAP1- MKN45 vs. vector-MKN45		
JAK1	Janus kinase 1 (a protein tyrosine kinase)	protein-tyrosine kinase	2.968	1.623
FH	fumarate hydratase	Krebs cycle	11.98	1.3983
MAP3K2	mitogen-activated protein kinase kinase kinase 2	MAP kinase signaling pathway	3.245	1.525
SKAP2	src kinase associated phosphoprotein 2	Src family kinases	2.989	1.565
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	phosphoinositide 3-kinase (PI3K) family	2.753	1.752
RFC3	replication factor C (activator 1) 3, 38kDa	replication	2.660	1.649

PLAU	plasminogen activator, urokinase	cell migration and proliferation	2.749	2.655
CDK8	cyclin-dependent kinase 8	cell cycle	2.882	1.556
RASAL2	RAS protein activator like 2	inhibitory regulator of the Ras-cyclic AMP pathway	2.182	1.623
FGFR2	fibroblast growth factor receptor 2	proliferation	2.130	2.001

Table 4.14 **Top 10 genetic networks identified by Ingenuity Pathway analysis.** Genes in boldface were identified by microarray analysis to be expressed differentially more than 1.5-fold and had an opposite expression pattern between YAP1-MKN45 and siYAP1-AGS cells. Other genes were either not on the expression array or not significantly regulated.

Network	Molecules in Network	Score	Focus genes	Top Functions
1	**ATP9A**, Caspase, Ck2, **CRKRS**, **DAZAP2 (includes EG:9802)**, **DCTD**, **DLAT**, Dnajb1-Hsp70, FSH, **GMCL1**, **HNRNPU**, Hsp70, **HSPA4**, **HSPA8**, **MAPK14**, MIR124, **NUCKS1**, **PPFIBP1**, **PRUNE**, **RBMS1**, RNA polymerase II, **RPS27A**, **SEPT2**, **SEPT11**, **SLC30A7**, **SNCAIP**, **STIP1**, **SYPL1**, **TCF7L2 (includes EG:6934)**, **TFRC**, **TMPO**, **TRIM23**, **UBE2D1**, Ubiquitin, **XRCC5**	41	27	Digestive System Development and Function, Cancer, Gastrointestinal Disease
2	**ADAM9**, **ADAM10**, Alcohol group acceptor phosphotransferase, AMPK, Calpain, **CDK8**, **CSDA**, Cytochrome c, DNA-directed DNA polymerase, Fgfr, Fibrinogen, **IFT57**, Integrin, **ITGB1**, Laminin, **LGALS8**, **MAD2L2**, **MAPK1**, Metalloprotease, **NEK2**, **PDE4C**,	36	25	Nervous System Development and Function, Tissue Development, Cancer

	PIK3CB, **POLH**, **POLQ**, **PPID**, **PRIM2**, **PRKAA1**, **PRKAB2**, **PRKAG2**, **PRPF4B**, **REV3L**, **RFC3**, **TNKS2**, **TRIB2**, **YLPM1**			
3	**ADM**, Akt, Alpha Actinin, **BIRC5**, **CCT2**, Cyclin E, **DAPP1**, **ELAVL1**, **FGFR1**, **FLT1**, **IL13RA1**, Importin beta, **JAK1**, **NRP1**, **NUP153**, **PCSK5**, Pdgf, Pdgf Ab, PDGF BB, **PDGFA**, **PIGF**, PLC gamma, **PTK2**, **RALGPS1**, **RHOU**, **SAV1**, **SOCS2**, STAT, **STK4**, **SWAP70**, **TNPO1**, **TPD52**, Trk Receptor, Vegf, **ZBTB10**	33	24	Cardiovascular System Development and Function, Organ Development, Visual System Development and Function
4	BCR, CD3, **CHI3L1**, Collagen(s), **CRK**, **CTH**, **DIXDC1**, **DOCK1**, **EMP1**, ERK, **LIG3**, Mek, **NAMPT**, **NF1**, Pak, Pkg, Pki, **PKIB**, **PSAT1**, Ptk, Rac, **RAC3**, **RIT1**, **RP6-213H19.1**, **RPS6KA5**, **SCLT1**, **SFRS4**, **SFRS12**, **SFRS2IP**, **SIP1**, Sos, **SOS2**, **SUV39H2**, TCR, **TRIO**	32	23	Cancer, Cellular Movement, Neurological Disease
5	ALP, **ARPC5**, **BMP2**, Creb, Cyclin A, **CYP51A1**, E2f, **ERCC8**, Histone h3, Histone h4, HMG CoA synthase, **HMGCS1**, Interferon alpha, **LOC26010**, **MTUS1**, PI3K, **PRMT5**, Rb, **SAE1**, **SKP2**, **SMARCA2**, **SNAPC3**, **SP1**,	32	23	Gene Expression, Cell Cycle, Nucleic Acid Metabolism

6	SWI-SNF, **TAF5**, **TAF15**, **TBL1X**, **TBL1XR1**, **TFCP2**, **TOB1**, **TYMS**, tyrosine kinase, **WDR1**, **WWC1**, **ZNF160** **ACSL1**, Actin, ADCY, Alpha catenin, Calcineurin protein(s), Calmodulin, CaMKII, **CAP2**, **CDC2L5**, **CHML**, **CLDN1**, **CTNNA1**, **CTSL2**, **CXADR**, **DLG1**, Dynamin, F Actin, hCG, Hsp90, **IPP**, **ITCH**, **JUB**, **OCLN**, P38 MAPK, **PGGT1B**, Proteasome, **RAB5A**, **RAB6A**, **RAB8B**, **RPAP3**, **TCF7**, **TJP1**, **TNNC1**, **TNNI3**, Transferase	30	22	Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cellular Function and Maintenance
7	**ABHD6**, **ADA**, **ANG**, **APLP2**, **ARFRP1**, **ATG10**, **ATG5 (includes EG:9474)**, **AZI2**, **CAV1**, ERK1/2, **FABP5**, Fgf, **FGFBP1**, **FGFR2**, **FIGNL1**, Gpcr, Hydrolase, IFN Beta, IgG, IL12 (complex), **INSIG1**, Mapk, **NLN**, **PCMT1**, Pka, PLA2, **PLA2G12A**, PLC, Pld, **PTEN**, **RAP2B**, **REXO2**, **RHOA**, **STOML2**, Tgf beta	29	22	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
8	**AKAP13**, Ap1, **CD55**, Clathrin, **DYX1C1**, **GNE**, Growth hormone, **HADHA**, IL1, Insulin, Jnk, **KIF13A**, LDL, **LIPA (includes EG:3988)**, **MAP3K1**, Nfat (family), **PCSK9**, **PGK1**, **PIK3C2A**, Pkc(s), **PSPC1**, Ras, Ras homolog,	26	22	Cellular Function and Maintenance, Lipid Metabolism, Small Molecule Biochemistry

