Association of *TP53* Mutations With Stem Cell-Like Gene Expression and Survival of Patients With Hepatocellular Carcinoma

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BACKGROUND & AIMS: Mutations in *TP53*, a tumor suppressor gene, are associated with prognosis of many cancers. However, the prognostic values of *TP53* mutation sites are not known for patients with hepatocellular carcinoma (HCC) because of heterogeneity in their geographic and etiologic backgrounds. **METHODS:** *TP53* mutations were investigated in a total of 409 HCC patients, including Chinese (n = 336) and white (n = 73) patients, using the direct sequencing method. **RESULTS:** A total of 125 *TP53* mutations were found in Chinese patients with HCC (37.2%). HCC patients with *TP53* mutations had a shorter overall survival time compared with patients with wild-type *TP53* (hazard ratio [HR], 1.86; 95% confidence interval [CI]: 1.37–2.52; *P* < .001). The hot spot mutations R249S and V157F were significantly associated with worse prognosis in univariate (HR, 2.11; 95% CI: 1.51–2.94; *P* < .001) and multivariate analyses (HR, 1.79; 95% CI: 1.29–2.51; *P* < .001). Gene expression analysis revealed the existence of stem cell-like traits in tumors with *TP53* mutations. These findings were validated in breast and lung tumor samples with *TP53* mutations. **CONCLUSIONS: *TP53* mutations, particularly the hot spot mutations R249S and V157F, are associated with poor prognosis for patients with HCC. The acquisition of stem cell-like gene expression traits might contribute to the aggressive behavior of tumors with *TP53* mutation.**

Keywords: Liver Cancer; p53; Gene Expression Patterns; Cancer Stem Cells.

Hepatocellular carcinoma (HCC) is one of the most common cancers with dismal clinical outcome accounting for the third highest cause of cancer-related deaths worldwide.¹ Significantly, China alone accounts for more than 55% of liver cancer in the world,¹ and the incidence of HCC continues to increase in the United States and Western Europe.² In addition, the clinical heterogeneity of HCC and the lack of good diagnostic markers and treatment options have rendered this disease a major health problem.

TP53 is a multifunctional transcription factor that, along with number of other functions, regulates genes

involved in cell cycle arrest, apoptosis, and senescence in response to various types of stress. Mutations of *TP53* are frequently found in human cancers and are generally associated with poor prognosis. In HCC, several studies have shown the association of *TP53* mutations with poor survival.^{3–5} However, these studies were limited to specific populations with small sample size. In addition, overwhelming evidence demonstrated multifactorial association of *TP53* mutation spectra with environmental and etiologic factors, resulting in variations among geographic regions and racial and ethnic groups.^{6,7} In particular, the mutation at codon 249 position (R249S) is prevalent in China and sub-Saharan Africa.⁸ This site preference is known to associate with the dietary exposure to aflatoxin B1 and hepatitis B virus (HBV) infection.⁹ Therefore, the prognostic value of *TP53* mutations needs to be evaluated in the context of such confounding factors. Furthermore, *TP53* mutations in different structural and functional regions of *TP53* are associated with different prognostic values in diverse cancer types.^{10–12} However, the prognostic values of the different sites of *TP53* mutations in HCC have not yet been rigorously evaluated.

In this study, we investigated the prognostic significance of the *TP53* mutations in a Chinese cohort of HCC patients and evaluated the impact of individual mutations on prognosis. We found that hot spot mutations at codons 249 and 157 are significantly associated with patients' survival. Additionally, we demonstrated that the *TP53* mutated tumors express "stemness"-related genes suggesting a potential progenitor cell origin. These results have both biologic and clinical implications for *TP53* mutated HCC.

Abbreviations used in this paper: AFP, alpha-feto protein; cDNA, complementary DNA; CI, confidence interval; ES, embryonic stem; HB, hepatoblast; HB_DOWN, hepatoblast downregulated genes; HB_UP, hepatoblast over-expressed genes; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazards ratio; PCR, polymerase chain reaction.

