Mutation of *p53* in Recurrent Hepatocellular Carcinoma and Its Association with the Expression of ZBP-89

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***p53* has recently been identified as a downstream target of ZBP-89, a zinc finger transcription factor. ZBP-89 promotes growth arrest through stabilization of the *p53* protein. The aim of this study is to determine the status of the *p53* gene in recurrent human hepatocellular carcinoma (HCC) and test the link between the expression of ZBP-89 and the *p53* gene. The results showed that mutations in the *p53* gene were frequently detected in recurrent HCC. The interval between surgical resection and the recurrence of HCC was significantly longer in patients with the wild-type *p53* gene than those with mutations, strongly suggesting a pathological role for the mutant *p53* gene in HCC recurrence. Among those positive for the *p53* protein, nearly 85% (18 of 21) showed nuclear localization of the *p53* protein while only about 14% (3 of 21) were positive for the *p53* protein in the cytoplasm. ZBP-89 co-localized with *p53* in the nucleus in about 67% (12 of 18) of all cases positive for the nuclear *p53* protein, suggesting that ZBP-89 may play a role in the nuclear accumulation of the *p53* protein in a subset of recurrent HCC. With accumulation of *p53* protein in the nucleus, tumor cells undergo apoptosis and thus are more susceptible to radiotherapy and chemotherapy. Therefore, co-localization of *p53* protein with ZBP-89 may define a subgroup of recurrent HCC that is more sensitive to treatment. (Am J Pathol 2003; 162:1823–1829)**

The *p53* gene functions as a tumor suppressor and plays a central role in regulating transcriptional activation of crucial growth regulatory genes that control cell cycle

progression and cell division. The *p53* protein is normally present at low levels in the cell, but it can be up-regulated by stimuli such as DNA damage, hypoxia, viral proteins, or the deregulated cell cycle progression caused by ectopic oncogene expression.¹ The biological consequence of *p53* up-regulation is the induction of pathways that lead to either cell cycle arrest or apoptosis.

Mutations in the *p53* gene abrogate its normal functions, leading to genomic instability and loss of growth control. Many studies have shown that *p53* is frequently mutated in human hepatocellular carcinoma (HCC).^{2–5} Although altered *p53* expression has been reported to correlate with advanced tumor grade, progression, therapy, and survival,^{4–7} information on how the mutations in the *p53* gene is linked to recurrence of HCC is limited and the expression of ZBP-89, a four-zinc finger transcription factor that regulates the expression of several genes related to cell growth through binding to GC-rich DNA elements,¹¹ has not been reported in HCC. HCC is an extremely aggressive disease and the mortality of this disease is very high. Surgical resection of the tumor is currently the most effective treatment for HCC. With the advance of early diagnosis, the short-term prognosis has been improved. However, recurrent tumor after surgical resection remains a serious obstacle to improve the prognosis of patients with HCC. Furthermore, recurrent tumors usually grow faster and have lower chemo- or radiosensitivity, which is believed to be affected by the status of the *p53* gene.^{9,10}

Our recent studies have further revealed that the inhibitory mechanism of ZBP-89 involves activation of p21^{waf1} stabilization of *p53*.^{12,13} ZBP-89 stabilizes *p53* through direct protein contact, which leads to retention of *p53* in the nucleus. Moreover, tumor cells with a mutation in the *p53* gene are resistant to ZBP-89-mediated stabilization.¹³ In the present study we examine the pattern of *p53*

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Table 1. Sequences of Primers Used in *p53* Mutation Analysis

Name	Sequence	Exon
P53-1	5'-cac gct tcc ctg gat tgg-3'	2, 3, 4
P53-2	5'-tca acc cac agc tgc aca-3'	5
P53-3	5'-ccc tgc cct caa caa gat-3'	6, 7
P53-4	5'-acg cac ctc aaa gct gtt-3'	7, 8
P53-5	5'-gcc cat cct cac cat cat-3'	8, 9
P53-6	5'-gga ggg agg ctg tca gtg-3'	9, 10

mutations and ZBP-89 expression to test the possible correlation between ZBP-89 and *p53* in recurrent HCC.

Materials and Methods

Tissue Samples

Thirty-three Hong Kong Chinese patients with HCC were selected for the study. These patients were found to have recurrent intrahepatic or extrahepatic tumors after surgical resection of primary tumor at Prince of Wales Hospital, Hong Kong. All patients studied were found to be positive for hepatitis B virus. The tumors were confirmed by compatible histopathology. The interval between resection surgery and the appearance of recurrence ranged from 2 to 31 months (mean 6.8 months).