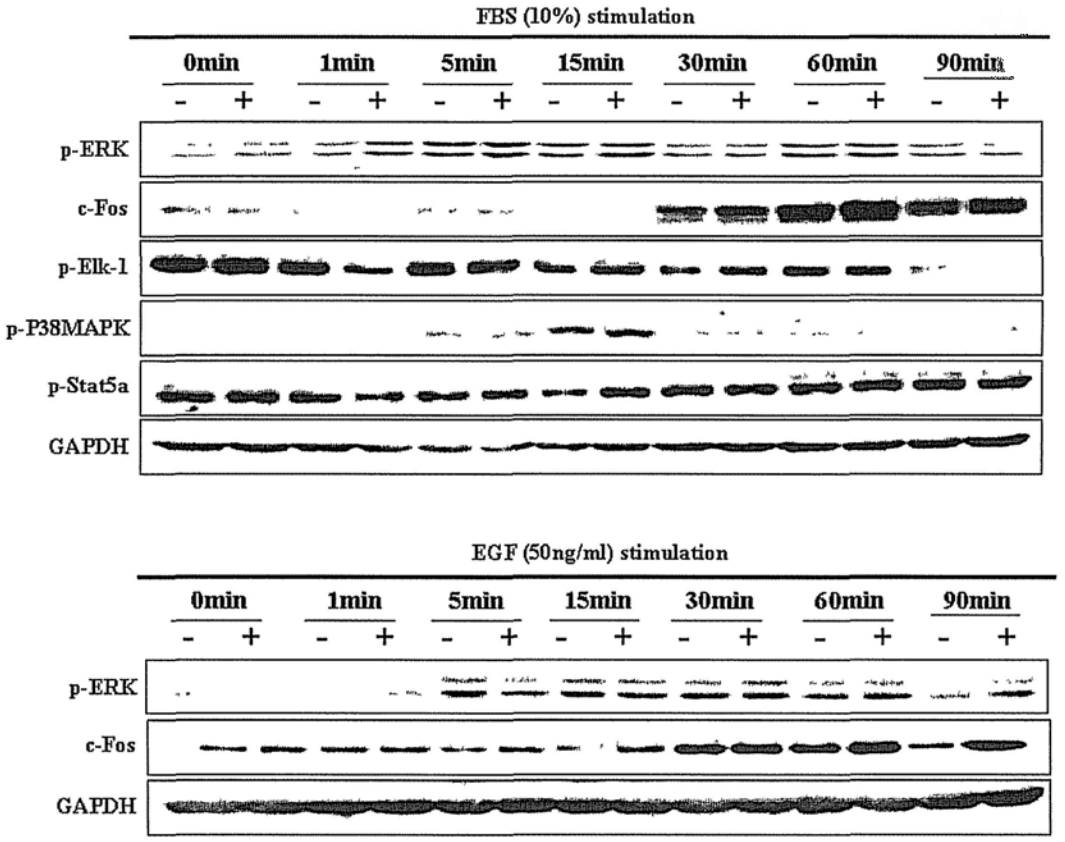
	RHOF, Sapk, **SCAMP1**, **SFPQ**, **SNAP23**, STAT5a/b, **STX3**, **TCF12**, **THRA (includes EG:7067)**, **TLK1**, **TXNIP**, **VAMP3**			
9	14-3-3, ADRB, APC, **ARHGEF12**, **ARRB1**, **CAMK2D**, **CCNG2**, **CDC25C**, **CDC42BPA**, Cofilin, **DBT**, Filamin, Gef, **LRRFIP1**, **MAD2L1**, MAP2K1/2, Mlc, NFkB (complex), **PLCE1**, PP1/PP2A, PP2A, **PPP2R1B**, **PPP2R2A**, **PPP2R5C**, **PRDX2**, **PTPLAD1**, Raf, Rap1, **RRAS2**, Scf, Shc, **SLPI**, **SMG7**, **SRRM2**, **WTAP**	24	20	Cancer, Cell Cycle, Reproductive System Disease
10	**ACLY**, **ACOT2**, ANKHD1, ATXN1, C17ORF81, **ERCC5**, **EXOSC2**, **EXOSC5**, **HELQ**, HNF4A, L2HGDH, MRPL4, N4BP2L2, **NCK1**, **NME1**, **NME7**, NRBF2, **NTHL1**, NXT2, PPARA, RAB1B (includes EG:81876), RARA, RBL1, retinoic acid, RNMTL1, RTN4IP1, RXRA, **SLC17A5**, **SLC22A3**, **SLC39A6**, **TP53**, **YBX1**, **ZNF277**	18	16	Gene Expression, Cell Cycle, Infection Mechanism

4.3.8 Activation of early response pathway by YAP1

To further address the effect of YAP1 expression on the activation of MAPK signaling, we stimulated YAP1-MKN45 and vector-MKN45 cells with serum after starvation for 24 hours. Western blot analysis was then performed to assess the level of phosphorylated-ERK1/2 and c-Fos (Figure 4.42). Stronger ERK1/2 activation at 1 to 15 minutes post serum stimulation was observed in YAP1-expressing cells compared with the vector-transfected cells. C-Fos was induced by serum at 30 minutes and maximal at 60 minutes. Ectopic expression of YAP1 resulted in a much stronger c-Fos induction in GCA cells. The MEK inhibitor PD98095 inhibited ERK activation and c-Fos induction (Figure 4.43), indicating that YAP1 regulated the serum-induced c-Fos induction though MAPK pathway. The enhanced capacity on c-Fos induction was also observed when stimulated the cells with epidermal growth factor (Figure 4.42). We knocked down YAP1 by siRNA in AGS and MKN1 cells. A reduced c-Fos expression was observed upon serum stimulation in siYAP1 treated cells (Figure 4.44). This finding further supported that YAP1 was responsible for the enhanced serum/EGF-induced c-Fos expression in GCA cells.

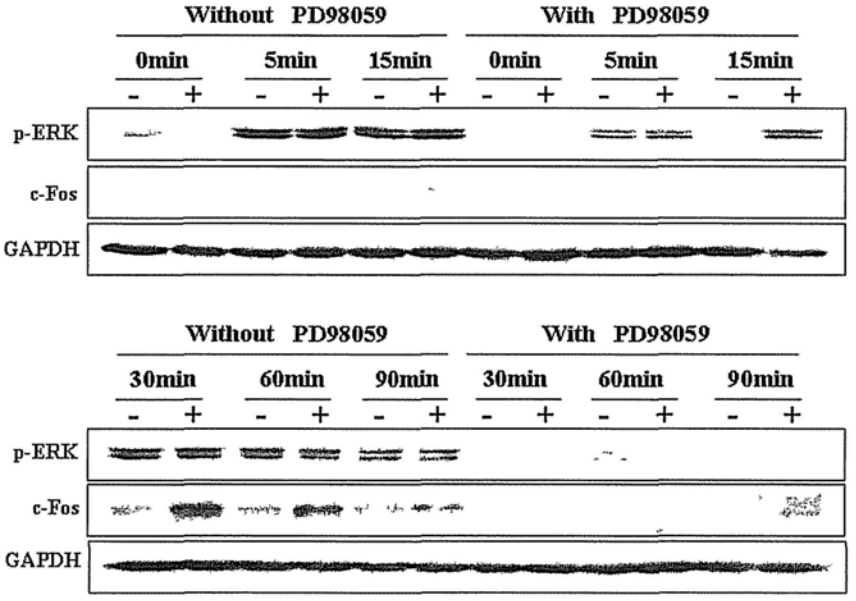
C-Fos is an immediate early gene whose expression is a key switch in cellular regulation. Together with c-Jun, it forms the AP-1 complex required for the transcription of many genes important for cell growth, differentiation and transformation. All these data strongly suggested that YAP1 played oncogeneic role in gastric tumorigenesis. Our data suggested that c-Fos is a target for the

function of YAP1. The early gene responses are representative of the events that initiate progression through the cell cycle. YAP1 might exert its growth promoting effect at least partly by up-regulating c-Fos in GCA cells.



"-"means empty vector control and "+" means YAP1-MKN45 stable clones

Figure 4.42 **YAP1 enhanced serum / EGF stimulated c-Fos induction.** YAP1-expressing MKN45 cells (+) and vector-transfected control cells (-) were cultured without serum for 24 hours and then stimulated with serum or epidermal growth factor (EGF) for 0, 1, 5, 15, 30, 60, 90 minutes. Cells were immediately washed with cold phosphate-buffered saline and collected by scraping. The cell lysates were analyzed for MAP kinase pathway by Western blotting using anti-phospho-ERK1/2 and c-Fos. Representative of three independent experiments was shown.



"-"means empty vector control and "+" means YAP1-MKN45 stable clones

Figure 4.43 **PD98059 inhibited ERK activation and c-Fos induction.** Treatment with 20μM of MEK inhibitor PD98059 for 2 hours prior to stimulation suppressed ERK1/2 phosphorylation and c-Fos induction in MKN45 cells.

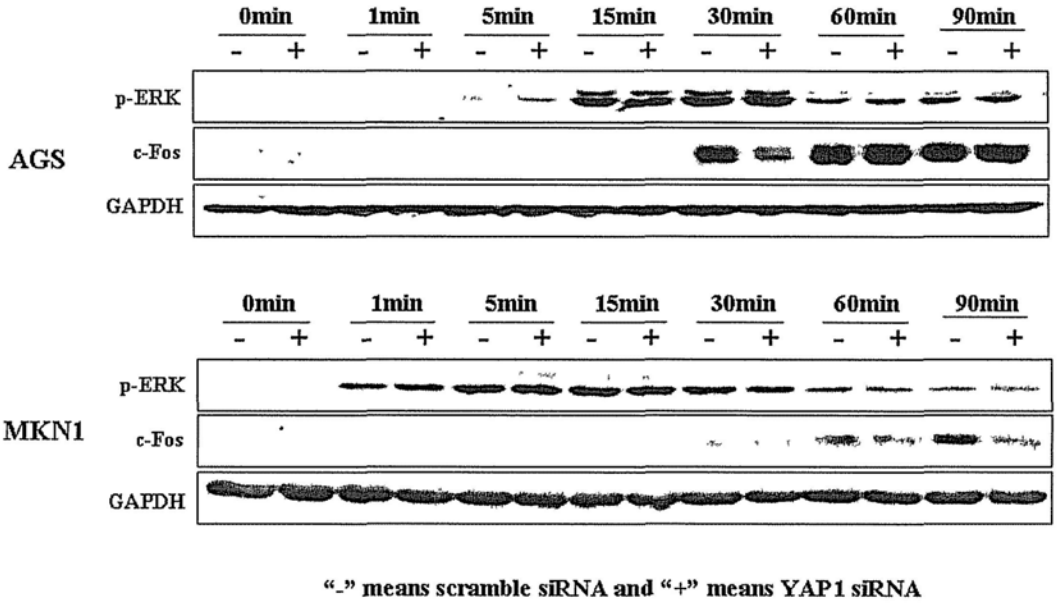


Figure 4.44 **siYAP1 reduced c-Fos expression upon serum stimulation.** AGS and MKN1 cells transfected with YAP1 siRNA (+) or scramble siRNA (-) were cultured without serum for 24 hours and then stimulated with 10% serum for 0, 1, 5, 15, 30, 60, 90 minutes. Cells were immediately washed with cold phosphate-buffered saline and collected by scraping. The cell lysates were analyzed for MAP kinase pathway by Western blotting using anti-phospho-ERK1/2 and c-Fos. Representative of three independent experiments was shown.