Materials and Methods

Direct Sequencing of TP53

A total of 409 HCC patients including 336 Chinese patients collected from Qidong (n = 120) and Shanghai (n = 216) areas and 73 white patients from Belgium (n = 47) and United States (n = 26) were analyzed. Direct sequencing of *TP53* exons 5 through 8 was performed using complimentary DNA (cDNA) obtained from frozen tissue specimens by reverse transcription polymerase chain reaction (PCR). Genomic DNAs were used in cases where cDNA samples were not available. Sequencing reaction was performed using ABI 3130xl Genetic Analyzer with BigDye terminator sequencing reaction kit version 1.1 (Applied Biosystems, Carlsbad, CA). The primers for sequencing reaction and PCR are summarized in [Supplementary Table 1](#).

Compiling Gene Expression Data Sets

Gene expression data sets from the Laboratory of Experimental Carcinogenesis (GSE1898 and GSE4024), GSE5975,¹³ and a publicly available data E-TABM-36¹⁴ were compiled together. For each data set, average expression levels of each array and gene were set to 0, and each array was scaled to have standard deviation 1. For multiple tagged gene features for the same Entrez Gene identifier, the gene feature with the highest magnitude (ie, sum of square of each sample) was used as a representative gene feature. Nontumor samples or the samples without *TP53* mutations were excluded, and, finally, 366 HCC profiles were compiled. For external validation, breast cancer data (GSE3494, n = 251)¹⁵ with 2 platforms (HGU-133A and HGU-133B) of Affymetrix (Santa Clara, CA) and a compiled lung cancer data set of GSE11969 (n = 149)¹⁶ and GSE8569 (n = 69) were used. Preprocessing of each data set was performed by the same method as described above.

Enrichment of Gene Expression Signatures

The enrichment of gene sets in individual tumors was determined as described previously.¹⁷ Briefly, for each array, we assessed the fraction of up- or down-regulated genes with a fold difference greater than 2 in each tested gene set. The gene set enrichment was calculated by hypergeometric test with a threshold of $P < .01$ for significant enrichment. The enrichment scores S_{up} and S_{down} for a given signature were calculated as $-\log_{10}$ (P value) from up- and down-regulated gene sets, respectively. The enrichment score S was defined as S_{up} if $S_{up} > S_{down}$ and S_{down} if $S_{up} < S_{down}$. The enrichment patterns across groups were determined by calculating fraction of the significantly enriched samples in a group, and the significance of the group enrichment was calculated by hypergeometric test.

Embryonic stem (ES) cell-related signatures (ie, ES1, ES2, NOS, NOS_TF, and the target genes for Nanog, Oct4, Sox2, Myc1, and Myc2) and proliferation-related

gene signature (prol) were obtained from the previous publication site.¹⁷ NOS includes the target genes for Nanog, Oct4, and Sox2. NOS_TF includes a subset of NOS activation targets encoding transcription regulators. We also obtained rat hepatoblast (HB) gene signatures (n = 191), which contained over-expressed (HB_UP) and down-regulated (HB_DOWN) genes in early fetal liver development.¹⁸ For the validation using breast and lung cancer data, Gene Set Enrichment Analysis (version 2.0) was applied with default parameters.¹⁹

Real-Time Quantitative PCR

The same amount of first-strand cDNA from each sample was used to detect the messenger RNA expression levels using specific primers (*CD24* sense 5'-ACAGCCAGTCTCTTCGTGGT-3', antisense 5'-CCTGT-TTTTCCTTGCCACAT-3'; alpha-feto protein (*AFP*) sense 5'-AGCTTGGTGGTGGATGAAAC-3', antisense 5'-CCC-TCTTCAGCAAAGCAGAC-3'; glyceraldehyde-3-phosphate dehydrogenase sense 5'-ACCCAGAAGACTGTGGATGG-3', antisense 5'-TTCTAGACGGCAGGTCAGGT-3'). PCR was carried out with a SYBR Green Master Mix (Applied Biosystems, Foster City, CA) of 40 cycles for 10 minutes at 95°C, 15 seconds at 95°C, 30 seconds at 60°C, with a final dissociation step of 15 seconds at 95°C, 20 seconds at 60°C, 15 seconds at 95°C using ABI 7000 real-time PCR system (Applied Biosystems). Each sample was assayed in duplicate, and a control normal liver DNA was included in every assay. The messenger RNA expression levels were normalized with that of the *glyceraldehyde-3-phosphate dehydrogenase*, and the relative values compared with a normal liver sample were calculated ($-\Delta\Delta CT$).