Tissue samples were collected at the time of surgical resection of primary tumors. After collection, samples were immediately stored in liquid nitrogen (-160°C) until experiments were performed.

Immunohistochemistry

Tissues underwent immunohistochemical processing, were embedded in paraffin, and were formalin-fixed for 30 hours to preserve the antigen for immunohistochemistry. Tissues were sectioned at 4 mm thick. Immunostaining was performed on paraffin sections according to our previous publications.^{14,15} The ZBP-89 antibody was raised against amino acids 1–521 of rat ZBP-89 (dilution 1:1000).¹⁴ The *p53* antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a concentration of 1:200. Negative controls were prepared by using PBS instead of the primary antibody.

DNA Extraction

Genomic DNA was isolated from frozen 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 1% agarose gel and the concentration of DNA was determined spectrophotometrically at 260 nm.

Analysis of *p53* Mutation

p53 mutations in exons 2 to 8 were analyzed by direct sequencing. The primers listed in Table 1 were designed for sequencing relevant exons. The sequencing was

Table 2. Mutation of the *p53* Gene in Recurrent HCC

Case	Exon	Codon	Nucleotide changes	Amino acid substitution
1	6	193	CAT → CCT	His → Pro
2	5	157	GTC → TTC	Val → Phe
3	8	274	GTT → TTT	Val → Phe
4	7	245	GGC → GAC	Gly → Asp
5	4	68	GAG → TAG	Glu → STOP
6	7	249	AGG → AGT	Arg → Ser
7	5	157	GTC → TTC	Val → Phe
8	5	175	CGC → CAC	Arg → His
9	3	25	TGAAAAC → AG	Frameshift
10	7	242	TGC → TAC	Cys → Tyr
11	7	249	AGG → AGT	Arg → Ser
12	7	249	AGG → AGT	Arg → Ser
13	7	249	AGG → AGT	Arg → Ser
14	8	281	GAC → GAG	Asp → Glu
15	7	249	AGG → AGT	Arg → Ser
16	5	159	GCC → CCC	Ala → Pro

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.

Immunoprecipitation

Hep 3B cells, transfected with wild-type or mutant *p53*, were infected with ZBP-89-containing recombinant adenovirus.^{12,13} Co-precipitation of *p53* and ZBP-89 was carried out using whole-cell lysate and specific antibodies as described above. The first antibody (*p53*)-associated proteins were precipitated with protein A-Sepharose. After washing, immunoprecipitates were separated and immunoblotted with the second antibody (ZBP-89). Hep 3B cells is a hepatocarcinoma cell line, whose *p53* gene has been deleted.⁸

Statistical Analysis

Student's *t*-test or Mann-Whitney *U*-test was used for continuous variables. χ^2 or Fisher exact test was carried out for categorical variables. Regression and correlation analyses of parametric and nonparametric data were performed by Pearson and Spearman methods respectively. A *P* value of less than 0.05 was considered statistically significant.

Results

Mutations in the *p53* Gene

Mutations in the *p53* gene were frequently detected in recurrent HCC. Of 33 recurrent cases studied, 16 (48.5%) had mutations in the *p53* gene. All except one mutation detected was a single nucleotide mismatch that occurred in the core domain between codons 68 and 281 (from exon 4 to exon 8) (Table 2). One mutation in exon 3 resulted from a deletion of 7 nucleotides with a 2-nucleotide insertion. Among all mutations detected, 7 cases (43.8%) were located in exon 7, especially in codon 249,

Table 3. Demographic Data on Patients with Recurrent HCC

Demographic data	p53 Mutation (n = 16)	p53 Wild-type (n = 17)	Significance (P)
Age (years)			>0.05
Range	32–74	37–75	
Mean	50.3	54.5	
Sex			>0.05
Male	13	14	
Female	3	3	
Histological differentiation			>0.05
Well	5	6	>0.05
Moderately	9	10	>0.05
Poorly	1	1	
AFP (ng/ml)			0.0063
Range	3–13,500	10–27,170	
Mean	1366.4	2337.5	
HBsAg			>0.05
Positive	11	15	
Negative	4	2	
HCV			>0.05
Positive	2	1	
Negative	14	16	
Liver cirrhosis			>0.05
Positive	12	10	
Negative	4	7	
Portal involvement			>0.05
Positive	3	3	
Negative	13	14	

which accounted for 5 cases (31.3%) of all mutations found.

