ZBP-89 Expression in Hepatocellular Carcinoma and Its Interaction with Mutant p53

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Abstract

ZBP-89, a Krüppel-type zinc-finger transcription factor, participates in the regulation of cell growth and cell death through regulating other cellular protein, such as p53 and $p21^{Waf1}$ (p21). ZBP-89 is elevated in some cancers, including gastric cancer, colorectal cancer and breast cancer. Interestingly, p53 mutants may interact with ZBP-89 and contribute to its transcriptional regulation of p21. However, to date, ZBP-89 expression in hepatocellular carcinoma (HCC) is not well documented. Its interaction with p53 mutants and the consequence of such an interaction in cancer treatments are, as well, poorly understood. Therefore, in this study, we aimed to examine ZBP-89 expression and its clinical significance in HCC, and to investigate its relationship with p53 mutants and its significance in cancer treatments.

Firstly, we examined ZBP-89 expression in 5 types of HCC cell lines and 182 HCC tissue samples by RT–PCR, Western blot and immunofluorescent staining. Our results showed that the expression of ZBP-89 was higher in HCC than the adjacent non-tumour liver at both mRNA and protein levels. ZBP-89 was localized in the nucleus in most HCC tissue samples but was found in the cytoplasm in 11.5% (21 of 182) of HCC tissues. Survival in patients with tumours showing high ZBP-89 expression was better than in those with low expression. High ZBP-89 expression tended to be more common in WHO grade I than grades II-IV HCC. There was a significant association between HBV positivity and high ZBP-89 expression. Colony

formation was dramatically reduced in those HCC cell lines with ZBP-89 overexpression; and this reduction appeared to correlate with increased apoptosis, which was evident by the increase in the cleaved PARP. The likely mechanisms responsible for the ZBP-89-mediated the reduction of colony formation may involve increased p53 or/and p21 expression.

Secondly, we demonstrated that ZBP-89 was essentially required in HDACi-mediated p21 up-regulation. ZBP-89 over-expression induced PARP cleavage and enhanced the lethal effectiveness of Trichostatin A (TSA). p53 mutant $p53^{G245D}$ but not $p53^{R273C}$, identified in HCC patients from Hong Kong, had a similar pattern of subcellular localization to ZBP-89 in HCC tissues, and abrogated butyrate-mediated induction of p21 by directly binding to ZBP-89 and preventing its translocation from the cytoplasm to the nucleus. Functionally, the cytoplasmic accumulation of ZBP-89 by $p53^{G245D}$ significantly protected cells from TSA-induced death. The activation of several apoptotic proteins, such as caspase 8, Bid and PARP, was involved in $p53^{G245D}$–mediated protection.

Taken together, we concluded that ZBP-89 may have anti-tumour properties and is a potential biomarker for prognosis of HCC. Furthermore, this study has suggested a novel role of $p53^{G245D}$ in the shuttling of ZBP-89 from the nucleus to the cytoplasm, and therefore established a plausible link between the mutant p53 binding to ZBP-89 and a decreased chemosensitivity of HCC cells.

摘要

通過調節如 p53 和 $p21^{Waf1}$ (p21)等多種細胞內蛋白的表達，轉錄因子 ZBP-89 參與了細胞生長與死亡的調控。研究表明，ZBP-89 在胃癌，結直腸癌和乳腺癌等多種癌組織中高表達。p53 突變體可與 ZBP-89 相互作用並干涉了 ZBP-89 對 p21 的調控。但是，ZBP-89 在肝細胞癌(HCC)中的表達至今仍未被研究，而其與 p53 突變體在肝細胞癌中的相互作用及其意義也並未被人們所研究清楚。 因此，本研究將通過檢測 ZBP-89 在肝細胞癌中的表達，與相關病理臨床參數進行統計學分析，以確定 ZBP-89 在肝細胞癌中的表達及其意義。另外，本研究同時將檢測 ZBP-89 與 p53 突變體的相互作用，並探討其對腫瘤治療的意義。

首先，我們應用了反轉錄聚合酶鏈式反應，免疫印跡和免疫螢光等方法檢測了 ZBP-89 在 5 種肝細胞癌細胞和 182 對肝細胞癌組織中的表達。結果顯示，在 mRNA 和蛋白水準上，ZBP-89 在肝癌細胞株中的表達高於其在正常細胞株中的表達，同時，ZBP-89 在肝癌組織中的表達也顯著高於其在相應癌旁組織中的表達。 ZBP-89 主要定位於細胞核，但在 11.5%的癌組織中，ZBP-89 定位於細胞漿。生存分析結果表明，伴有 ZBP-89 高表達的肝癌病人預後較好。低分級(WHO grade I)的肝癌病人容易伴有 ZBP-89 高表達；同時，ZBP-89 的高表達 HBV 陽性感染統計學相關。此外，細胞克隆形成實驗發現過表達 ZBP-89 可顯著減少肝癌細胞克隆形成數量。進一步研究表明 ZBP-89 過表達可引起細胞凋亡，其機制可能爲 ZBP-89 上調了 p53 和/或 p21 的表達，啓動了凋亡蛋白 PARP，從而導致了細胞凋亡。

然後，我們採取了 MTT 細胞增殖檢測，TUNEL 細胞凋亡檢測，免疫印跡，

免疫螢光和免疫共沉澱等實驗手段檢測ZBP-89與p53突變體的相互作用及其意義。結果發現組蛋白去乙醯化酶抑制劑介導的p21上調是ZBP-89依賴，ZBP-89高表達增強了Trichostatin A (TSA)的致死效應。此外，p53突變體$p53^{G245D}$，發現于香港肝癌病人，與ZBP-89在肝癌組織中共定位。$p53^{G245D}$在肝癌細胞中與ZBP-89相互作用並將ZBP-89從細胞核轉運至細胞漿中。功能研究表明通過與ZBP-89相互作用，$p53^{G245D}$抑制了Butyrate介導的p21上調，並且減少了TSA介導的細胞死亡。機制研究表明，$p53^{G245D}$通過有效抑制了caspase 8, Bid and PARP等凋亡蛋白的激活。

綜上所述，ZBP-89可以抑制腫瘤生長，發揮抑癌蛋白功能，並可能成爲肝細胞癌的預後指標。此外，本研究表明$p53^{G245D}$可通過與ZBP-89相互結合並轉運其至細胞漿中，從而降低了化療藥物，如乙醯化酶抑制劑的抗癌效價。因此，ZBP-89與p53突變體的相互作用可能成爲增強肝癌化療敏感性的潛在靶點。

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Symbols and Abbreviations

Symbols

α	Alpha
β	Beta
γ	Gamma
ζ	Zeta
°C	Degree Celsius

Abbreviations

aa	amino acid
AFP	alpha-fetoprotein
ATCC	american type culture collection
ATM	ataxia-telangiectasia mutated
Bcl-2	B-cell leukemia/lymphoma 2
bHLH	basic Helix-Loop-Helix proteins
bp	base pair
caspase	cysteine aspases
CDK	cyclin dependent kinases
cDNA	complementary deoxyribonucleic Acid
cisplatin	cis-diamminedichloroplatinum
DAB	3, '3-diaminobenzidine tetrahydrochloride

DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-binding domain
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoside
DNA	deoxynucleic acid
EBV	epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra-acetic Acid
ENA-78	epithelial neutrophil-activating peptide-78
ERK	extracellular signal-Related Kinase
ESC	embryonic stem cells
FBS	fetal bovine serum
GHR	growth hormone receptor
GOF	gain-of-function
HBV	hepatitis B virus
HBx	HBV X protein
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDACi	histone deacetylase inhibitor
HEPES	N-2-hydroxy-ethyl-piperazine-N'-2-Ethane-sulfonic acid
HRP	horse radish peroxidase
IAP	intestinal alkaline phosphatase

IF	immunofluorescence
IFNγ	interferon-gamma
IHC	immunohistochemistry
IP	immunoprecipitation
JKTBP1	heterogeneous nuclear ribonucleoprotein D-link protein
JNK	c-Jun N-terminal Kinase
lck	lymphocyte-specific protein-tyrosine kinase
LOF	loss-of-function
MAPK	mitogen-activated protein kinase
MDM2	mouse double-minute 2
mL	milliliter
mM	millimolar
MMP-3	matrix metallopeptidase 3
mRNA	messenger ribonucleic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazo-lium bromide
NaB	sodium butyrate
NES	nuclear export signal
NLS	nuclear localization signals
ODC	ornithine decarboxylase
p21	$p21^{Wafl}$
PAGE	polyacrylamide gel electrophoresis

PARP	poly (ADP-ribose) polymerase
PBS	phosphatebuffered saline
PEST	proline, glutamic acid, serine, threonine
PFA	parafomaldehyde
PMSF	Phenylmethyl-sulphonyl Fluoride
PR	proline-rich domain
Reg	regulatory domain
RNA	ribonucleic acid
RT–PCR	reverse-transcription PCR
SDS	sodium dodecyl sulfate
si-ZBP-89	small interfering RNA for ZBP-89
STAT	signal transducer and activator of transcription
TA	transactivation domain
tBid	truncated Bid
TBS	tris-Buffered Saline
Tet	tetramerization
Tris	tris(hydroxymethyl)aminomethane
TSA	trichostatin A
TUNEL	terminal deoxynucleotidyl transferase-dUTP nick end labeling
v/v	volume by volume
WB	western blot

WHO	world health organization
YY1	Yin Yang 1
ZBP-89	Zinc-finger Binding Protein-89
$ZNF148^{TgVZ}$	transgenic villin-ZNF148

Chapter 1: General introduction

Hepatocellular carcinoma (HCC) is one of the sixth most common malignancies and the third cause of cancer-related death worldwide (Parkin et al., 2005). Incidence of HCC has been rising and is expected to further increase in the near future (Tanaka et al., 2002). In developing countries, hepatitis B virus (HBV) infection is the leading risk factor, whereas hepatitis C virus (HCV) is the main cause of HCC in developed areas including the United States and Europe. Liver cirrhosis is the cause of the majority of HCC patients (Fattovich et al., 1995). There has been major progress in the understanding of HCC and therapeutic options in the past 2 decades, which substantially altered the clinical management of patients with HCC.

ZBP-89, a Krüppel-type zinc finger transcription factor that binds to GC-rich sequences, is involved in regulation of cell growth and cell death. It maps to chromosome 3q21 and is composed of 794 residues. Having bifunctional regulatory domains, ZBP-89 may function as a transcriptional activator or repressor of variety of genes such as p16 and vimentin. ZBP-89 arrests cell proliferation through its interactions with p53 and $p21^{waf1}$. It is able to stabilize p53 through directly binding, and enhance p53 transcriptional activity by retaining it in the nucleus. In addition, ZBP-89 potentiates in butyrate-induced endogenous $p21^{waf1}$ up-regulation. ZBP-89 is usually over-expressed in human cancer cells, where it can efficiently induce apoptosis through p53-dependent and -independent mechanisms. Moreover, ZBP-89 is capable of enhancing killing effects of several anti-cancer drugs. Therefore, ZBP-89 may be served as a potential target in cancer therapy.

p53, encoded by the human gene *TP53*, is well known as a stress response protein. It exerts functions primarily as a tumor suppressor, regulating plenty of genes in response to a variety of cellular stimuli, such as DNA damage and oncogenic signals. p53 is usually activated by the above signals mainly through post-translational modifications which augment the level of p53 protein and its transactivation activity. Structurally, p53, similar to the other transcription factors, bares an amino-terminal transactivation domain (TA), a core DNA-binding domain (DBD) and carboxy-terminal tetramerization (Tet) a proline-rich domain (PR) and a carboxy-terminal regulatory domain (Reg). Activated p53 suppresses cellular transformation mainly by inducing growth arrest, apoptosis, DNA repair and differentiation in damaged cells. p53 function is almost always compromised in tumor cells, as a result of somatic mutations, which occur in over half of human cancers and constitute a cornerstone in tumorigenesis.

1.1 Hepatocellular carcinoma (HCC), one of the major cancer killers

1.1.1 HCC epidemiology

Hepatocellular carcinoma is a malignancy of worldwide significance and is currently one of the most common solid tumors and the third leading cause of cancer-related death (Parkin et al., 2005). The incidence of HCC geographically varies, due to the large heterogeneity of the penetration of the risk factors within the population. About 80% of new cases occur in developing countries, but the incidence is increasing in economically developed regions, including Japan, Western Europe, and the United States (Bosch et al., 2004; Deuffic et al., 1998; Erichsen et al., 2008; McGlynn et al., 2001). Because of late diagnosis and advanced liver cirrhosis, only limited treatment options with marginal clinical benefits have been available in up to 70% of patients. Furthermore, HCC is a representative of looming epidemic for which the medical oncology community is largely unprepared. Despite the many treatment options, the prognosis of HCC remains unsatisfactory. More than 75% of HCC patients present with advanced or unresectable disease (Nagasue et al., 1993). Even for the rest patients who undergo resection, the recurrence rates can be as high as 50% at 2 years (Poon et al., 1999; Yamamoto et al., 1996). The underlying liver dysfunction, aggressive nature of the disease, lack of general consensus regarding treatment, and lethal threats to our lives contribute to the emergency of systemic study and development of effective treatment for HCC.

1.1.2 HCC etiology

Unfortunately, to date, the molecular events related to the development and progression of HCC have not been well studied. A hypothesis accepted commonly presents a step-by-step process (Buendia, 2000; Theise et al., 2002). Firstly, external stimuli induce genetic alterations in mature hepatocytes, which may result in cell death, proliferation, and monoclonal populations (Thorgeirsson and Grisham, 2002). The formed populations will harbor dysplastic hepatocytes, and then evolve to highgrade dysplastic nodules that are thought as truly preneoplastic lesions that may develop into malignant tumors (Borzio et al., 2003). Once highgrade dysplastic nodules become less differentiated, more pathological changes, such as neovessel formation, angiogenesis, tissue invasion, and metastasis, will gradually show up. Later on, the neoplastic cells become undifferentiated and are able to invade vessels and spread outside the liver, characteristics that define end-stage disease.

HCC develops commonly, but not exclusively, in a setting of liver cell injury, which leads to inflammation, hepatocyte regeneration, liver matrix remodeling, fibrosis, and ultimately, cirrhosis. Lots of efforts have been made to reveal the genetic damage at different evolutionary stages of HCC (Block et al., 2003; Kern et al., 2002; Liu and Kao, 2007). However, no homogeneous pattern has been defined. Recent studies have suggested that the pathway leading to HCC differs from etiology (Hsu et al., 2000; Llovet et al., 2003). Virus-related HCC exhibits a more intense genetic

instability, while non-virus- related HCC presents less instability but frequently bares beta-catenin mutations and Wnt activation. Studied focus on the mechanisms through which HCC develops indicated that altered expressions of factors that are involved in cell proliferation, cell signaling transduction, cell cycle control and apoptosis contribute to the tumourgenesis and developing of HCC (Addeo et al., 2009; Azechi et al., 2001; Calvisi et al., 2007; Thorgeirsson and Grisham, 2002; Wagayama et al., 2002). Identification of key molecular targets and pathways involved in hepatocarcinogenesis has significant therapeutic implications: the arrival of many molecularly targeted agents in the clinic provides the rationale and opportunity for study of these agents in HCC. The vast majority of HCC is attributed to hepatitis B virus (HBV) and hepatitis C virus (HCV), but other risk factors include alcohol abuse, hemochromatosis, fatty liver disease, androgenic steroid use, and other metabolic disorders. The mechanisms by which these varied etiologies lead to cirrhosis and HCC are still not well understood.

1.1.3 HCC treatments

To date, the only curative treatment option for HCC is liver transplantation, with the current 1- and 5-year survival rates of 77.0% and 61.1%, respectively. The 5-year survival rate was less than 30% in 1980s. This improvement was due to the incorporation of the Milan criteria which suggests the long-term survival after HCC liver transplantation is highest in HCC patients with either a single lesion ≤ 5 cm or three lesions ≤ 3 cm each and without gross vascular invasion. Generally, patients

with cirrhosis are not suitable for surgical resection because of the connections between poor prognosis and cirrhosis. However, even for those that undergo resection, the recurrence rates can be as high as up to 75% at 5 years. Thomas et al. summarized the treatment options for HCC (Table 1.1).

About 80% of HCC patients have advanced disease at presentation, and based on the number, size, and location of lesions, and the severity of underlying cirrhosis, are not candidates for transplantation, surgical resection, or liver-directed therapies. However, systemic chemotherapy is abjectively quite ineffective in HCC. Therefore, it is very urgent and important to investigate the mechanism via which HCC resists chemotherapeutic treatment.

Table 1.1 Summary of Treatment Options for HCC

Treatment Option	Comments
Liver transplantation	Historically low survival rates (20%-36%) recent improvement (61.1%; 1996-2001), likely related to adoption of Milan criteria at US transplantation centers
	Currently HCC represents 20+% of liver transplantations performed annually in the United States
Surgical resection	Historical 5-year survival rates 30%-40%
	Recent series indicates 5-year PFS as high as 48%; majority of patients develop recurrence or second primary tumors
	Resection in cirrhotic patients carries high morbidity and mortality
TACE	Multiple trials show objective tumor responses and slowed tumor progression but questionable survival benefit compared to supportive care; greatest benefit seen in patients with preserved liver function, absence of vascular invasion, and smallest tumors
	Modest survival benefit demonstrated for repeated TACE (82% 1-year survival) v supportive care (63%) in patients with preserved liver function, PS 0, and small tumor burden; improvement in 1-year survival from 32% in controls (supportive care) to 57% for TACE shown in randomized study of 279 primarily HBV-positive patients with tumors < 7 cm
Intra-arterial iodine-131–lipiodol administration	Efficacy demonstrated in unresectable patients, those with portal vein thrombus, and as adjuvant therapy in resected patients
Percutaneous treatments (ethanol injection, radiofrequency ablation)	PEI well tolerated, high RR in small (< 3 cm) solitary tumors; no randomized trial comparing resection to percutaneous treatments; recurrence rates similar to those for postresection
Hormonal therapy	Antiestrogen therapy with tamoxifen studied in several trials, mixed results across studies, but generally considered ineffective
	Octreotide (somatostatin analogue) showed 13-month MS v 4-month MS in untreated patients in a small randomized study; results not reproduced
Chemotherapy	Adjuvant: No randomized trials showing benefit of neoadjuvant or adjuvant systemic therapy in HCC; single trial showed decrease in new tumors in patients receiving oral synthetic retinoid for 12 months after resection/ablation; results not reproduced
	Palliative: Regimens that included doxorubicin, cisplatin, fluorouracil, interferon, epirubicin, or taxol, as single agents or in combination, have not shown any survival benefit (RR, 0%-25%); a few isolated major responses allowed patients to undergo partial hepatectomy; no published results from any randomized trial of systemic chemotherapy

Abbreviations: HCC, hepatocellular carcinoma; TACE, transarterial embolization/chemoembolization; PFS, progression-free survival; PS, performance status: HBV, hepatitis B virus; PEI, percutaneous ethanol injection; RR, response rate; MS, median survival.

1.2 Transcription factor ZBP-89 in cancer growth and apoptosis

Studies have revealed that Zinc-finger Binding Protein-89 (ZBP-89) possesses multiple functions, including the transcriptional regulation of genes, cell growth arrest and cell death (Bai et al., 2004). Due to the ability of ZBP-89 to form direct protein complexes with known tumor suppressor factors, such as p53(Bai and Merchant, 2001), p300 (Bai and Merchant, 2000), ataxia-telangiectasia mutated (ATM) (Bai and Merchant, 2007), ZBP-89 is thought to participate in the tumor development. Increasing efforts have been made to reveal the role of ZBP-89 in human diseases, especially cancer, and data appear to suggest that ZBP-89 is a target in cancer therapy. This review will summarize the recent development in this direction.

1.2.1 ZBP-89 structure

ZBP-89 (also known as Zfp-148, BFCOL1, ZNF-148 and BERF-1) belongs to the Krüppel-type (Cys2-His2-type) zinc finger family which includes WT-1 (Cheng et al., 2000), GATA-1 (Guyot et al., 2006; Woo et al., 2008), Sp1 (Cheng et al., 2000; Keates et al., 2001) and Egr1 (Fukada and Tonks, 2001; Lantinga-van Leeuwen et al., 2005). ZBP-89 was originally cloned by the screening of an expression library with a GC-rich epidermal growth factor element from *gastrin* gene (Merchant et al., 1996) and thought to be ubiquitously expressed at low levels. It maps to chromosome 3q21 and is composed of 794 residues (Law et al., 1998a). Interestingly, human ZBP-89 localizes just proximal to a leukemia break points and translocations cluster region at chromosome 3q21 (Pekarsky et al., 1995), suggesting that mutations in ZBP-89 may

be involved in tumorigenesis probably via interacting with other related factors such as SCL/Tal (Endoh et al., 2002; Kassouf et al., 2008). The intron/exon organization and nucleotide sequence of *ZBP-89* gene are highly conserved (Feo et al., 2001). Both mouse and human *ZBP-89* genes consist of 9 exons, and the coding is organized in a domain-specific manner, each encoding a distinct functional domain. There are four Krüppel-type zinc fingers residing within the N-terminus of ZBP-89 protein (Feo et al., 2001). Besides the proximal zinc-finger domain, a glutamic acid–rich domain lies within the first 100 residues, followed by basic domains that flank the zinc-finger DNA binding region. A third basic domain plus serine-rich and PEST (proline, glutamic acid, serine, threonine) domains are located in the distal 250 residues (Figure 1.1). To date, ZBP-99, one other member of the ZBP family, has been cloned, and it has the homologous zinc-finger domain with ZBP-89 but a greater molecular weight (Law et al., 1999; Lisowsky et al., 1999). A human ZBP-89 splice isoform ZBP-89-DN, which lacks amino terminal residues 1-127 of the full-length protein, has also been identified (Law et al., 2006a). ZBP-89-DN mRNA is co-expressed with its ZBP-89-FL cognate at low levels in many normal and transformed human tissues (Milona et al., 2003).

ZBP-89 has bifunctional regulatory domains, which suggest that ZBP-89 may function transcriptionally as either an activator or a repressor. ZBP-89 is a transcriptional repressor in the expression of vimentin (Salmon and Zehner, 2009; Wu et al., 2004; Wu et al., 2007; Zhang et al., 2003) , epithelial neutrophil-activating peptide-78 (ENA-78) (Keates et al., 2001), β_2-integrin CD11b (Park et al., 2003) , gastrin (Ashcroft et al., 2004; Bai et al., 2002; Holley-Guthrie et al., 2005) , ornithine decarboxylase (ODC) (Dawson et al., 2001; Law et al., 1998b), SOX18 (Petrovic et

al., 2009) , p16 (Feng et al., 2009) , Pax7 (Salmon et al., 2009b) and bovine adrenodoxin (Cheng et al., 2000) genes. However, for other genes such as $p21^{waf1}$ (Bai et al., 2006; Bai and Merchant, 2007; Hasegawa et al., 1999; Tvrdik et al., 2006), the T cell α- and β-receptor (Wang et al., 1993), the lymphocyte-specific protein-tyrosine kinase (*lck*) (Yamada et al., 2001), type 1 collagen (Hasegawa et al., 2000), intestinal alkaline phosphatase (*IAP*) (Malo et al., 2006), growth hormone receptor (*GHR*) (Thimmarayappa et al., 2006; Xu et al., 2006) and stromelysin (Moran et al., 2005), it is a gene activator. The exact mechanism how ZBP-89 functions as both an activator and a repressor is still unknown. It is widely believed that whether ZBP-89 binding stimulates or inhibits gene transcription is dependent on the sequence content. The mechanism by which ZBP-89 represses transcription is not well known. However, ZBP-89 can directly inhibit transcription, as in the cases of the gastrin gene, or indirectly inhibit transcription through competition with Sp1 for binding to overlapping binding sites, such as in the case of the *ODC* gene, or through blocking the activity of Sp1 by protein–protein interaction, such as in the case of the vimentin gene. Although much less is known about how ZBP-89 activates transcription, ZBP-89 activates the human $p21^{waf1}$ promoter through cooperation with the transcriptional co-activator p300 (Bai et al., 2002; Bai and Merchant, 2000).

1.2.2 ZBP-89 and cell growth

It is well studied that eukaryotic transcription factors containing Krüppel-type zinc finger are involved in various processes of cell growth and differentiation and many of these factors exhibit highly restricted patterns of expression in adult tissues and during development (Suske, 1999; Turner and Crossley, 1999). Previous studies of ZBP-89 function in cells have demonstrated that ZBP-89 arrested cell proliferation

through its interactions with p53 (Bai and Merchant, 2001; Chen et al., 2003) and $p21^{waf1}$ (Tvrdik et al., 2006), indicating the key roles of p53 and $p21^{waf1}$ in ZBP-89-mediated regulation of cell proliferation.

p53, a transcriptional factor, can induce cell growth arrest and/or apoptosis through activation or repression of downstream target genes. Given its critical function, the expression of p53 protein under normal physiological conditions must be tightly regulated. Early analysis suggested that ZBP-89 was involved in inhibiting cell growth by activating p53 (Bai and Merchant, 2001). Over-expression of ZBP-89 by adenovirus infection induced growth arrest and apoptosis in AGS cells, accompanied by wild-type p53 protein accumulation. Besides, ZBP-89 is evident to stabilize p53 through directly binding, and enhance its transcriptional activity by retaining p53 in the nucleus. Specifically the DNA-binding and carboxy-terminal domains of p53 were shown to bind the zinc-finger containing DNA binding domain of ZBP-89, suggesting that the stabilization is independent of mdm2 and $p19^{ARF}$. After infection with Ad5-ZBP-89 in AGS cells, the percentages of G0/G1 and G2/M phase cells were increased from 50 to 62% and 22 to 30%, respectively, whereas the S phase cells dramatically decreased from 27% to 7%. In our study, we discovered that the percentage of Hep G2 cells treated with Ad5-ZBP-89 in G2/M phase was markedly reduced, while the number of cells in S phase was significantly increased (Chen et al., 2009). However, the percentage of the cells in the G0/G1 phase was not different. It is known that G2/M checkpoint is essential to the growth of tumor cells (Schwartz and Shah, 2005). Therefore, G2/M checkpoint abrogation by ZBP-89 may impact on cell death or proliferation. Similarly, some other well-known anti-tumor agents such as cisplatin (Albertella et al., 2005), irofulven (Serova et al., 2006), okadaic acid

(Traore et al., 2001) and topotecan (Redkar et al., 2004) also arrest cells in S phase. Thus, the ability of ZBP-89 to abolish hepatocellular carcinoma (HCC) cells in the G2/M phase may contribute to its ability to function as a tumor growth inhibitor and is highly comparable to other potent anti-tumor agents. However, in a p53 null cell line, the cell cycle inhibition observed with ZBP-89 over-expression was abolished (Ma et al., 2008), which indicated that the growth arrest caused by ZBP-89 is p53 dependent. This finding is in line with other reports (Remington et al., 1997; Takeuchi et al., 2003).

Histone deacetylase inhibitors (HDACi) induce growth arrest, differentiation and apoptosis, particularly in the colon where they are potential chemotherapeutic agents (Zhou and Zhu, 2009). The cyclin dependent kinase inhibitor $p21^{waf1}$ is a key mediator of HDACi action (Dagtas et al., 2009). $p21^{waf1}$ controls progression through the cell cycle by binding to cyclin/CDK complexes. This control functions through both p53 dependent and independent mechanisms (Gartel and Tyner, 1999). Inducible p53-independent regulation of $p21^{waf1}$ transcription is mediated through its proximal GC-rich sites (Owen et al., 1998). It is reported that ZBP-89 can directly or indirectly regulate $p21^{waf1}$ (Bai et al., 2006; Merchant et al., 2003). Over-expression of ZBP-89 in colon cancer HT-29 cells revealed that ZBP-89 potentiated endogenous $p21^{waf1}$ induction by butyrate (a member of HDACi). The amino-terminal domain of ZBP-89 binds to the region of the $p21^{waf1}$ promoter from -245 to -215 in a butyrate-dependent manner. HDACi treatment of colonic cells promotes the formation of an ATM/ZBP-89/p300 complex on the $p21^{waf1}$ proximal promoter, and this multi-molecular complex plays an important role in the induction of $p21^{waf1}$ expression *in vitro* and *in vivo* by HDACi (Bai and Merchant, 2007). Reduction of

ZBP-89 or ATM with siRNA blocks HDACi-induced $p21^{waf1}$ expression. Moreover, ATM can phosphorylate ZBP-89 at Ser202 by association with the zinc finger and amino terminal domains of ZBP-89 in an HDACi-dependent manner. Histone acetylase coactivator p300 binds the N-terminal domain of ZBP-89 and the C-terminal domain of p53 (Zhao et al., 2006).

1.2.3 ZBP-89 and apoptosis

In addition to its role in cell growth, ZBP-89 can also initiate programmed cell death/apoptosis. Interestingly increased susceptibility to chemotherapy-induced cell death has been shown in both p53-null cells with elevated ZBP-89 levels and cells with an accumulation of p53 (wild-type) enhanced by ZBP-89. ZBP-89 induces caspase activation in AGS cells, subsequently mediates PARP cleavage and represses mitochondrial Bcl-2 family survival factors such as Mcl-1 and Bcl-xL (Bai et al., 2004). The mechanism responsible for the suppression of apoptosis by Mcl-1 is known to be p53-independent (Croxton et al., 2002; Lin et al., 2001). The reduction of Bcl-xL promotes p53-independent apoptosis (Komatsu et al., 2000; Strasberg Rieber et al., 2001). Moreover, the inhibition of ZBP-89 by siRNA decreased Bid and Bax expression (Bai et al., 2004), suggesting that ZBP-89 is required to maintain the basal levels of these two proapoptotic proteins. These findings indicate that ZBP-89 plays an important role in mitochondrial-mediated apoptosis.

The induction of apoptosis by p53-independent pathways is less well defined. Extracellular signals including pro-inflammatory cytokines and the withdrawal of growth factors can induce certain molecular mechanisms that may trigger p53-independent apoptotic pathways (Panjala et al., 2009; Vincent et al., 2009),

resulting in destabilization of mitochondrial membranes and ultimately cell death. For example, the pro-inflammatory cytokine interferon-gamma (IFNγ) induces apoptosis to sensitize cancer cells to death signals (Amin et al., 2006). IFNγ-induced phosphorylation of STAT1 triggers apoptosis. ZBP-89 binds to a GC-rich element from +171 to +179 of STAT1 and is required for the constitutive expression of STAT1 (Bai and Merchant, 2003). Reduction of ZBP-89 with its siRNA attenuated both basal and IFNγ-induced STAT1 expression and subsequently diminished the activation of apoptotic molecules, such as caspase-3 and PARP. Through interacting with STAT1, ZBP-89 effectively contributes to IFNγ-mediated apoptosis.

Another example is that ectopic ZBP-89 expression can induce activation of all three MAP kinase subfamilies (Bai et al., 2004), Extracellular signal-Related Kinase (ERK) 1/2, c-Jun N-terminal Kinase (JNK) 1/2, and p38 MAP kinase (p38). Utilizing specific inhibitors and dominant negative constructs to block kinase activity indicated that both ERK1/2 and p38 were dispensable for ZBP-89-induced apoptosis. However, JNK1/2 inhibition led to a significant decrease in ZBP-89-induced apoptosis. Furthermore, p53 null cells infected with adenoviral ZBP-89 show activation of JNK1/2. Therefore ZBP-89-induced apoptosis requires JNK1/2 and this pathway is p53-independent.

In some circumstances, when ZBP-89 is over-expressed, p53 protein expression is also increased. Thus, ZBP-89 may also induce apoptosis through p53-related pathway. ZBP-89 potentiated p53-mediated cell death induced by 10nM staurosporine and 100nM etoposide (Okada et al., 2006). In our study, ZBP-89 greatly enhanced the killing effectiveness of 5-fluorouracil or staurosporine in HCC

cells (Chen et al., 2009). It is reported that zfp148$^{+/-}$ embryonic stem (ES) cells are resistant to the growth-inhibitory effect of serum starvation (Takeuchi et al., 2003), which arrests wild-type ES cells, and thus they continue to proliferate. And in zfp148$^{+/-}$ ES cells, the phosphorylation of p53 at Ser15 is reduced, suggesting that leading ES cells to unregulated cell growth is through a p53-dependent mechanism. In this case, ZBP-89 appears to serve as a tumor suppressor. The anti-tumor effect of ZBP-89 has been further confirmed in a transgenic villin-ZNF148 (ZNF148^{TgVZ}) mouse experiment showing that the incidence of intestinal adenoma is reduced by 50% in the mice with ZNF148 over-expression and that the over-expression of ZNF148 is correlated with increased DNA fragmentation (Law et al., 2006b). In a later study, mice with the overexpression of ZBP-89-DN, a splice isoform of ZBP-89 that is lack of p300 binding domain, experienced growth delay, reduced viability and increased susceptibility to dextran sodium sulfate colitis. The 2-year survival ratios were 20:20 ZBP-89FL/FL, 39:40 ZBP-89FL/DN and 5:16 ZBP-89DN/DN mice in this experiment (Law et al., 2006a). Therefore, ZBP-89 is a potential target for tumor chemotherapy.

Taken together, ZBP-89 has been shown to be an important regulator in apoptosis and cell growth. Since ZBP-89 can induce apoptosis through both p53-dependent and -independent mechanisms, it will be more effective, at least in some cases, for the anti-tumor drugs to target ZBP-89 rather than p53 to kill the cancer cells, especially for tumors that are less sensitive to chemotherapy such as lung cancer and HCC.