Statistical Analysis

Survival analysis was performed by Kaplan-Meier method and log-rank test. The association of clinical features with *TP53* mutations was analyzed by Fisher exact test. Univariate and multivariate analyses for survival data were performed by using Cox proportional hazard models. Quantitative PCR result was analyzed by Mann-Whitney U test.

Results

Profiling of TP53 Mutation Status in HCC

A total of 134 mutations were found in 409 HCC patients ([Supplementary Table 2](#)). Of these, 1 case of silent mutation, 2 cases of nonsynonymous single nucleotide polymorphism (R213R), and 1 case of a deletion at splicing site were excluded from the analysis. For tumors with more than 1 mutation, the mutations were counted as a single event. In the Chinese cohort (n = 334), 125 cases of *TP53* mutations (37.4%) including missense (120 cases), 4 nonsense, and 1 frame shift mutations were found. The patients from Qidong showed higher mutation rate compared with the patients from Shanghai

Table 1. Univariate Analysis for Selected Clinical Features of HCC

	Number of patients	Hazard ratio	95% CI lower	95% CI upper	P value
Mutation, any type					
Sex (male/female)	282/48	1.23	0.78	1.93	.369
Age (<50/≥50, y)	173/157	1.30	0.95	1.76	.097
AFP (≥300/<300 ng/mL)	170/152	2.13	1.54	2.95	<.001
Size (≥/<5 cm)	177/152	1.94	1.41	2.66	<.001
Presence of cirrhosis (yes/no)	249/80	0.79	0.57	1.11	.182
Grade (III, IV/I, II)	184/108	1.82	1.28	2.58	<.001
HBV (+/-)	296/38	0.60	0.38	0.93	.022
HCV (+/-)	5/329	0.35	0.05	2.48	.269
TP53 mutation types					
Any types	125/209	1.86	1.37	2.53	<.001
R249S	77/209 ^a	1.98	1.41	2.80	<.001
V157F	8/209 ^a	4.25	1.95	9.27	<.001
Hot spot mutations	84/209	2.11	1.51	2.94	<.001
Non-hot spot mutations	41/209	1.44	0.89	2.31	.134

^aOne case of double mutation with R249S and V157F was counted as a separate event.

areas (54.1% and 28.0%, respectively). In accordance with previous studies,^{6,20} codon 249 of *TP53* (R249S) was the most common mutation (24.4%, 77 cases) both in the samples from Qidong (36.6%, 44 cases) and Shanghai (14.4%, 31 cases). Codon 157 (V157F) was the second most common mutation site (2.4%, 8 cases), particularly in Qidong province (7 cases). By contrast, white HCC patients showed low mutation rate (7.0%, 5 out of 71) with mutations at codon 249 (3 cases) and codon 157 (2 cases).

Clinicopathologic Features Related to TP53 Mutation Status

Because of the very high heterogeneity between the mutation status of Chinese and white people, which could introduce bias, survival analysis was confined to the Chinese cohort. The survival data were available in a total of 329 out of the 334 Chinese patients. Follow-up time was truncated to 5 years after surgical resection. Univariate Cox regression analysis was performed to

characterize the prognostic association of clinicopathologic features. Of the clinical features, serum AFP (>300 ng/mL), tumor size (>5 cm), and tumor grade (>III, IV) were significantly associated with the increased risk of death ($P < .001$, Table 1).

Kaplan-Meier plot analysis showed that the presence of *TP53* mutations is significantly associated with shorter overall survival (hazard ratio [HR], 1.86; 95% confidence interval [CI]: 1.37–2.52; $P < .001$) (Figure 1A). Interestingly, when we examined the prognostic values of individual mutations, we found that highly frequent mutations, ie, hot spot mutations (mutation rate >2%), at codon 249 and codon 157 are significantly associated with worse survival (HR, 1.98; 95% CI: 1.41–2.80; $P = 7.1 \times 10^{-5}$; HR, 4.25; 95% CI: 1.97–9.27; $P < .001$, respectively) (Figure 1B). Grouping the patients with hot spot mutations (84/125, 67.2% cases with mutations) also showed shorter survival than patients with the wild-type *TP53* ($P < .001$) (Figure 1C). Multivariate analysis

Figure 1. Prognostic values of *TP53* mutation sites. (A) Kaplan-Meier plot for survival between the tumors of mutation-type (MT) and wild-type (WT) *TP53*. (B) Kaplan-Meier plot for survival between the tumors with R249S and V157F mutations. (C) Kaplan-Meier plot for survival between the tumors with hot spot (ie, R249S and V157F) and non-hot spot mutations. The + symbols in panels indicate censored data.