Patient demographics are shown in Table 3. The patients with recurrent HCC and mutations in the *p53* gene were younger than those with the wild-type *p53* gene. However, the difference did not reach statistical significance, probably due to the wide variations in age, which ranged from 14 to 75 years. The level of α -fetoprotein (AFP) in patients with mutations in the *p53* gene was significantly lower than those without mutations. There was no difference in other clinical values between patients with and without mutations in the *p53* genes.

Association of *p53* Mutations with Tumor Recurrence

The interval between surgical resection and the appearance of recurrence was significantly longer in patients with the wild-type *p53* gene than those with mutations in the *p53* gene (Figure 1), strongly suggesting a pathological role for the *p53* gene in HCC recurrence. Since codon 249 in exon 7 was the most frequent site for mutation, we specifically calculated the interval for recurrent HCC with this particular mutation. The mean interval for recurrent HCC with a mutation in codon 249 of exon 7 was 5.4 months, which was much shorter than that in all other codons (9.6 months, $P < 0.05$). The detailed distribution of intervals between the two groups of recurrent HCC is shown in Figure 2.

Expression of ZBP-89 in HCC

The frequency of ZBP-89 protein detection was significantly higher in cancerous tissues (28 cases) than in

noncancerous ones (18 cases, $P = 0.0148$). ZBP-89 protein was detected in both the cytoplasm and the nucleus. The intensity was stronger in the nucleus than in the cytoplasm in HCC patients regardless of the presence of *p53* mutations (Figure 3A). However, the expression of ZBP-89 was more readily detected in the cytoplasm of recurrent HCC when *p53* mutations were present (Figure 3A) whereas it was more likely to be present in the nucleus of recurrent HCC when there were no mutations in the *p53* gene (Figure 3B).

Figure 1. Mean interval of recurrence in HCC patients with mutations in *p53* gene was significantly shorter than those without mutations in *p53*.

Figure 2. Distribution of interval in recurrent HCC with or without mutations in *p53*.

Co-Localization of *p53* and ZBP-89 in Hepatoma Cells

The expression of *p53* protein was detected in 21 of 33 cases (63.6%). The positive rate of *p53* protein detection was significantly higher in recurrent HCC patients with mutations in the *p53* gene than those without ($P < 0.01$). All 16 cases of recurrent HCC with *p53* mutations showed *p53* signals and only 5 cases with *p53* expression did not have underlying mutations in the *p53* gene. *p53* protein was more likely to accumulate in the cytoplasm of recurrent HCC if mutations were present in the *p53* gene (Figure 3C). Nuclear localization of *p53* was also observed, especially in those with the wild-type *p53* gene (Figure 3D). Since ZBP-89 was able to bind to *p53* and co-localize to the nucleus,¹³ we performed double-staining of both proteins in tissues positive for *p53* protein. Indeed, ZBP-89 was present in the nuclear compartment where *p53* protein was found (Figure 3E). In most cases where ZBP-89 and *p53* proteins co-localized in the nucleus, there were no mutations in the *p53* genes. Co-localization of ZBP-89 and *p53* proteins was also observed in the cytoplasm. Cytoplasmic co-localization was detected in recurrent HCC tumors primarily with mutant *p53* gene. However, ZBP-89 localization could also be observed with some tumors with cytoplasmic wild-type *p53* (Figure 3F).

Direct Interaction Between ZBP-89 and *p53*

Since ZBP-89 and *p53* were co-localized in most recurrent HCC, we examined whether this occurred by direct contact. Co-immunoprecipitation was performed to assess whether both proteins could bind each other. Hep 3B, a *p53*-negative liver cancer cell line,⁸ was infected with ZBP-89-containing recombinant adenovirus. Cell extracts were subjected to immunoprecipitation with the

p53 antibody followed by immunoblotting with ZBP-89 antibody. Figure 4 shows that ZBP-89 co-precipitated with wild-type *p53* and some *p53* mutants whose mutations occurred in the N-terminal domains. Mutations in the *p53* transactivation domain (35 frameshift) and PXXP domain (G68A) did not affect the binding between ZBP89 and *p53*. All mutations in the DNA binding domain, except those located in the most N-terminal region of DNA binding domain (V157P and A159P), abolished the direct interaction between ZBP-89 and *p53*.