1.2.4 ZBP-89 and myogenesis

Several reports have revealed that ZBP-89 functions in differentiation. Feo et al.

reported that ZBP-89 mRNA is up-regulated at day 12–14 in mouse embryos differentiation stage (Feo et al., 2001). While in adult mice, ZBP-89 mRNA's level is low in the adult heart and testis, intermediate in skeletal muscle and spleen, and high in brain, kidney, lung and liver. Notable differences in ZBP-89 mRNA levels are found during differentiation in Salmon's study (Salmon et al., 2009b). In Takeuchi's study, highly expressed in the neural tube and in male and female gonads during mouse embryogenesis, Zfp148 is documented to regulate the differentiation of fetal germ cells through affecting the phosphorylation of p53 at Ser15 (Takeuchi et al., 2003). Apart from above, ZBP-89 is also identified as a regulator in myogenesis and hematopoiesis, to be involved in animal differentiation.

Myogenesis, involving the complex interplay between the down-regulation of non-muscle genes and the up-regulation of muscle-specific genes, is a two-step process consisting of determination, where precursor cells commit to a muscle lineage, followed by differentiation of committed myoblasts to myotubes. The basic Helix-Loop-Helix proteins (bHLH), Myf5, MRF4, MyoD and myogenin are found to be important in the myogenesis process. Besides, a number of different kinases such as CamK II and PKC-ζ, for which ZBP-89 contains phosphorylation sites, contribute a lot to the myogenic program. During myogenesis, ZBP-89 interacts with YY-1 and heterogeneous nuclear ribonucleoprotein D-link protein (JKTBP1) to participate in the regulation of *Murine Cytochrome c Oxidase Vb* gene expression whose transcription is induced 5–7-fold during differentiation of C2C12 myoblasts into

myotubes (Boopathi et al., 2004). ZBP-89 has been implicated to be a negative regulator of β-enolase, a member of relatively small group of muscle-specific genes expressed in proliferating myoblasts, as well as other muscle-specific genes during induced myogenesis of C2C12 myocytes (Salmon and Zehner, 2009). Interestingly, ZBP-89 is demonstrated to "turn off" the intermediate filament protein vimentin during myogenesis, to be replaced by desmin, the muscle-specific intermediate filament protein (Salmon and Zehner, 2009). Over-expression and elimination of ZBP-89 are evidenced that ZBP-89 contributes substantially to the myogenic program in C2C12 skeletal muscle cells by modulating the bHLH myogenic regulatory factors Pax7 and MyoD, and by regulating their down-stream targets such as Cyclin D1 and Rb protein that are known to contribute to differentiation (Salmon et al., 2009b).

1.2.5 ZBP-89 and hematopoiesis

Hematopoiesis can also be seen as a two-step process: predominantly generating erythrocytes and primitive myeloid cells, and producing long-term hematopoietic stem cells. Hematopoietic development is closely linked to that of blood vessels and the two processes are regulated in large part by transcription factors that control cell fate decisions and cellular differentiation.

It has been demonstrated that during hematopoiesis, ZBP-89 represses *CD11b* gene expression during the further differentiation of monocytes into macrophages (Park et

al., 2003). Integrin CD11b is a differentiation marker of the myelomonocytic lineage and an important mediator of inflammation. Over-expression of ZBP-89 in U937 cells reduces CD11b promoter activity while U937 cells differentiate into monocyte-like cells. To further explore ZBP-89's role in hematopoiesis *in vivo*, the same team reported that elimination of ZBP-89 in zebrafish embryos that may disrupt both primitive and definitive hematopoiesis leads to a bloodless phenotype, while over-expression of ZBP-89 in mouse ESCs resultes in increased hematopoiesis but reduced sprouting angiogenesis (Li et al., 2006). Furthermore, injection of ZBP-89 mRNA into cloche zebrafish embryos, lack of the hematopoietic and endothelial lineages, rescues hematopoiesis but not vasculogenesis. Forced expression of ZBP-89 induces the expansion of hematopoietic progenitors in wild-type zebrafish and in mouse embryonic stem cell cultures but inhibits angiogenesis *in vivo* and *in vitro* (Li et al., 2006).

In another study, Woo et al. reported that ZBP-89 is involved in megakaryocytic and erythroid development (Woo et al., 2008). ZBP-89 is identified as a component of multiprotein complexes involving GATA-1 and its essential cofactor friend of GATA-1(FOG-1). In a follow-up study, a functional ZBP-89 binding site that mediates *Gata-1* gene expression during hematopoietic development is characterized. The G5 string is necessary for the *Gata-1* gene expression *in vivo* and ZBP-89 is the functional trans-acting factor for this *cis*-acting region. GATA-1 is a lineage-restricted transcription factor required for normal erythropoiesis and

megakaryopoiesis. FOG-1 is also required for hematopoiesis, as FOG-1 deficient mice display erythropoietic defects that largely mimic those of GATA1-null mice. ZBP-89 transcriptionally regulates GATA-1 and they co-occupy the cis-regulatory elements of certain erythroid and megakaryocyte-specific genes. Loss-of-function studies in zebrafish and mice indicate the requirement for ZBP-89 in megakaryopoiesis and definitive erythropoiesis but not primitive erythropoiesis.

1.2.6 ZBP-89 and cancer

ZBP-89 has been reported to be involved in many cellular functions related to cancer, including cell growth, differentiation, transformation, myogenesis, hematopoiesis and senescence (Figure 1.2). However, to date, its role in cancer has not been systemically reviewed. It has been shown that ZBP-89 was elevated in some tumor tissues and cell lines, including gastric cancer (Taniuchi et al., 1997), colorectal cancer (Moran et al., 2005), breast cancer (Serova et al., 2006), melanoma (Strasberg Rieber et al., 2001), and HCC (Chen et al., 2003). ZBP-89 protein is elevated in a variety of gastrointestinal cancers (Wakabayashi et al., 2005). However, in some cases, ZBP-89 is undetectable. For example, ZBP-89 is normally expressed in pancreatic islets and ducts but it disappears in about 70% of pancreatic adenocarcinomas (Bai et al., 2002). Importantly, ZBP-89 may play a role at the early stage of gastric cancer development, as its level is elevated in pre-malignant states of gastric cancers (Taniuchi et al., 1997). Moreover, ZBP-89 can inhibit the proliferation of human stomach adenocarcinoma (AGS) cells and repress the

expression of gastrin (Law et al., 1998a). Consistent with the inhibitory role of ZBP-89, Remington et al. reported that over-expression of ZBP-89 resulted in DNA synthesis inhibition and S phase arrest in gastric cancer GH4 cells (Remington et al., 1997). The involvement of ZBP-89 in the gastro-tomorigenesis is further supported by finding that the Epstein-Barr virus (EBV) *BMRF1* gene increases the binding of ZBP-89 to the gastrin promoter (Holley-Guthrie et al., 2005). It is known that EBV infection is an etiological cause for a subset of gastric cancer (Fukayama et al., 2008), and that gastrin is up-regulated in a variety of pre-malignant conditions and established gastric cancers (Chao and Hellmich).

In human lung cancer, ZBP-89 and HDAC3 form a complex to facilitate the latter to bind to the $p16^{INK4a}$ promoter to repress its transcription (Feng et al., 2009). It is thus concluded that ZBP-89 is able to restrain senescence through epigenetically down-regulating the expression of p16 which functions as a negative regulator of cyclin–CDK complexes, blocks cell cycle progression, and inhibits retinoblastoma protein phosphorylation (Ohtani et al., 2001). This finding suggests that ZBP-89 may facilitate the proliferation of lung cancer cells.

In the analysis of gene expression in Dukes' B colon cancer, ZBP-89 was shown to be down-regulated (Bandres et al., 2007). The amount of down-regulation of ZBP-89 protein was associated with recurrence in these patients (Bandres et al., 2007), suggesting a negative correlation between the level of ZBP-89 and the tumor

recurrence. This concept is further supported in mouse model of intestinal adenoma, in which overexpression of ZBP-89 can reduce adenoma burden by 50% (Law et al., 2006b). Such an inhibitory effect of ZBP-89 is executed via suppressing cellular proliferation and inducing apoptosis. Intereestingly, the level of ZBP-89 is increased when cells are exposed to butyrate, a fiber derived fermentation product in colon, which is capable of maintaining epithelial cell differentiation (Siavoshian et al., 2000). This finding suggests that ZBP-89 may significantly contribute to the mechanism responsible for the prevention of high fiber diet-associated colorectal cancer development. Taken together, it appears that ZBP-89 may serve as a tumor suppressor in colorectal cancer.

ZBP-89 has been reported to regulate the expression of various molecules that are involved in the tumor growth, invasion and metastasis (Figure 1.3). For example, ZBP-89 up-regulates the expression of matrix metallopeptidase 3 (stromelysin 1, MMP-3) through activating the 5T promoter in gastric carcinoma (Moran et al., 2005). The role of ZBP-89 in regulation of MMP-3 is further supported by the fact that mutations in the polymorphic region of the MMP-3 promoter that includes ZBP-89's binding element impair its basal and induced transcriptional activity in MSI-H colorectal tumors (Borghaei et al., 2009). Since MMP-3 is involved in tumor angiogenesis, invasion and metastasis (Juncker-Jensen et al., 2009; Tang et al.), it is likely that ZBP-89 participates in certain tumor development and metastasis by regulating MMP-3. In contrast to the up-regulatory role of ZBP-89 in MMP-3,

ZBP-89 inhibits the expression of two other molecules, ornithine decarboxylase (ODC) (Dawson et al., 2001; Law et al., 1999) and vimentin (Zhang et al., 2003). The elevation of both ODC and vimentin positively contributes to the tumor development, invasion and metastasis (Ehrnstrom et al., 2008; Manni et al., 2004; Ngan et al., 2007; Wei et al., 2008), whereas the inhibition of both may result in the tumor growth arrest (Elmets and Athar; Wu et al., 2009). In addition, there are reports showing that over-expression of ODC in fibroblasts induces neoplastic transformation (Iwata et al., 1999), suggesting a possible link between ZBP-89 and the inhibition of tumor transformation via reducing ODC expression. Studies have also looked into the mechanism leading the inhibition of vimentin by ZBP-89. Wieczorek et al. identified ZBP-89 as repressor of *vimentin* gene through recruiting HDAC1 to its promoter. Zhang et al. utilized deletion constructs to confirm that the glutamine-rich region of Sp1 is required to enhance vimentin transcription, whereas the N-terminus of ZBP-89 is required to interact with Sp1 and repress gene expression. Wu et al. demonstrated that Stat3 and ZBP-89 can interact in the nucleus and that the activated Stat3 can overcome ZBP-89-mediated repression on vimentin.

In recurrent human HCC, regardless of p53 status, ZBP-89 protein was significantly higher in cancerous tissues than non-cancerous tissues (Chen et al., 2003). For the patients with wild-type p53, co-localization between ZBP-89 and p53 in the nucleus was shown in 67% of these cases. ZBP-89 expression was more likely to be higher in the nucleus when wild-type p53 was present, while the cytoplasmic co-localization

of ZBP-89 was detected in recurrent HCC tumors primarily with mutant p53 gene. The data suggest a role for ZBP-89 in the nuclear retention of p53 in a subset of recurrent HCC. Similar to HCC, in other cancer cells p53 accumulation in the nucleus is associated with the increasing susceptibility to chemotherapy and radiotherapy (Goldberg et al., 2004; Lan et al., 2007; Yamashita et al., 2006). Therefore, co-localization of p53 protein with ZBP-89 may define a subgroup of cancer cells that are more sensitive to anti-tumor treatment.

The development of cancer is closely associated with cell differentiation. Various carcinogenic factors can block cell differentiation, causing the cells to retain embryonic capabilities, to proliferate in an uncontrolled manner and thus to initiate cancer (Britschgi and Fey, 2009; von Wangenheim and Peterson, 2008). In line with the laboratory data, clinically differentiation and malignancy are inversely correlated (Fusenig et al., 1995). In order to correct this malignant process, a number of approaches have been developed to reprogram tumor cells so that cell differentiation will be resumed (Botrugno et al., 2009). Although the role of ZBP-89 in cancer cell differentiation has not been directly studied, ZBP-89 has been shown to regulate differentiation in a number of cells including embryonic cells, fetal germ cells, epithelial cells, and skeletal muscle cells (Bai et al., 2002; Ohneda et al., 2009; Park et al., 2003; Salmon et al., 2009a; Salmon and Zehner, 2009; Woo et al., 2008) . To support the role of ZBP-89 in cell differentiation, studies have also shown that ZBP-89 can regulate myogenesis, hematopoiesis, erythrogenesis and

megakaryogenesis, at least in part, via differentiation (Boopathi et al., 2004; Li et al., 2006; Ohneda et al., 2009; Salmon et al., 2009a; Salmon and Zehner, 2009; Woo et al., 2008). Although the forced expression of ZBP-89 induces the expansion of hematopoietic progenitors, it suppresses angiogenesis, the finding of which appears to be in line with the role of ZBP-89 in inhibition of tumor cell invasion and metastasis via interacting with MMP-3, ODC and vimentin (Li et al., 2006; Ohneda et al., 2009). It is known that one of ZBP-89 functions is to facilitate HDAC to the promoter of tumor-related genes (Bai et al., 2006; Bai and Merchant, 2007; Wu et al., 2007). Interestingly, the HDAC inhibitors (HDACi) are able to resume the process of maturation in undifferentiated cancer cells, and as such, HDACi have been introduced as differentiating agents in several clinical trials of cancers (Botrugno et al., 2009). Obviously, further experiments are needed to clarify the relationship among ZBP-89, HDACi and differentiation in cancer cells.

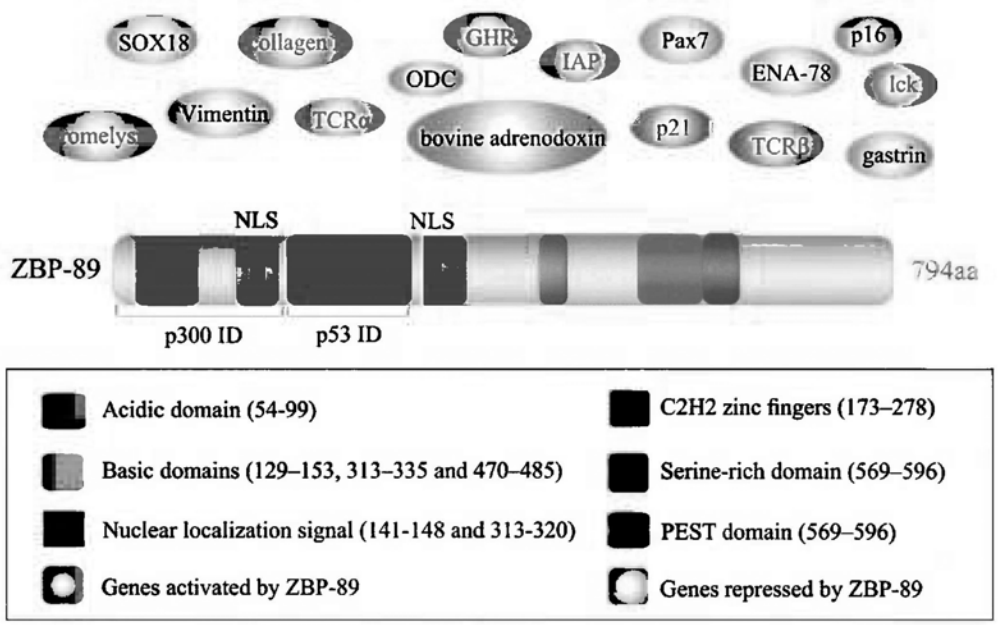

Figure 1.1 Structure of ZBP-89. The structural domains of ZBP-89 include the acidic domain, basic domains, four C2H2 zinc fingers, a serine-rich domain and a PEST domain. Interacting Domains (ID) with p53 and p300 are indicated. Some genes that are regulated by ZBP-89 are also shown.

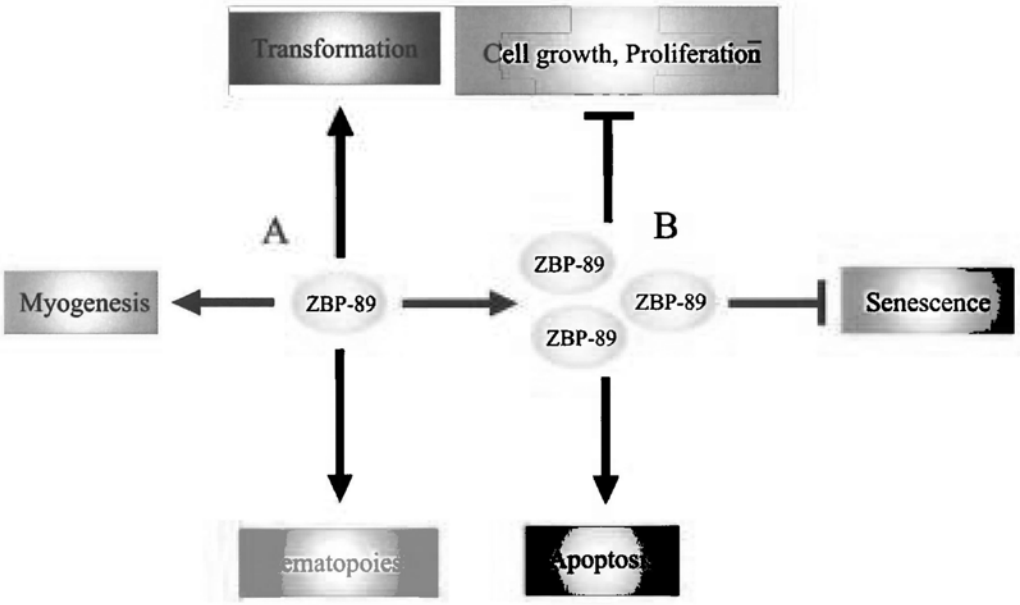

Figure 1.2 Schematic illustration of ZBP-89 functions. A. ZBP-89 is ubiquitously expressed at low levels in most of the normal proliferating cells, participating in cell function of differentiation, transformation, myogenesis and hematopoiesis. **B.** In tumor cells, ZBP-89 is often over-expressed and can exert its tumor suppressor potential by both arresting cell proliferation and inducing apoptosis.

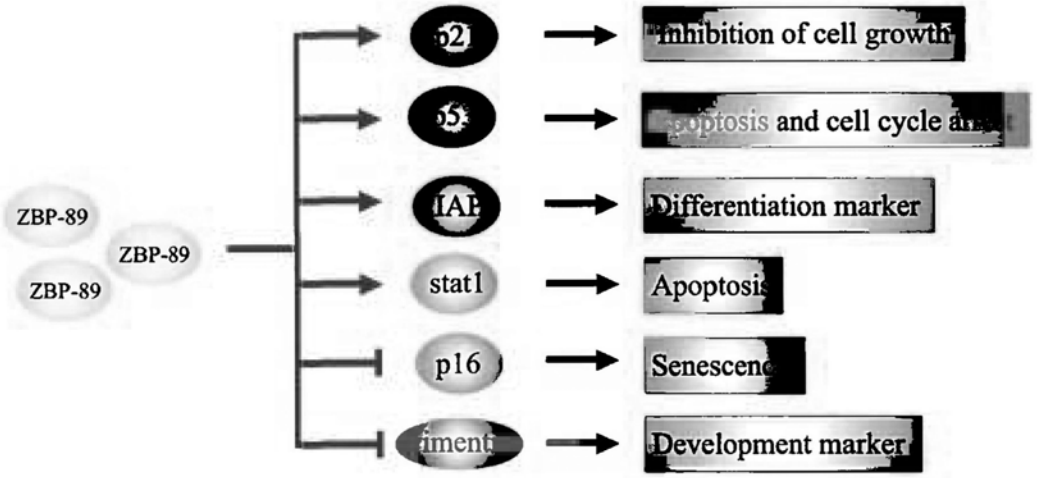

Figure 1.3 Over-expressed ZBP-89 shows tumor suppressor potential. Over-expressed ZBP-89 shows tumor suppressor potential by initiating apoptosis through stabilizing p53, up-regulating $p21^{waf1}$ and activating stat1. Moreover, ZBP-89 participates in cell development by regulating intestinal alkaline phosphatase (IAP) and vimentin. ZBP-89 also modulates cell senescence by repression of p16 expression.

1.3 Mutant p53, a potential therapeutic target in cancers

1.3.1 p53 research history

To date, field of p53 study has been ignited for over 30 years. p53 was firstly discovered in Simian virus 40-transformed cells to interact with the SV40 T antigen (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). At the very beginning, p53 was considered as an oncoprotein because of its frequent presence in various cancer cells. Till the late 1980s, p53 was revealed to be in fact a tumor suppressor and often mutated in cancers cells to exert oncogenic functions (Finlay et al., 1989). Since then, a dramatic upsurge of interest has been raised to study the role of p53 in cancer cells.

Based on the effects on the thermodynamic stability of p53, the majority of *p53* mutations can be divided into two main categories that are usually in terms of 'DNA-contact' and 'conformational' mutations (Bullock and Fersht, 2001). The former includes mutations within the DNA binding domain, such as G245D and R273C. The later comprises mutations that cause local or global conformational distortions, such as G245S and R175H respectively.

1.3.2 Regulation of p53 mutants

The cell and molecular biology of p53 has been studied for over 30 years. As a tumor suppressor, p53 inhibits cell proliferation and transformation mainly by inductions of

growth arrest, apoptosis, and DNA repair (Figure 1.4). Its protein levels which is mediated primarily by the ubiquitin ligase mouse double-minute 2 (MDM2) by regulating its degradation, have been demonstrated to control the activity of p53 (Haupt et al., 1997). Interestingly, MDM2 is at the meantime a target gene of p53 (Barak et al., 1993). The induction of MDM2 by p53 leads to the negative feedback loop to balance the levels of both proteins.

Since p53 is always frequently detected in cancer cells, more and more studies have accumulated to assess the status of p53 by gene sequencing and related methods. Sequencing studies show that *p53* mutations present in approximately half of all human cancers(Goh et al.). The frequencies of reported *p53* mutations vary considerably between cancer types, ranging from 10% to 50–70% (Maslon and Hupp). Over 70% of the mutations are missense mutations and usually abrogate its sequencespecific DNA-binding activity, falling within its central DNA-binding domain (DBD), more than 30% of which are in one of six 'hotspot' codons (Figure 1.5 and Table 1.2).

The regulation of mutant p53 is still not well understood. Increasing evidences indicate that mutant p53 proteins contribute to transformation, metastasis and drug resistance partly by inhibiting functions of wild-type p53 and p53 family members, such as p63 and p73 (Figure 1.4) (Deyoung and Ellisen, 2007; Di Como et al., 1999; Flores et al., 2005; Li and Prives, 2007). Although mutant p53 serves as a substrate

for MDM2 (Peng et al., 2001a; Peng et al., 2001b; Zhu et al., 2001) and interacts with heat-shock proteins such as HSP90 (Lin et al., 2008; Muller et al., 2005; Peng et al., 2001b) and HSP70 (Diaz et al.; King et al., 2001), the mechanism by which it is stabilized and the causes of such heterogeneous expression remain unknown.

1.3.3 Gain-of-Function of p53 mutants

Loss-of-function (LOF) in p53 mutations destabilize thermodynamically the DNA-binding domain, resulting in not only reducing the expression of genes that are transactivated by p53 but also derepressing genes that are normally suppressed by p53. However, ample data indicate that despite of LOF, mutant p53 proteins gain new abilities to promote tumorigenesis, termed 'gain-of-function (GOF)'.

As the notion that p53 mutants augment the oncogenic potential, the previously unstudied field of molecular modulators of mutant p53 level and activity has attracted more and more interest. For incidence, p53 mutants interact with the oncogenic Ras protein to transform primary rat cells *in vitro* (Yao et al., 2005). The interaction between mutant p53 and the pivotal tumor-suppressor PTEN which results in promotion of tumourgenesis is another one of the interesting examples (Kato et al., 2000; Kim et al., 2007). PTEN has further reported to stabilize mutant p53, which may form a positive feedback loop to enhance carcinogenesis (Li et al., 2008).

In addition, p53 mutants increase the resistance of cancer cells to apoptosis. Conferring resistance to apoptosis to cancer cells if one of distinctive features of mutant p53 GOF. Lotem et al. firstly reported such notion, showing that mutant p53 protected leukemic cells from c-Myc-induced apoptosis (Lotem and Sachs, 1993). Of particular interest, overexpression of mutant p53 proteins remarkably renders cells more resistant to cell death caused by anticancer agents, whereas knockdown of endogenous mutant p53 proteins sensitizes cells to death. For examples, Guida et al. reported inhibition of mutant p53 using peptide aptamer promotes apoptosis in tumor cells (Guida et al., 2008). Lambert et al. demonstrated that reactivation of mutant p53 by PRIMA-1MET induced apoptosis in various human cancer cells (Lambert et al.). Xu et al. reported that targeting mutant p53 with CP-31398 caused reactive oxygen species-dependent apoptosis in rhabdomyosarcoma (Xu et al.). Bykov et al. reported that reactivation of mutant p53 by maleimide analogs led to induction of apoptosis in human tumor cells (Bykov et al., 2005). Tsang et al. showed that p53-R175H mutant gained new function in regulation of doxorubicin-induced apoptosis (Tsang et al., 2005). However, the GOF of mutant p53 varies from different types of mutations (Xu, 2008). In one study, systematic mutations of the R175 hotspot codon in human *p53* have different impacts on cancer cells. R175C mutant seemed to be wild-type in its phenotype; R175K, R175P, R175I and R175S mediated only cell cycle arrest; R175N and R175T induced both cell cycle arrest and apoptosis; and R175Y, R175W, R175D and R175F did not exert any oncogenic functions.

Importantly, the subcellular localization of mutant p53 affects its oncogenic abilities. It has been showed that the localization of mutant p53 depends on the mutation type, the cellular context and the variety of stimuli signals. For most cases, mutant p53 proteins often present in the nucleus of cancer cells, whereas in some cases it localizes to the cytoplasm. Studies revealed that MDM2-dependent and MDM2-independent ubiquitylation may regulate the nuclear export of p53 mutants, and especially of conformational mutants, probably by exposing their c-terminal nuclear export signal. Functionally, nuclear p53 mutants play roles in transcriptionally regulate genes related to apoptosis and proliferation and cytoplasmic p53 mutants can inhibit autophagy in cancer cells (Morselli et al., 2008), which constitutes a new oncogenic function of p53 mutants and highlights the importantce of cytoplasmic p53 mutants in cell death and carcinogenesis.

1.3.4 p53 mutants and cancer

The gain-of-function of p53 mutants can be responsibility for the significant correlation between the expression of p53 mutants and the poor prognosis of cancer patients. For example, R175H mutation is correlated with the poor prognosis of small-cell lung carcinomas (Stricker et al., 1996). Expression of p53 mutants is correlated with the poor prognosis in soft tissue sarcomas. These examples suggest reliable associations between mutant p53 proteins and clinicopathological parameters, with the general trend being that p53 mutants connect with poor overall survival, as well as with high drug resistance. However, there are still many reports showing lack

of such connections, even opposite trends. In the breast, head and neck, bladder, colorectum and the haematopoietic systems, 65%–90% of studies reported mutant p53 proteins significantly associated with poor prognosis; whereas in brain, lung and ovarian cancers, more than half of the studies showed no significant association between p53 mutants and prognosis (Maslon and Hupp). In a few studies, the expression levels of p53 mutants are associated with good prognosis. Therefore, the relationship between the expressions of p53 mutants and the clinicpathological parameters seems to be complicated, depending to certain extend on the property of mutant p53.

According to the current trend of p53 research, a therapeutic strategy is to reactivate the mutant p53 in order to induce tumour clearance. Numerous molecules designed to arouse the mutant p53 response are currently in clinical trials. Since mutant p53 appear in up to 40% of human cancers, uncovering the potential impacts of p53 mutants on pathology and therapeutic response is becoming more and more important and attracting increasing attentions.

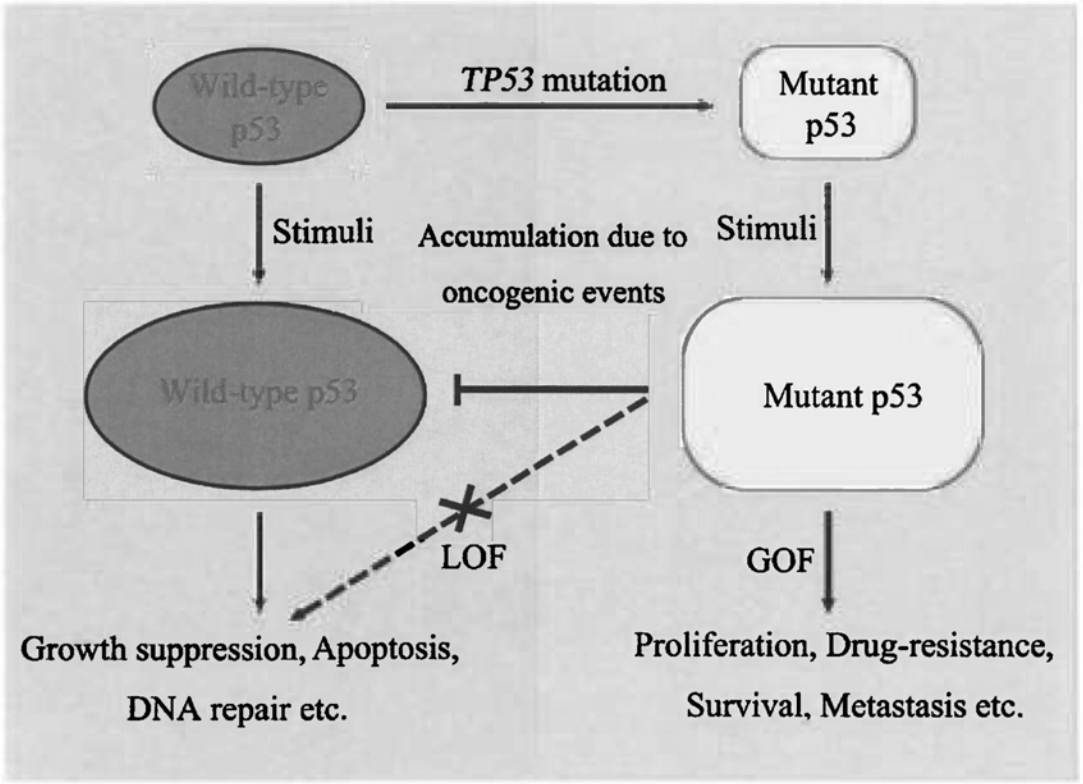

Figure 1.4 Functions of wild-type p53 and mutant p53. *TP53* gene mutation leads to production of mutant p53. In response to oncogenic events, such as external stimuli, both wild-type and mutant p53 are accumulated to play important roles in cell proliferation and death. Under normal circumstances, p53 exerts antitumour functions by induction of growth suppression, apoptosis and DNA repair, while mutant p53 functions as an oncoprotein by increasing drug-resistance, cell proliferation and metastasis. (LOF, loss-of-function; GOF, gain-on-function)

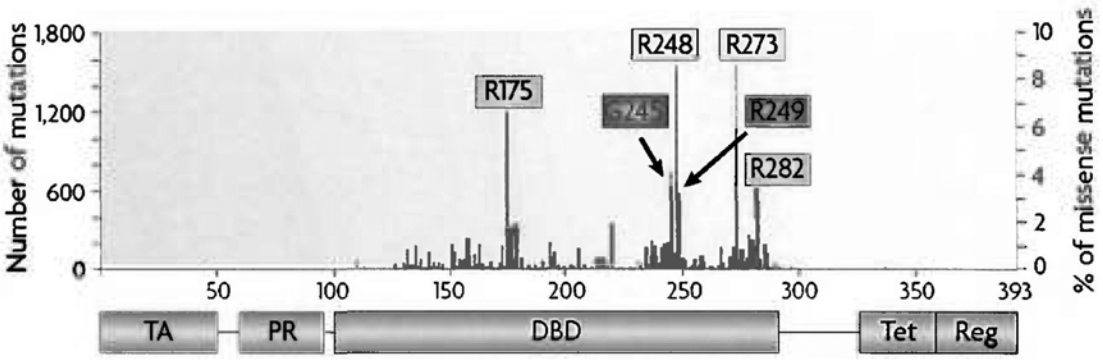

Figure 1.5 The distribution of reported missense mutations along the 393 amino-acid sequence of p53. The six most common hotspot mutations are highlighted in yellow for DNA-contact mutations, green for locally distorted mutants and blue for globally denatured mutants. The domain architecture of p53 is aligned below. Note that the depicted enrichment of mutations in the DNA-binding domain (DBD) is probably an overestimation as in many studies only the core domain exons of TP53 are sequenced, thus, mutations outside this region are overlooked. PR, proline-rich domain; Reg, carboxy-terminal regulatory domain; TA, transactivation domain; Tet, tetramerization domain.

Table 1.2 p53 'hotspot' mutants.

p53 mutants	Frequency in database*	Frequency in our study
R175H	944	2
R175L	24	0
R175C	23	0
R175S	10	0
R175P	7	0
G245S	343	0
G245D	134	2
G245C	76	0
G245V	64	0
R248Q	682	0
R248W	597	1
R248L	95	0
R248G	19	0
R248P	15	0
R249S	351	7
R249T	23	0
R273H	636	1
R273C	536	2
R273L	119	0
R273P	30	0
R282W	497	1
R282Q	23	0

* Data from IARC TP53 Database (R10, July 2005).