Table 2. Multivariate Analysis for the Selected Clinical Features of HCC

	Hazard ratio	95% CI lower	95% CI upper	P value
Mutation, any type				
Sex (male/female)	1.09	0.67	1.79	.730
Age (<50/≥50, y)	0.91	0.65	1.27	.580
AFP (≥300/<300 ng/mL)	1.76	1.24	2.50	.002
Size (≥5/<5 cm)	1.68	1.19	2.38	.004
Presence of cirrhosis (yes/no)	0.99	0.68	1.43	.950
Grade (III, IV/I, II)	1.73	1.21	2.48	.003
TP53 mutation, any type	1.79	1.29	2.51	<.001
Mutation category				
Sex (male/female)	1.07	0.65	1.75	.800
Age (<50/≥50, y)	0.89	0.64	1.25	.510
AFP (≥300/<300 ng/mL)	1.77	1.24	2.52	.002
Size (≥5/<5 cm)	1.71	1.21	2.43	.003
Presence of cirrhosis (yes/no)	1.03	0.71	1.50	.880
Grade (III, IV/I, II)	1.70	1.19	2.44	.004
Hot spot mutations	1.97	1.36	2.86	<.001
Non-hot spot mutations	1.47	0.88	2.44	.140

including *TP53* mutations also revealed that only the hot spot mutations are independent risk factors for overall survival (HR, 1.79; 95% CI: 1.29–2.51; $P < .001$, Table 2). These results suggest that the hot spot mutations mainly contribute to the heterogeneous progression of HCC.

Previously, classification of *TP53* mutations based on the structural properties revealed distinct prognostic values in diffuse large B-cell lymphoma and squamous cell carcinoma of the head and neck.^{10,11} We therefore evaluated whether the structural classifications of *TP53* mutations have different prognostic values in HCC. The *TP53* mutations were categorized based on the structural properties as described in the *TP53* mutation database of International Agency for Research on Cancer (Geneva, Switzerland; <http://www.p53.iarc.fr>). The HCC patients with mutations at the DNA binding motifs (loop-L3) and S4 β -sandwich regions showed a significantly shorter survival compared with the wild-type *TP53* patients (Supplementary Table 3). These results seem to be largely due to the profound prognostic values of the 2 hot spot mutations at codons 249 and 157, which are located in L3 and S4, respectively. However, mutations located outside the L3 or S4 were also associated with a poor prognosis albeit with a marginal significance ($P = .053$ and $P = .048$, respectively), indicating that these structural properties may not be proficient in classifying the prognostic outcome. We also applied another classification of disruptive vs nondisruptive mutations based on the mutation location and the predicted amino acid alterations.¹¹ Differing from the previous analysis, both disruptive and nondisruptive mutations were associated with a worse prognosis ($P < .001$ and $P = .048$, respectively, Supplementary Table 3), which might be due to the nondisruptive nature of V157F mutation. These findings suggest that the hot spot mutation frequency rather than structural or functional

classifications of *TP53* mutations is a more significant prognostic indicator at least in the Chinese cohort.

The association of *TP53* mutations with clinical features was also investigated (Supplementary Table 4). *TP53* mutations were more frequent in the patients with high serum AFP level (>300 ng/mL) and patients without cirrhosis ($P < .05$). Hotspot mutations were more frequent in young patients ($P = .019$) and patients without cirrhosis ($P = .007$). The association of *TP53* mutation with HBV infection has been reported previously^{6,21}; however, we could not assess this finding because most of the patients in our cohort were HBV infected.

Next, we tested whether a combination of clinicopathologic features and *TP53* mutations could improve the stratification of the prognostic subgroups. Patients with *TP53* mutations could be stratified according to a poor survival in each of the subpopulations with a low tumor grade (I, II), low serum AFP level (<300 ng/mL), large tumor size (>5 cm), or presence of cirrhosis (Figure 2). The patients with hot spot *TP53* mutations showed similar prognostic values in these subpopulations (Supplementary Figure 1). These results suggest that a combined application of *TP53* mutation status with the conventional clinical features would be a practical approach to predict the patient's survival.