Discussion

It is well known that mutations in the *p53* gene are closely associated with HCC.²⁻⁵ However, the pattern of mutations varies, depending on the geographic areas and types of patients selected. The present study focuses on recurrent HCC in a Chinese population in Hong Kong. Within the population examined, mutations in the *p53* gene were detected in 48.5% of recurrent HCC, which is much higher than the 23.1% reported in a previous study of HCC in Hong Kong Chinese.¹⁶ The pattern of *p53* mutations detected between these two studies was also different. In the present study, mutations in exon 7 of the *p53* gene, which is a hot spot in many studies performed in Asia,^{17,18} accounted for 43.8% of all alterations found, and therefore the present study suggests that the exon 7 should be considered a prevalent site of *p53* mutation. In the previous study, mutations in exon 7 of the *p53* gene were detected in 33.3% of cases; however, these two studies may not be comparable, as one was performed on newly diagnosed and recurrent HCC patients in general while the other was conducted only on recurrent HCC.

The mean interval between surgical resection and the appearance of recurrence in patients with mutations in the *p53* gene was 7.8 months, whereas those with the wild-type *p53* gene was about two-fold as long (16.4 months). This finding establishes a potential link between mutations in the *p53* gene and recurrent tumor. Such a link may be of clinical significance if it can be further confirmed in a large number of patients. The behaviors of the recurrent tumor usually differ from that of the primary tumor. Of particular importance, recurrent tumors grow faster and are less sensitive to chemotherapy and radiotherapy.^{10,11,19,20} These features may be strongly linked to the function of *p53* protein. Mutant *p53* provides tumor cells with a selective survival advantage during non-surgical therapy and thus decrease the sensitivity to chemo- and radiodrugs.^{21,22} Functioning as a transcription factor, *p53* binds to DNA in a sequence-specific manner and regulates the transcription of the gene responsible for the promotion of apoptosis and the inhibition of angiogenesis,²³ both of which are believed to be critical for the prevention or the inhibition of the development of tumors. This concept is supported by both animal and human data. Although *p53*-null mice are able to develop normally, they are susceptible to an array of spontaneous tumors in early adult life.²⁴ When the wild-type *p53* allele is lost, a variety of mesenchymal and epithelial tumors develop in patients with Li-Fraumeni

Figure 3. Immunohistochemical analysis of *p53* and ZBP-89 in liver tissues obtained from recurrent HCC with or without mutations in *p53*. **A:** Expression of ZBP-89 in recurrent HCC with mutant *p53* gene. ZBP-89 was stained in brown. **B:** Expression of ZBP-89 in recurrent HCC with wild-type *p53* gene. ZBP-89 was stained in brown. **C:** Expression of *p53* in recurrent HCC with mutant *p53* gene. *p53* was stained in brown. **D:** Expression of *p53* in recurrent HCC with wild-type *p53* gene. *p53* was stained in brown. **E:** Co-expression of ZBP-89 and *p53* in recurrent HCC with wild-type *p53* gene. ZBP-89 was stained in brown and *p53* in blue. **F:** Co-expression of ZBP-89 and *p53* in recurrent HCC with mutant *p53* gene. ZBP-89 was stained in brown and *p53* in blue. Magnification, $\times 400$.

Figure 4. Co-immunoprecipitation of ZBP-89 and *p53*. Hep 3B cells, transfected with various forms of *p53* (lanes 1–11) or vector (lane 12), were infected with Ad5-ZBP-89. After cell lysis, the extract was react with *p53* antibody (the first antibody) followed by ZBP-89 antibody (the second antibody).

syndrome, which carries heterozygous *p53* mutations in the germline.²⁵ In recurrent HCC, *p53* dysfunction caused by the mutation may create a similar scenario to the one observed in *p53*-null mice or *p53* allele deletion, in which the growth of a tumor is promoted. If this is true, it would be easier to understand why HCC patients with a mutant *p53* gene are more likely to have tumor recurrence within a short interval after surgical resection for the primary tumor.