Chapter 2: Increased expression of ZBP-89 and its prognostic significance in hepatocellular carcinoma

2.1 Introduction

Hepatocellular carcinoma (HCC) is frequently associated with liver cirrhosis and liver dysfunction, making the treatment of HCC more difficult than many other cancers.(Abdalla and Vauthey, 2004) To better management of HCC, a great effort has been made to establish a reliable and accurate marker for its prognosis (Braillon, 2009; Thomas and Zhu, 2005; Worns et al., 2009). In addition to clinicopathological parameters including tumor size, histological grade, stage, and vascular invasion, (Liu et al., 2009; Yun et al., 2007) several biological molecules, such as the transcription factor YY1 (Gordon et al., 2006) and smoothened (Sicklick et al., 2006), have been used for HCC prognosis. However, the accuracy and reproducibility of these markers remain either unsatisfactory or unclear.

ZBP-89, a zinc-finger transcription factor that binds to GC-rich sequences, is involved in the regulation of cell growth and cell death (Zhang et al.). ZBP-89 can activate or repress transcription to regulate the expression of many growth-related genes, such as gastrin (Law et al., 1998b) and vimentin (Wu et al., 2007). ZBP-89 can form protein complexes with well-known tumor suppressor factors, such as p53 (Bai and Merchant, 2001) and p300 (Bai and Merchant, 2000). To date, ZBP-89 has been shown to be elevated in some cancers, including gastric cancer (Vesely et al., 2009), colorectal cancer (Moran et al., 2005) and breast cancer (Frensing et al., 2008).

Moreover, evidence has shown that ZBP-89 participates in many cellular functions related to cancer development and growth, including differentiation, myogenesis, hematopoiesis, transformation and senescence (Feng et al., 2009; Li et al., 2006; Salmon et al., 2009b). The data appear to suggest that ZBP-89 is a target in cancer therapy.

Our previous study has shown that ZBP-89 was expressed in a subset of recurrent HCC and co-localized with p53 mutants in nucleus and cytoplasm (Chen et al., 2003). However, the expression of ZBP-89 in HCC and its relationship with clinicopathological features of HCC are still not understood. In this chapter, we intended to examine the expression of ZBP-89 in HCC cell lines and HCC tissues, analyze the relationship between its expression and the clinicopathological features, and determine whether the altered ZBP-89 expression could affect the HCC colony formation.

2.2 Materials and methods

2.2.1 Cell cultures

Expression of ZBP-89 was examined in HCC cells and normal liver cells. HCC cells (Hep3B, PLC/PRF/5, Huh-7, HepG2 and SK-Hep-1) were purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium. The non-tumorigenic immortalized liver cells (MiHA), kindly provided by XY Guan from the University of Hong Kong, were maintained in Minimum Essential Medium.

2.2.2 Clinical samples

Total 182 HCC tissues and the adjacent liver tissues were sectioned for immunohistochemistry (IHC). Among these samples, 159 were collected from the Cancer Center of Sun Yat-Sen University between 1997 and 2009, and 23 from Prince of Wales Hospital, the Chinese University of Hong Kong, Hong Kong between 2000 and 2008. 14 pairs of HCC tissues collected from Prince of Wales Hospital were used for RT-PCR and Western blot. The use of the human specimens in the study was approved by the Independent Ethics Committees of the Sun Yat-Sen University and the Chinese University of Hong Kong.

2.2.3 Reverse-transcription PCR (RT–PCR)

Total RNA was extracted from cells using the Trizol (Gibco, Carlsbad, CA) according to the manufacturer's instruction. One microgram of RNA sample was reverse transcribed with oligo-dT primers (Promega, Madison, WI) to obtain single-stranded cDNA. One-tenth of the product was used as template in PCR

amplification. The following primers were used: ZBP-89 forward, 5'- CGC TGT GAT GAA TGT GGT GAT GAG AC -3'; ZBP-89 reverse, 5'-CCC AGC TCT ATT ATC ATT TAC ATT C -3'; GAPDH forward, 5'-AAA TCC CAT CAC CAT CTT CC-3'; and GAPDH reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3'. The PCR products were analyzed by 1.0% agarose gel electrophoresis. The PCR signals were analyzed using the Quantity One™ Software (Bio-Rad, Hercules, CA).

2.2.4 Western blot analysis

Forty microgram of proteins were boiled with 6x SDS loading buffer and then fractionated by SDS-PAGE (Xiao et al., 2009). The protein was transferred onto PVDF membrane, which was then incubated with specific primary antibody to ZBP-89 and GAPDH, followed by a horse radish peroxidase-conjugated anti-rabbit antibodies and ECL detection reagent (Amersham Life Science, Piscataway, NJ).

2.2.5 Immunohistochemistry

IHC was performed according to the published procedure (Chen et al., 2003). In each slide 1000 cells were randomly selected, counted, and scored. The intensity of IHC staining was defined as: (+), less than 10% of the tumor cells were positive; (++), 10%-50% of the tumor cells were positive; (+++), more than 50% the tumor cells were positive; (-), negative staining. Negative and weakly positive stainings were defined as low expression, whereas intermediately and strongly positive stainings as high expression. All sections were observed under light microscopy and the intensity was assessed by two pathologists, separately.

2.2.6 Immunofluorescence

Immunofluorescent staining was performed as previously described (Park et al., 2003). Cells grown on cover slips were fixed for 20 min in PBS containing 4% parafomaldehyde, permeabilized in 0.1% Triton X-100 for 2 x 5 min and incubated in blocking buffer (3% donkey serum in TBS) for 1 h. Cells were then incubated in antibody dilution buffer (3% bovine serum albumin in TBS) containing the indicated primary antibody for 2 h in room temperature and then washed extensively in PBS before being incubated with the appropriate fluorochrome-conjugated secondary antibody for 1 h. DNA was stained by 4',6-diamidino-2-phenylindole (DAPI).

2.2.7 Transfection and colony formation assay

Cells were plated in six-well plates and transiently transfected with pCMV-vector, pCMV-ZBP-89 (full length, FL), pCMV-ZBP-89-Δ6-180, pCMV-ZBP-89-Δ180-298, or pCMV-ZBP-89-Δ298-447 according to the manufacture's instruction of Lipofectamine 2000 (Sigma, MO). After transfection for indicated periods, cells were lysed by RIPA buffer to examine the levels of cleaved PARP, p53, p21 and Bid using Western blot as described above. To test the ability of colony formation, 1000 cells of each type were cultured in 60mm dishes and exposed to fresh media with G418 for 15 days. Colonies were stained with trypan blue and counted.

2.2.8 Statistical analysis

Statistical analysis was performed to determine the relationship between the clinicopathological parameters including sex, age, tumor size, number of tumors, hepatitis B virus (HBV), pathologic grade, serum level of alpha-fetoprotein (AFP), and the expression of ZBP-89 by Pearson's chi-square test or Yates' chi-square test.

Overall survival was assessed by the Kaplan-Meier method, and log-rank test were used to analyze survival curves. Statistical significance was set at $P<0.05$.

2.3 Results

2.3.1 Expression of ZBP-89 in HCC cell lines

Initially, total RNA and protein were prepared from normal liver cells (MiHA) and HCC cells (Hep 3B, PLC/PRF/5, Huh-7, Hep G2 and SK-Hep-1) to examine the expression of ZBP-89. Semi-quantitative polymerase chain reaction was preformed to investigate the level of ZBP-89 mRNA expression. Using GAPDH, a house keeping gene as a control, ZBP-89 mRNA levels were compared among 5 human hepatocellular carcinoma cell lines and the normal liver cell. Besides, Western blot and immunofluorescent staining analysis were also utilized to examine the expressions and cellular localizations of ZBP-89 in HCC and normal liver cells.

Results showed that the expression of ZBP-89 in HCC cells was noticeable higher than that in normal cells, at both mRNA and protein levels (Figure 2.1). Furthermore, ZBP-89 was mainly present in the nucleus in HCC cells except PLC/PRF/5 in which ZBP-89 partly localized in the cytoplasm (Figure 2.2). The intensity of staining indicated that the level of ZBP-89 was different in these cells, the change of which is basically consistent with the level of ZBP-89 detected by RT-PCR and Western blot (Figures 2.1).

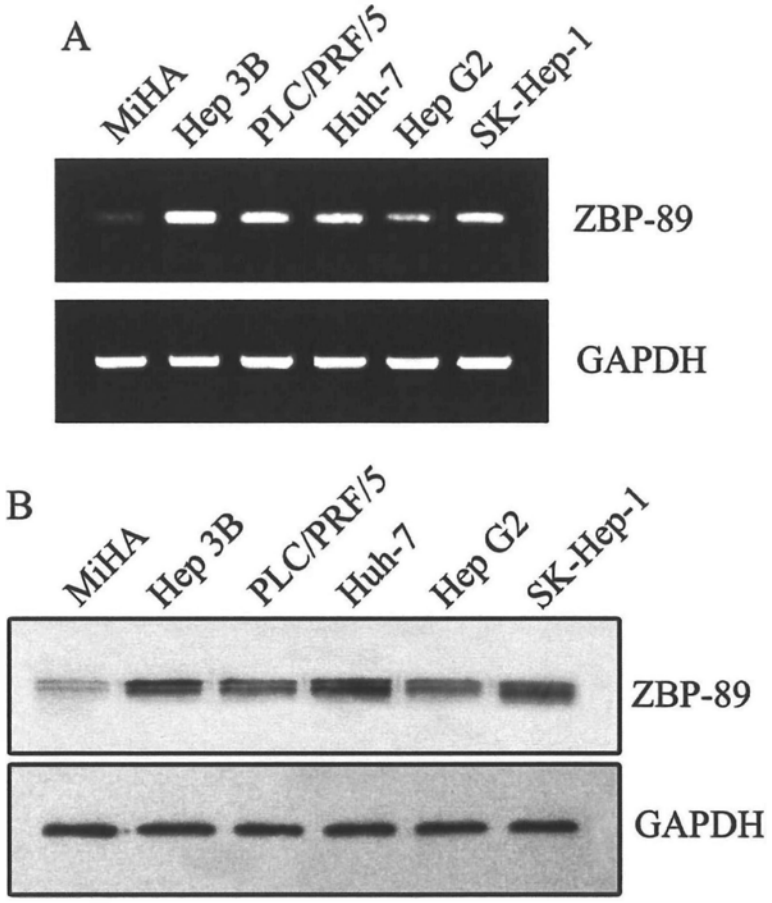

Figure 2.1 The expressions of ZBP-89 mRNA and protein in HCC cells by RT-PCR and western blot. A. Total RNAs were extracted from normal liver MiHA cells and HCC cells (Hep 3B, PLC/PRF/5, Huh-7, Hep G2, and SK-Hep-1). RT-PCR was performed to measure the expression of ZBP-89 mRNA. **B.** The expression of ZBP-89 protein in above cells was examined by Western blot. (GAPDH, a housekeeping gene that serves as a control)

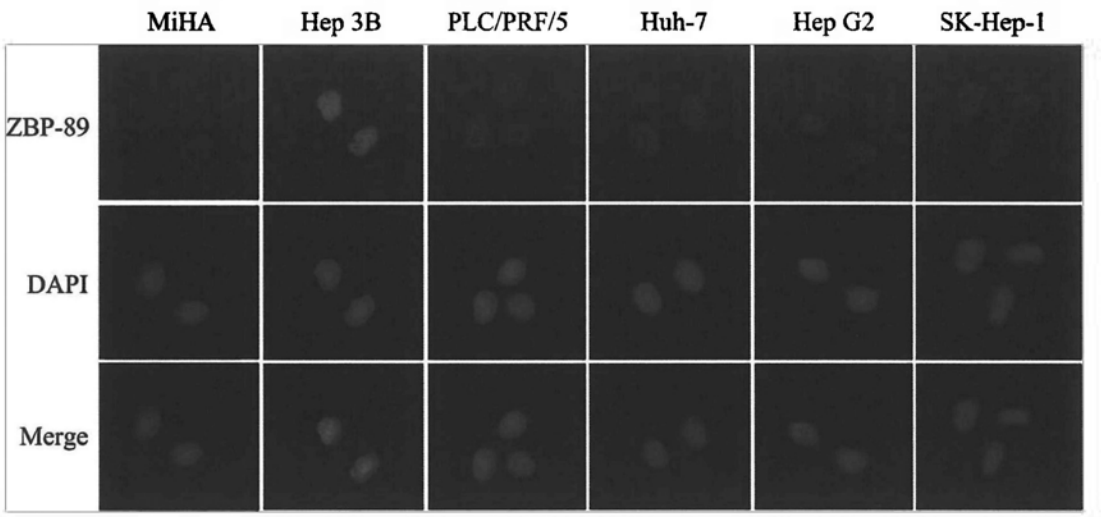

Figure 2.2 The expressions of ZBP-89 mRNA and protein in HCC cells by immunofluorescence. Normal liver MiHA cells and HCC cells (Hep 3B, PLC/PRF/5, Huh-7, Hep G2, and SK-Hep-1) were fixed for 20 min in PBS containing 4% PFA, permeabilized in 0.1% Triton X-100 for 2 x 5 min and incubated in blocking buffer for 1 h. Cells were then incubated in antibody dilution buffer for ZBP-89 for 2 h in room temperature and then incubated with the appropriate fluorochrome-conjugated secondary antibody for 1 h. DNA was stained DAPI. The amount and subcellular localization of ZBP-89 were observed under fluorescent microscope in cells (All fields ×400).

2.3.2 Analysis of ZBP-89 expression in HCC tissues by RT-PCT and Western blot

In addition to the expressions of ZBP-89 in HCC cells, we also examined its expressions in fresh HCC tissues. Fourteen paired HCC tissues were collected firstly and then lysised either in RIPA buffer to prepare proteins or in Trizol reagents to obtain total RNA. Next, using GAPDH for control, RT-PCR, Western blot, and immunofluorescence were perform to detect the expressions of ZBP-89 in the paired HCC tissues to assess the different expression patterns of ZBP-89 in HCC and the corresponding adjacent normal tissues.

RT–PCR results showed that the level of ZBP-89 mRNA was higher in HCC tumorous tissues than in the corresponding nontumorous tissues (Figure 2.3A). Overall, compared with the adjacent nontumorous tissues, the average level of ZBP-89 mRNA was 5.89 fold higher in the tumorous tissues (Figure 2.3B). Therefore, the data indicated that ZBP-89 was over-expressed in HCC tumorous tissues. The finding of RT-PCR was basically confirmed by Western blot, which showed that ZBP-89 expression was increased in the tumorous tissues by 4.46 fold on average, compared with that in the nontumorous tissues (Figures 2.4).

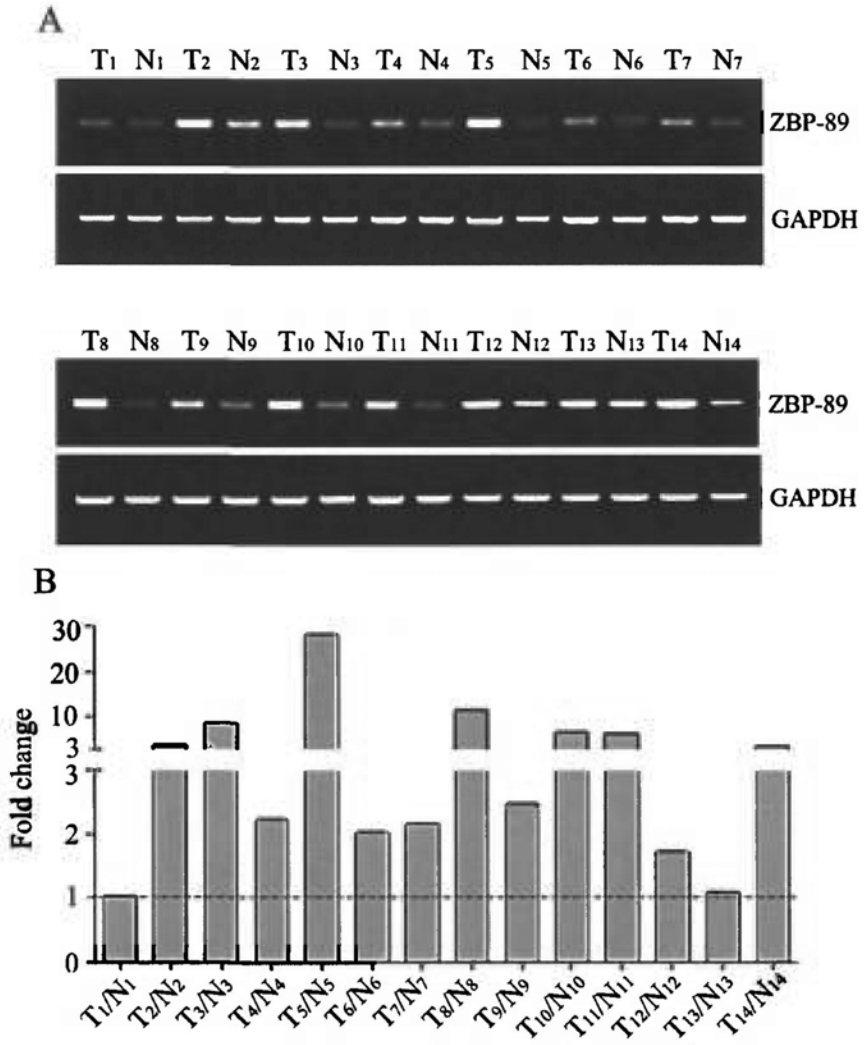

Figure 2.3 The expression of ZBP-89 mRNA and protein in HCC fresh tissues by RT-PCR. A. Total RNAs were prepared in 14 pairs of tumorous tissues (T) and the corresponding nontumorous tissues adjacent to the tumor (N) by Trizol reagent and then subjected to RT-PCR. **B.** The altered expression of ZBP-89 mRNA in each pair of the sample was determined by the fold change of T over N.

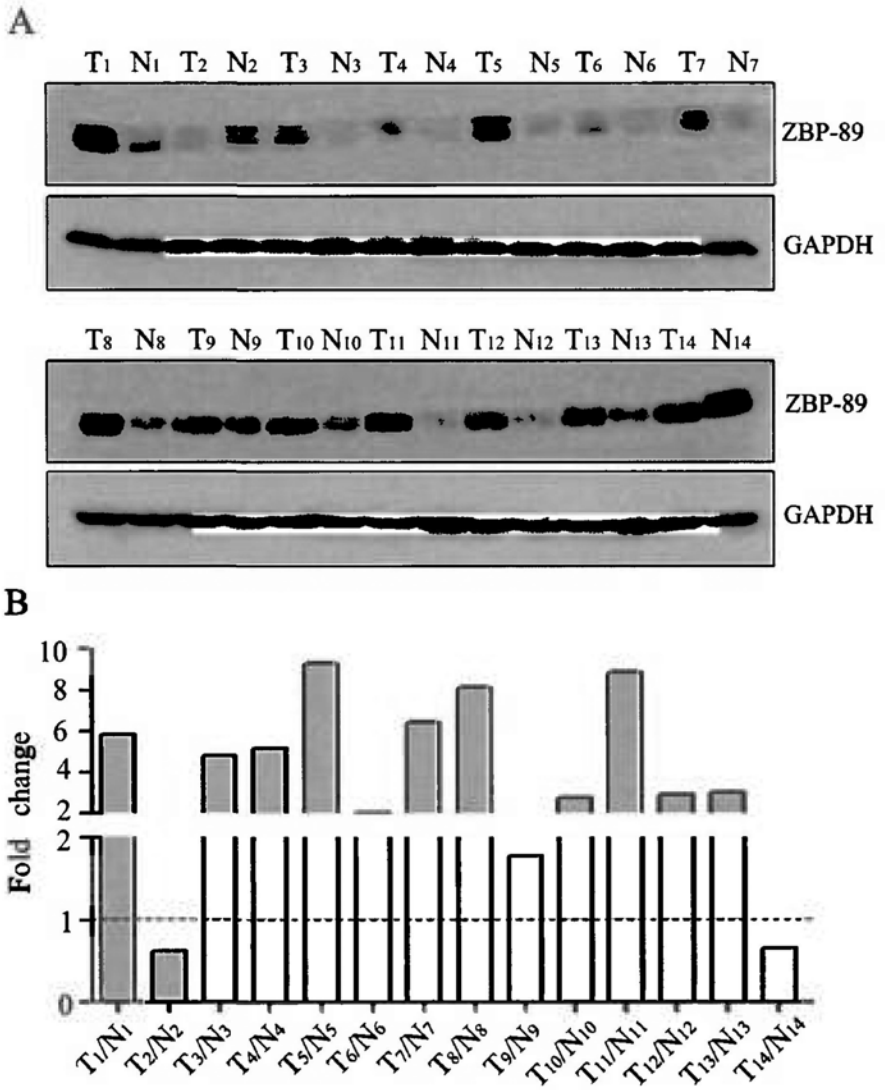

Figure 2.4 The expression of ZBP-89 mRNA and protein in HCC fresh tissues by Western blot. A. 14 pairs of the fresh tumorous tissues (T) and the corresponding nontumorous tissues adjacent to the tumor (N) were lysed by RIPA buffer. The expression of ZBP-89 protein was examined by Western blot. **B.** The fold change in each pair of T over N was calculated and shown in histogram to indicate the up-regulated expression of ZBP-89 protein in the tumorous tissues.

2.3.3 Analysis of ZBP-89 expression in HCC tissues by immunohistochemistry

To assess ZBP-89 expression in HCC *in situ*, we performed IHC staining of ZBP-89 protein in a series of 182 HCC tissues and their corresponding nontumorous tissues. Results revealed that ZBP-89 was expressed in 91.3% (166 of 182) of tumorous and 65.4% (119 of 182) of nontumorous tissues. The representative results were shown in Figure 2.5 and 2.6. In 77.4% (141 of 182) of cases, ZBP-89 levels were higher in the tumorous tissues than in the corresponding nontumorous tissues adjacent to the tumor (Figure 2.7C). Furthermore, the high expression of ZBP-89 was found in 67.2% of the tumorous tissues but only in 26.7% of the nontumorous tissues. The difference between them was statistically significant ($P<0.001$). The median percentage of ZBP-89 expression calculated by the staining intensity score was listed in Figure 2.7D.

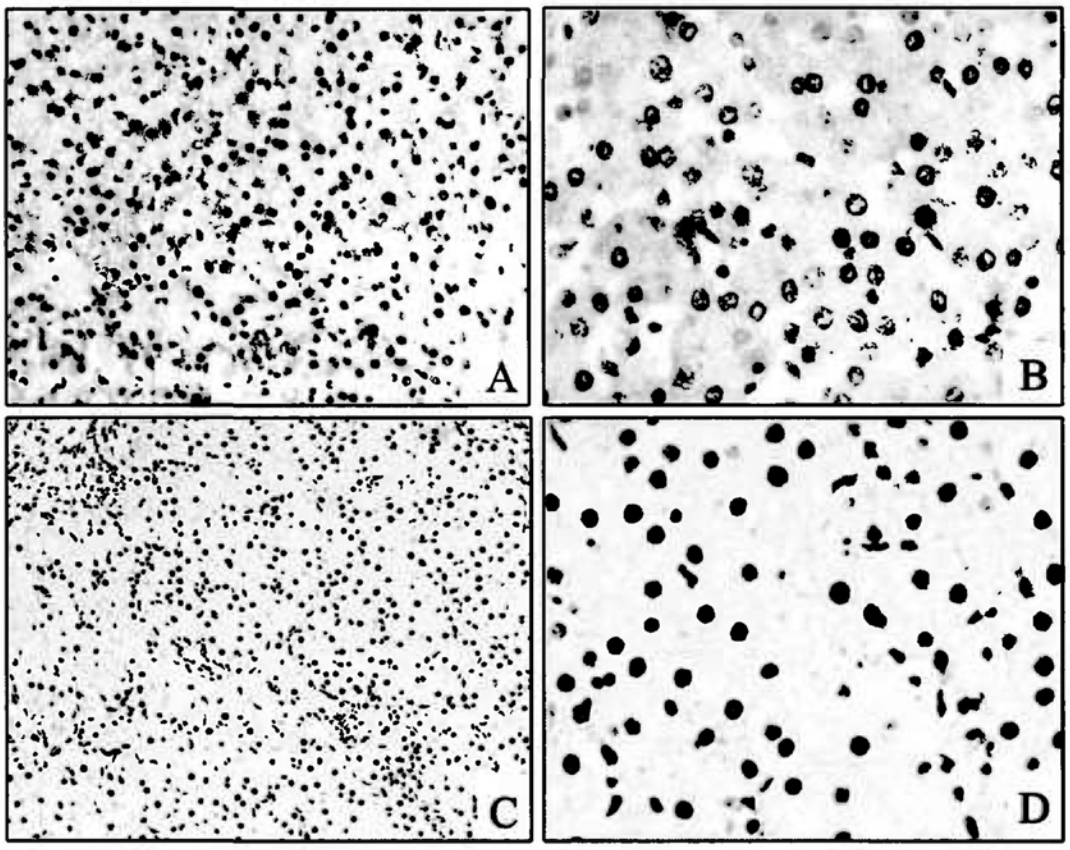

Figure 2.5 Immunohistochemical staining of ZBP-89 protein in normal liver tissue samples. Negative **(A, B)** and positive **(C, D)** stainings of ZBP-89 in the liver tissues adjacent to the tumor were shown. (Left panel fields ×100. Right panel fields ×400.)

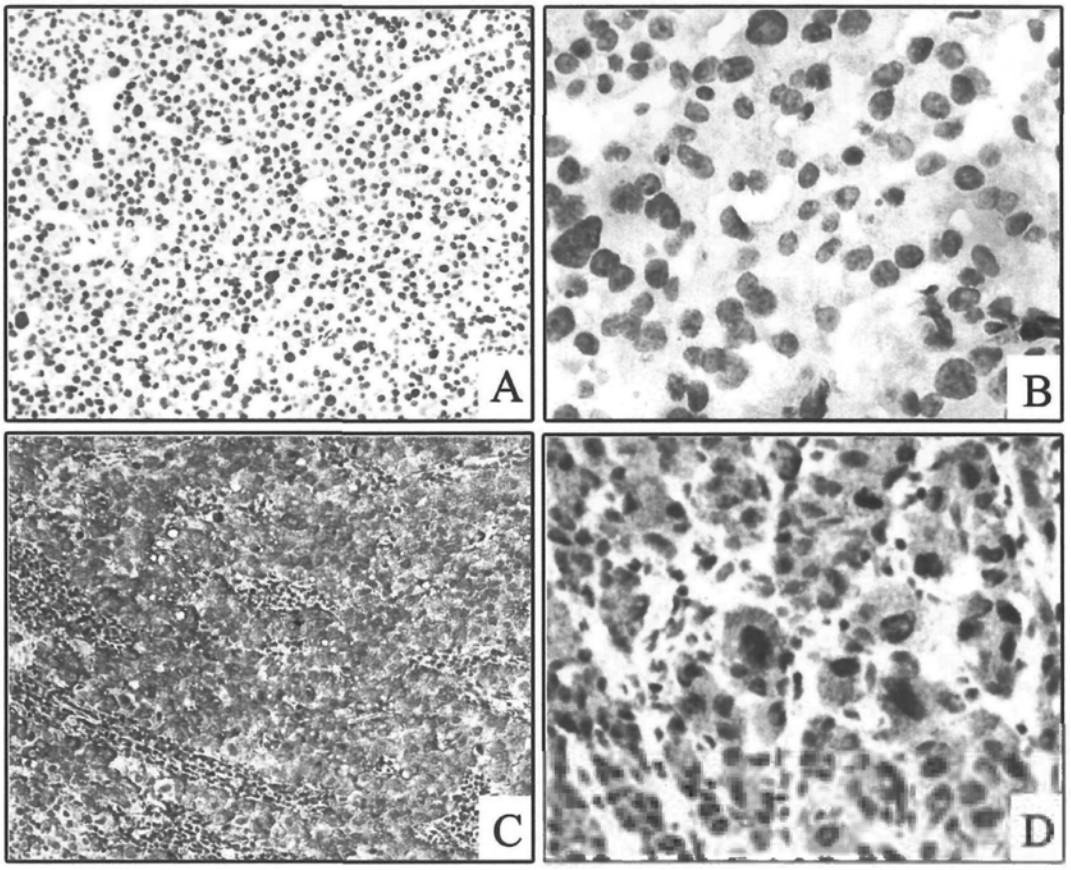

Figure 2.6 Immunohistochemical staining of ZBP-89 protein in HCC samples. Over expressions of ZBP-89 in HCC were present in both nucleus and cytoplasm. The expression of ZBP-89 in nucleus **(A, B)** or cytoplasm **(C, D)** was observed in the HCC tissues. (Left panel fields ×100. Right panel fields ×400.)

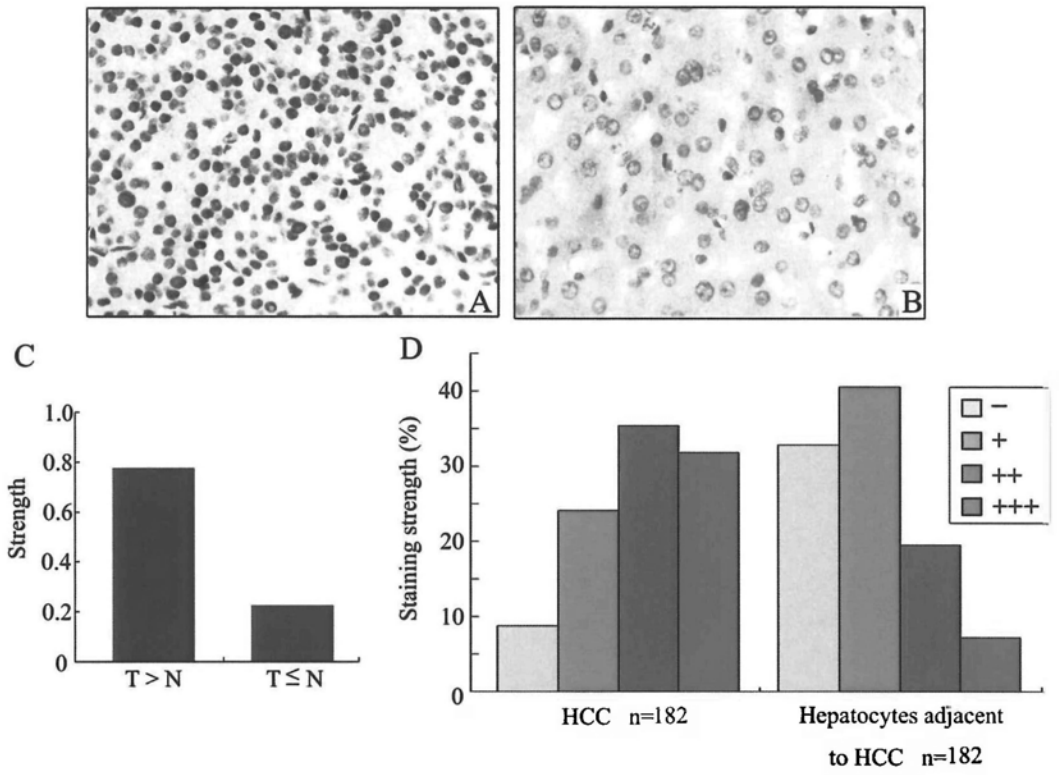

Figure 2.7 Comparison of the nuclear ZBP-89 staining intensity between the tumorous tissues (T) and the corresponding nontumorous tissues adjacent to the tumor (N). The expression of the nuclear ZBP-89 was much stronger in the tumorous cells (**A**) than in nontumorous hepatocytes adjacent to the tumor (**B**). ZBP-89 expression in the tumorous tissues and the corresponding nontumorous tissues was compared (**C**). The level of ZBP-89 staining intensity in the nucleus was scored in both T and N and the percentage of the median staining intensity at each level was calculated (**D**). (All fields ×400.)

2.3.4 Subcellular localization of ZBP-89

The subcellular expression of ZBP-89 was examined by IHC staining. ZBP-89 was found to localize predominantly in the nucleus in both nontumorous tissues and tumorous tissues (Figure 2.6 and 2.7) in most of cases, which was confirmed by the immunofluorescence results (Figure 2.8). However, in addition to the nuclear localization, ZBP-89 was also present in the cytoplasm in 11.5% (21 of 182) of the tumorous tissues (Figure 2.8C), which was hardly observed in the nontumorous tissues.