4.3.9 YAP1 involved in Notch Signaling pathway

The Notch signaling pathway regulates several cellular processes through control of diverse mechanisms, such as proliferation, cell cycle arrest, differentiation, cell survival, apoptosis, epithelial-to-mesenchymal transition and angiogenesis, even cell fate decision and maintenance of stem cells (Bouras et al., 2008; Dreesen and Brivanlou, 2007; Miele et al., 2006; Shi and Harris, 2006). Disruption of controlled Notch activation could promote oncogenesis. The activated Notch receptor may function as oncogene or tumor suppressor to modulate tumorigenesis (Yeh et al., 2009). However, the regulatory mechanisms of Notch signaling pathway in tumorigenesis is still unclear.

In gastric cancers, the canonical Notch signaling pathway to inhibit chief cell differentiation is frequently activated. It has been reported that Notch 1, Notch 2 and Notch 3 were expressed in all of the eight gastric cancer cell lines examined (Sekine et al., 2006). In Drosophila, the cell surface localization of the Notch receptor is also increased in mutant clones, opening the possibility that aberrant receptor signaling may participate in overgrowth of hippo-deficient tissue (Genevet et al., 2009). In a transgenic mice model, YAP1 stimulates Notch signaling, and administration of gamma-secretase inhibitors suppressed the intestinal dysplasia caused by YAP1 (Camargo et al., 2007).

In this part, we first evaluated expression level of some components of canonical Notch signaling pathway in GCA cell lines and normal gastric mucosal tissues

(Figure 4.45). Numb and cleaved-Notch 1 were detected in all GCA cell lines but not in normal gastric samples by Western blotting. Expression of cleaved Notch 3 was noted in all GCA cells lines and normal gastric mucosal samples. Cleaved Notch 4 could be detected in 5 out of 9 GCA cell lines and all normal gastric tissues.

Knocking down Notch 3 could induce the downregulation of YAP1 mRNA level in AGS cell line, but not in MKN1 (Figure 4.46), suggesting that it is cell context-dependent. These findings indicate that YAP1 might be involved in the Notch signaling pathway. The Notch inhibitor Numb is a docking protein with phosphotyrosine-binding domain (PTB) and multiple SH3-binding proline rich regions. It binds to the WW domain of mammalian E3 ubiquitin ligase Itch and targets Notch receptors for Itch-dependent ubiquitination. So we will next examine that if YAP1 knockdown or overexpression could influence Numb and Notch 3.

We found that after knocking down YAP1 in AGS and MKN1, Notch 3 was downregulated in day 2 but upregulated in day 3 from protein level, which appears to be a dynamic change (Figure 4.48). From mRNA level, the downstream protein of Notch, HES1 and Hey1 was downregulated in day 1 but upregulated in Day 3 (Figure 4.47). In AGS, knocking down YAP1 could also induce Numb upregulation (Figure 4.48).

In stable clones, YAP1-MKN45 has low Numb but high Notch 3 expression level. On the contrary vector-MKN45 has high Numb expression but low Notch 3 expression, suggesting that YAP1 could enhance Notch 3 level through downregulation of Numb. In xenograft tissue, no expression difference in Numb and Notch 3 between YAP1-MKN45 and empty vector control (Figure 4.48).

The role of Notch signaling pathway in gastric tumor is very complicated, and its regulatory mechanism remains elusive at present (Yeh et al., 2009). In this preliminary study, we demonstrated that YAP1 might be involved in Notch signaling pathway in GCA. The precise interaction between YAP1 and the Notch signaling pathway deserve further investigations in future.

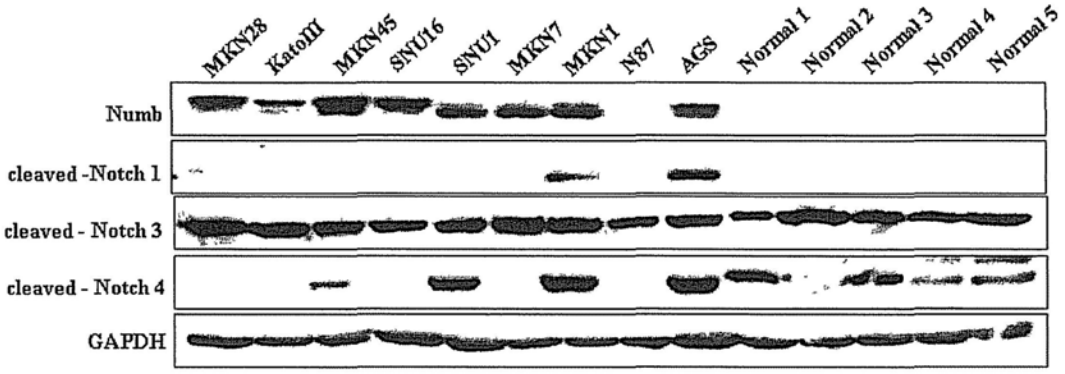


Figure 4.45 **Expression of Numb, activated Notch 1, 3, and 4 were determined by Western blotting in 9 GCA call lines and 5 normal gastric mucosal samples.**

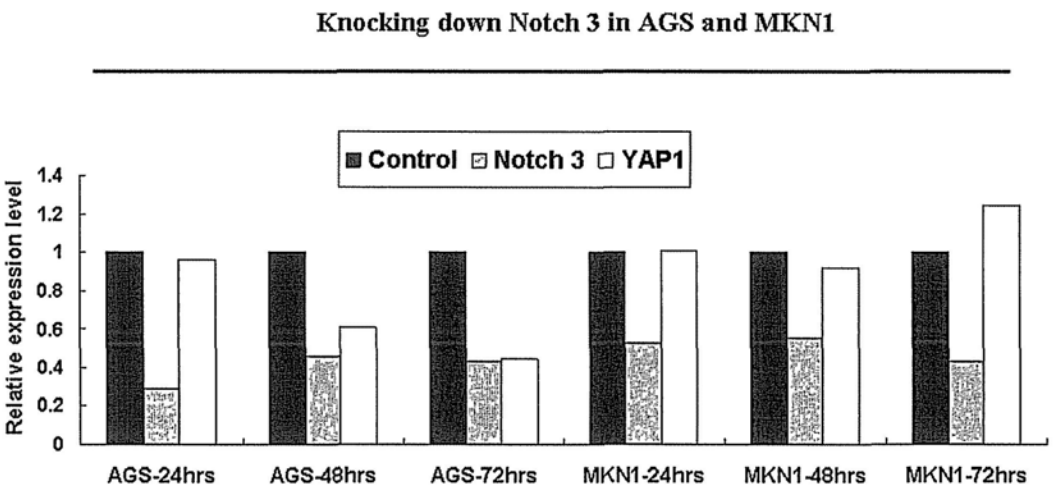


Figure 4.46 **Synchronous down-regulation of YAP1 upon Notch 3 knockdown in GCA cell line AGS.** AGS and MKN1 cells were transfected with Notch 3 siRNA. Cells were harvested after 24 and 48 hours and Notch 3 and YAP1 mRNA level were determined by qRT-PCR.