Figure 2. Prognostic value of combined status of *TP53* mutation and other clinical features. (A–D) Kaplan–Meier plots for survival between patients with mutation-type (MT) and wild-type (WT) *TP53* in subpopulations of patients who have a low tumor grade (I, II) (A), low serum AFP level (<300 ng/mL) (B), large tumor size (>5 cm) (C), or cirrhosis (D). The + symbols in panels indicate censored data.

Gene Expression Profiles Reflect the Prognostic Value of *TP53* Mutation Sites

Gene expression profile analysis was performed to characterize further the tumors according to the *TP53* mutation status. A total of 366 HCC gene expression profiles having *TP53* mutation status were compiled (see Materials and Methods section). It includes 238 Chinese samples with 89 cases of *TP53* mutation and 128 white samples with 19 cases of *TP53* mutation. We performed an unbiased estimation of the functional gene set enrichments using Gene Ontology (<http://www.geneontology.org/>) hierarchy. Considering the complexity of the *TP53* biology and molecular heterogeneity of each sample, gene set enrichment analyses were performed on the individual HCC samples not the groups (see Materials and Methods section for details). The unbiased screening of the gene set enrichment revealed a profound increase in proliferation-related gene expression activity in the *TP53* mutated tumors with the enrichment of transcription/cell cycle-related functions (Supplementary Figure 2A). By contrast, the wild-type tumors were enriched with the metabolism-related genes, which may indicate a conservation of liver functions. The same patterns were observed in both Chinese ($n = 238$) and white ($n = 128$) cohorts. The comparison of hot spot and non-hot spot mutations was performed using the Chinese cohort because of the marked difference in the mutation frequency in the white cohort. Consistent with the different prognostic values, the proliferation-related genes were prevalently enriched in the hot spot-mutated tumors compared with the non-hot spot mutated tumors (Supplementary Figure 2B). These data indicate that the distinct prognostic significance of *TP53* mutations, particularly the hot spot mutations, is a consequence of the molecular alterations rather than by-chance casual association with the clinical data.

TP53 Mutations Are Associated With Stem Cell-Like Gene Expression Traits

It has been suggested that the tumors originated from the stem/progenitor cells acquire a more aggressive phenotype in numerous cancer types.^{17,22} Moreover, several recent studies have shown association between *TP53* mutations and the “stemness” of cancer cells (see also Discussion section). To address this issue in human tumor specimens, we measured the expression of stemness genes by the enrichment scores of multiple stem cell-related genes rather than the expression of individual stem cell marker genes as described in previous study.¹⁷ It has been suggested that detection of the activity of such stemness-related complex networks rather than the expression of individual genes is more important because different ES regulators may activate stem-cell regulatory networks in each tumors. Enrichment of various ES and HB-related gene signatures (for details see Materials and Methods section) was calculated in individual tumors of

the HCC data set ($n = 366$). We found that the ES-related and HB signatures were concomitantly enriched in the *TP53* mutated tumors but repressed in the wild-type tumors (Figure 3A). To exclude the contribution of the proliferation-related genes in the stem cell signature, we tested the enrichment of the signatures after subtracting the proliferation-related genes as described previously.¹⁷ The results were similar suggesting that the enrichment of the stem cell-related signatures in the *TP53* mutated tumors was independent of the expression of proliferation-related genes (Figure 3A, right). In addition, when the tumors were classified according to the status of stem cell-like expression (ie, HB and ES) and hot spot *TP53* mutation, they showed correlation with patient's survival, supporting the prognostic significance of *TP53* mutation and stem cell traits (Figure 3B). Classification with mutations with any types (MT vs WT) also demonstrated a similar result (Supplementary Figure 3).

The generality of our finding was evaluated by extending our analysis to the public data sets of breast cancer ($n = 251$) and lung cancer ($n = 218$) using the Gene Set Enrichment Analysis method.¹⁹ Similar to HCC, there was a significant up-regulation of ES trait in the *TP53* mutated tumors compared with wild-type breast and lung cancers (Figure 3B and C and Supplementary Table 5). These findings suggest that the stem cell-like traits are a common feature of tumors harboring *TP53* mutations.