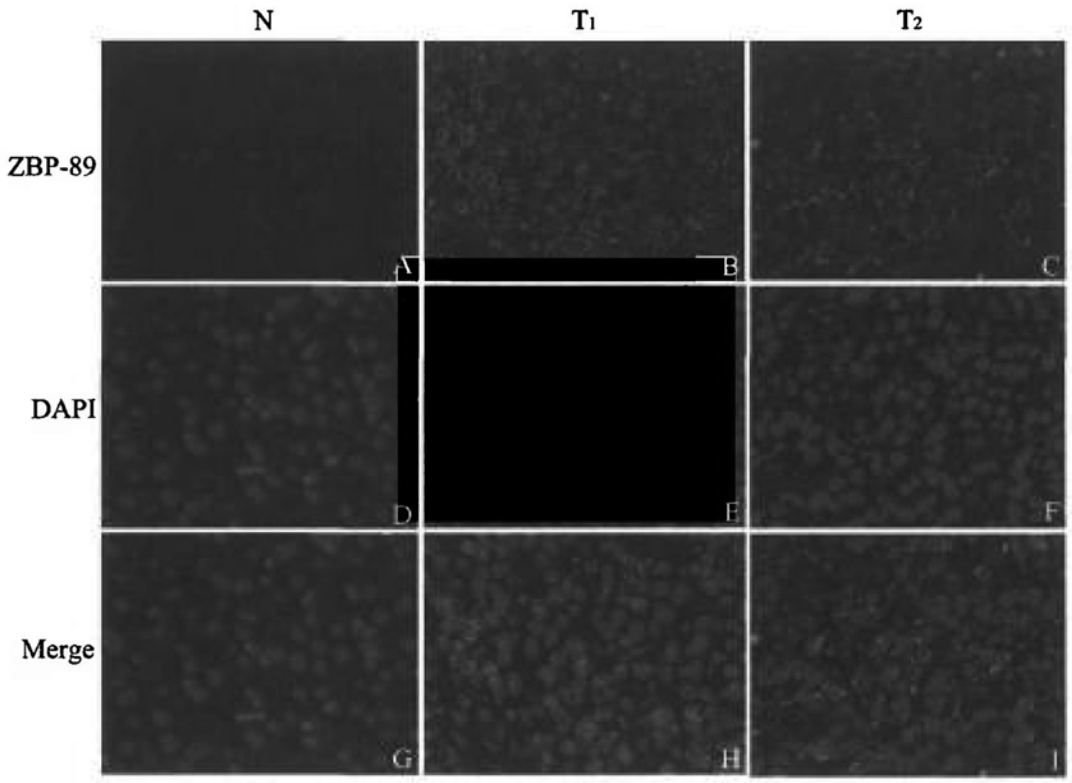

Figure 2.8 Immunofluorescent staining of ZBP-89 protein in HCC samples. Rhodamine (red) signals indicate ZBP-89 **(A, B, C)**, and 4', 6-diamidino-2-phenylindole (DAPI) (blue) signals indicate the nucleus **(D, E, F)**. Merge pictures showed that ZBP-89 was highly expressed and mainly localized within the nucleus **(G, H)** or partly presented in the cytoplasm **(I)** of HCC tissues. (N: nontumorous tissues; T1: tumorous tissues with the nucleus ZBP-89; T2: tumorous tissues with the cytoplasmic ZBP-89; all fields ×400.).

2.3.5 Relationship between ZBP-89 expression and clinicopathological parameters

The relationship between the ZBP-89 expression and clinicopathological parameters including the patient's sex, age, tumor size, number of tumors, serum level of AFP, histological grade, and status of HBV was analyzed in 182 cases of HCC. HCC cases were divided into two subgroups: "Low ZBP-89 expression" and "High ZBP-89 expression" as defined in the IHC section of Materials and Methods.

A significant correlation was found between ZBP-89 expression and two clinicopathological parameters (the histological grade and the status of HBV) (both $P<0.05$). Patients with a low pathologic grade appeared to express more ZBP-89 in the tumorous tissues than patients with a high pathologic grade, suggesting that patients may benefit from the up-regulation of ZBP-89. For the relationship with HBV, the expression of ZBP-89 was higher in patients infected with HBV, suggesting that HBV may play a role in regulation of ZBP-89. There were no statistical connections between ZBP-89 expression and the rest clinicopathological parameters ($P>0.05$) (Table 2.1).

Table 1: Correlation between ZBP-89 expression and clinicopathological parameters.

Variable	Cases (n=182)	ZBP-89 expression		*P* value*
		Low (n=56)	High (n=126)	
Sex — no. (%)				0.541
Female	16 (8.8)	6	10	
Male	166 (91.2)	50	116	
Missing data	0			
Age — no. (%)				0.861
≤50	106 (58.2)	32	74	
>50	70 (38.5)	22	48	
Missing data	6 (3.3)	2	4	
Hepatitis B virus — no. (%)				0.028*
Negative	14 (7.7)	8	6	
Positive	166 (91.2)	48	118	
Missing data	2(1.1%)	2		
Tumor size — no. (%)				0.530
≤5cm	62 (34.1)	21	41	
>5cm	116 (63.7)	34	82	
Missing data	4 (2.2)	1	3	
Multinodular tumor — no. (%)				0.668
No	143 (78.6)	45	98	
Yes	36 (19.8)	10	26	
Missing data	3 (1.6)	1	2	
Alpha-fetoprotein — no. (%)				0.403
Negative, ≤20 ng/ml	61 (33.5)	21	40	
Positive, >20 ng/ml	113 (62.1)	32	81	
Missing data	8 (4.4)	3	5	
Histological grade — no. (%) ¶				0.030*
I	27 (14.8)	3	24	
II – IV	155 (85.2)	53	102	
Missing data	0			

* A *P* value of less than 0.05 indicated the statistical significance. *P* values were calculated by Pearson's chi-square test, except for histological grade, which was calculated by the Yates' chi-square test.

¶ Histological grade was determined with reference to World Health Organization classification published in 2002.

2.3.6 Relationship between ZBP-89 expression in HCC and prognosis

One hundred and twenty five deaths were recorded in the 182 cases tested. The mean survival was 37.4 months, with a range of 1 to 72 months. The median survival was 38 months. The association between ZBP-89 expression in HCC and the survival period of patients was determined by Kaplan-Meier survival analysis (Figure 2.9). Interestingly, when ZBP-89 expression in the tumorous tissues was used as a marker, the survival in patients with the high expression tended to be longer than in patients with the low expression (log-rank test; $P = 0.016$, Figure 2.9A). In contrast, when ZBP-89 expression in the nontumorous tissues adjacent to tumors was used as a marker, patients with the low expression survived longer than those with the high expression (log-rank test; $P = 0.034$, Figure 2.9B).

We further examined the connection between the survival period and the intensity of ZBP-89 expression in details. Results confirmed that the higher ZBP-89 expression in the tumorous tissues, the longer the survival (log-rank test; $P = 0.028$, Figure 2.10A). But the survival was found no difference among three subgroup cases with different intensities of ZBP-89 staining in nontumorous tissues (log-rank test; $P = 0.360$, Figure 2.10B). Nevertheless, our data suggest that the expression of ZBP-89 in HCC tissues may have a clinical value for HCC prognosis.

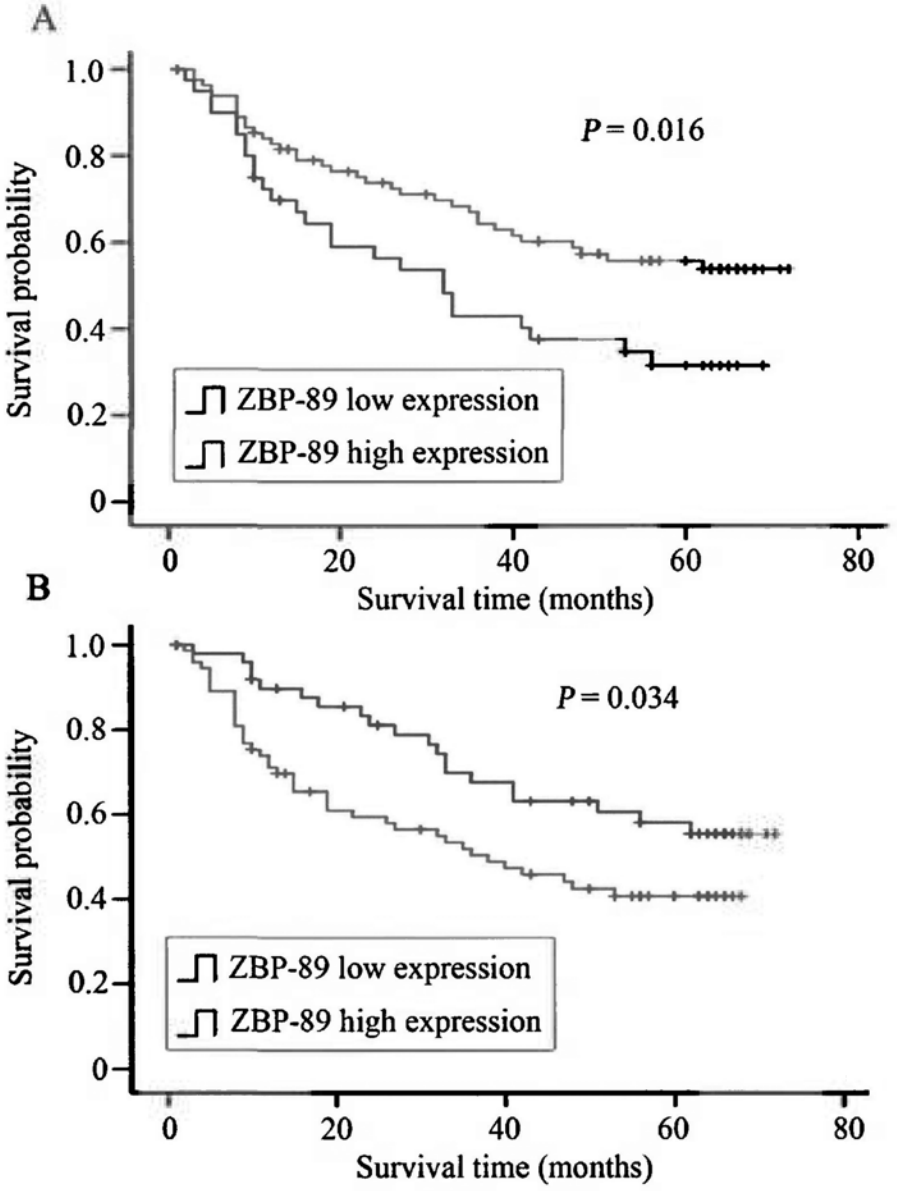

Figure 2.9 Relationship between ZBP-89 expression and patient survival. The overall survival curve was analyzed with Kaplan–Meier method to determine the influence of ZBP-89 expression on survival of HCC patients. There was a significant difference in survival between patients with low ZBP-89 expression and those with high ZBP-89 expression in the tumorous tissues (P=0.016) (**A**) and in the nontumorous hepatocytes adjacent to the tumor (P=0.034) (**B**).

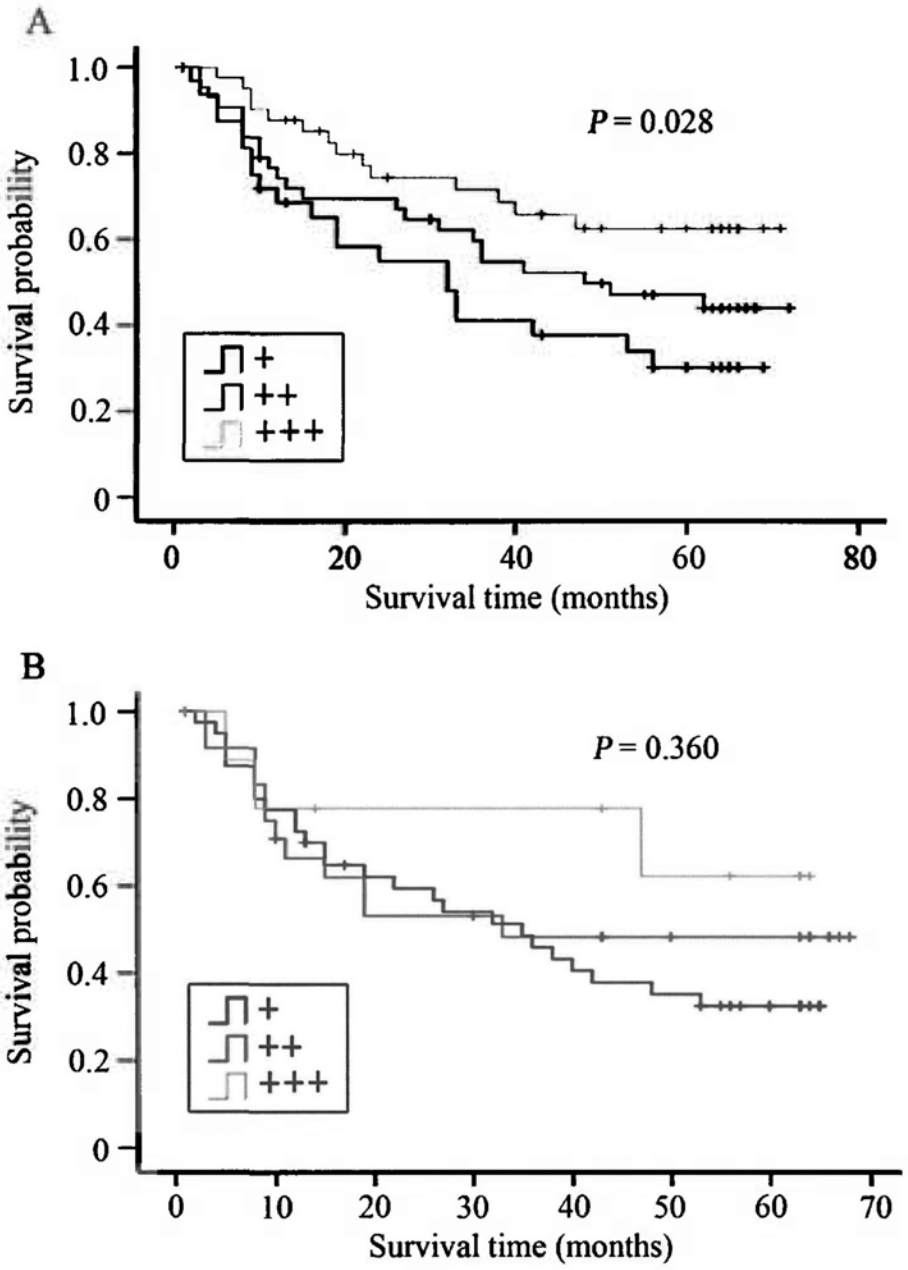

Figure 2.10 Relationship between different ZBP-89 expression in HCC and patient survival. The overall survival curve was analyzed with Kaplan–Meier method to determine the influence of ZBP-89 expression on survival of HCC patients. Survival periods were significantly different among three subgroups of patients with different levels of ZBP-89 in the tumorous tissues (P=0.028) (**C**) but not in the nontumorous tissues adjacent to cancer (P=0.360) (**D**).

2.3.7 HCC colony size and ZBP-89 over-expression

HCC cells with over-expression of ZBP-89-FL and ZBP-89-deletion mutants were subjected to the colony formation (Figure 2.11). Cells in control groups formed a number of visible colonies in 15 days. Compared with the control, the number of colonies formed by the cells with over-expression of ZBP-89-FL, ZBP-89-Δ180-298, and ZBP-89-Δ298-447 was significantly lower and the size was much smaller. The number of colonies formed by cells with ZBP-89-Δ6-180 was fewer than that in control cells but more than in the cells with ZBP-89-FL (Figure 2.12, all $P<0.01$). In order to check the possible mechanism through which ZBP-89 inhibited the colony formation, the levels of PARP, p53, p21 and Bid were measured in cells transfected with ZBP-89-FL and ZBP-89-deletion mutants (Figure 2.13). The expression of wild-type p53 but not Bid was increased in HepG2 cells containing ZBP-89-FL and ZBP-89-Δ6-180, whereas the level of mutant p53 was not changed in PLC/PRF/5 cells. p21 was up-regulated by ZBP-89-FL and ZBP-89-deletion mutants in a p53-independent manner. Cleaved PARP was increased by over-expression of ZBP-89-FL in all three cells, suggesting the occurrence of apoptosis.

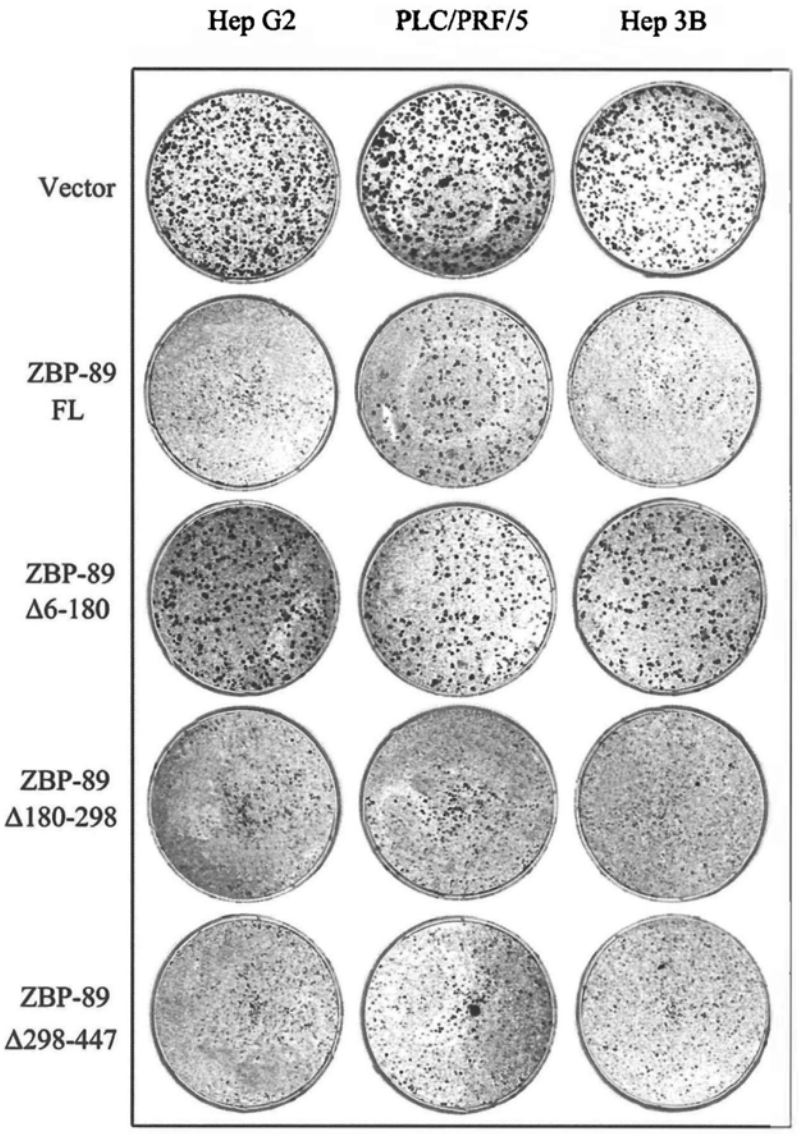

Figure 2.11 Over-expression of ZBP-89 in HCC cells reduces colony formations. HCC cells (HepG2, Hep3B and PLC/PRF/5) were transfected with pCMV-vector (negative control), pCMV-ZBP-89 (Full length, FL), pCMV-ZBP-89-Δ6-180 (ZBP-89 deletion mutant, lack of amino acid 6-180), pCMV-ZBP-89-Δ180-298 (ZBP-89 deletion mutant, lack of amino acid 180-298), or pCMV-ZBP-89-Δ298-447 (ZBP-89 deletion mutant, lack of amino acid 298-447), and then selected by the antibiotic G418 for 4 weeks to establish stable cells. Stable cells were seeded into 60mm dishes and continually selected by G418 for 15 days. Colonies were stained with trypan blue.

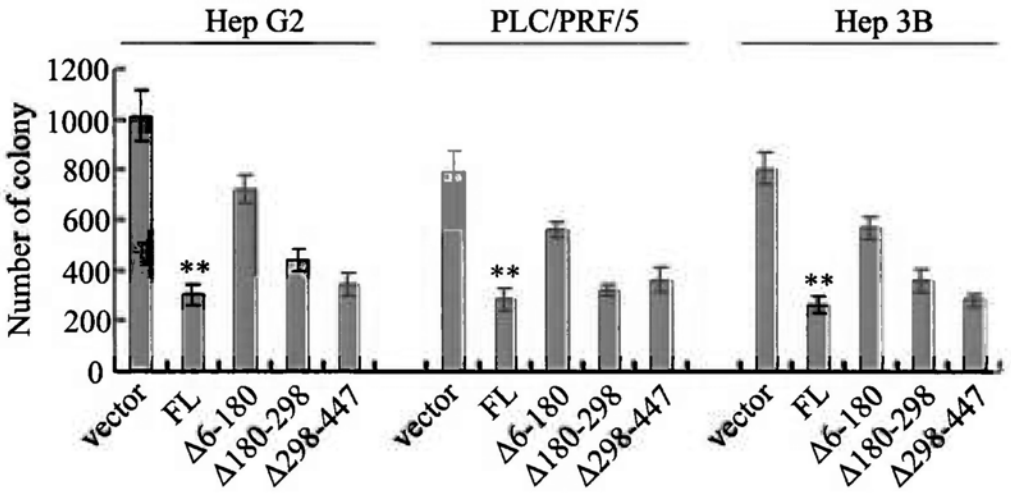

Figure 2.12 The number of colonies in each dish is determined. Stable cells were seeded into 60mm dishes and continually selected by G418 for 15 days. Colonies were stained with trypan blue. The experiment was performed in triplicate independently three times. (**, $P < 0.01$ versus the control group).

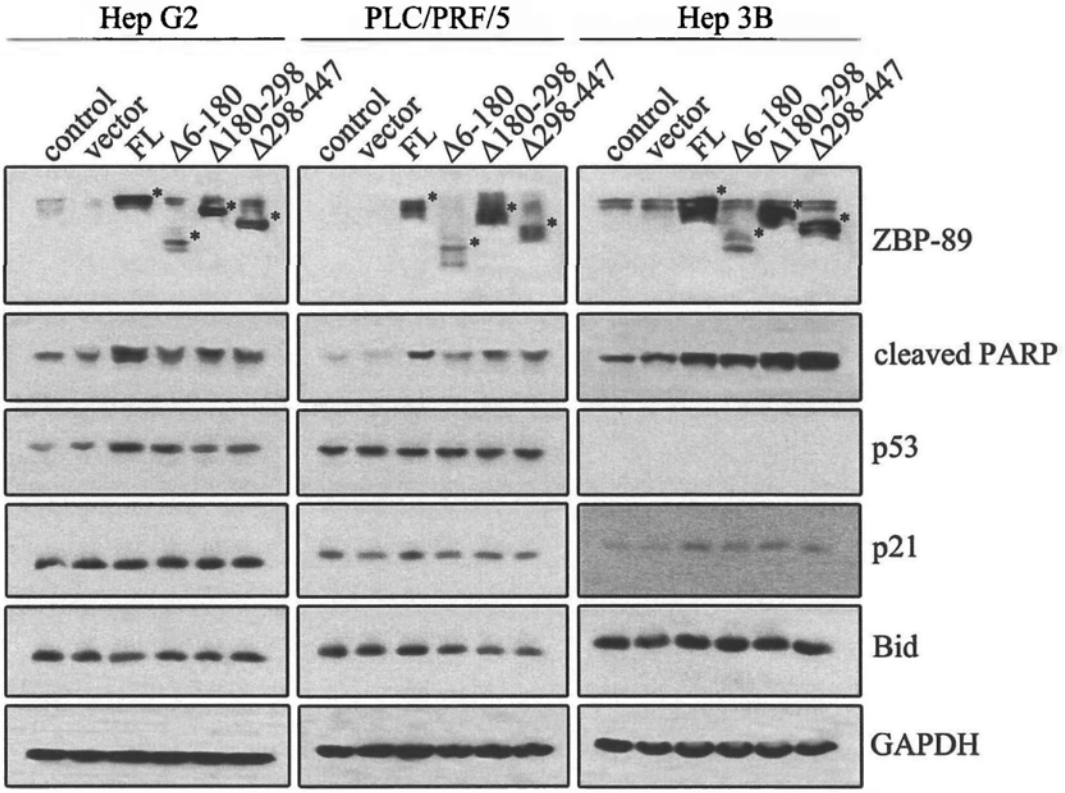

Figure 2.13 Overexpression of ZBP-89 in HCC cells induced apoptosis. HCC cells (HepG2, Hep3B and PLC/PRF/5) were transfected with pCMV-vector (negative control), pCMV-ZBP-89, pCMV-ZBP-89-Δ6-180, pCMV-ZBP-89-Δ180-298, or pCMV-ZBP-89-Δ298-447 for 48 h. Effects of ZBP-89 over-expression (full length and three deletion mutants, indicated by the star symbol) on apoptotic molecules were determined after the transfection by Western blot.

2.4 Discussion

ZBP-89 has been shown to be elevated in some cancers including gastric cancer, colorectal cancer and breast cancer, but reduced in some other diseases, such as pancreatic adenocarcinomas (Bai et al., 2002) and Dukes' B colon cancer (Bandres et al., 2007). However, the role of ZBP-89 in these cancers is not well studied. Because of the ability of interacting with known tumor suppressor factors, such as p53, p21 and ataxia-telangiectasia mutated (ATM) (Bai et al., 2006; Bai and Merchant, 2001, 2007), ZBP-89 is considered as a tumor suppressor in most of the circumstances. ZBP-89, over-expressed in gastrointestinal cancer cell lines, inhibits cellular proliferation and induced apoptosis (Bai et al., 2004). A transgenic villin-ZNF148 ($ZNF148^{TgVZ}$) mouse experiment further confirms the anti-tumor effect of ZBP-89, showing that the incidence of intestinal adenoma is reduced by 50% in the mice with ZNF148 (ZBP-89) over-expression (Law et al., 2006b). However, reasons that ZBP-89 is inconsistently expressed in different types of cancers are still unknown.

The development of cancer is closely associated with cell differentiation. Studies have shown that ZBP-89 can regulate hematopoiesis, myogenesis, erythrogenesis and megakaryogenesis, at least in part, via the regulation of differentiation in a number of cells including embryonic cells, fetal germ cells, epithelial cells and skeletal muscle cells (Bai et al., 2002; Park et al., 2003; Salmon et al., 2009b; Woo et al., 2008). Similarly, ZBP-89 was thought to take part in gastric cancer development, as its level is elevated in pre-malignant states of gastric cancers (Taniuchi et al., 1997). In the present study, the high expression of ZBP-89 was connected with the histological grade of HCC, indicating that patients with a low histological grade were more likely

with high ZBP-89 expression. ZBP-89 may contribute to the degree of tumor malignancy by modulating p53, since the histological grade of HCC can be affected by p53 (Aoki et al., 2002; Zhang et al., 2009), and ZBP-89 can regulate p53 as demonstrated in the previous reports (Yu et al., 2009; Zhang et al.).

Interestingly, our previous study of p53 in HCC showed that patients with a low pathologic grade had a higher tendency to express p53. Moreover, the high expression rates of ZBP-89 and p53 were 77.4% and 70.7%, respectively. It has been reported that ZBP-89 can directly bind to and stabilize p53 by retaining it in the nucleus (Bai and Merchant, 2001). In our early study, the cytoplasmic co-localization of ZBP-89 with some p53 mutants was detected in recurrent HCC tumors (Chen et al., 2003), suggesting that the localization of ZBP-89 may be affected by certain p53 mutants. p53 is mutated in 24-69% of HCC (El-Shanawani et al., 2006). In the present study, IHC staining revealed the presence of ZBP-89 protein in both the nucleus and the cytoplasm. However, the cytoplasmic ZBP-89 was only found in about 10% HCC cases. This finding is somehow in agreement with our early data that some p53 mutants may interact with ZBP-89. The role of the cytoplasmic ZBP-89, however, has not been identified and is needed more investigations. One of the hypotheses is that certain p53 mutants may transfer ZBP-89 out of the nucleus where ZBP-89 interacts with p53, p21 and many other genes that have been implicated in the response to chemotherapy. As a result, tumor cells become less sensitive to chemotherapeutic drugs.

HBV infection is an etiological cause for a subset of HCC (Liu and Kao, 2007). Here in our study, patients with HBV infection had more chances to be with high

expression of ZBP-89. In other studies, Epstein–Barr virus (EBV) *BMRF1* gene can enhance the binding of ZBP-89 to the gastrin promoter (Holley-Guthrie et al., 2005), suggesting that ZBP-89 function could be affected by virus. In our previous study, HBV X-protein (HBx), a well-known hepatocarcinogenesis (Datta et al., 2007), has been shown to be responsible for the decreased expression of Bid (Chen et al., 2001) whose basal expression is maintained by ZBP-89. Bai *et al.* reported that deletion of Bid impeded cell proliferation and hepatic carcinogenesis (Bai et al., 2005). Thus, it is possible that ZBP-89 interacts with HBx to modulate the expression of Bid and subsequently participates in hepatocarcinogenesis.

One of the main findings in this study is that the high expression of ZBP-89 in HCC is significantly associated with the better survival of HCC patients. This is the first report of relationship of ZBP-89 expression and survival in patients. Law *et al.* once reported that survival could be prolonged by more than fifty percent when ZBP-89 was over-expressed in animals (Law et al., 2006b). Our data indicated that the higher ZBP-89 was expressed in HCC tissues, the longer patients survived. The high recurrence rate of HCC is the main reason that causes the unsatisfactory outcome in HCC treatment. Although we do not have data to support the correlation between ZBP-89 expression and HCC recurrence, a negative correlation between the level of ZBP-89 and the recurrence of Dukes' B colon cancer has been reported (Bandres et al., 2007). p53 stabilization by ZBP-89 may contribute to the low recurrence of HCC and thus the longer survival in patients, supported by our early study showing that the mean interval between surgical resection and the appearance of recurrence in patients with HCC was prolonged when there was wild-type p53 that could bind to ZBP-89 (Chen et al., 2003). Furthermore, ZBP-89 has been shown to sensitize tumor

cells to anti-tumor drugs as the increased susceptibility to chemotherapy-induced cell death has been shown in cells with ZBP-89 over-expression (Chen et al., 2009; Law et al., 2006a; Okada et al., 2006). In this study, the result of the colony formation assay has also demonstrated an inhibitory role of ZBP-89. The reduction of the colony formation by ZBP-89 appears to be associated with the elevation of p21 and cleaved PARP since these changes were evident in the present study. The ability of ZBP-89 to reduce tumor colony formation and to sensitize tumor cells to anticancer agents should benefit patients with HCC. Taken together, these data may explain why patients with the high expression of ZBP-89 in HCC tissues have a longer lived period. The findings may also suggest that it will be more effective, at least in some cases, if the antitumor drugs can enhance the level of ZBP-89 in the cancer cells.

There are limited reports looking into the relationship between overall survival and tumor proteins expressed in the nontumorous tissues adjacent to the tumor. In our present study, we showed that, contrary to the situation in ZBP-89 expression in tumor tissues, the high expression of ZBP-89 in the nontumorous tissues was negatively correlated with survival periods of HCC patients. Therefore, the prognostic significance of ZBP-89 expression in the tumorous and nontumorous tissues is totally different, indicating that one has to carefully evaluate the location of ZBP-89 expression when considering it as a biomarker for the prognosis in HCC.

One of the interesting findings in this study is that ZBP-89-Δ6-180 could abrogate the PARP cleavage. The cells with ZBP-89-Δ6-180 formed more colonies than the cells with ZBP-89-FL. Bai *et al.* reported that ZBP-89 interacted with p300 through N-terminus to regulate p21 following Butyrate treatment (Bai and Merchant, 2000).

Thus, the region of 6-180 aa in ZBP-89 may be essential for cell growth regulation. ZBP-89-Δ180-298 is the domain through which ZBP-89 interacts with p53 that is reported to be involved in apoptosis. However, in our study, overexpression of ZBP-89-Δ180-298 did not possess the similar effects of overexpression of ZBP-89-Δ6-180. Furthermore, though p53 was increased in ZBP-89 overexpression HepG2 cells, in Hep3B cells which do not have p53 expression, PARP activation was not abroshed by ZBP-89-Δ180-298 exression. These data may suggest that ZBP-89-induced apoptosis is p53-independent. Additional experiments need to confirm or further define the interaction between ZBP-89 and p53.

Chapter 3: $p53^{G245D}$ influences effects of HDACi on liver cancer cell growth by modulating the cellular localization of ZBP-89

3.1 Introduction

Histone deacetylase inhibitors (HDACi) have been reported to induce cell growth arrest, differentiation and apoptosis in tumor cells (Zhou and Zhu, 2009). Histone deacetylase, the target of HDACi, consists of 18 described family members which have been divided into four general classes. Class I includes HDACs 1, 2, 3, and 8, located within the cell nucleus; class II includes the HDACs 4, 5, 6, 7, 9, and 10; class III HDACs, consisting of the NAD+-dependant Sirtuin family 1 to 7; and class IV HDAC 11. HDACIs generally bear three structural characteristics: a zinc binding moiety, an opposite capping group, and a straight chain alkyl, vinyl or aryl linker connecting the two.

HDACi play roles in alterating patterns of acetylation of histones and many non-histone proteins involved in gene expression, apoptosis, cell cycle progression, cell migration, mitotic division, redox pathways, DNA repair, and angiogenesis (Table 3.1). HDACi-mediated transactivation of a specific gene or set of genes is responsible for the inhibition of cell cycle progression or induction of apoptosis (Deubzer et al., 2008). Sodium butyrate (NaB, Figure 3.1), a short chain fatty acid and a type of HDACi, whose action is mimicked by trichostatin A (TSA, Figure 3.2), a structurally unrelated HDAC inhibitor, is produced in the colon by the breakdown

of dietary fiber and transported to the liver directly via the portal vein (Bloemen et al., 2009). HDAC inhibitors arrest the growth of cancer cells. The growth-inhibitory effect of NaB against cancer cells has been attributed to its ability to induce cell cycle arrest and differentiation by up-regulating $p21^{Waf1}$ (p21) in a p53-independent manner (Archer et al., 1998; Chopin et al., 2004). TSA effectively induces apoptosis in various cancer cells even at nanomolar concentrations, and efforts have been made to investigate the regulatory effects of TSA on the cell-cycle progression, differentiation, and apoptosis (Maecker et al., 2002; Setiadi et al., 2008). Therefore, to date, the responses observed in studies using HDACi as a single agent have predominantly been seen in advanced hematologic malignancies, with few seen in solid tumors (Table 3.2).