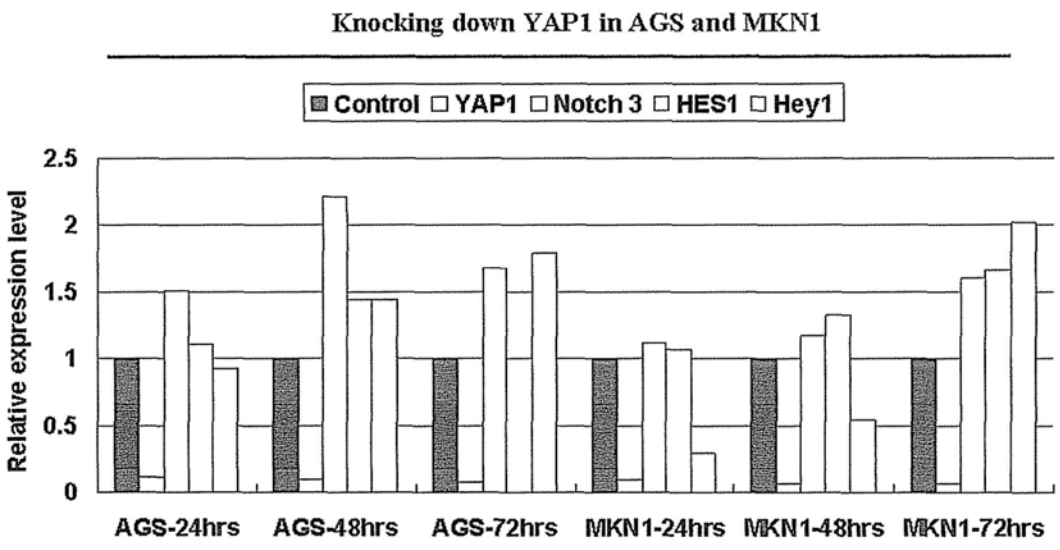


Figure 4.47 **The effect of YAP1 knockdown on Notch signaling pathway in GCA cell lines.** GCA cell lines AGS and MKN1 were transfected with YAP1 siRNA. Cells were harvested 24, 48 and 72 hours after transfection. The mRNA level of Notch 3, HES1 and Hey1 were determined by qRT-PCR.

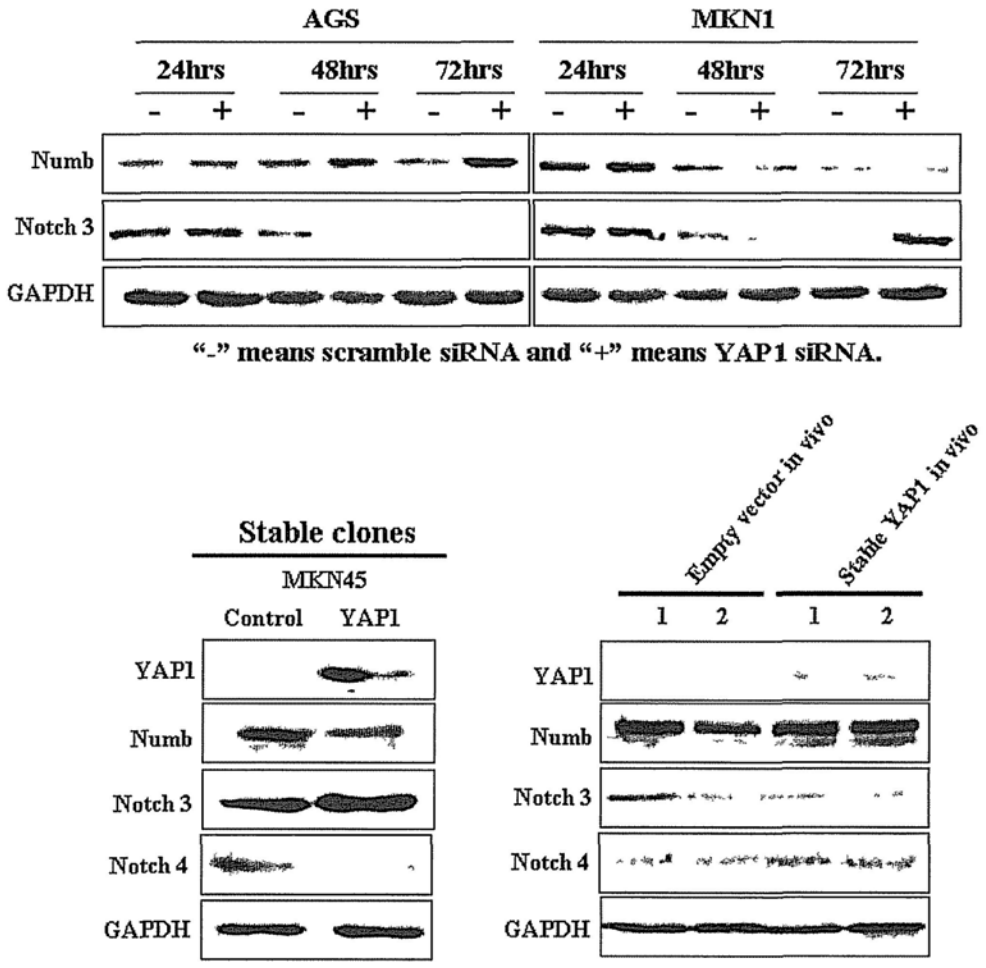


Figure 4.48 **The effect of YAP1 expression on Notch proteins.** Upper panel: GCA cell lines AGS and MKN1 were transfected with YAP1 siRNA and the cells were harvested 24, 48 and 72 hours after transfection. The protein level of Numb and Notch 3 were determined by Western blotting. Lower panel: Numb, Notch 3 and Notch 4 were determined by Western blotting in MKN45 cells stably expressing YAP1 (lower left panel), and YAP1-MKN45 and vector-MKN45 xenograft tumors.

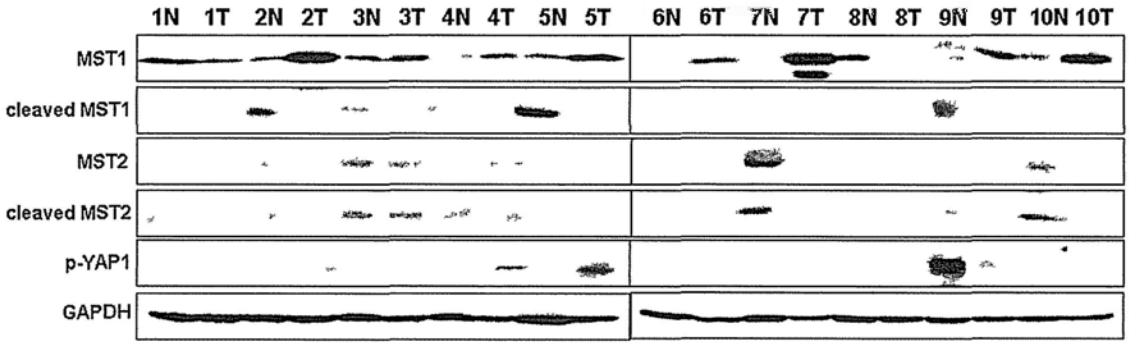
4.3.10 YAP1 was regulated by Hippo signaling pathway in gastric cancer

The mammalian Hippo orthologs MST1/MST2 protein kinases suppress the oncogenic activity of YAP1 oncogene by promoting YAP1 S127 phosphorylation and subsequent cytoplasmic retention. Loss of function of the growth inhibitory components of the Hippo pathway that results in defects in organ size regulation and massive overgrowth has been reported in hepatocellular carcinoma (Zhou et al., 2009). However, the significance of Hippo pathway in gastric cancer has not been established.

We examined the MST1 and MST2 protein expression in paired primary gastric cancer and non-cancerous gastric mucosa. The cleaved, activated MST1 and MST2 were lost in 9 and 5 out of 10 gastric cancers, respectively (Figure 4.49). The finding suggested that the MST-YAP1 pathway might be disrupted in a substantial fraction of gastric cancers.

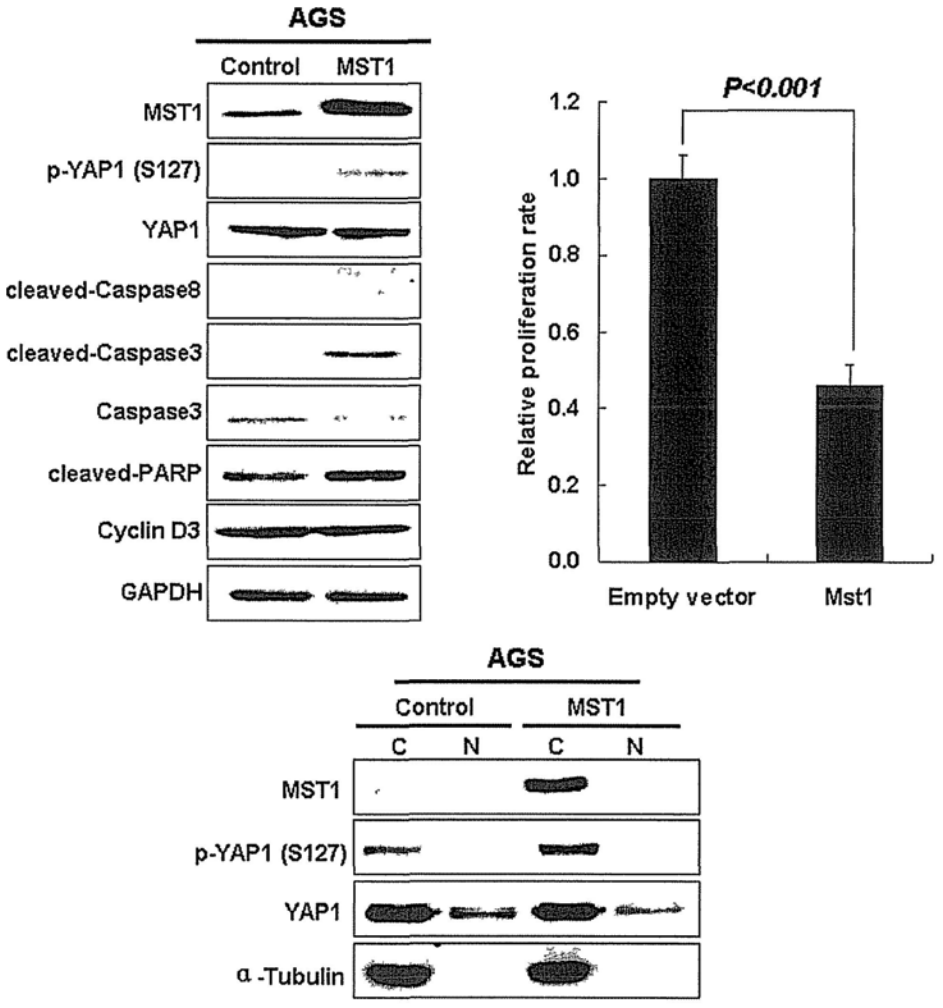
To further investigate whether MST1 acts upstream of YAP1 and suppresses the oncogenic activity of YAP1 in gastric cancer, we transfected MST1 expression vector into AGS cells. Ectopic expression of MST1 suppressed cell proliferation, and induced apoptosis through consecutively activated cleaved-Caspase8, cleaved-Caspase3 and cleaved-PARP (Figure 4.50). Furthermore, MST1 overexpression promoted the phosphorylation and cytoplasmic translocation of p-YAP1 (S127) as demonstrated by the increased p-YAP1 S127 in cytoplasmic fraction upon MST1 ectopic expression, hence quenched the oncogenic function