Validation of Stem Cell-Like Gene Expression in *TP53* Mutated Tumors

Next, we evaluated the expression of stemness-related genes and identified 14 differentially overexpressed genes in mutated tumors compared with wild-type tumors by performing 10,000 permuted 2-sample *t* test ($P < .01$) with greater than 2-fold difference (Supplementary Table 6). Notably, *CD24* highly expressed in *TP53* mutated as compared with wild-type tumors. *CD24* has been proposed as a stemness marker in numerous types of cancers including ovary,²³ pancreas,²⁴ and colon cancers.²⁵ In addition, *AFP* was found in the gene list, which is recognized as a cancer stem cell marker in combination with epithelial cell adhesion molecule in HCC.²⁶ To confirm our finding, the expression levels of *CD24* and *AFP* were measured by real-time quantitative PCR on 75 samples of mutant type (MT, $n = 31$) and wild-type (WT, $n = 44$) HCC. Significant overexpression of *CD24* and *AFP* was found in the mutant as compared with the wild-type tumors (Mann-Whitney *U* test, $P < .0001$, Figure 4A and B). Taken together, our results support the association of *TP53* mutations with the enriched expression of stemness-related traits.

Discussion

In this study, we demonstrate that the *TP53* mutations in Chinese HCC are associated with poor clinical

Figure 3. Expression of stem cell-like traits in *TP53* mutated tumors. (A) The enrichment scores for the ES signatures and HB signatures in the compiled HCC data set are shown on the *left*; the enrichment in groups of mutation type (MT) and wild type (WT) is shown in the *middle*; and the enrichments of ES signature without the proliferation-related genes (noprol) are shown on the *right*. The enrichment scores in each sample and group represent $-\log_{10}(P \text{ value})$ calculated by hypergeometric test. (B) Based on the expression status of ES1 (*upper*) or HB (*lower*) signatures and the hot spot *TP53* mutation status, the 366 patients were stratified into 4 groups. Forty-two cases of non-hot spot mutations were not included in the analysis. ES+ represent the tumors that were enriched with ES1 signature ($P < .01$), whereas other tumors were indicated as ES-. HB+ represents the tumors that were positively enriched with HB_UP and negatively enriched with HB_DOWN signatures ($P < .01$), whereas other tumors were indicated as HB-. (C) The plots showed the enrichment scores (ES) for the ES1 signature in breast cancers (*upper*), and lung cancers (*lower*) were calculated by Gene Set Enrichment Analysis method.

outcome. In particular, the hot spot mutations, ie, R249S and V157F, are strongly associated with bad prognosis. Evaluation of site-specific prognostic values of mutations may be helpful in delineating mutant p53 functions as well as their clinical implication.

The reason(s) for the worse prognostic feature associated with the hot spot mutations of *TP53* is unclear. It

has been suggested that the hot spot mutations, arising from the clonal expansion of the mutated cells during cancer progression, can provide a selective advantage for tumor cell survival²⁷ and/or that they are more deleterious than the non-hot spot mutations.²⁸ Indeed, accumulating evidence supports the functional alterations of the hot spot mutations, ie, R249S and V157F.^{29–31} It is also

Figure 4. Validation of *CD24* and *AFP* expression by quantitative polymerase chain reaction. (A and B) Expression of *CD24* (A) and *AFP* (B) levels in mutant type (MT, *n* = 31) and wild-type (WT, *n* = 44) HCCs were measured by quantitative polymerase chain reaction. Statistical significance was tested by 2-tailed Mann–Whitney *U* test. Median and interquartile ranges were indicated by horizontal lines.

possible that the different mode of actions can contribute to their prognostic distinction. R249S is frequently found, both in normal and in cirrhotic liver, in high aflatoxin B1 exposure area, suggesting that R249S mutation occurs at the early stage of hepatocarcinogenesis, whereas other mutations seem to be more prevalent at later stages.^{32,33} Similarly, in lung cancer, V157F is frequently found in close association with cigarette smoking.²⁷ These hot spot-specific features caused by the environmental exposure of mutagens result in G to T transversion and may be involved in the development of more aggressive phenotypes. However, considering the complexity of p53 biology, further studies are needed to delineate the mechanisms by which the hot spot mutations result in a worse prognosis.