ZBP-89 (BFCOL1, BERF1, ZNF-148 or Zfp-148), a Krüppel-type zinc-finger transcription factor that binds to GC-rich sequences, is universally expressed and involved in the regulation of cell growth and cell death (Zhang et al.). ZBP-89 is usually over-expressed in human cancer cells, where it can efficiently induce apoptosis through both p53-dependent and -independent mechanisms (Bai and Merchant, 2001; Bai et al., 2004). By regulating genes such as gastrin and ornithine decarboxylase, ZBP-89 participates in cell proliferation and growth (Law et al., 1998b; Merchant et al., 1996). It is reported that ZBP-89 can directly and indirectly regulate p21. ZBP-89 binds to the region of the p21 promoter from -245 to -215 through its amino-terminus in a butyrate-dependent manner (Bai et al., 2006). HDACi treatment of colonic cells promotes the formation of an ATM/ZBP-89/p300 complex on the p21 proximal promoter, and this multi-molecular complex plays an important role in the induction of p21 expression *in vitro* and *in vivo* (Bai and

Merchant, 2000). The reduction of ZBP-89 or ATM with siRNA blocks HDACi-induced p21 expression (Bai et al., 2006; Bai and Merchant, 2007).

The transcription factor p53 induces cell growth arrest and/or apoptosis through activation or repression of downstream target genes (Kruse and Gu, 2009; Yang et al., 2009). However, p53 is frequently inactivated in the vast majority of human cancers. Missense mutations in the *p53* gene are present in more than 50% of human cancers, including hepatocellular carcinoma (HCC). Most of mutations in p53 localize in the DNA-binding domain, leading to genomic instability and loss of growth control (Brosh and Rotter, 2009). ZBP-89 stabilizes p53 through direct protein binding, which results in retention of p53 in the nucleus (Bai and Merchant, 2001). Moreover, ZBP-89 also interacts with some p53 mutants (Chen et al., 2003; Okada et al., 2006). However, tumor cells with a mutation in the p53 gene may resist to ZBP-89-mediated stabilization. Mutant p53 may shift the subnuclear location of ZBP-89 to the nuclear periphery, which is a domain rich in heterochromatin (Merchant et al., 2003). In our previous study, the cytoplasmic localization of ZBP-89 was found in recurrent HCC tumors primarily with p53 mutants (Chen et al., 2003). In another study, ZBP-89 was shown to potentiate p53-mediated cell death induced by staurosporine and etoposide, which was not seen in the presence of the $p53^{R273H}$ mutation (Okada et al., 2006). However, the role of mutant p53 in the ZBP-89/p53 complex has not been identified.

In this chapter, we found that $p53^{G245D}$ but not $p53^{R273C}$ abrogated p21 up-regulation induced by NaB treatment. $p53^{G245D}$ physically bound to ZBP-89 and transferred it from the nucleus to the cytoplasm. Moreover, cells that possess $p53^{G245D}$ were

resistant to the lethal effect of TSA treatment. These data may reveal a novel pathway in which mutant p53 enables tumor cells to resist chemotherapy. The findings deem to be of significance in clinical guidance for HCC treatment.

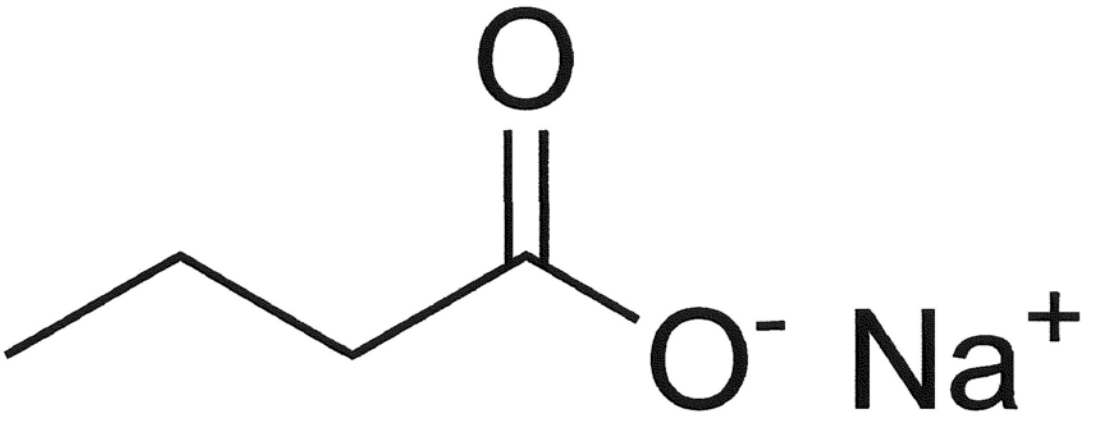

Figure 3.1 Chemical structure of sodium butyrate (NaB). Sodium butyrate is a compound with formula $Na(C_3H_7COO)$. It is the sodium salt of butyric acid. It has various effects on cultured mammalian cells including inhibition of proliferation, induction of differentiation and induction or repression of gene expression. As such, it can be used in lab to bring about any of these effects. Specifically, butyrate treatment of cells results in histone hyperacetylation, and butyrate itself inhibits HDAC activity. Butyrate has been an essential vehicle for determining the role of histone acetylation in chromatin structure and function.

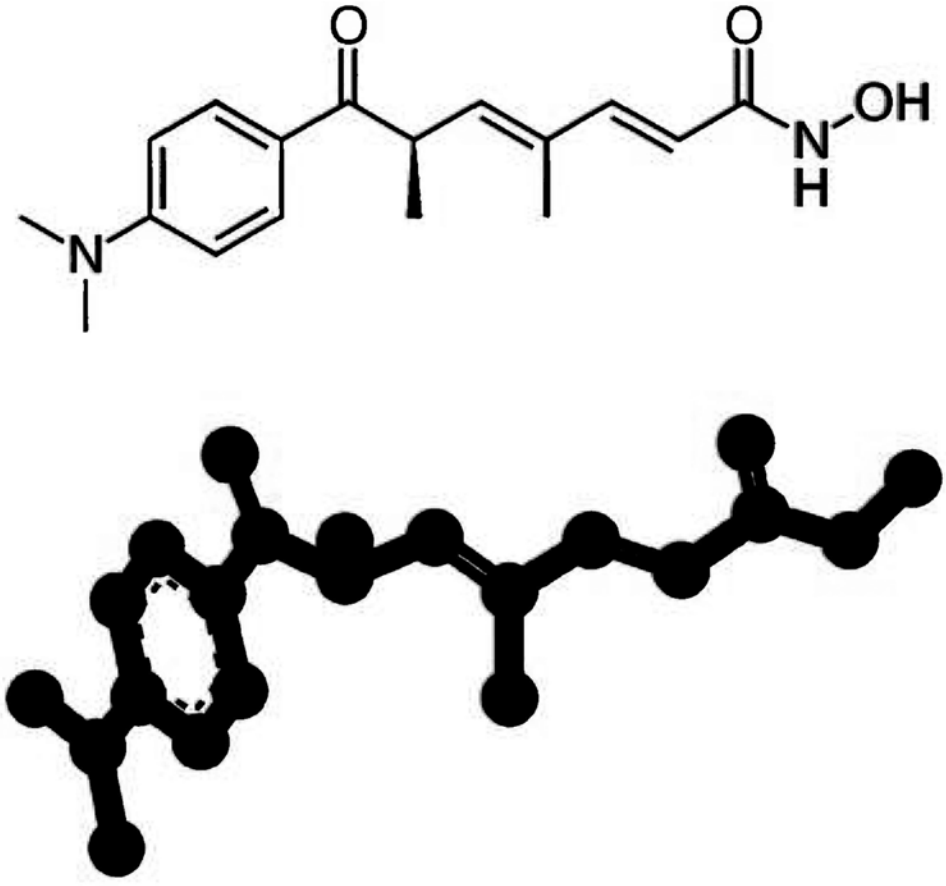

Figure 3.2 Chemical structure of Trichostatin A (TSA). TSA is an organic compound that serves as an antifungal antibiotic and selectively inhibits the class I and II mammalian histone deacetylase (HDAC) families of enzymes, but not class III HDACs (i.e., Sirtuins). TSA inhibits the eukaryotic cell cycle during the beginning of the growth stage. TSA can be used to alter gene expression by interfering with the removal of acetyl groups from histones and therefore altering the ability of DNA transcription factors to access the DNA molecules inside chromatin. TSA has some potential as an anti-cancer drug. One suggested mechanism is that TSA promotes the expression of apoptosis-related genes, leading to cancerous cells surviving at lower rates, thus slowing the progression of cancer.

Table 3.1 HDACi and genomic instability

Strutural class	HDACi	Targets (HDAC, class)	Clinical trialsb (phase)	Effects
Hydroxamic acid	TSA	I and II	ND	DDR, MD
	SAHA	I and II	FDA approval	DDR, OS, MD
	LAQ824	I and II	I	DDR, MD
	SBHA	ND	ND	MD
	PCI-24781	ND	ND	DDR
	LBH589	I and II	I/II	DDR
Short-chain fatty acids	Sodium butyrate	I and II a	ND	MD
	Valproic acid	I and IIa	I	DDR
	AN-9	ND	I/II	DDR
	OSU-HDAC42	ND	ND	DDR
Benzamides	MS-275	I	I/II	DDR, OS
Cyclic peptides	Depsipeptide	ND	I/II	DDR
	Apicidin	ND	ND	MD
	HC-toxin	ND	ND	DDR
Thiolate	NCH-51	ND	ND	OS

Abbreviations: ND, not determined; DDR, DNA damage and repair; OS, oxidative stress; MD, mitosis defects.

Table 3.2 Key trials of HDACi in hematologic malignancies. (Prince et al., 2009)

Agent	Disease	Phase	Sample size	Response
Vorinostat (SAHA)	HL/DLBCL/CTCL	I	35	CR 1, PR 4, SD 3
	CTCL	II	33	PR 8 (SS 4)
	CTCL	IIb	74	CR 1, PR 21
	Advanced leukemia/MDS	I	41	CR 2, CRi 2, HI 3
Romidepsin (depsipeptide)	AML/CLL	I	20	Nil
	CTCL	II	71	CR 6, PR 24, SD 19
	CTCL	II	92	CR 7, PR 10
	PTCL	II	43	PR 10, CR 7
MGCD0103	Advanced leukemia/MDS	I	29	CR 3
	Advanced leukemia/MDS	I	19	SD 4
	HL	II	33	CR 2, PR 6
	DLBCL/FL	II	50	CR 1, PR 4, SD 22
Panobinostat (LBH589)	Advanced leukemia/MDS	I	15	HI 1
	CTCL	II	95	CR 4, Skin CR 2, PR
	AML/MF/MM/HL/NHL	I/II	146*	CR 3, PR 17, SD 14
	NHL/solid tumors	I	19*	CR 2, PR 2
Belinostat	NHL/CLL/MM	I	16	SD 5
Entinostat	Advanced leukemia/MDS	I	38	Nil

Abbreviations: MF, myelofibrosis; HI, hematologic response; CTCL, cutaneous T-cell lymphoma; CR, complete remission; SD, sustained stable disease; HL, hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; AML, acute myeloid leukemia; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; PR, partial responses.

*Ongoing.

3.2 Materials and Methods

3.2.1 HCC Cells and Reagents

The HCC cell lines (Hep3B, PLC/PRF/5 and HepG2) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 mg/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The p53 status in these three HCC cell lines is different. p53 is deleted in Hep3B cells, and mutated at R249S in PLC/PRF/5 cells but HepG2 cells have wt p53. In some experiments, p53-nagtive Hep3B cells were transfected with pcDNA 3.1 vector, pcDNA 3.1-$p53^{G245D}$ or pcDNA 3.1-$p53^{R273C}$ using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After transfection for indicated times, the cells were selected by G418 to establish stable lines. Sodium butyrate (NaB dissolved in H_2O) and trichostatin A (TSA dissolved in DMSO) (the DMSO final concentration is less than 0.1%) were purchased from Sigma.

3.2.2 Antibodies

The antibodies used in this study were as follows: primary antibodies for ZBP-89, Bid, p53, actin, GAPDH, lamin B, PARP and Bcl-xL were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA), and the antibodies for Caspase 8, Caspase 3 and p21 were from Cell Signaling (Danvers, MA). p53 mutant antibodies for the immunoprecipitation (IP) experiment were purchased from Calbiochem (Gibbstown, NJ).

3.2.3 MTT

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazo-lium bromide (MTT) assay. Briefly, 8×10^3 of cells were seeded onto 96-well plates for 24 h followed by incubation with various doses of NaB and TSA for indicated time. After adding 100 µl/well of MTT solution, the cells were incubated for another 2 h. Supernatants were then removed and the formazan crystals were dissolved in 100 ul/well DMSO. The absorbance at 570 nm/630 nm of each sample was measured using multilabel plate reader (PerkinElmer, MA). Three independent experiments were performed.

3.2.4 DNA Extraction

Genomic DNA was isolated from frozen HCC tissues by proteinase K digestion and spin columns using QIAamp DNA mini kit (QIAGEN, Hilden, Germany). The quality of DNA was checked by running DNA on a 1% agarose gel and the concentration of DNA was determined spectrophotometrically at 260 nm.

3.2.5 Analysis of p53 Mutation

p53 mutations were analyzed by direct sequencing. The primers for sequencing were designed as: 5'-acg cac ctc aaa gct gtt-3' and 5'-gcc cat cct cac cat cat-3'. The sequencing was done by ABI PRISM 310 Genetic Analyzer using BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA). Experiment was performed according to the manufacturer's instructions.

3.2.6 Reverse Transcription PCR

Experiments performed as described. Total RNA was extracted from cells using Trizol (Gibco, Carlsbad, CA) according to the manufacturer's instructions. One microgram of RNA sample was reverse transcribed with oligo-dT primers (Promega, Madison, WI) to obtain single-stranded cDNA. One-tenth of the product was used as template in PCR amplification for 28 cycles in a thermal cycler. The following primers were used: ZBP-89 forward, 5'- CGC TGT GAT GAA TGT GGT GAT GAG AC -3'; ZBP-89 reverse, 5'-CCC AGC TCT ATT ATC ATT TAC ATT C -3'; p53 forward, 5'-TGC GTG TGG AGT ATT TGG ATG-3', p53 reverse, 5'-TGG TAC AGT CAG AGC CAA CCA G-3'; p21 forward, 5'-GTT CCT TGT GGA GCC GGA GC-3', p21 reverse, 5'-GGT ACA AGA CAG TGA CAG GTC-3'; GAPDH forward, 5'-AAA TCC CAT CAC CAT CTT CC-3'; and GAPDH reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3'. The PCR products were analyzed by 1% agarose gel electrophoresis. The abundance of PCR signals was determined using the Quantity One™ Software (Bio-Rad, Hercules, CA).

3.2.7 Immunohistochemistry

Formalin-fixed and paraffin-embedded HCC sections (The use of the human specimens in the study was approved by the Independent Ethics Committees of The Chinese University of Hong Kong) with a thickness of 4 μm were dewaxed in xylene and graded alcohols, hydrated, and washed in phosphatebuffered saline (PBS). After pretreatment in a microwave oven, endogenous peroxidase was inhibited by 3% hydrogen peroxide in methanol for 20 min, followed by avidin-biotin blocking using a biotin-blocking kit (DAKO, Carpinteria, CA). Slides were then incubated with

mouse anti-p53 or rabbit anti-ZBP-89 antibodies for 4 h in a moist chamber at room temperature, washed in PBS, and incubated with biotinylated goat anti-rabbit/mouse antibodies. Slides were developed with the Dako Liquid 3, '3-diaminobenzidine tetrahydrochloride (DAB) + Substrate-chromogen System and counterstained with hematoxylin.

3.2.8 Immunofluorescence

Cells grown on cover slips were fixed for 20 min in PBS containing 4% parafomaldehyde, permeabilized in 0.1% Triton X-100 for 2 x 5 min and incubated in blocking buffer (3% donkey serum in TBS) for 1 h. Cells were then incubated in antibody dilution buffer (3% bovine serum albumin in TBS) containing the indicated primary antibody for 2 h in room temperature and then washed extensively in PBS before being incubated with the appropriate fluorochrome-conjugated secondary antibody for 1 h. DNA was stained by 4',6-diamidino-2-phenylindole (DAPI).

3.2.9 Immunoprecipitation and Western blot

Experiments were performed as described in our previous study (Xiao et al., 2009). Briefly, for immunoprecipitation (IP), cells were lysised in RIPA buffer supplemented with proteinase inhibitor cocktail. Specific antibodies were added for 2 to 3 h, and then protein A/G beads were added for an additional 2 h. Precipitated proteins were dissolved the in sodium dodecyl sulfate (SDS) loading buffer and fractionated by SDS polyacrylamide gel electrophoresis (PAGE). For Western blot (WB), cellular lysate or IP complex were boiled with 6x SDS loading buffer and then fractionated by SDS-PAGE. The proteins were transferred to PVDF membrane, which was then incubated with a primary specific antibody in 5% milk, followed by

a horse radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibodies and ECL detection reagent (Amersham Life Science, Piscataway, NJ).

3.2.10 Nuclear Extraction

To isolate nuclear and cytoplasmic fractions, a nuclear extraction kit (Panomics, Fremont, CA) was used. Briefly, cells were rinsed in ice-cold PBS twice and then lysised in buffer A (100 mM HEPES, pH 7.9; 100 mM KCl; 100 mM EDTA) plus protease inhibitor cocktail and shaken at 200 g for 10 min. After centrifuging at 15,000 g for 4 min at 4°C, the supernatant (cytosolic fraction) was saved. Pellet was resuspended in buffer B (100 mM HEPES, pH 7.9; 2 M NaCl; 5 mM EDTA; 50% Glycerol) plus protease inhibitor cocktail. Solutions were shaken at 200 g for 2 h at 4°C and then centrifuged at 15,000 g for 10 min at 4°C. The supernatants obtained were referred to as the nuclear fraction.

3.2.11 RNA Interference

Small interfering RNA for ZBP-89 (si-ZBP-89) and nonspecific siRNA (si-control) were synthesized as 5'-AAG ATC GAA GTA TGC CTC ACC TT-3' and 5'-AAG ATC GAA CGT GTC CTC ACC TT-3' respectively. For RNA interference, 2 x 10^5 of Hep 3B cells were seeded into 6-well plates 24 h prior to transfection. For each well, 50 nM siRNA was transfected into cells by Lipofectamine 2000 according to the protocol. After 24 h, the cells were ready for gene knockdown analysis.

3.2.12 *In Situ* Cell Death Detection

Labeling of fragmented DNA was performed with TUNEL staining (green fluorescence), using *In Situ* Cell Death Detection Kit (Roche, Indianapolis, IN).

3.2.13 TUNEL assay

Apoptosis assay was performed using APO-DIRECTTM TUNEL ASSAY kit (Chemicon, Temecula, CA). Cells were harvested and fixed in 1% paraformaldehyde for 60 min at 4°C, followed by a second fixation in 70% (v/v) ethanol overnight at -20°C. Then the cells were treated with various reagents for a designed period according to the manufacture's instruction. Finally, the cells were analyzed by flow cytometry using FACS Vantage machine (Becton Dickinson, Franklin Lakes, NJ). The Cell Quest software (Verity Software House, Topsham, ME) was used to analyze the data.

3.2.14 Statistical analysis

Differences between groups were examined for statistical significance using Student's t-test. All *P*-values are two-sided and $P < 0.05$ was considered as statistically significant. All statistical calculations were performed with the SPSS software (version 13.0, IL). The data were presented as mean ± SD from at least three independent experiments.

3.3 Results

3.3.1 Cytotoxicities of NaB and TSA in HCC cells

We firstly assessed the cytotoxicity of NaB and TSA in HCC cells that carry different status of p53. Compared with Hep3B cells ($p53^{-/-}$), NaB (Figure 3.3) or TSA (Figure 3.4) treatment caused more death in PLC/PRF/5 cells ($p53^{R249S}$) but less death in HepG2 cells ($p53^{WT}$). Furthermore, the effects of both agents were in a dose- and time-dependent manner. It was noted that at 24 h culture, TSA at 500 nM markedly reduced cell viabilities of all 3 HCC cell lines whereas NaB, even at high dose (10 mM), did not cause significant cell death. However, prolonged treatment with 4mM NaB (72 h culture) induced cell growth inhibition.

The effects of both agents on the levels of PARP (an apoptotic marker) and p21 (a cell cycle inhibitor) were next examined (Figure 3.5). Following 4mM NaB treatment, p21 was gradually induced but PARP was not activated. However, with 500 nM TSA treatment, p21 was firstly increased, but at the time when PARP was cleaved, it was dramatically decreased in Hep3B and PLC/PRF/5 cells but not obviously in HepG2 cells (Figure 3.5). Moreover, the levels of the cleaved PARP appeared to match the percentage of cell viability reduction in all three HCC cell lines. Based on the above data, 4 mM NaB and 500 nM TSA were respectively used in the subsequent cell cycle arrest and apoptosis experiments.

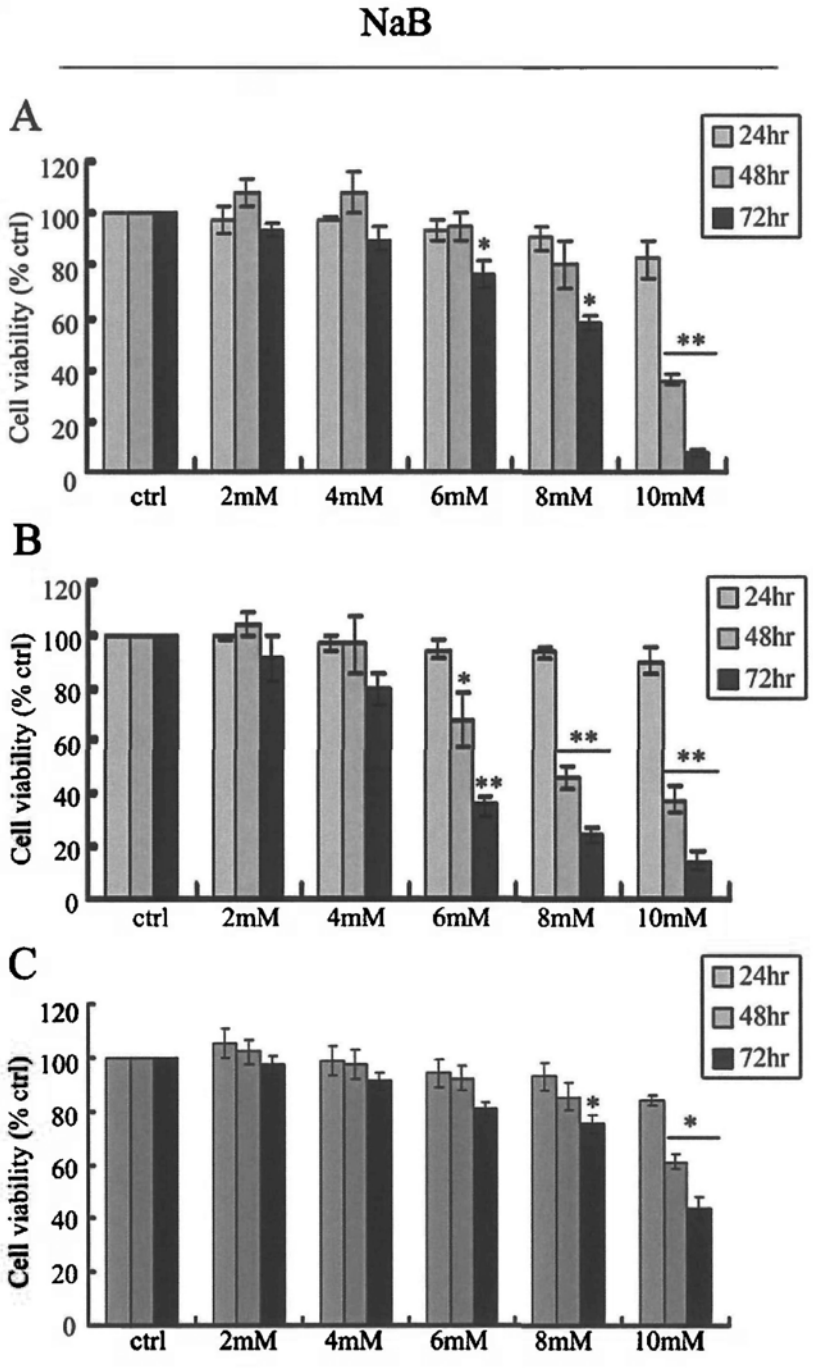

Figure 3.3 Cytotoxicity of NaB in HCC cells. Cells (**A.** Hep3B; **B.** PLC/PRF/5; **C.** HepG2) were seeded into 96-well plates and treated with various concentrations of NaB (0-10 mM) for indicated periods. The cell viability was determined by MTT assay. The data represent mean ± SD of three independent experiments. $*P < 0.05$ and $**P < 0.01$, versus the control group.

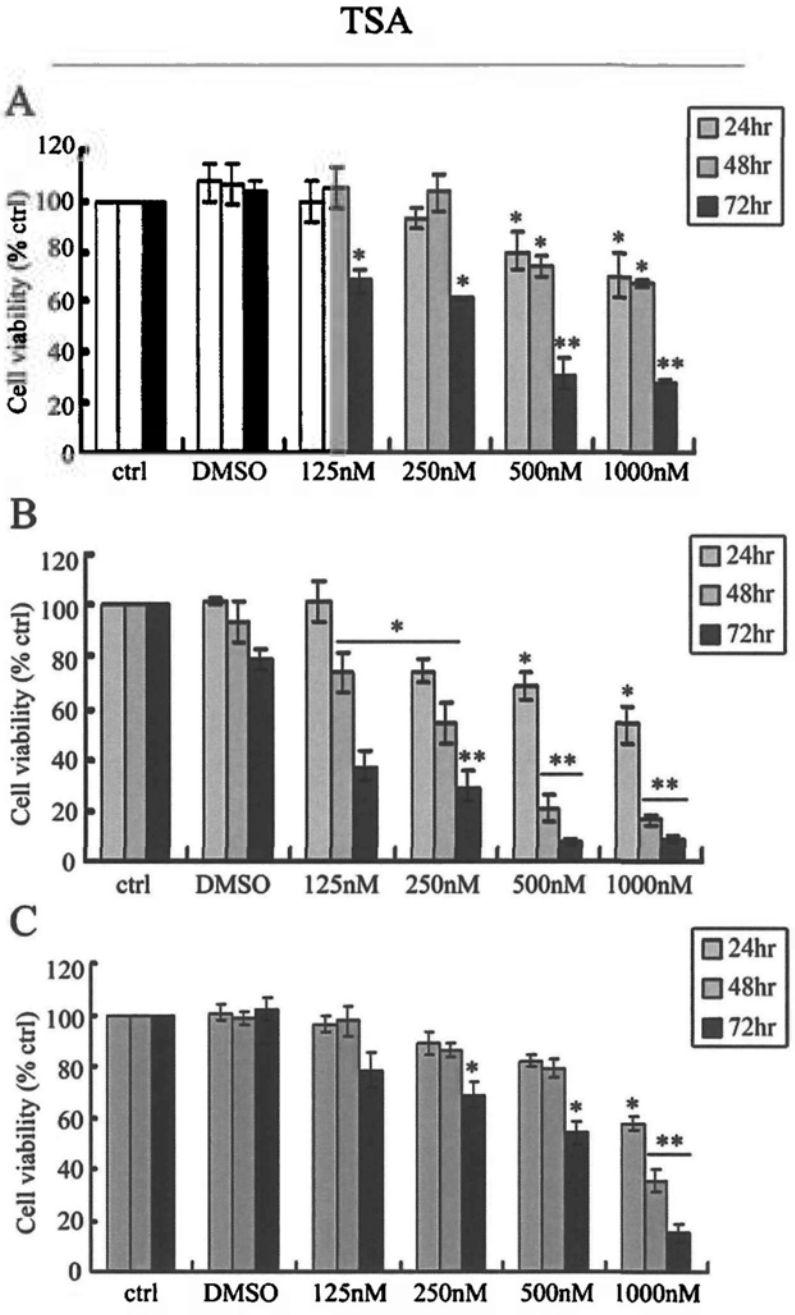

Figure 3.4 Cytotoxicity of TSA in HCC cells. Cells (**A.** Hep3B; **B.** PLC/PRF/5; **C.** HepG2) were seeded into 96-well plates and treated with various concentrations of TSA (0-1000 nM) for indicated periods. The cell viability was determined by MTT assay. The data represent mean ± SD of three independent experiments. $*P < 0.05$ and $**P < 0.01$, versus the control group.

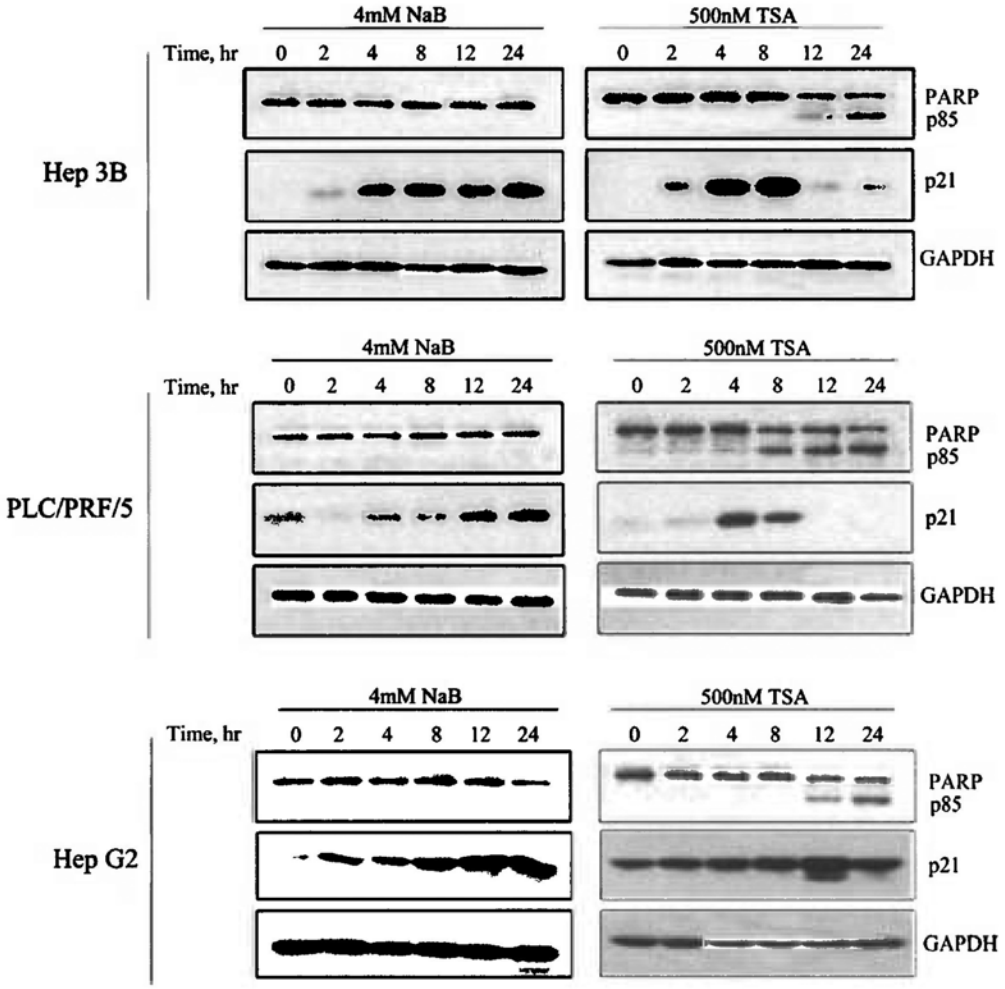

Figure 3.5 The effects of both agents on PARP and p21. Cells were seeded into 6-well plates and then treated with either 4 mM NaB or 500 nM TSA for 24 hr. Protein were collected by RIPA buffer and then subjected to Western blot analysis to examine the levels of apoptotic marker PARP and cell cycle inhibitor p21.

3.3.2 NaB-mediated p21 up-regulation requires ZBP-89

Next, we examined the effect of NaB on ZBP-89 and p21. NaB rapidly induced ZBP-89 in the early hour incubation (Figure 3.6). The induction appeared to be plateaued at 24-48 h after the treatment and the level of ZBP-89 was even decreased after 48 h in PLC/PRF/5 and HepG2 cells (Figure 3.7). The expression of p21 was continually increased by NaB following 24-96 h incubation in Hep3B cells. However, the level of p21 was peaked at 48 h incubation and subsequently decreased in PLC/PRF/5 and HepG2 cells, the pattern of which is similar to the changes seen in ZBP-89. Furthermore, caspase 3 was activated in all 3 HCC cell lines but the activation of caspase 3 was found earlier (at 24 h) in PLC/PRF/5 cells than the other 2 HCC cell lines (Figure 3.7). These data, plus MTT results, indicate that NaB may inhibit HCC cells without the presence of p53，however, wt p53 and mutant p53 can potentially influence the effect of NaB.

As shown in Figure 3.7, ZBP-89 seemed to be involved in NaB-induced p21 up-regulation. To test this hypothesis, we knocked down ZBP-89 with two different siRNAs. Our results showed that both ZBP-89 siRNAs effectively abolished NaB-induced expression of p21 (Figure 3.8). Therefore, it is concluded that ZBP-89 is required for NaB-mediated p21 induction.