of YAP1 in the nucleus (Figure 4.50). Loss of function of the upstream growth inhibitory components of the Hippo pathway might result in constitutive activation of YAP1 and therefore contribute to carcinogenesis.



"N" means normal gastric epithelium and "T" means paired tumor

Figure 4.49 **MST1 and MST2 in 10 paired primary GCA samples.** The cleaved 34KDa, activated MST1 was lost in nearly all gastric cancer tissue (9/10), whereas 7 paired non-cancerous tissues retained activated MST1 catalytic fragment. Non-cancerous tissue showed stronger expression both for the total MST2 (5/10) and cleaved activated MST2 (6/10) compared to tumor tissue.



"C" means cytoplasmic lysate and "N" means nuclear lysate

Figure 4.50 **Exogenous MST1 overexpression in AGS.** Upper left panel: Western blotting showing overexpression of MST1 in AGS cells enhanced YAP1 phosphorylation at S127, activated cleaved-Caspase8, cleaved-Caspase3 and cleaved-PARP. Upper-Right panel: Overexpression of MST1 suppressed cell proliferation in AGS cells (*P<0.001*). Lower panel: Overexpressed MST1 mainly localized in the cytoplasm and its overexpression could induce accumulation of p-YAP1(S127) in the cytoplasm and attenuate nuclear localization of total YAP1.

CHAPTER 5. DISCUSSION

5.1 Array-CGH and mRNA expression microarray profiling in GCA

Array-CGH is a powerful technique in the field of cancer research (Buffart et al., 2007; Lilljebjorn et al., 2007; Qian et al., 2008; Sung et al., 2007; Zanazzi et al., 2007). The results of high-resolution array-CGH analysis in GCA cell lines show good concordance with those of conventional CGH and low-resolution array-CGH (Morohara et al., 2005; Takada et al., 2005; Tsukamoto et al., 2008; Varis et al., 2002; Vauhkonen et al., 2006). Array-CGH can reproducibly detect copy number gains and losses using small amount of whole genome sample. The result showed that array-CGH was a powerful technique in identification of gene copy number change. With high throughput methods with high resolution and sensitivity, array-CGH provided valuable clues to the identification of novel tumor suppressor genes and oncogenes associated with GCA. Some novel target genes identified in this study that showed amplification or deletion deserved further investigations.

Array-CGH provides a genome-wide perspective to observe DNA level changes in the cancer cells, but the significance of these changes remains to be elucidated in RNA and protein level determinations of specific gene expression (Ulger et al., 2003). A gene copy number change is likely biologically more meaningful if it is directly related to a change in gene expression (Kim et al., 2007). However,

studies about the relationship between copy number changes and gene expression changes are very limited (Kim et al., 2007; Varis et al., 2002; Zanazzi et al., 2007; Zhu et al., 2004), despite lots of genome-scale analysis of DNA copy number changes and expression profiling in GCA. For that, we also analyzed the gene-expression profiles of the GCA.. Large scale of correlations between DNA copy number changes and expression level variations were calculated for a common set of genes identified from array-CGH and gene expression profiling. For the genes showing negative correlation between copy number change and expression, such as DNA copy number gain with reduced mRNA expression level, the underlying mechanism deserved further investigations. mRNA expression was altered not only by DNA copy number changes, but also by many other different mechanisms, like transcription factors, epigenetic modifications, mutations, polymorphisms and post-transcriptional regulation (Albertson et al., 2003).

Many tumors had similar clinicopathologic parameters but have different outcomes or responses to therapy (Alizadeh et al., 2001), so combination of array-CGH and RNA expression studies had great potential for discovery of prognostic molecular markers. GCA was currently being dissected at the molecular level with genomic and proteomic tools to draw novel molecular signatures that could be helpful in early diagnosis or treatment (Fernandez et al., 2009; Hippo et al., 2002; Yokoyama et al., 2008; Yu et al., 2009). In our experiment, array-CGH was first applied as an initial screening tool for the

preliminary selection of copy number-changed genes and employed other molecular tools for the quantitation of the target genes. Gene copy number changes were able to distinguish cancerous and normal tissues and would have potential clinical application.. When combined with expression microarray profiling data, array-CGH could show us those genes that were differentially expressed between normal gastric tissues and gastric tumor. Integrated approach of studying a chromosome plus expression facilitated the understanding of GCA carcinogenesis. Some novel genes were identified showing high frequency of DNA copy number changes. The results provided a list of putative candidate oncogenes and tumor suppressors that could help to elucidate GCA at the molecular level (Kim et al., 2007).

5.2 YAP1 in GCA

5.2.1 Up-regulation of YAP1 in GCA

YAP1, a 65KDa protein, was originally identified as a transcription factor that binds to the SH3 domain of the YES kinase (a Src protein kinase) (Sudol, 1994). This protein contains a WW domain that is found in various structural, regulatory and signaling molecules and may be involved in protein-protein interaction. The Drosophila ortholog of YAP, Yorkie (Yki) (Zhang et al., 2009a), activates proliferation by inducing the expression of cyclin E and inhibits apoptosis by induction of the caspase-inhibitor protein DIAP1 (Drosophila inhibitor of apoptosis) (Dong et al., 2007). More recently YAP1 has been identified as a candidate oncogene that promotes tumorigenesis in many different

types of cancer. Some observations point to a potential oncogenic role for YAP1 in human cancers as a downstream effector of the Hpo-Sav-Wts signaling pathway and as a critical regulator of cellular proliferation and apoptosis. Lowe et al have reported the oncogenic activity for YAP1 in a mouse model of hepatocellular carcinoma where YAP1 amplification contributes to development of tumors (Zender et al., 2006). In nontransformed mammary epithelial cells, overexpression of human YAP1 induces epithelial to mesenchymal transition, suppression of apoptosis, growth factor-independent proliferation, and anchorage independent growth in soft agar (Overholtzer et al., 2006). Carmargo et al showed that YAP1 could increase organ size and expand undifferentiated progenitor cells (Camargo et al., 2007). However, YAP1 was also implicated in the tumor-suppressor pathway, such as RASSF1A-MST2-LAST1 pathway. The phosphorylated YAP1 binds to p73 and induces transcription of PUMA (Matallanas et al., 2007). Moreover, there are data suggested that YAP1 could switch between p73-mediated proapoptotic and growth arrest target genes based on its phosphorylation state and the modification of a transcription coactivator, namely the DNA damage-induced phosphorylation of YAP1 by c-Ab1. (Levy et al., 2008a; Levy et al., 2008b).

There were several papers reporting the amplifications of 11q22 in several different types of human cancers (Imoto et al., 2001; Overholtzer et al., 2006; Yokoyama et al., 2008; Zender et al., 2006), including liver cancer, breast cancer and malignant pleural mesotheliomas. In 11q22, several candidate oncogenes

were amplified in this region, such as YAP1 and cIAP1 (BIRC2). These two genes may be cooperated in tumorigenesis, because they shared similar copy number change and expression level and Lowe et al reported that these two genes cooperated to promote tumorigenesis (Zender et al., 2006). In our array-CGH data of GCA cell lines, we found YAP1 also had copy number gain in some cell lines, such as MKN7, MKN1 and N87. The copy number change (Table 5.1) and expression level of cIAP1 and YAP1 (Figure 5.1) were also very similar in GCA. This may be because these two genes were located in the same chromosome loci, so they underwent similar copy number change and expression. So it is possible that in GCA YAP1 and cIAP1 could cooperate in gastric tumorigenesis as they were in liver cancer. Apart from this, YAP1 in MKN45 has an intragenic homozygous deletion. This homozygous deletion of YAP1 in MKN45 was not seen in any other GCA cell lines examined.