The central domain of *p53*, which consists of amino acids 100 to 293, is required for sequence-specific DNA binding.²³ All *p53* gene mutations except two found in recurrent HCC of this study are located in the DNA-binding domain. Mutations in the central domain alter either the structural integrity of the domain or the amino acids that directly contact the DNA, as demonstrated by the study of the crystal structure of the *p53* protein.²⁶ The N terminus of the *p53* protein encoded by exons 2 to 4 has an abundance of acidic amino acids that are involved in the transcriptional function of *p53*.²³ It is reported that multiple point mutations in this region are usually required to block the transcriptional transactivation of the *p53* gene.²⁷ In the present study, two patterns of mutant *p53* gene in this region were found in two patients with recurrent HCC, one was a single point mutation in exon 4 and the other was a frameshift in exon 3. The interval between surgical resection and the appearance of tumor in these two patients were 12 months and 5 months respectively, which were much shorter than the mean 16.4 months in those patients without mutations in the *p53* gene. Therefore, we believe that these two *p53* mutants within the N-terminus are pathologically significant. This is consistent with the report which indicated that a point mutation in codon 68 of exon 4 decreased approximately 35% transcriptional activity of the *p53* gene.²⁸

The *p53* nuclear import or retention is essential for its normal function in growth inhibition or induction of apoptosis.^{23,29} A defect in the regulation of *p53* nuclear import or export may result in tumorigenesis. Dysfunctional transportation of *p53* between the nucleus and the cytoplasm is known to occur in a subset of human tumors and in such a situation, *p53* is sequestered either in the cytoplasm or in the nucleus.^{30,31} In normal cells, the wild-type *p53* protein is kept at a low concentration by rapid degradation. Therefore, the wild-type *p53* protein is usually undetected or detected at a very low level. However, when there are mutations within the *p53* gene, they result in a dysfunctional protein product with a prolonged

half-life that enables them to accumulate in the cell. Accumulation of *p53* protein either in the cytoplasm or in the nucleus is considered a pathological index.²³ In the present study, nearly 58% (19 of 33) of recurrent HCC showed a nuclear localization of *p53* protein and about 9% (3 of 33) was positive for the *p53* protein in the cytoplasm. Detailed analysis revealed that accumulation of *p53* protein in the nucleus occurred in recurrent HCC regardless of whether the *p53* gene was mutated, whereas the cytoplasmic *p53* protein was found in all recurrent HCC with mutant *p53* gene. Mechanisms responsible for the sequestration of a particular mutant *p53* protein in the nucleus or the cytoplasm are very complicated and not yet completely known.

The nuclear localization signals are located within the carboxyl terminus of *p53* protein (amino acids 293 to 393).²³ Mutations in this nuclear signal region can affect the nuclear import or export of *p53*. However, none of the mutations detected in the current study were within this nuclear signal region. Recently, we have demonstrated that ZBP-89 protein can stabilize *p53* through a direct protein contact, leading to its retention in the nucleus.¹³ In the present study it was found that ZBP-89 co-localized with *p53* in the nucleus in about 63% (12 of 19) of all cases positive for the nuclear *p53* protein, suggesting that ZBP-89 may play a role in the nuclear accumulation of *p53* protein in a subset of recurrent HCC. Indeed, ZBP-89 is able to bind wild-type *p53* and some forms of *p53* mutants, as demonstrated by co-immunoprecipitation experiment. Mutation within *p53* non-binding N-terminal domains retains its ability to bind ZBP-89, whereas mutation in DNA binding domain (after codon 175) abolishes the direct interaction between ZBP-89 and *p53*. These *p53* mutations may thus eliminate ZBP-89-mediated stabilization of *p53*.¹³ In agreement with the co-immunoprecipitation experiment reported here, ZBP-89 was found to co-localize not only with wild-type *p53* but also with the mutant protein. This further confirms that the ZBP-89-interaction domain in some mutant *p53* proteins remains intact. Co-localization of ZBP-89 and *p53* in the nucleus may be clinically significant in certain types of patients who possess functional *p53*. The function of *p53* protein depends on the nuclear localization.²³ With accumulation of *p53* protein in the nucleus, tumor cells are more likely to undergo apoptosis and are thus more susceptible to radiotherapy and chemotherapy.^{9,10} Therefore, by co-localizing *p53* protein, the expression of ZBP-89 may define a subgroup of recurrent HCC that is more suitable to receive radiotherapy and chemotherapy.

The present study also revealed that the level of serum α -fetoprotein (AFP) was much lower in recurrent HCC with mutant *p53* than those without. This finding is not consistent with an *in vitro* study that demonstrated that wild-type *p53* inhibits the expression of AFP and that mutant one loses its ability to repress AFP production.³¹ We are unable to explain the difference at the present, but it may be due to the different model of experiments, one is *in vivo* and the other *in vitro*. Although AFP is a well-established conventional tumor marker for HCC, there is a report indicating that recurrent HCC may not

have an increase in serum α -fetoprotein level.³² Also, serum AFP is in general not disease-stage-dependent.³³

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