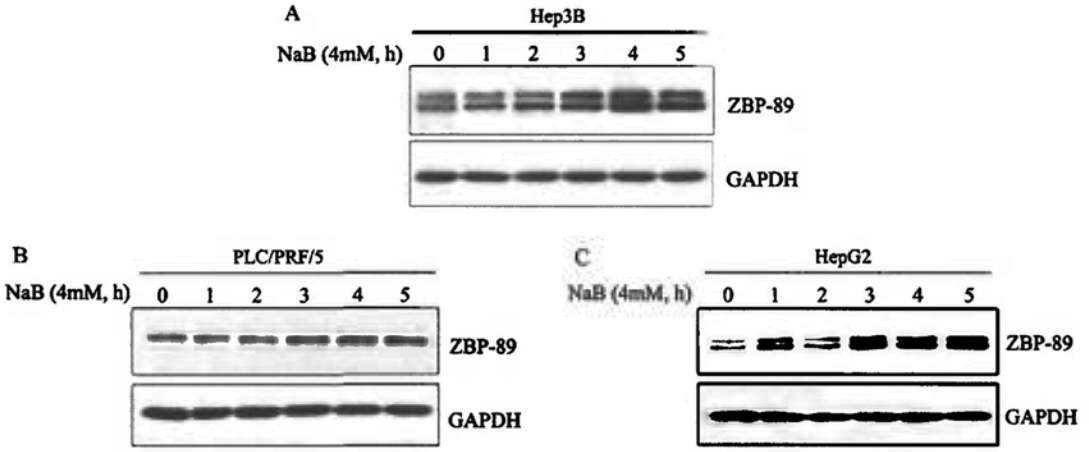

Figure 3.6 NaB treatment induces ZBP-89 expression in HCC cells. Hep3B, PLC/PRF/5, and HepG2 cells were seeded into 35 mm dishes and then incubated with 4 mM NaB for indicated periods. Cells were lysised in RIPA buffer to obtain total protein. The expressions of ZBP-89 in NaB-treated HCC cells were detected by Western blot and representative results are shown.

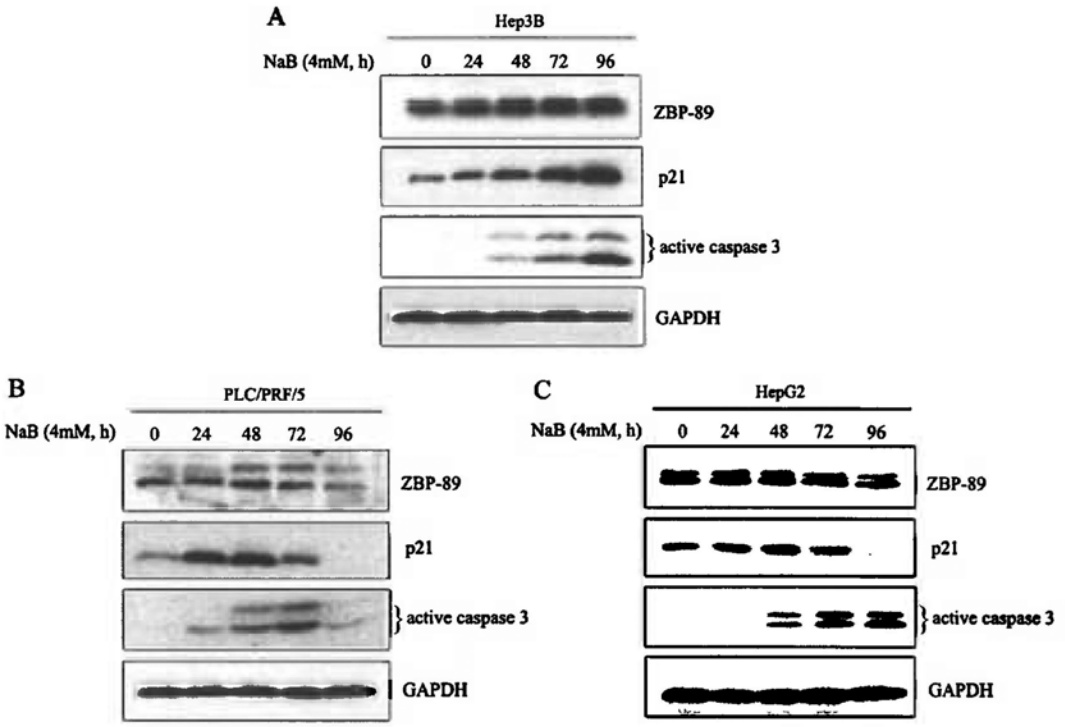

Figure 3.7 NaB treatment up-regulates p21. Hep3B, PLC/PRF/5, and HepG2 cells were seeded into 35 mm dishes and then treated with 4 mM NaB for indicated periods. The increased levels of p21 as well as active caspase-3 were detected and representative results are shown.

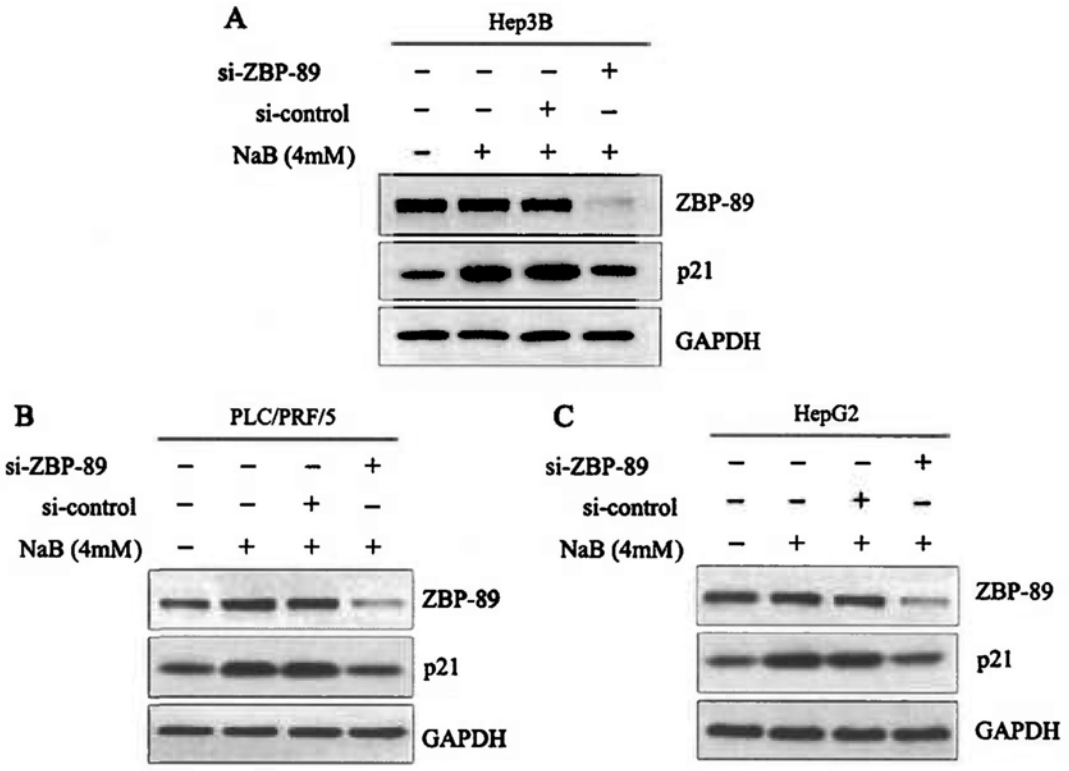

Figure 3.8 NaB-induced p21 up-regulation requires ZBP-89. Hep3B, PLC/PRF/5, and HepG2 cells were transfected with ZBP-89 siRNA or control siRNA for 24 h, followed by 4 mM NaB-treatment for another 24 h. The levels of ZBP-89 and p21 proteins were examined.

2.3.3 p53 mutants expressed in HCC tissues

According to our previous studies, p53 was frequently mutated in HCC patients, especially in exons 7 and 8 (Chen et al., 2003) (Table 3.3). Some of the p53 mutants were demonstrated to interact with ZBP-89. Therefore, we intended to determine whether p53 mutants identified in Hong Kong Chinese HCC patients would affect functions of ZBP-89.

We next constructed plasmids expressing the identified p53 mutants, using pcDNA 3.1 vector. To test the expressions of p53 mutants *in vitro*, we transfected the plasmids that express different 10 types of p53 mutants into Hep3B cells in which *p53* gene is knocked out, after the sequencing of prepared plasmids. Stable cell lines were established by G418 screening. Results showed that different p53 mutants possessed altered expressions. The expressions of p21 in stable cell lines were also examined, showing that none of the p53 mutants affected p21 expression (Figure 3.9).

Next we intended to determine whether p53 mutants respond to HDACi treatments. Cytotoxicities of NaB and TSA in stable cells expressing p53 mutants were determined by MTT assays (Figure 3.10). Interestingly, it seemed cells expressing $p53^{G245D}$ were less sensitive to TSA treatments, especially compared to those cells with $p53^{WT}$ and $p53^{R273C}$. To further test the effects of p53 mutants on HDACi, we treated the stable cells with either 4 mM NaB or 500 nM TSA for 24 hr and examined the expressions of p21, PARP and caspases. Results indicated that $p53^{G245D}$ might abrogate the inductions of p21, cleavages of PARP and caspases (Figure 3.11 and 3.12), which may suggest $p53^{G245D}$ resists cells to HDACi treatments.

Table 3.3 Mutations of the *p53* gene in HCC samples

p53 mutant	Codon	Exon	Nucleotide changes	Amino acid substitution
1	132	5	AAG → AGG	Lys → Arg (K→R)
2	157	5	GTC → TTC	Val → Phe (V→F)
3	163	5	TAC → TGC	Tyr → Cys (Y→C)
4	163	5	TAC → TCC	Tyr → Ser (Y→S)
5	175	5	CGC → CAC	Arg → His (R→H)
6	242	7	TGC → TAC	Cys → Tyr (C→Y)
7	245	7	GGC → GAC	Gly → Asp (G→D)
8	248	7	CGG → TGG	Arg → Trp (R→W)
9	249	7	AGG → AGT	Arg → Ser (R→S)
10	273	8	CGT → TGT	Arg → Cys (R→C)

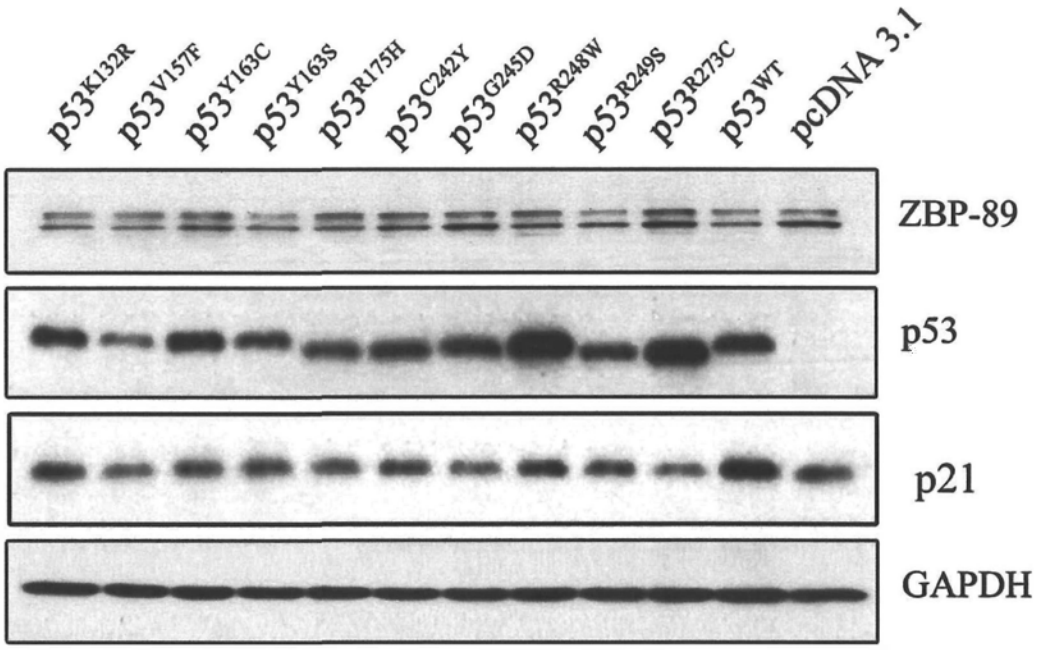

Figure 3.9 p53 mutants in HCC cells. Plasmids encoded 10 types of p53 mutants identified in Hong Kong HCC patients and wild-type p53 were transfected into Hep3B cells that express no p53 proteins for 24 hours. Cells were then selected by the antibiotic G418 for 4 weeks to establish stable cells. Proteins obtained from the stable cells were subjected to Western blot to examine the expressions of ZBP-89, p53 and p21. GAPDH was served as control.

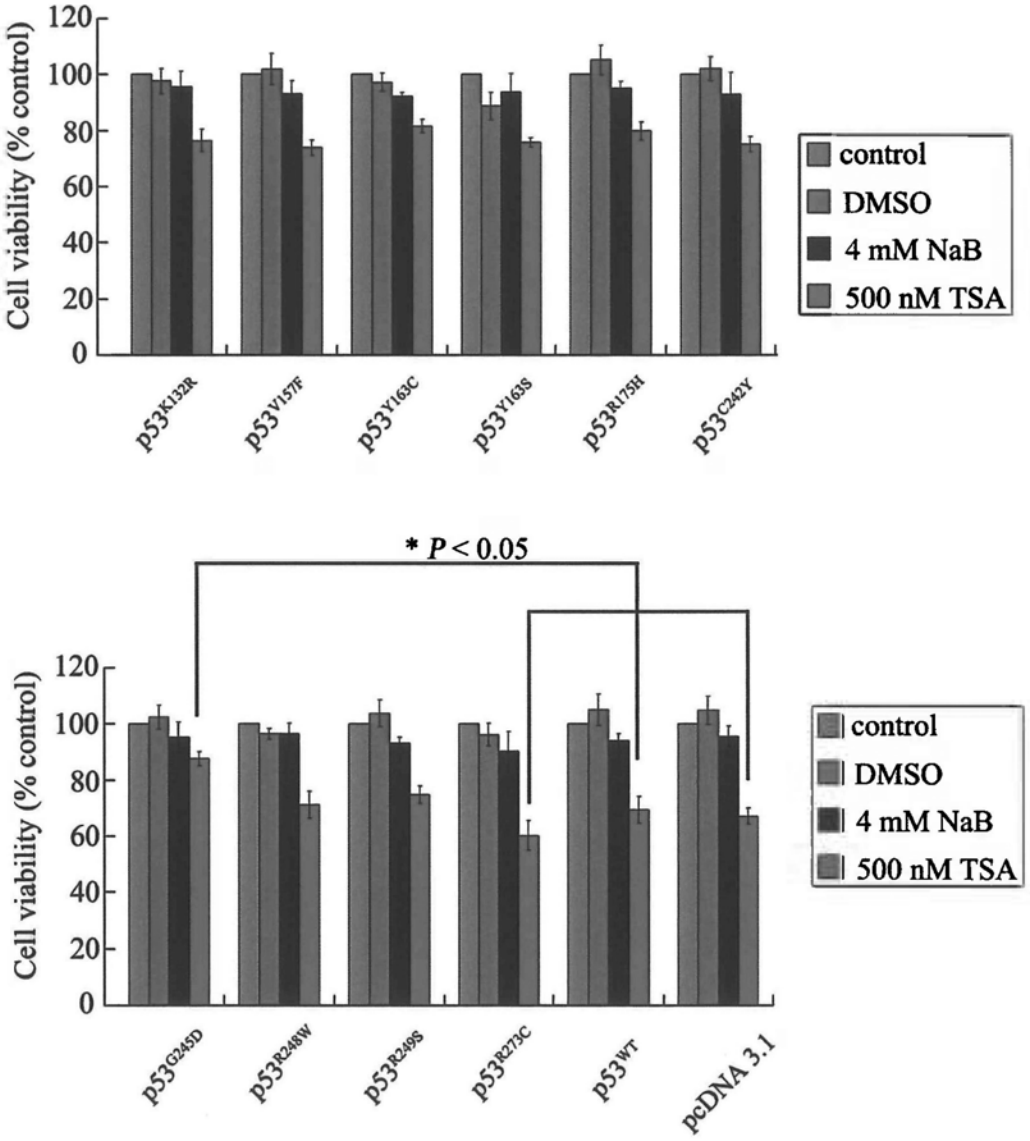

Figure 3.10 Cytotoxicities of NaB and TSA in cells expressing p53 mutants. Cells expressing p53 mutants and wild-type p53 were seeded into 96-well plates and treated with 4 mM NaB or 500 nM TSA for 24 hr. The cell viabilities were determined by MTT assay. The data represent mean ± SD of three independent experiments. *$P < 0.05$.

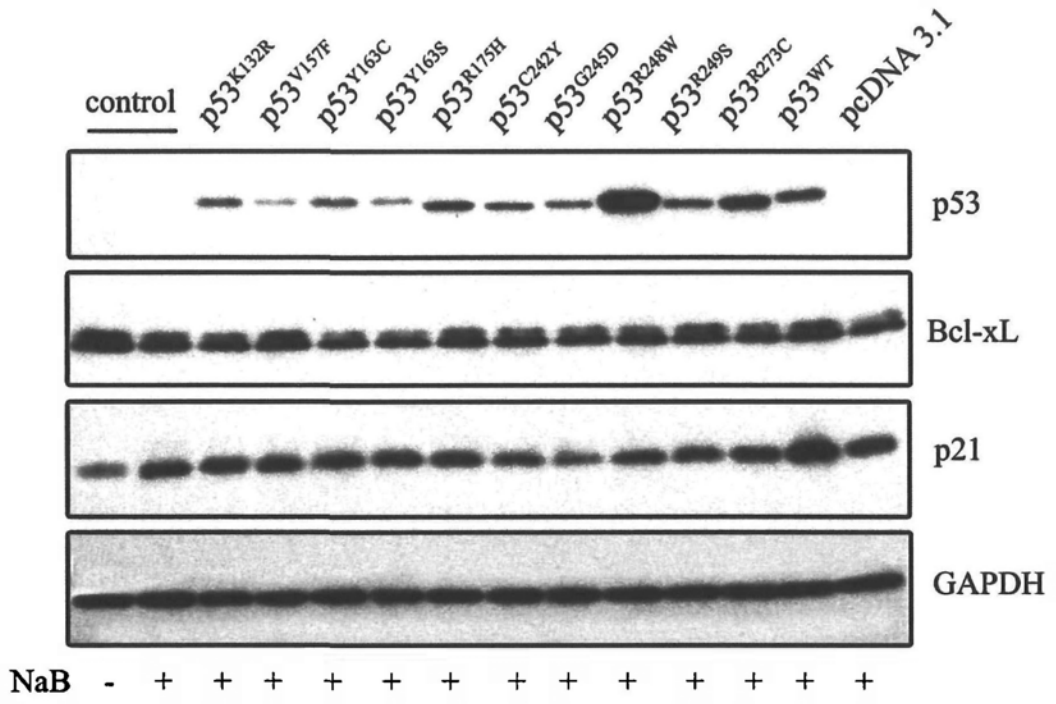

Figure 3.11 Effects of p53 mutants on NaB-induced p21 up-regulation. Stable cells expressing different p53 mutants were treated with 4 mM NaB for 24 hr. Cells were then lysised in RIPA buffer. And the expressions of p53, Bcl-xL, and p21 were detected to determine the effects of p53 mutants on p21 up-regulation induced by NaB treatment. Bcl-xL was used as a positive control for NaB treatment, as it has been proved to be down-regulated in Hep3B cells treated with NaB.

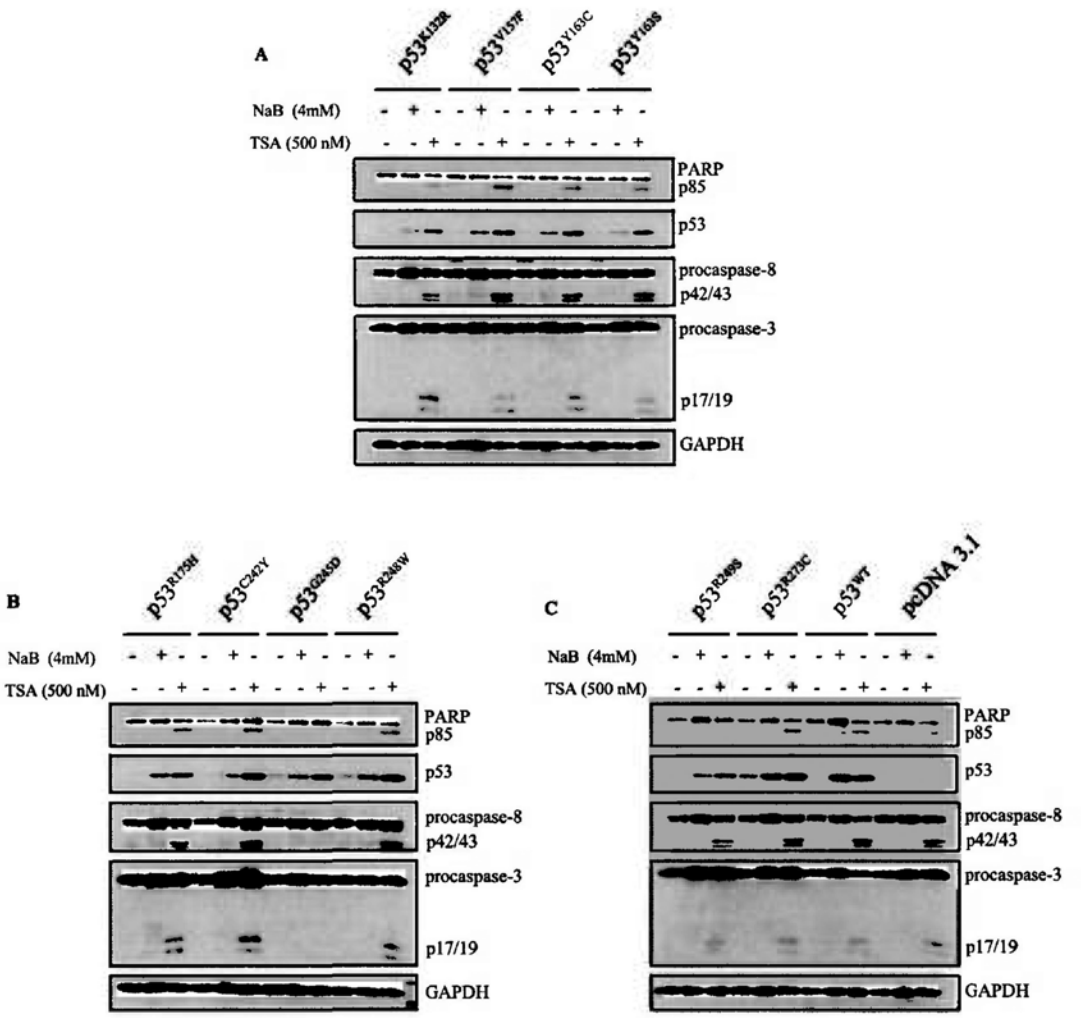

Figure 3.12 Effects of p53 mutants on TSA-induced cleavages of PARP and caspases. Stable cells were treated with either 4 mM NaB or 500 nM TSA for 24 hr. Proteins were collected by RIPA and subjected to Western blot analysis to examine the activations of PARP and caspases.

2.3.4 $p53^{G245D}$ and $p53^{R273C}$ were identified in HCC tissues

We next further confirmed the expressions of $p53^{G245D}$ and $p53^{R273C}$ in HCC tissues. Firstly, DNAs isolated from HCC tissues were subjected to analysis of p53 mutations. Among various p53 mutants identified, $p53^{G245D}$ (case #44) and $p53^{R273C}$ (case #94) were frequently found. Also, $p53^{WT}$ was found in patients such as the case #122. For $p53^{G245D}$, mutation (GGC → GAC) was found in codon 245, located in exon 7 (Figure 3.13A). For $p53^{R273C}$, mutation (CGT → TGT) was detected in codon 273, located in exon 8 (Figure 3.13B).

Next, we examine the expressions of both p53 mutants in HCC tissues by immunohistochemistry. $p53^{G245D}$ was present in the cytoplasm and nucleus, whereas $p53^{R273C}$ was mainly detected in nucleus, which is similar to the case of $p53^{WT}$. Interestingly, the majority of ZBP-89 was found to localize in cytoplasm when $p53^{G245D}$ was present. However, in the cases of $p53^{R273C}$ and $p53^{WT}$, ZBP-89 was expressed mainly in the nucleus (Figure 3.14). These data may suggest that the localization of ZBP-89 may be affected by p53 mutants.

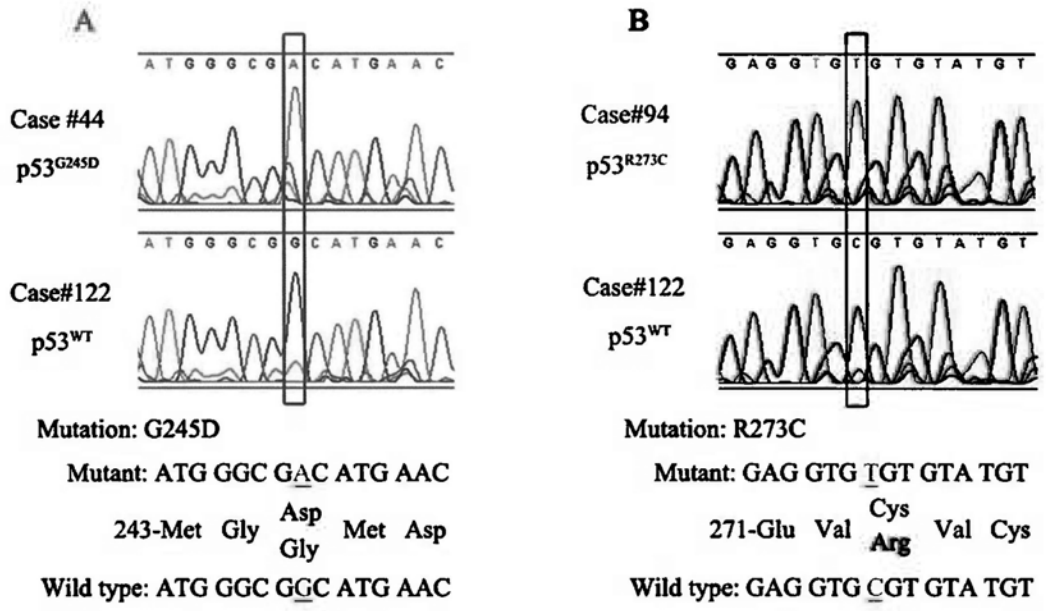

Figure 3.13 p53 mutants are identified in HCC. A. A p53 mutant, $p53^{G245D}$, was identified in liver tissues of HCC patients. DNAs extracted from frozen HCC tissues of Case #44 and #122 were subjected to sequencing analysis. The sequencing diagram is shown. Nucleotide and amino acid sequences of both wild-type and mutant p53 are also presented. **B.** Another type of p53 mutant, $p53^{R273C}$, was found in HCC tissues of Case #94.

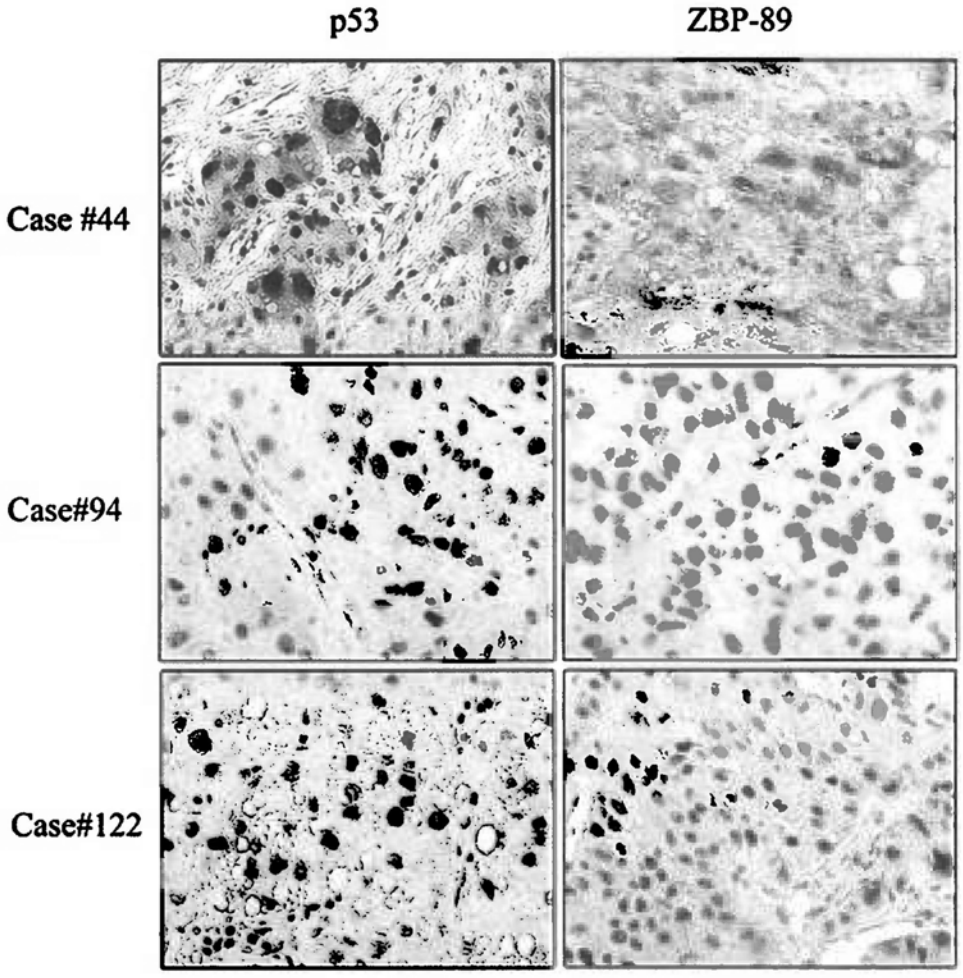

Figure 3.14 Expressions of both p53 mutants in HCC tissues. HCC tissues of Case #44, Case #94 and Case #122 were fixed in 4% PFA and then subjected to immunohistochemistry to detect the expressions of p53 mutants and ZBP-89. The representative microphotographs of p53 and ZBP-89 immunohistochemical staining in HCC tissues were shown (original magnification, 400×).

2.3.5 $p53^{G245D}$ binds to ZBP-89 and transports it to cytoplasm

Some p53 mutants were demonstrated to interact with ZBP-89 in our and others' previous studies (Chen et al., 2003; Okada et al., 2006). To examine whether the p53 mutants found in this study were associated with ZBP-89, $p53^{G245D}$ as well as $p53^{R273C}$ were transfected into Hep3B cells to establish stable cell lines. Using a conformation specific antibody that recognizes p53 mutant, we found that $p53^{G245D}$, but not $p53^{R273C}$, directly bound to ZBP-89 *in vitro* (Figure 3.15A). Furthermore, ZBP-89 was present in $p53^{G245D}$ immunoprecipitates, but not in normal mouse IgG precipitates. To further confirm the interaction of $p53^{G245D}$ and ZBP-89, plasmids harboring FLAG- ZBP-89 and HA-$p53^{G245D}$ were constructed. Hep3B cells were transfected with above plamids. Cell lysate was analyzed by immunoprecipitation with antibodies against FLAG and HA. Immunoblotting analysis revealed the binding of FLAG- ZBP-89 and HA-$p53^{G245D}$ but not $p53^{R273C}$ (Figure 3.15B).

ZBP-89 primarily localized to the nucleus under normal conditions, whereas p53 mutants can localize to either the nucleus or the cytoplasm or both sites simultaneously, depending on the site mutated. As shown in Figure 3.16, ZBP-89 had diffuse fractions in the cytoplasm and co-localized with $p53^{G245D}$, which could not be observed in the control and $p53^{R273C}$ groups. The findings suggest that the localization of ZBP-89 might be modulated by $p53^{G245D}$.

To further investigate the effect of $p53^{G245D}$ on the localization of ZBP-89, we isolated the cytoplasmic and nuclear fractions then examined the expression patterns of ZBP-89 and p53 mutants in both fractions. Our results showed that $p53^{G245D}$ was mainly localized in the cytoplasm and nucleus, while $p53^{R273C}$ remained exclusively

in the nucleus. ZBP-89 expression was not affected by p53 mutants, but its distribution between the nucleus and the cytoplasm was affected. Using differential centrifugation to separate the nucleus from the cytoplasm of Hep3B cells transfected with either p53 mutant, we confirmed preferential distribution of ZBP-89 to the cytoplasm with $p53^{G245D}$ and to the nucleus with $p53^{R273C}$ or the vector control (Figure 3.17).