Table 5.1 **Array-CGH data of cIAP1.** The copy number change of cIAP1 was similar with YAP1. NGT: normal gastric tissue.

Array-CGH data of cIAP1										
Probe	**MKN45**	**MKN28**	**KatoIII**	**AGS**	**N87**	**SUN1**	**SNU16**	**MKN1**	**MKN7**	**NGT**
1	-1.87155	-1.14597	0.241082	-0.92801	0.66578	-0.3297	-0.19793	0.529842	0.585088	0
2	-1.57135	-1.16898	-0.39298	-0.79692	1.107924	0.16785	0.177223	0.846264	0.873278	0
3	-1.40758	-1.18867	-0.09408	-0.59309	1.082915	0.131518	0.279577	0.675271	0.825081	0
4	-0.67919	0.005617	0.541545	-0.10725	0.898373	0.093426	-0.02742	0.037573	0.149058	0
5	-0.59572	0.075827	0.547936	-0.12849	0.999152	0.024458	0.057921	-0.00593	0.167251	0
6	-0.4251	0.228704	0.50573	-0.11673	0.941085	0.051017	0.029202	0.042803	0.072844	0

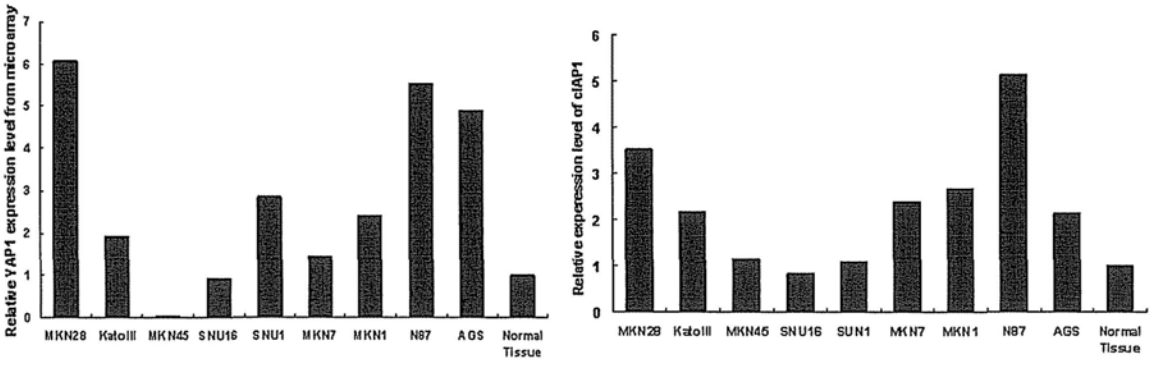


Figure 5.1 **Relative mRNA expression levels of YAP1 and cIAP1 in GCA cell lines.** These two genes shared the similar expression level in GCA cell lines.

YAP1 was up-regulated in some solid tumors, such as lung, colon, liver, ovarian cancer and medulloblastoma (Dong et al., 2007; Fernandez et al., 2009; Steinhardt et al., 2008a, b; Xu et al., 2009; Zender et al., 2006) and could predict poor prognosis. The tumorigenicity of YAP1 seems controversial and may be cell type dependent (Bertini et al., 2009; Matallanas et al., 2008). The possible role of YAP1 in gastric cancer carcinogenesis is unclear. YAP1 protein was confirmed up-regulated in gastric cancers. In GCA cell lines, both qRT-PCR and Western blotting methods confirmed that YAP1 was upregulated in both RNA and protein levels compared with non-cancerous gastric tissues. Copy numbers of YAP1 seemed normal in GCA cell lines such as MKN28 and SNU1, but the YAP1 expression in these two cell lines were very high. Thus, apart from gene copy number gains, other mechanisms are involved in the up-regulation of YAP1. We also determined the YAP1 mRNA expression in 30 primary human GCAs and paired non-cancerous gastric mucosal tissues by qRT–PCR. When compared to paired non-cancerous gastric mucosal tissues, 11 (36.7%) cases showed more than 1.5-fold up-regulation in tumor tissues. However, the mean level of YAP1 mRNA expression in 30 tumor tissues was not significantly higher than that in the normal tissues (3.1 (0.19 – 16.43) vs. 2.5 (0.16 -8.46), *P=0.134*, Figure 5.2). In addition, no mutations were detected in the coding sequence and adjacent exon and intron boundaries of YAP1 DNA in 8 gastric cancer cell lines and 10 primary gastric tumors, except MKN45 cells, in which an intragenic homozygous deletion was observed. Therefore, post translational modification or inhibition of YAP1 degradation machinery was thought to be involved in the

over-expression of YAP1 protein in GCAs.

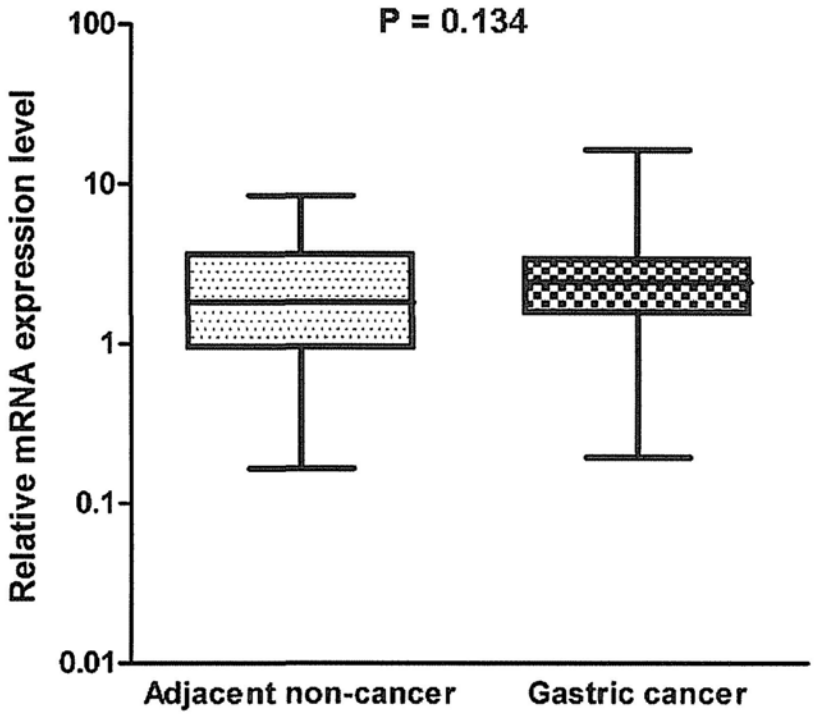


Figure 5.2 **qRT-PCR of 30 pairs of gastric tumor / normal adjacent tissues.** The box plot showed the YAP1 mRNA expression level from 30 pairs of gastric adenocarcinoma and paired non-cancerous gastric mucosa (*P=0.134*). There was no significant difference for mRNA level of YAP1 between GCAs and non-cancerous gastric tissues.

IHC analysis on primary gastric cancers also demonstrated the up-regulation of YAP1 in gastric cancer. Our finding was in keeping with a previous report that 48% of the gastric tumor showed overexpression of YAP1 in a tissue microarray study (Da et al., 2009). Total YAP1 could be detected both in cytoplasm and nucleus. We found that YAP1 nuclear accumulation predicted a poorer prognosis in patients with GCA, especially for early stage patients (stage I & II). The significant prognostic value of nuclear YAP1 accumulation was further supported by the expanded cohort of stage I and II gastric cancer. Our data provided the first evidence that YAP1 could be used as a potential prognostic biomarker, especially for patients with early stage disease. In our study, no correlation between YAP1 nuclear accumulations and clinicopathologic features including Helicobacter pylori infection were found, suggesting that YAP1 up-regulation may not be directly related to these features. Nevertheless, the possible pathogenic link between Helicobacter pylori infection and YAP1 activation cannot be excluded and this requires further investigations. Expression of YAP1 in intestinal metaplasia has been reported and suggested that YAP1 might be also involved in early gastric carcinogenesis.