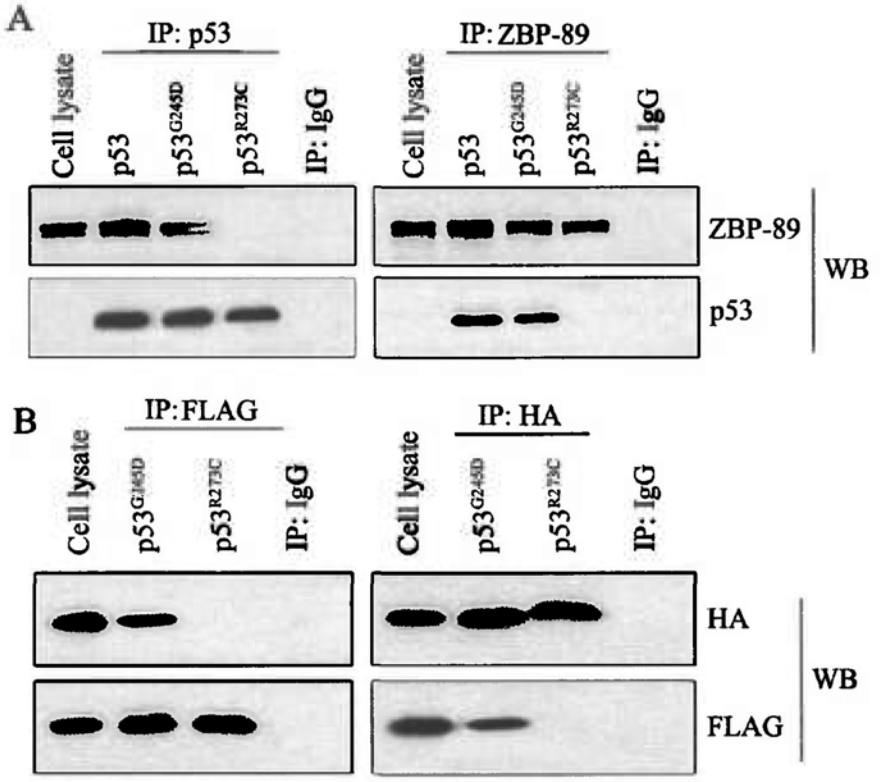

Figure 3.15 $p53^{G245D}$ directly binds to ZBP-89 *in vitro*. **A.** Hep3B cells were transfected with pcDNA 3.1-$p53^{WT}$, pcDNA 3.1-$p53^{G245D}$ or pcDNA 3.1-$p53^{R273C}$ for 36 h. Total proteins were extracted by RIPA plus protease cocktail. A primary antibody that recognizes ZBP-89 or p53 mutants was added to the pre-cleared proteins. After incubated overnight at 4°C, the protein-antibody complex was deposited by Protein A/G-agarose. The whole complex was boiled and Western blot was performed to check $p53^{G245D}$- or/and $p53^{R273C}$- binding proteins. (IgG served as a negative control for antibodies). **B.** $p53^{G245D}$ and $p53^{R273C}$ were tagged with HA. ZBP-89 was fused to FLAG. Plasmids were then transfected into Hep3B cells. Immunoprecipitations were performed to examine the interaction between $p53^{G245D}$ and ZBP-89, using antibodies that recognize HA and FLAG.

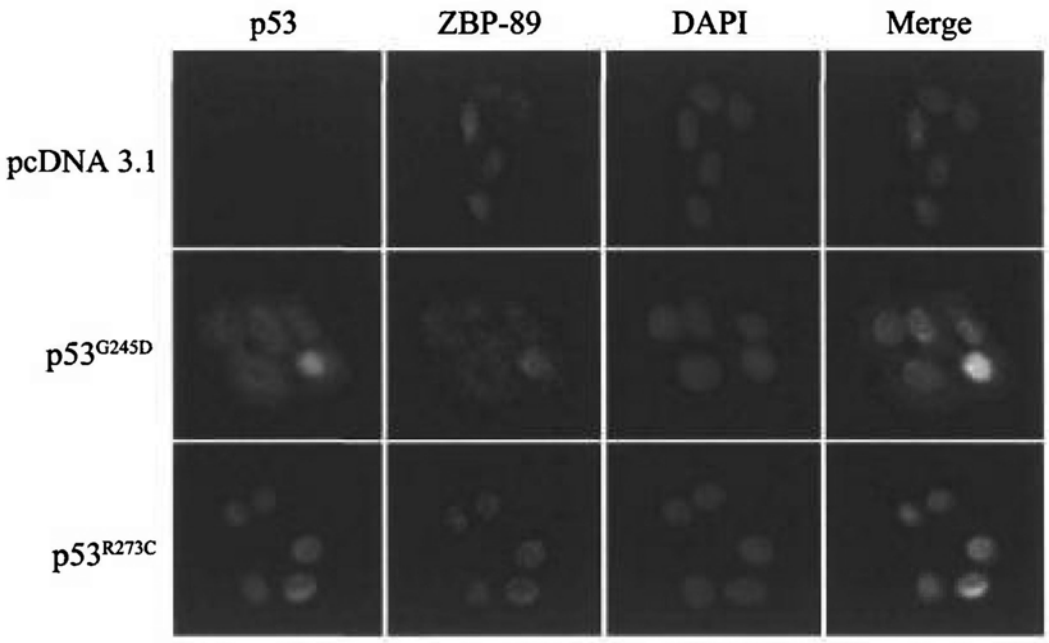

Figure 3.16 Immunofluorescence data indicate the co-localization of p53 mutants and ZBP-89. Cells expressing $p53^{G245D}$ or $p53^{R273C}$ were fixed by 4% PFA and incubated with p53 and ZBP-89 antibodies overnight at 4°C. After being stained with fluorescence secondary antibodies and DAPI, cells were observed under fluorescence microscope.

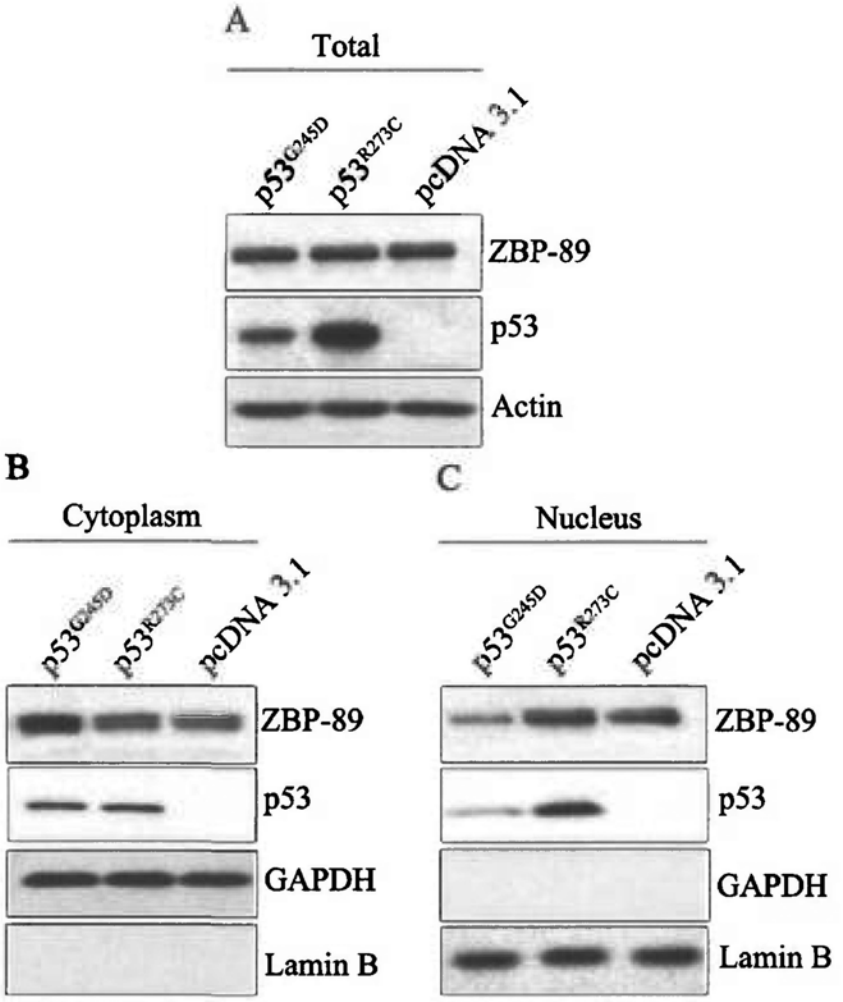

Figure 3.17 $p53^{G245D}$ but not $p53^{R273C}$ alters the cellular localization of ZBP-89. Fraction experiments show that $p53^{G245D}$ affects cellular localization of ZBP-89. Cells expressing $p53^{G245D}$ or $p53^{R273C}$ were fractioned into cytoplasm and nucleus. Levels of p53 and ZBP-89 in each fraction were detected. GAPDH and lamin B were used as the cytoplasmic and the nuclear markers, respectively.

2.3.6 $p53^{G245D}$ abrogates p21 up-regulation induced by NaB

Since $p53^{G245D}$ was capable of altering the cellular localization of ZBP-89 that was essential for NaB-induced p21 induction, we next examined if $p53^{G245D}$ influenced the effect of NaB in HCC cells. The mRNA and protein levels of p53 and p21 in stable cell lines were determined by RT-PCR and Western blot (Figure 3.18), respectively. Results demonstrated that neither of these two p53 mutants affected p21 mRNA or protein expression.

Next, cells were treated with 4 mM NaB for 24 h, and the levels of ZBP-89, p53 and p21 proteins were measured by Western blot. NaB induced p21 in the presence of $p53^{R273C}$. However, there was no significant induction of p21 expression at mRNA and protein levels following NaB treatment in HCC cells with $p53^{G245D}$ (Figure 3.19). Therefore, $p53^{G245D}$, but not $p53^{R273C}$, was able to abrogate NaB-mediated induction of p21 mRNA and protein. Both mutants did not affect the amount expression of ZBP-89.

To further confirm the negative effect of $p53^{G245D}$ on NaB-mediated p21 up-regulation, we measured the change of cell cycle using flow cytometry. In control and $p53^{R273C}$ groups, after 4 mM NaB treatment for 24 h, the percentages of G0/G1 phase cells were dramatically increased from 51% ± 3.2% to 71.3% ± 3% and from 51% ± 3.2% to 80.5% ± 1.4%, respectively, whereas the percentages of G0/G1 phase cells were hardly increased in $p53^{G245D}$ group (Figure 3.20).

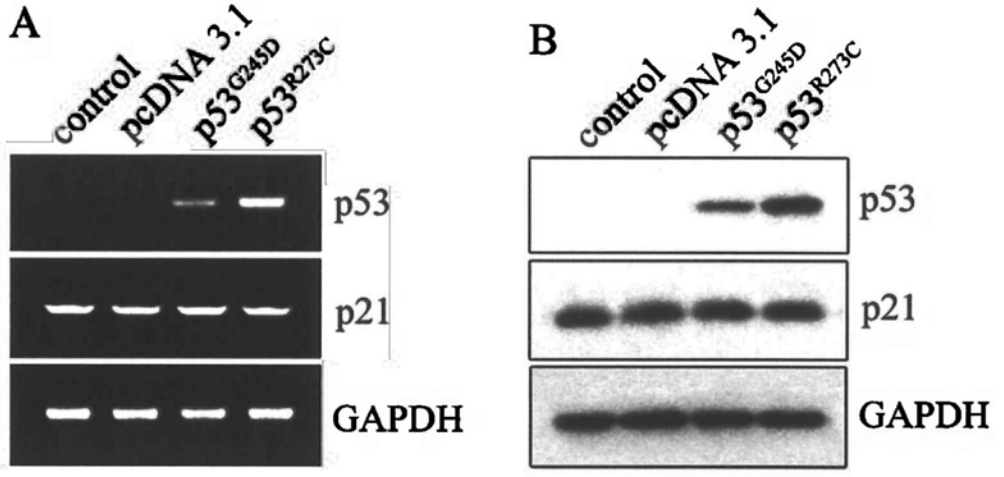

Figure 3.18 Both $p53^{G245D}$ and $p53^{R273C}$ do not affect the expression of p21. Hep3B cells were transfected with pcDNA 3.1-$p53^{G245D}$ and pcDNA 3.1-$p53^{R273C}$ to establish stable cell lines. p53 and p21 were determined at both mRNA **(A)** and protein levels **(B)**.

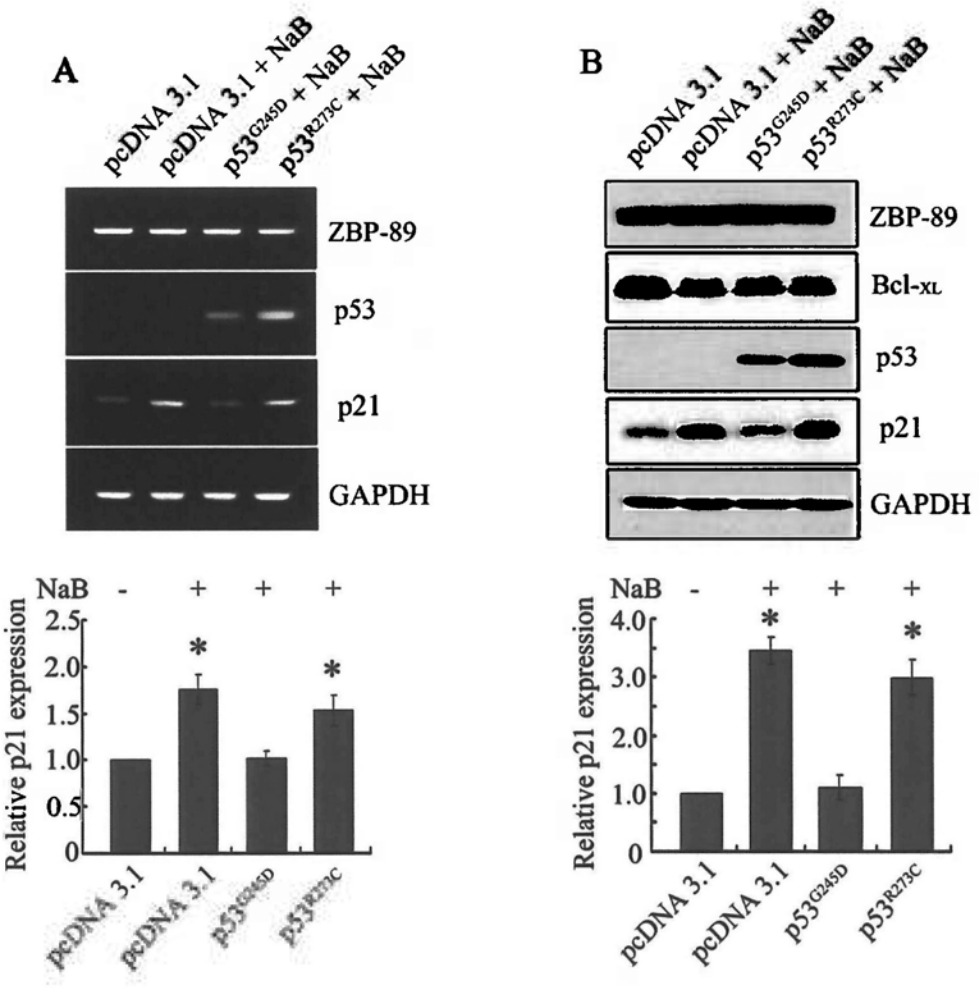

Figure 3.19 p53^{G245D} abrogates NaB-mediated p21-upregulation. A. Stable cells were treated with 4 mM NaB for 24 h. Total RNA was extracted by Trizol reagent. RT-PCR was performed to measure the expression of ZBP-89, p53 and p21. (Upper panel: representative results of 3 independent experiments. Bottom panel: relative expression of p21 mRNA.). **B.** 30 μg of proteins extracted from cells treated as described in **A** were used in Western blot experiments. Levels of ZBP-89, p53 and p21 proteins were detected. Bcl-xL was used as a positive control for NaB treatment, as it has been proved to be down-regulated in Hep3B cells treated with NaB. (Upper panel: representative results of 3 independent experiments. Bottom panel: relative expression of p21 protein.). *$P < 0.05$ versus the control group.

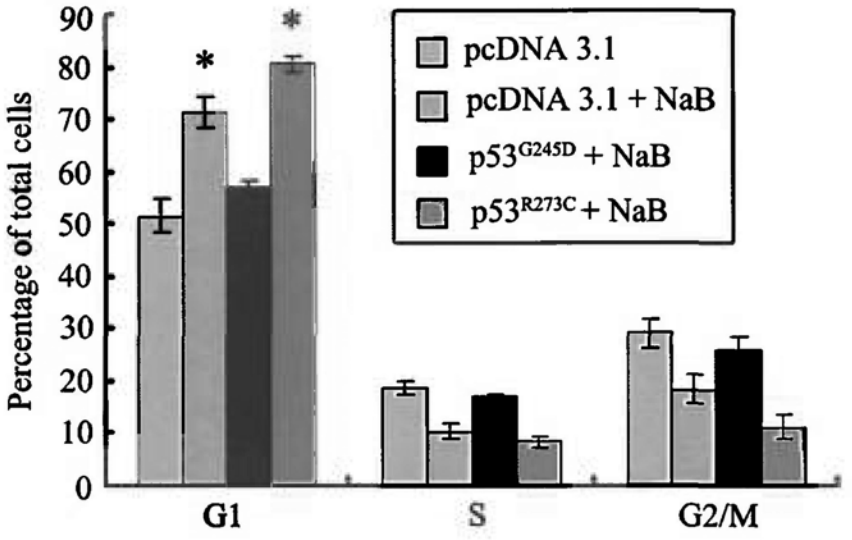

Figure 3.20 $p53^{G245D}$ abrogates G1 phase arrest induced by NaB. Cells that express $p53^{R273C}$ or $p53^{G245D}$ were treated with 4 mM NaB for 24 h. The number of cells in each cell cycle was counted by flow cytometry. The data represent mean ± SD of three independent experiments. *$P < 0.05$ versus the control group.

2.3.7 $p53^{G245D}$ protects cells from death induced by TSA

Next, we tested if $p53^{G245D}$ influenced TSA-induced apoptosis. We first demonstrated reduced viability with cells transfected with $p53^{R273C}$ but not $p53^{G245D}$ (Figure 3.21). Compared to the control and $p53^{R273C}$ groups, $p53^{G245D}$ significantly reduced cell death induced by TSA compared with $p53^{R273C}$. Furthermore, apoptotic bodies are decreased in $p53^{G245D}$-expressing cells (Figure 3.22). The finding was further confirmed by TUNEL assays (Figure 3.23 and 3.24). After the 24 or 48 h treatment with 500 nM TSA, we observed apoptosis in all three groups of transfected cells but the apoptotic levels were quite different. For instance, with the 24 h treatment, the percentage of apoptotic cells in the vector control, $p53^{G245D}$ and $p53^{R273C}$ expressing cells were 33.1%, 17.4% and 56.7%, respectively. At 48 h after TSA treatment, the corresponding apoptotic rates were 53.1%, 30.9% and 71.4% respectively (Figure 3.23). All these results indicate that the functions of $p53^{G245D}$ and $p53^{R273C}$ are totally different, with the former protected cells from TSA-mediated apoptosis whereas the latter enhanced apoptosis.

In most cases, apoptosis is mediated by activated caspases. Our Western blot analysis indicated that the caspase family was involved in TSA-mediated apoptosis (Figure 3.25). Following TSA treatment, caspase 8 was activated and Bid was cleaved, which leaded to the activation of caspase 3 and finally PARP, a major target of the executioner caspase. PARP cleavage was significantly reduced in cells expressing $p53^{G245D}$, but increased in cells expressing $p53^{R273C}$. These data confirm that $p53^{G245D}$ enables cells resistant to TSA treatment by modulating the apoptotic proteins.

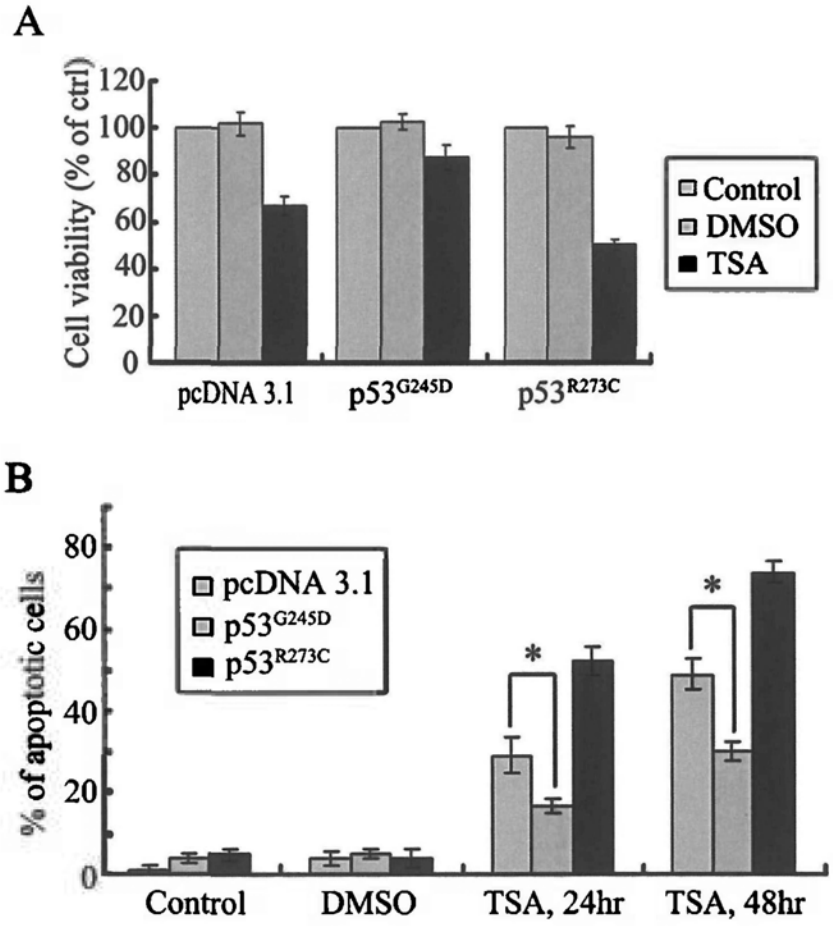

Figure 3.21 $p53^{G245D}$ protects cells from TSA treatment to which $p53^{R273C}$ sensitizes cells. A. Cytotoxicity of 500 nM TSA in Hep3B cells that express p53 mutant. Hep3B cells were transfected with pcDNA 3.1-$p53^{G245D}$ or pcDNA 3.1-$p53^{R273C}$ for 24 h, and then treated with 500 nM TSA for another 24 h. Cell viabilities were detected by MTT assay. **B**. Apoptotic effect of TSA on cells that express $p53^{G245D}$ or $p53^{R273C}$ was measured by Flow Cytometry. The data represent mean ± SD of three independent experiments. $*P < 0.05$ versus the control group.

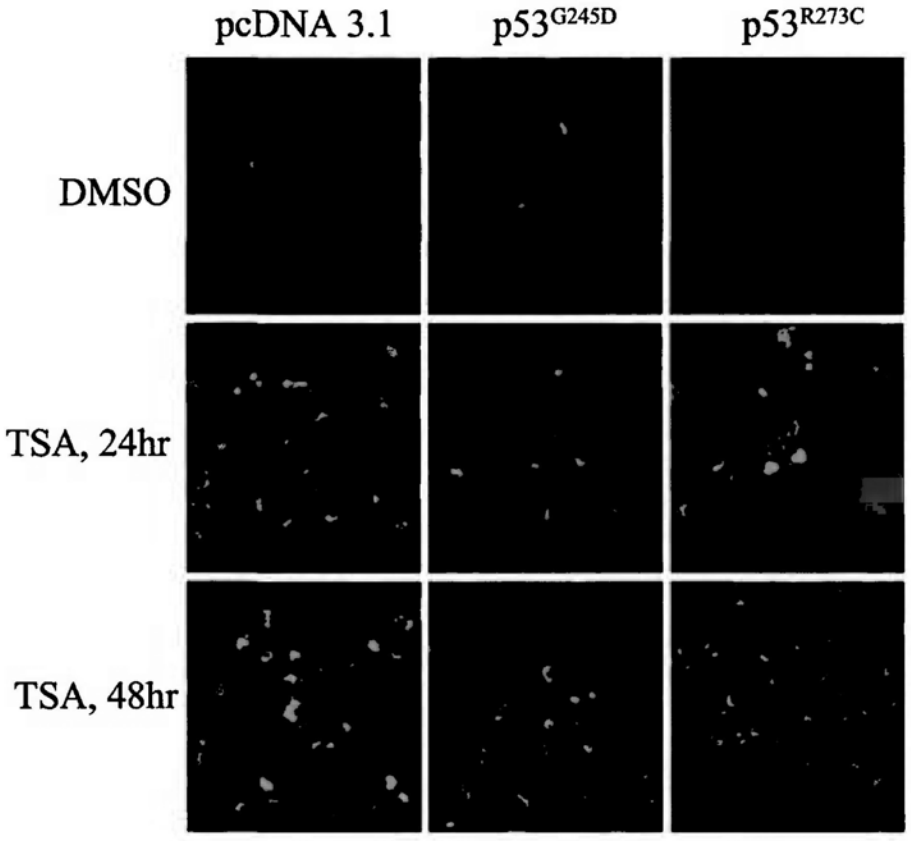

Figure 3.22 Apoptotic bodies are decreased in p53^{G245D}-expressing cells. Hep3B cells were transfected with pcDNA 3.1-p53^{G245D} or pcDNA 3.1-p53^{R273C} for 24 h, and then treated with 500 nM TSA for indicated periods.Cells were then fixed by 4% PFA and then stained with Hoechst. Apoptotic bodies were observed under fluorescent microscope. (Mag. ×400)

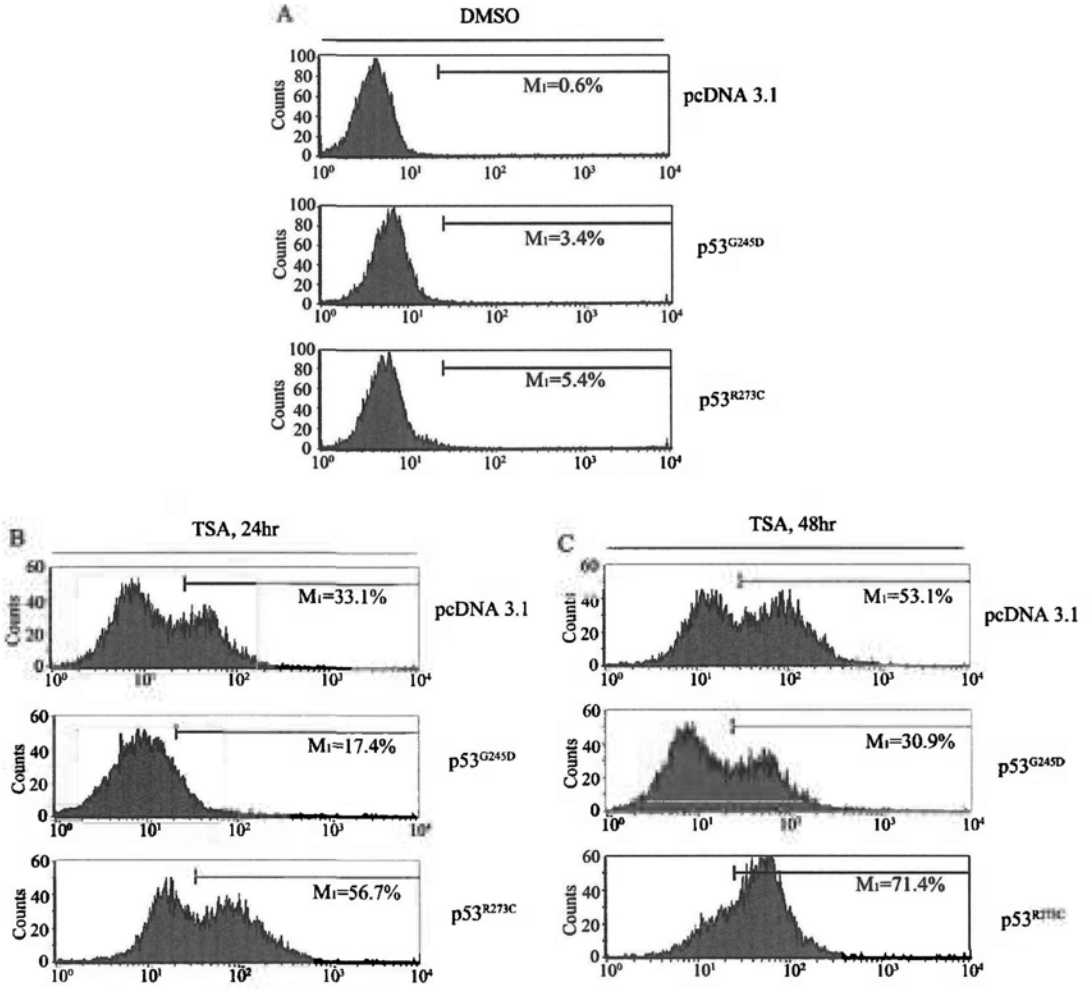

Figure 3.23 TUNEL assay results indicate that less apoptosis is induced in p53^{G245D}-expressing cells. Hep3B cells were transfected with pcDNA 3.1-p53^{G245D} or pcDNA 3.1-p53^{R273C} for 24 h, and then treated with 500 nM TSA for indicated periods. Cells were fixed and subjected to TUNEL assays by flow cytometry. Representative data were shown.

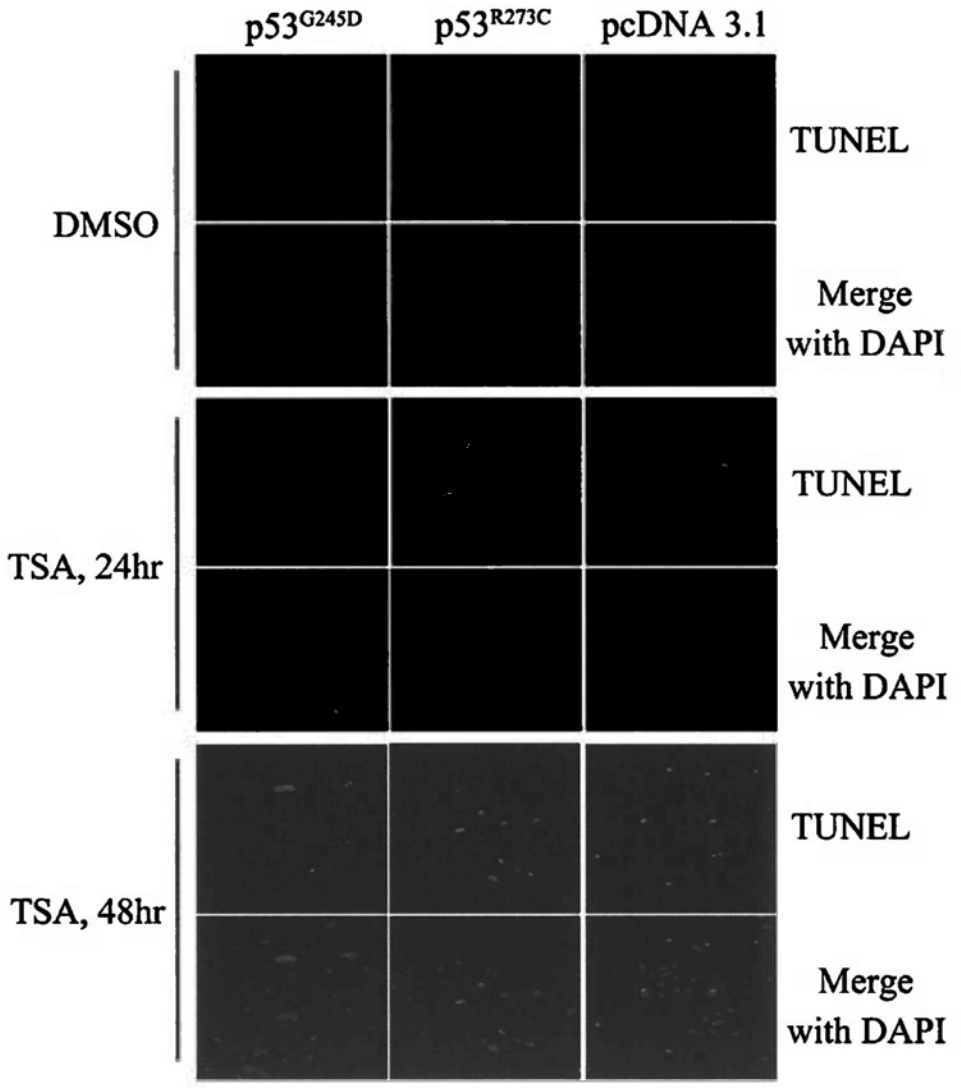

Figure 3.24 *In Situ* cell deaths are decreased in $p53^{G245D}$-expressing cells. The effects of $p53^{G245D}$ and $p53^{R273C}$ on TSA-mediated cell death were determined by TUNEL assay using *In Situ* Cell Death Detection Kit.