Due to YAP1 has WW domain structure, it has been reported to bind to and regulate the activities of various transcriptional regulators, including p73, Smad7 (Ferrigno et al., 2002), ErbB4, and several TEAD/TEF-type transcription factors (Ota and Sasaki, 2008; Zhao et al., 2009), and YAP1 has oncogenetic potential in mammals possibly through an association with RUNX2 and ErbB4 (Aqeilan et

al., 2005; Komuro et al., 2003). YAP1 could be phosphorylated at S127 by at least three proteins, including AKT (Basu et al., 2003), LATS1 (Dong et al., 2007; Hao et al., 2008; Zhao et al.; Zhao et al., 2007) and Merlin (Yokoyama et al., 2008), leading to its association with protein 14-3-3, which sequestrates YAP1 in the cytoplasm and inhibits its co-activator activity. One important aspect is the regulation of YAP subcellular localization. However, the function of YAP1 translocating into nucleus is very controversial or even contradictory because YAP1 in nucleus could induce cell proliferation, expand the organ size of mice liver, advance development of pronephric cysts of zebrafish (Skouloudaki et al., 2009), even induce tumorigenesis (Camargo et al., 2007; Dong et al., 2007; Zhao et al., 2007), or it would participate in P73-induced apoptosis (Matallanas et al., 2007). The more confusing point is LAST1 could phosphorylate YAP1 on different site, making YAP1 retention in the cytoplasm (Dong et al., 2007) or translocation into the nucleus (Matallanas et al., 2007). Our experimental data favored a tumorigenic role of YAP1 in gastric cancer. Its nuclear accumulation correlated with a poorer prognosis. This was in keeping with the findings of nuclear translocation of YAP1 could induce liver cancer formation as other paper reported (Dong et al., 2007).

5.2.2 Oncogenic roles of YAP1 in GCA

A series of functional studies were performed including proliferation rate, monolayer and anchorage independent soft agar colony formation, cell invasion and migration through knocking down or overexpressing YAP1 in GCA cell

lines and *in vivo* nude mice study. The results were supportive of an oncogenic role of YAP1 in gastric cancer. In transfection experiment, knocking down YAP1 could reduce cell proliferation rate, suppress cell migration and reduce colony formation significantly, whereas overexpression of YAP1 could advance cell proliferation and these result was concordant with other report of YAP1 in liver cancer (Zender et al., 2006). In YAP1 knocking down experiment, G1 phase arrest was probably induced by ERK phosphorylation. Although ERK is known to play a key role in the proliferation process and functioning as a positive regulator of cell cycle progression, the accumulated results showed that excessive activation of ERK may mediate cell cycle arrest (Camargo et al., 2007; Meloche and Pouyssegur, 2007). Moreover, several studies showed that this high intensity of phosphorylated ERK may induce the expression of the cyclin-dependent kinase inhibitor p21 and persistent accumulation of p21 has been associated with inhibition of CDK4 and CDK2 enzymatic activities and thus the resulting hypophosphorylation state of pRb and cell arrest at G1 phase (Han et al., 2005). However, in YAP1 overexpression assays, the overexpressed YAP1 would also activate MAPK, AKT even P38MAPK pathways, upregulate cyclin D1, CDK4 and CDK6 in transient expression, stable expression or even in nude mice model. In concordance with previous reports on mouse mammary tumors (Overholtzer et al., 2006) and human colorectal cancers (Camargo et al., 2007), our data indicated that YAP1 might increase cell proliferation through these pathways.

5.2.3 Possible down-steam events of YAP1.

To investigate the mechanism by which YAP1 regulated cell proliferation, microarrays was employed to identify YAP1-induced transcription targets in gastric cancer cells. By Ingenuity Pathway Analysis, we found that the MAPK family was significantly enriched in several networks. The MAPK pathway is a major intracellular signaling pathway involved in cell proliferation, differentiation, and tumorigenicity. A constitutive activation of RAF/MEK/ERK in MKN45 cells stably expressing YAP1 was confirmed and further demonstrated that YAP1 enhanced serum/EGF induced c-Fos expression in gastric cancer cells. C-Fos is a major target of MAPK activation. It was first identified as a cellular counterpart of a viral oncogene capable of cell transformation (Muller, 1986) and considered immediate early gene product that regulates gene expression required for progression through cell cycle (Brown et al., 1998). Induction of c-Fos by YAP1 might affect changes in cell phenotype that contributed to neoplastic transformation. The mechanisms by which YAP1 might activate MAPK pathway and regulate c-Fos required further investigations.

5.2.4 Regulation of YAP1 by Hippo pathway.

YAP1 is a negatively regulated downstream target of the Hippo signaling pathway. The mammalian Hippo orthologs MST1/MST2 protein kinases

suppress the oncogenic activity of YAP1 oncogene by promoting YAP1 S127 phosphorylation and subsequent cytoplasmic retention. Loss of function of the growth inhibitory components of the Hippo pathway might result in defects in organ size regulation and massive overgrowth (Zhou et al., 2009). The loss of cleaved activated MST1/2 in gastric cancer tissues was first identified, suggesting that the MST-YAP1 pathway was disrupted in a substantial fraction of gastric cancers. In addition, *in vitro* studies demonstrated that ectopic expression of MST1 suppressed cell proliferation, induced apoptosis in gastric cancer cells. Further experiments showed that MST1 promoted the phosphorylation of YAP1 on S127, enhanced its retention in the cytoplasm and therefore quenched its oncogenic function in the nucleus. The data suggested that activation of YAP1 in gastric cancer may be related to the disruption of Hippo pathway.

5.2.5 Postulation of the dynamic functions of YAP1.

There were some reports showing that YAP1 functioned as a tumor suppressor (Lapi et al., 2008; Levy et al., 2007, 2008a; Levy et al., 2008b; Matallanas et al., 2007; Strano and Blandino, 2007; Strano et al., 2005; Yuan et al., 2008). However, the proapoptotic function of YAP1 was strictly dependent on P73. In addition, in irradiated prostate carcinoma cells, YAP1 could interact with EGR-1 through its WW domain and enhanced the transcriptional activity of EGR-1 on the Bax promoter (Zagurovskaya et al., 2009). Thus, it might well be possible that the function of YAP1 would change dependent on the availability and

affinity of binding partners (Matallanas et al., 2008). Both parameters may be determined by the specific state of cellular signaling networks. Recent study even suggested that pathways were not fixed but can dynamically change depending on the context and circumstance (Santos et al., 2007). In GCA, YAP1 exhibited oncogenic properties according to our experimental findings. Many key players, e.g. RASSF1A and p73 which involved in the tumor suppressor function of YAP1, were downregulated or lost in GCA cell lines (Figure 5.3). It is possible that the anti-apoptotic pathways YAP1 involved was ablated or thwarted in GCA cells. In normal culture condition, YAP1 suppression apoptosis in MKN45 cells (Figure 5.4). When treated YAP1-MKN45 and vector-MKN45 with Cisplatin, we noted a significantly higher proportion of apoptotic cells were induced in YAP1-MKN45 than vector-MKN45 (Figure 5.5). The finding suggested that YAP1 facilitated apoptosis when challenged with apoptotic stimuli in GCA cells. The biological function of YAP1 may well be cell context and stimuli-dependent (Levy et al., 2007, 2008a; Matallanas et al., 2007; Strano et al., 2005).

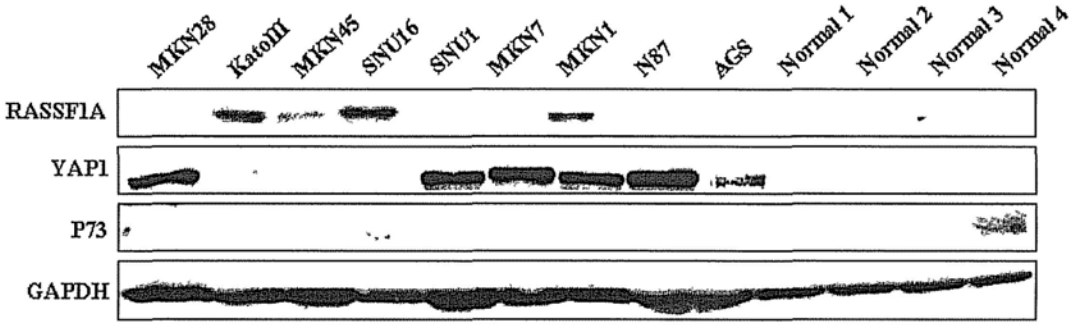
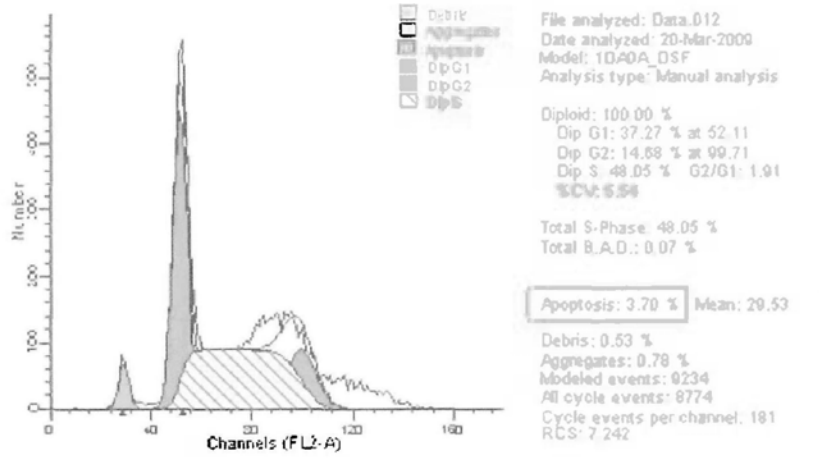


Figure 5.3 **RASSF1A and P73 in GCA cell lines.** RASSF1A and P73 protein expression level in GCA cell lines were detected by Western blot.

Vector-MKN45



YAP1-MKN45

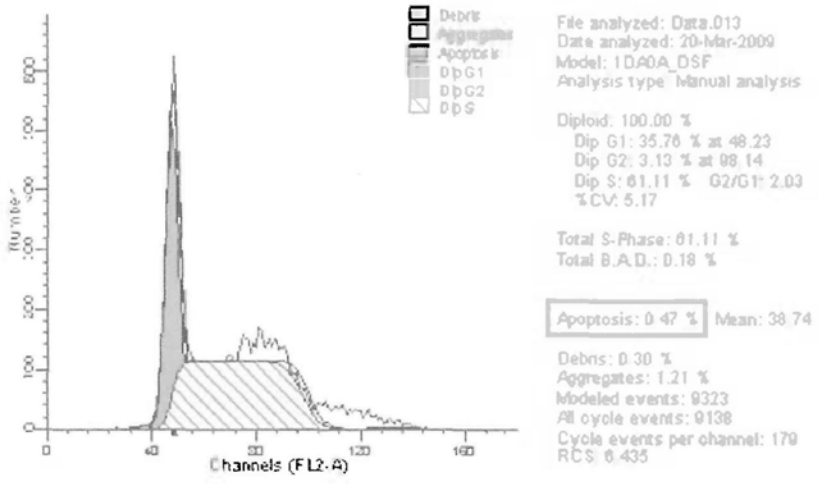
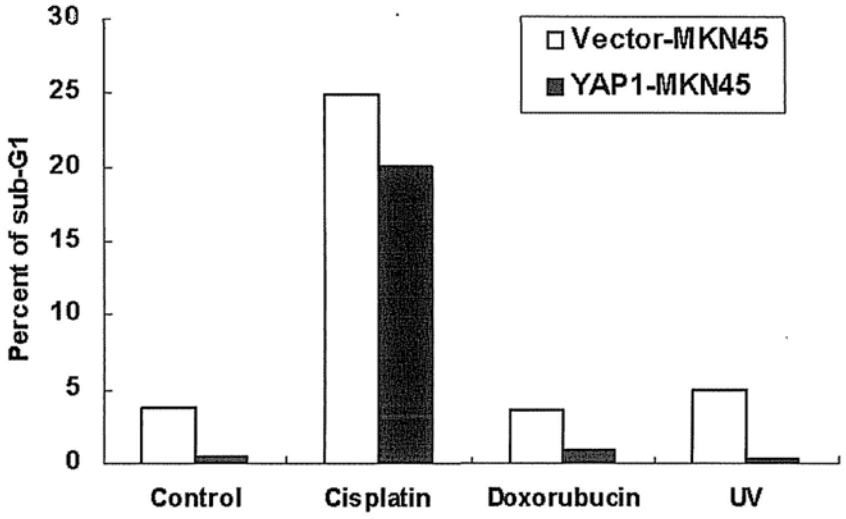


Figure 5.4 **Stable YAP1 expression could suppress apoptosis in MKN45 cells in normal culture condition.** The apoptosis as measured by sub-G1 fraction was 3.7% (upper panel) in vector-MKN45 and 0.47% in YAP1-MKN45 (lower panel).



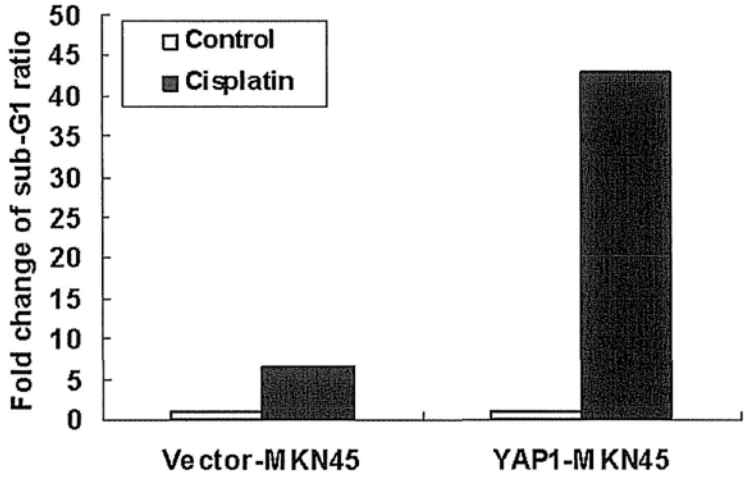


Figure 5.5 **Stable YAP1 expression could induce apoptosis under the stimulation of Cisplatin.** Vector-MKN45 and YAP1-MKN45 were treated with Cisplatin and the apoptosis was determined by sub-G1 fractions.

CHAPTER 6. SUMMARY AND FUTURE WORKS

Array-CGH was first used in this study to analyze each chromosome of tumor cells for genetic gains and losses. Our results of array-CGH analysis showed good concordance with those of conventional CGH. We identified some novel target genes that showed gene amplification or deletion in GCA cell lines which deserved further investigations. The DNA copy number changes were correlated with mRNA expression profiling. The selection of the genes that show a high correlation between copy number changes and expression changes is expected to identify differentially expressed candidate genes between normal gastric tissues and gastric cancers.

A comprehensive study was carried out to investigate the biological function of putative oncogene YAP1 in gastric cancer. YAP1 is a multifunctional protein that can interact with different transcription factors to activate gene expression. YAP1 protein expression was up-regulated in gastric cancer. Nuclear accumulation of YAP1 was associated with poor disease specific survival ($P=0.021$), especially in patients with early stage diseases ($P<0.001$). Knockdown YAP1 resulted in a significant reduction in proliferation, anchorage-dependent colony formation, cell invasion and cell motility. Ectopic YAP1 expression promoted anchorage-independent colony formation, induced a more invasive phenotype and accelerated cell growth both *in vitro* and *in vivo*. Microarray analysis highlighted the alteration of MAPK pathway by YAP1. We confirmed a

constitutive activation of RAF/MEK/ERK in YAP1-expressing MKN45 cells and further demonstrated that YAP1 enhanced serum/EGF induced c-Fos expression in gastric cancer cells. Our findings supported YAP1 is a functional oncogene in gastric cancer. We provided the first evidence that YAP1 exerted the oncogenic function by enhancing the capacity to activate the early response gene pathway. YAP1 could be a prognostic biomarker and potential therapeutic target for gastric cancer.

Our ongoing works and future studies include:

1. Confirmation and validation of the gene copy number changes using FISH for the candidate genes which showed high-level amplification or homozygous deletion in GCAs.

2. Further analysis of correlation between array-CGH and expression microarray in the GCA cell lines. We have selected several genes showed a higher negative correlation between copy-number change and expression level, including ASB2, TSPYL2, MFPR, ABTB1 and etc. Epigenetic regulation such as promoter hypermethylation or histone deacetylation may contribute to the reverse relationship.

3. Further investigate the function of YAP1. The cellular pathways YAP1 is involved in gastric cancer are not completely understood. It would be of interest

to have in-depth investigation of the Notch signaling pathway and NF-κB pathway. Further dissecting the oncogenetic functions of YAP1 in GCA should reveal new insights into the molecular mechanisms of gastric carcinogenesis and provide new therapeutic targets for GCA treatments.

As an adaptor protein, YAP1 can interact with multiple transcription factors and modulate their transcriptional activity. The choice of its partner transcription factors, and consequently the final outcome might be determined by multiple factors, e.g. cell context, type of stimuli, and regulation of upstream pathways. The TEAD proteins are major partners of YAP1 and are required for the YAP1-mediated gene expression that promotes proliferation and inhibits apoptosis. On the other hand, YAP1 physically interacts with p73α, p73β and p63α and promotes apoptosis following DNA damage. We showed in this study that YAP1 up-regulated in gastric carcinoma and functional studies supported a tumorigenic role of YAP1 in gastric carcinogenesis. Being an adaptor protein with the capacity to interact with multiple transcription factors, YAP1 is placed at the crossroads of multiple signaling pathways. It has been proposed that YAP1 regulates the balance between cell proliferation and apoptosis to maintain homeostasis. Further investigations are required to delineate the molecular mechanisms that underlie the roles of YAP1 in different cell context.

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