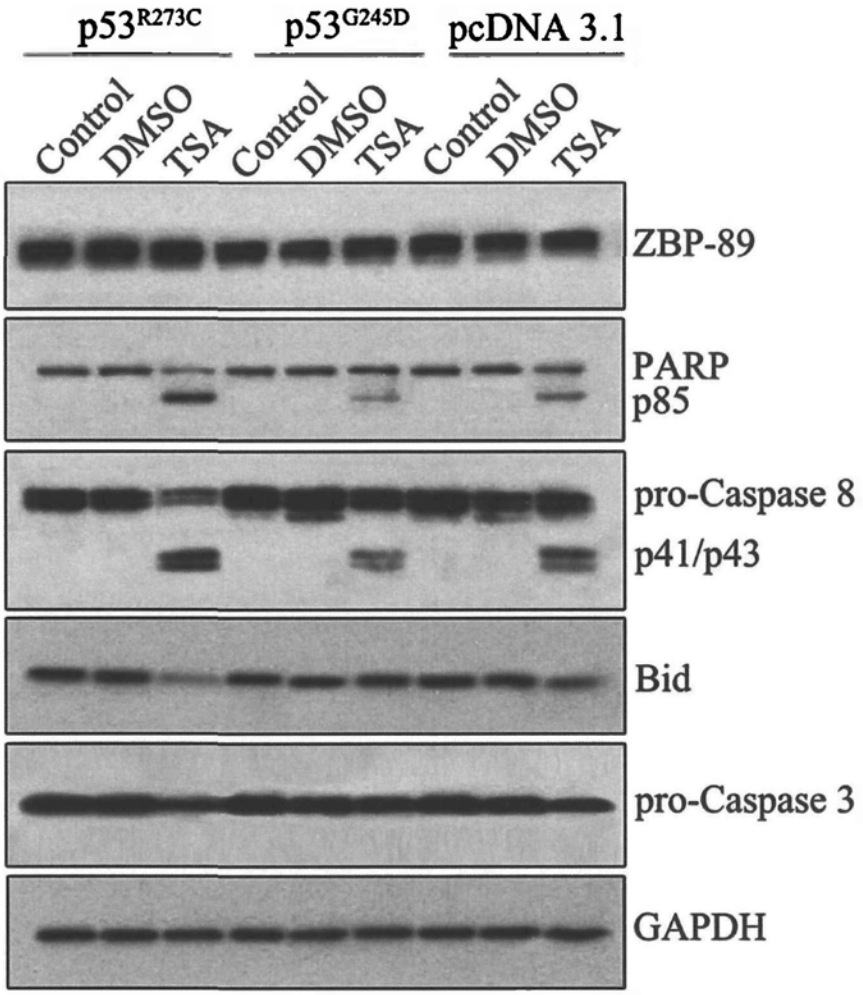

Figure 3.25 PARP cleavage and caspases-8 activation are involved in TSA-induced apoptosis. Some apoptotic molecules were detected after TSA treatment for 24 hr in p53 mutants expressing cells.

3.3.8 $p53^{G245D}$ prevents cells from ZBP-89-induced cell death.

We have previously reported that ZBP-89 over-expression resulted in apoptosis (Bai et al., 2004; Chen et al., 2009). Using plasmids that encode full length ZBP-89 and deletion mutant ZBP-89 which is based on the biological structure of ZBP-89 (Figure 3.26A), we found that ectopic expression of ZBP-89 activated PARP (Figure 3.26B), which may subsequently induce apoptosis. However, unlike the case of over-expression of ZBP-89-FL and ZBP-89-Δ298-447, excess expression of ZBP-89-Δ6-180 and ZBP-89-Δ180-298 proteins could not significantly increase PARP cleavage (Figure 3.26B).

In our early study, we have demonstrated that ZBP-89 enhanced the lethal effectiveness of 5-fluorouracil or staurosporine in HCC cells (Chen et al., 2009). Therefore, we checked whether ZBP-89 influences TSA treatment. Firstly, the cellular localizations of ZBP-89 deletion mutants were determined by immunofluorescent staining (Figure 3.27), since ZBP-89 functions depend on its position. Secondly, Western blot analysis was performed to examine the effects of ZBP-89 on TSA treatment. The result showed that cells with ZBP-89 and ZBP-89-Δ298-447 over-expression were more sensitive to TSA treatment, as these cells had a significant increase in cleaved PARP, cleaved caspase 8 and tBid. The levels of these apoptotic markers were not significantly altered in cells with over-expression of ZBP-89-Δ6-180 or ZBP-89-Δ180-298, compared with the control (pcDNA3.1+TSA) (Figure 3.28).

Next, we cotransfected $p53^{G245D}$ and ZBP-89-FL plasmids into Hep3B cells to further investigate whether $p53^{G245D}$ could affect ZBP-89-induced apoptosis. When ZBP-89

was over-expressed, the percentages of apoptotic cells in cells with and without $p53^{G245D}$ were 8.7% and 15.8%, respectively (Figure 3.29A). At 24 h after TSA treatment, the corresponding percentages of the apoptotic death in cells with and without $p53^{G245D}$ were 25.1% and 41.2%, respectively. However, in $p53^{R273C}$ expressing cells, the percentages of apoptotic cells were not significantly changed, compared with the control. There data suggest that $p53^{G245D}$ may protect cells from ZBP-89-induced apoptosis, and this finding was further confirmed by Western blot analysis that showed less PARP was activated in $p53^{G245D}$ expressing cells (Figure 3.29B).

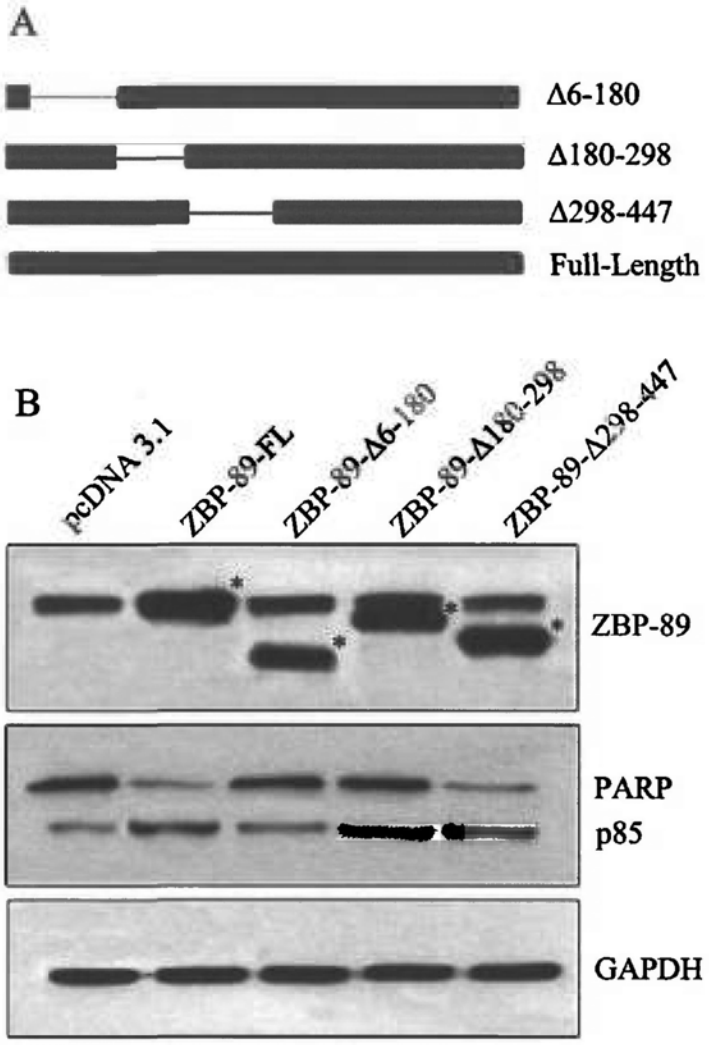

Figure 3.26 Ectopic expression of ZBP-89 in Hep3B induces PARP cleavage. A. the full-length ZBP-89 and deletion mutant ZBP-89 were cloned. **B.** Hep3B cells were transfected with ZBP-89 plasmids for 24 h and then the total protein was extract for Western blot. Expression of apoptotic marker PARP was measured.

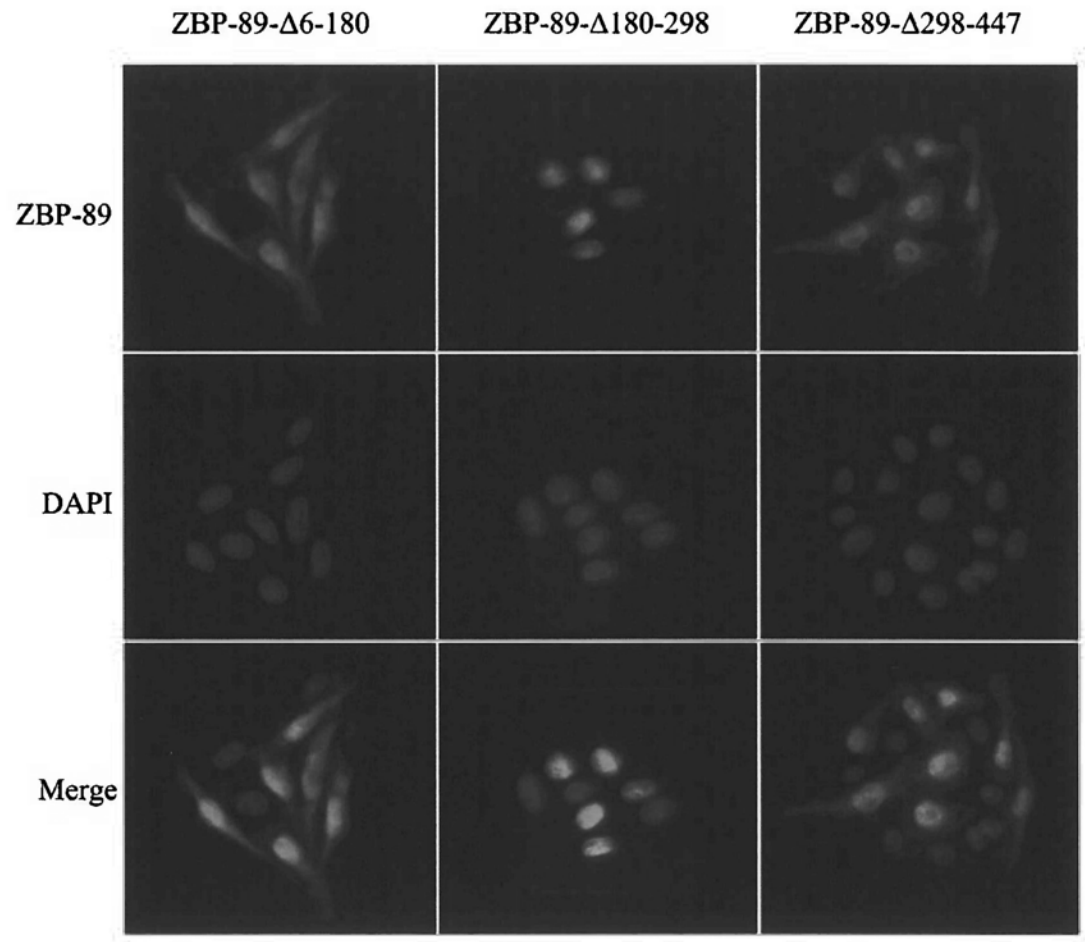

Figure 3.27 Expressions of ZBP-89 deletion mutants in HCC cells. ZBP-89 deletion mutants (ZBP-89-Δ6-180, ZBP-89-Δ180-298, and ZBP-89-Δ298-447) were transfected into Hep3B cells for 24 hours. Cells were fixed for 20 min in PBS containing 4% PFA, permeabilized in 0.1% Triton X-100 for 2 x 5 min and incubated in blocking buffer for 1 h. Cells were then incubated in antibody dilution buffer for ZBP-89 for 2 h in room temperature and then incubated with the appropriate fluorochrome-conjugated secondary antibody for 1 h. DNA was stained DAPI. The amount and subcellular localization of ZBP-89 mutants were observed under fluorescent microscope in cells (All fields ×400).

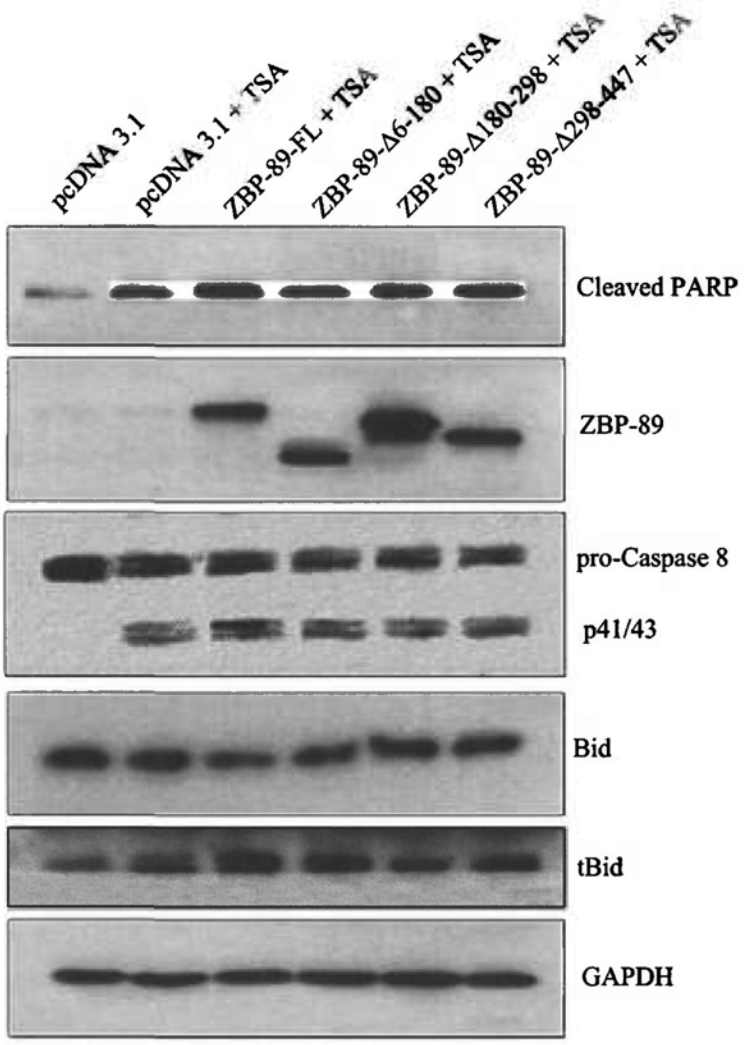

Figure 3.28 Over-expression of ZBP-89 enhances the effect of TSA. Cells were transfected with ZBP-89 plasmids for 24 h, and then treated with 500 nM TSA for another 24 h. The cleaved forms of PARP, caspase 8 and Bid were detected.

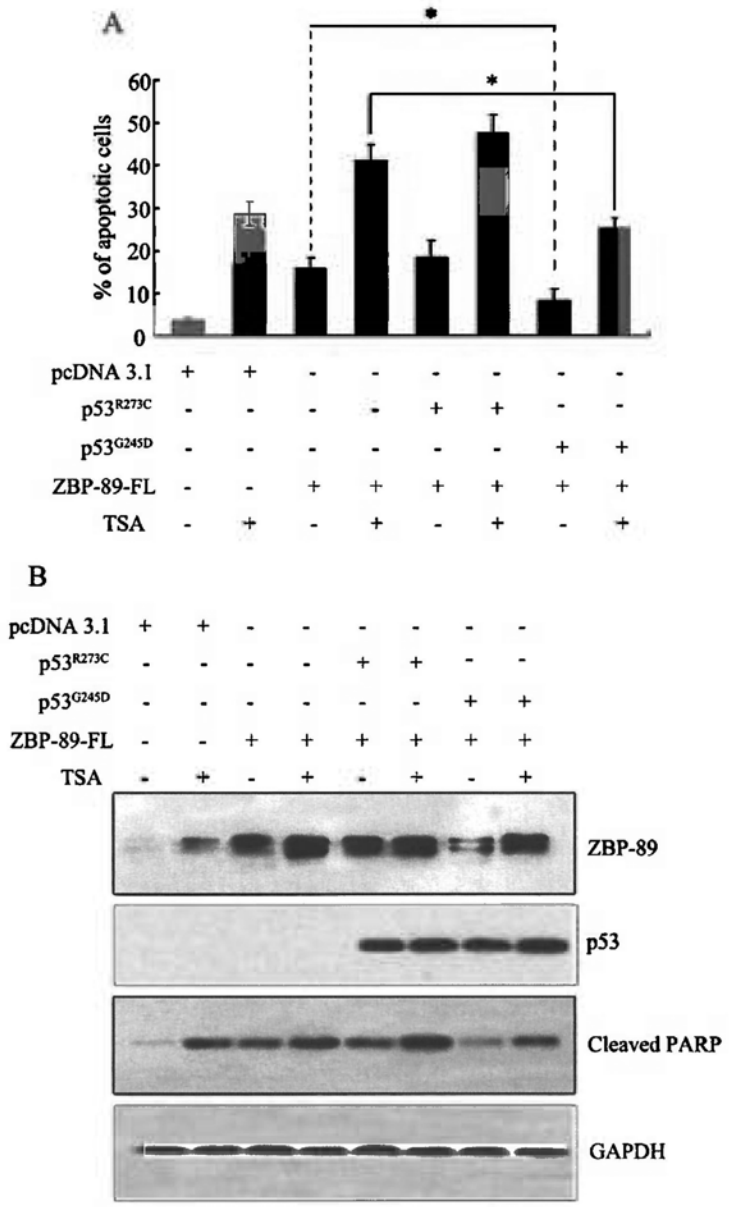

Figure 3.29 p53^{G245D} prevents cells from ZBP-89-induced cell death. A. TUNEL assays show that p53^{G245D} reduces ZBP-89-induced apoptosis. Plasmids encoding full-length ZBP-89, p53^{G245D}, or p53^{R273C} were transfected into Hep3B cells. After treatment with 500 nM TSA for 24 h, cells were subjected to TUNEL assays. **B.** Western blot analysis confirms the protective role of p53^{G245D} against ZBP-89-induced apoptosis. Proteins extracted from cells treated as described in **A** were prepared for detection of active PARP.

3.4 Discussion

The current study presents compelling evidence that mutant p53 modulates HDACi-mediated cell growth/death. Our data clearly show that HCC cells containing $p53^{G245D}$ and $p53^{R273C}$, two p53 mutants occurred frequently in the local HCC samples, respond differently to HDACi treatment, with the former being inhibitory but the latter being promotive. Since HCC cell lines naturally containing these two p53 mutants are not available, two approaches were used. First, we used HCC cells with p53 deletion (Hep3B), containing natural wt p53 (HepG2) and mutant $p53^{R249S}$ (PLC/PRF/5) to confirm that the sensitivity of HCC cells with different p53 status to HDACi is significantly different (Figure 3.30 and 3.31). Second, we used $p53^{G245D}$ and $p53^{R273C}$ plasmids to establish two stable HCC cell lines to explore the responsible mechanism. The findings reveal that $p53^{G245D}$, but not $p53^{R273C}$, attenuated NaB-mediated p21 induction by modulating the cellular localization of ZBP-89 that led to the inhibition of p21 transcription and eventually the prevention of the lethal effect of TSA on HCC cells. These data suggest a potential role of the mutant p53 in chemotherapy resistance via modulating ZBP-89 (Figure 3.31).

HDACi have been as the intense focus of possible therapies for cancers (Arts et al., 2009; Marks and Xu, 2009). Recently, accumulating evidence suggest HDACi can be used as a new class of anticancer drug due to their selective toxicity and synergistic activity with chemotherapeutic agents. Currently HDACi is in phase I of clinical trials (Cang et al., 2009). In our present study, NaB and TSA were shown to induce either cell cycle arrest or apoptosis. NaB, produced in colon and transported to liver, acutely increased the expression of ZBP-89, a transcriptional factor that regulates

cell growth, and subsequently induced p21 expression, resulting in G1 phase arrest. Zhou *et al.* reported that NaB regulates *α-fetoprotein* and *albumin* gene expression, increases the anti-tumor effect of interferon-α by enhancing the expression of STAT1 (Zhou et al., 2007). Recent studies have suggested that TSA has a promising therapeutic effect on cancer cells. We found that TSA at low doses efficiently induced cell death in HCC cells with p53 deletion or with certain p53 mutants including $p53^{R273C}$ and $p53^{R249S}$. These data confirm that both NaB and TSA are potential candidates as anticancer drugs, which may particularly benefit a subset of HCC that contain certain types of p53 mutants and are frequently resistant to conventional chemotherapy (Figure 3.31). The cyclin-dependent kinase inhibitor p21 is a key gene target that mediates HDACi action (Dagtas et al., 2009). ZBP-89 can directly or indirectly regulate p21 (Bai and Merchant, 2000, 2001). Our data showed that ZBP-89 siRNA effectively blocked NaB-induced p21, indicating that ZBP-89 is required for cell growth arrest mediated by HDACi in HCC cells. This result is reminiscent of early findings reported in colon cancer cells (Bai and Merchant, 2007).

Mutation of p53 is one of the most frequently altered genes found in human cancers. Most of the missense mutations possess point mutation and accumulate to high levels in tumor cells. These mutations influence the sequence-specific binding or the conformation of the mutant p53 protein, diminishing its ability to induce the transcription of target genes, resulting in the loss of function. However, in some circumstances, the p53 mutants can acquire novel oncogenic activities classified as gain of function (GOF) mutations. GOF mutations participate in tumorigenesis, tumor progression, and responsiveness to therapy (Brosh and Rotter, 2009;

Selivanova and Wiman, 2007). One mechanism resulting in GOF is through the interaction between p53 mutants and other proteins, for example the interaction between $p53^{G245D}$ and ZBP-89 as reported in this study. $p53^{G245D}$ is frequently found in human cancers and is associated with poor clinicopathologic parameters (Blokx et al., 2005; Catasus et al., 2009; Inazuka et al., 2000; Kato et al., 2000). In Kato's study, $p53^{G245D}$ was identified in a Gliomas patient with advanced stage (Kato et al., 2000). Catasus *et al.* reported that endometrial carcinomas patients with $p53^{G245D}$ were associated with poor prognosis (Catasus et al., 2009). We found that both wild type p53 and $p53^{G245D}$, but not $p53^{R273C}$, formed a complex with ZBP-89. We also observed that the majority of $p53^{G245D}$ was localized in the cytoplasm, whereas the majority of $p53^{R273C}$ was in the nucleus. Further analysis showed that ZBP-89, which is usually located in the nucleus (Hasegawa et al., 1997), was also found in the cytoplasm in cells that contained $p53^{G245D}$, suggesting that $p53^{G245D}$ may help to translocate ZBP-89 from the nucleus to the cytoplasm. This finding is not surprising since ZBP-89 contains two nuclear localization signals (NLS, 141-148 and 313-320) but no nuclear export signal (NES) (Zhang et al.), while $p53^{G245D}$ retains a functional NES (Inazuka et al., 2000). Therefore, once $p53^{G245D}$ binds to ZBP-89, ZBP-89 might be shuttled to the cytoplasm from the nucleus, which can reasonably explain the abrogation of NaB-mediated p21 up-regulation by ZBP-89 in $p53^{G245D}$-containing HCC cells.

In addition to the role of ZBP-89 in cell growth, it can also initiate programmed cell death. By interacting with STAT1, ZBP-89 contributes to IFNγ-mediated apoptosis (Bai and Merchant, 2003). Moreover, ZBP-89 plays a role in the mitochondrial-mediated apoptosis by maintaining the basal expression of Bid and

Bax (Bai et al., 2004). Over-expression of ZBP-89 is known to significantly promote cell death in five different types of HCC cells (Chen et al., 2009). However, over-expressions of ZBP-89-Δ6-180 and ZBP-89-Δ180-298 proteins did not induce apoptosis. This may suggest the N-terminus of ZBP-89 is essential for apoptosis induction. In fact, the interacting domains of p300 and p53 target in N-terminus of ZBP-89 and ZBP-89 transcriptionally regulates a number of factors that participate in cell growth and apoptosis via the C2H2 zinc finger domain (173-278) and the DNA binding domain (145-300) of ZBP-89 (Bai and Merchant, 2001, 2003, 2007; Bai et al., 2004).

Importantly, the nuclear localization of ZBP-89 is required for ZBP-89-mediated apoptosis. Therefore, the interaction between $p53^{G245D}$ and ZBP-89 should theoretically bear some functional consequences. Using TSA to induce the death of HCC cells, we have provided clear evidence that this concept is valid. HCC cells that contain $p53^{G245D}$ and show cytoplasmic ZBP-89 (less nuclear ZBP-89) were much more resistant to TSA-induced cell death as measured by MTT and TUNEL assays. However, this was not the case with $p53^{R273C}$, which did not bind to ZBP-89. The relationship between $p53^{G245D}$ and ZBP-89 in HCC cells somewhat resembles that between p53 mutants and p73 in some other types of cancer cells in which p53 mutants bind to p73, resulting in higher resistance to chemotherapeutic drugs (Flores et al., 2005; Li and Prives, 2007). Taken together, our data provide a plausible link between mutant $p53^{G245D}$ binding to ZBP-89 and a decreased chemosensitivity of HCC cells. It is known that both wild-type p53 and $p53^{G245D}$ bind ZBP-89 while $p53^{R273C}$ dismisses this binding ability. However, we still do not understand how p53 mutants exactly bind to ZBP-89, which is of further investigations.

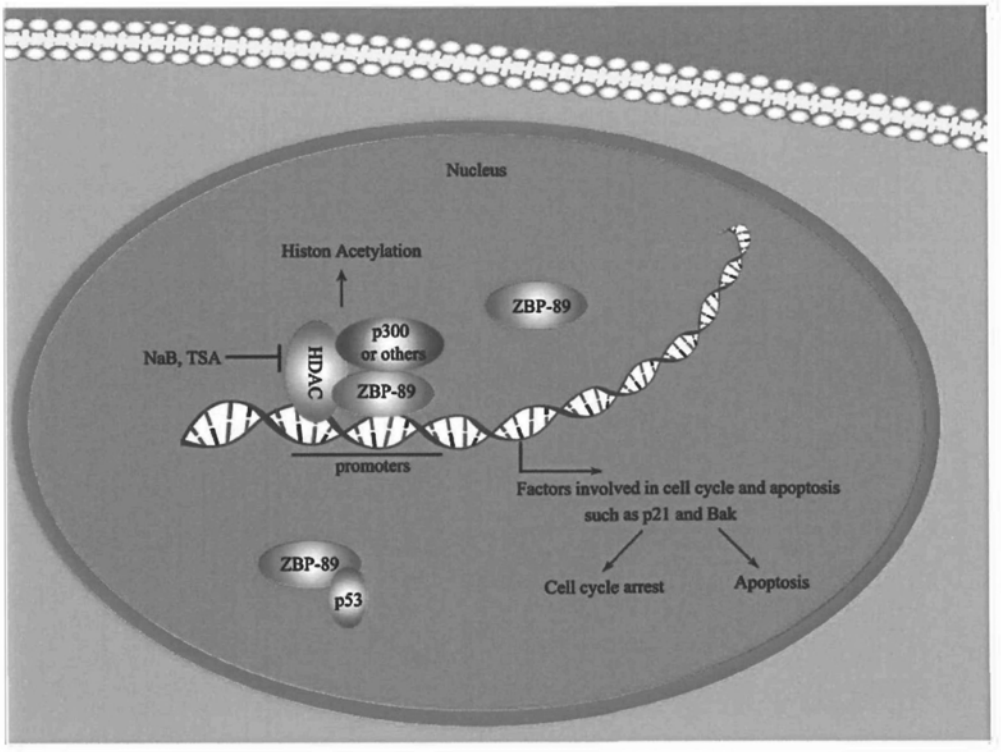

Figure 3.30 Mechanism through which ZBP-89 exerts its regulative functions. Cells with $p53^{R273C}$ mutant may function in some aspects similar to wild-type p53 but the detailed feature remains unclear.

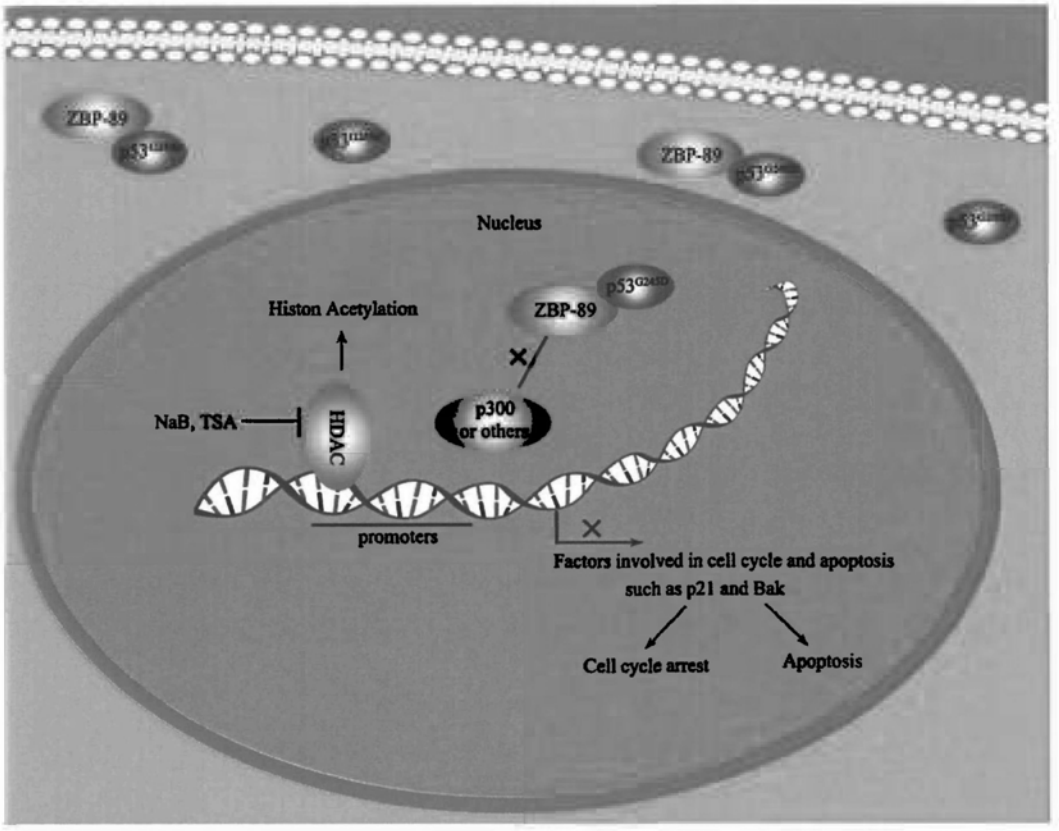

Figure 3.31 Potential mechanism through which $p53^{G245D}$ attenuates effects of HDACi. $p53^{G245D}$ physically binds to ZBP-89 and transfer it to cytoplasm, which might result in the failure of ZBP-89's function on transcriptionally regulating factors that are involved in cell cycle and apoptosis.

Chapter 4 Summaries and proposed future studies

4.1 Summaries

4.1.1 Expression of ZBP-89 in HCC

In summary, the increased expression of ZBP-89 was found in 5 HCC cell lines and over 70% of HCC tissues. ZBP-89 was localized mainly in the nucleus but in some cases in the cytoplasm. ZBP-89 expression was statistically correlated with two clinical parameters, the histological grade and the status of HBV. Furthermore, the over-expression of ZBP-89 in HCC cells noticeably reduced the tumor colony formation. Finally, the higher the expression of ZBP-89 in HCC tissues, the longer HCC patients survived, suggesting that ZBP-89 may be a potential biomarker for prognosis of HCC.

4.1.2 Interaction between ZBP-89 and p53 mutants in HCC

In this study, we found that $p53^{G245D}$ but not $p53^{R273C}$ abrogated p21 up-regulation induced by NaB treatment. $p53^{G245D}$ physically bound to ZBP-89 and transferred it from the nucleus to the cytoplasm. Moreover, cells that possess $p53^{G245D}$ were resistant to the lethal effect of TSA treatment. These data may reveal a novel pathway in which mutant p53 enables tumor cells to resist chemotherapy. The findings deem to be of significance in clinical guidance for HCC treatment.

4.2 Proposed future studies

4.2.1 ZBP-89 and Hepatitis B Virus

In this study, we found that ZBP-89 expression was significantly associated with the infection of HBV. In details, for the relationship with HBV, the expression of ZBP-89 was higher in patients infected with HBV, suggesting that HBV may play a role in regulation of ZBP-89. Since the size of HBV-negative samples was quite small (14/182), we were unable to detail the relationship between ZBP-89 and HBV. But interestingly, Bid, an important apoptotic factor, may be a link to connect ZBP-89 to HBV. In our previous study, HBx, which is considered to exert both antitumour and oncogenic functions, was demonstrated to contribute to the decreased expression of Bid (Chen et al., 2001). In another study, basal expression of Bid was reported to be ZBP-89-denpendent. Therefore, there may be a possibility that HBx influences the expression of Bid in HCC via its regulation of ZBP-89.

4.2.2 Localization of ZBP-89 in HCC

ZBP-89, which mainly present in nucleus, was demonstrated to localize in cytoplasm in 11.5% (21 of 182) of HCC tissues in this study. However, the significance of ZBP-89 in cytoplasm is unclear. Although ZBP-89 was shown to be translocated to cytoplasm from nucleus by p53 mutants, the relationship between ZBP-89 and p53 mutants in those HCC tissues which showed ZBP-89 cytoplasmic localizaiton has not been studied. Besides, the significance of cytoplasmic ZBP-89 expression in HCC and clinicpathological properties including the HBV infection and prognosis has not well investigated in our study. Therefore, it will be of great interst to look into the mechanism through which ZBP-89 localizes in cytoplasm and its

consequence in HCC.

4.2.3 ZBP-89 and p53 mutants

In this study, interaction between ZBP-89 and $p53^{G245D}$ was demonstrated *in vitro*. However, this interaction *in vivo* and the subsequent significance have not been studied. Furthermore, other p53 mutants that are involved in carcinogenesis and tumore development may also interact with ZBP-89, which is unfortunately not investigated in this study. Moreover, the interaction between ZBP-89 and p53 mutants in normal cells may be also of importance. The above aspects are on the list of our future studies.

4.2.4 ZBP-89 and Bid

As mentioned in the section of 'ZBP-89 and Hepatitis B Virus', ZBP-89 may be one of the candidates that regulate Bid. Being a transcriptional regulator that modulates numerous factors that are involved in proliferation and apoptosis, ZBP-89 may also transcriptionally regulate Bid to induce apoptosis. Though knock-down of ZBP-89 resulted in the decreased basal expression of Bid (Bai et al., 2004), how ZBP-89 exactly regulate Bid is still not well understood, which makes it potential future work.

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