We arbitrarily classified the tumors into hot spot vs non-hot spot mutated tumors with mutation frequency of 2%. However, the prognostic values of the hot spot *TP53* mutations are well reflected in their corresponding gene expression profiles. These data support the notion that the worse prognostic value of the hot spot mutations is not likely to be a biased observation because of the large number of cases or arbitrary classification. In addition, we found that the *TP53* mutations are accompanied by the expression of stem cell-like traits. Although further validation was not performed here for the causal link between *TP53* mutations and stem cell traits, it was already known that the p53 is involved in the stem cells' self-renewal by suppressing *NANOG* expression.³⁴ It has also been shown that neural stem cells harboring *TP53* mutations have increased potential to accumulate genetic lesions and generate glioblastomas.³⁵ Moreover, it was recently shown that *TP53* is critical for reprogramming of differentiated cells into pluripotent stem cells.³⁶ Similarly, our data support the view that the acquisition of stem cell-like traits in the *TP53* mutated tumors play an important role in the generation and/or progression of poor prognostic phenotype. Accordingly, the invalida-

tion of stem-stem traits might be a novel potential therapeutic strategy especially for the *TP53* mutated tumors.

In the present study, we observed the differential expression of the well-known putative cancer stem cell markers, ie, *CD24* and *AFP* in *TP53* mutated tumors compared with wild-type tumors; however, other putative liver cancer stem cell markers were not differentially expressed.^{37–39} This might be due to the population heterogeneity of the stem-like tumors as well as *TP53* mutated tumors activating different ES cell regulators. However, our data collectively suggest that the acquisition of stem-like trait in *TP53* mutated tumors is a likely explanation for the aggressive phenotype of *TP53* mutated tumors.

In conclusion, we demonstrate that the *TP53* mutations, particularly hot spot mutations, represent an independent risk factor for a shorter survival of the HCC patients. The acquisition of stem cell-like phenotype likely contributes to the aggressive behavior of *TP53* mutated tumors. Our results may therefore have prognostic and therapeutic implications for the future management of HCC patients with *TP53* mutations.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.11.034.

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Received January 19, 2010. Accepted November 10, 2010.

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Conflicts of interest

The authors disclose no conflicts.

Funding

Supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research, and a research grant of the Ajou University School of Medicine, Suwon, Korea (3-2010027-0, 2010).

Supplementary Table 1. List of Primer Sequences for *TP53* Mutation Analysis

Exons	Name	Sequence
Primers for genomic DNA		
Exon5	p53_5_S	5'-TCCTACAGTACTCCCCTGCC-3'
	p53_5_AS	5'-GCCCCAGCTGCTCACCATC-3'
Exon6	p53_6_S	5'-ACTGATTGCTCTTAGGTCTG-3'
	p53_6_AS	5'-AGTTGCAAACCAGACCTCAG-3'
Exon7	p53_7_S	5'-AGGTTGGCTCTGACTGTACC-3'
	p53_7_AS	5'-CTCCTGACCTGGAGTCTTCC-3'
Exon8	p53_8_S	5'-CTATCCTGAGTAGTGGTAATC-3'
	p53_8_AS	5'-GTCCTGCTTGCTTACCTCGC-3'
Primers for cDNA		
Exon5	p53_5_S	5'-GTCTGGGCTTCTTGCAATTCT-3'
	p53_5_AS	5'-ACACGCAAATTCCTTCCAC-3'
Exon6	p53_6_S	5'-CATGAGCGCTGCTCAGATAG-3'
	p53_6_AS	5'-GCCCATGCAGGAAGTGTTA-3'
Exon7	p53_7_S	5'-AGGTTGGCTCTGACTGTACC-3'
	p53_7_AS	5'-CTCCTGACCTGGAGTCTTCC-3'
Exon8	p53_8_S	5'-CCTCACCATCATCACACTGG-3'
	p53_8_AS	5'-CTTCTTTGGCTGGGGAGAG-3'

Supplementary Table 2. List of *TP53* Mutations in Chinese and White HCC Cohorts

No.	ID	Geographic region	Mutation	Wild type	Mutation type	Double mutation
1	HCC008	Belgium	R249S	AGG	AGT	R213R
2	HCC012	Belgium	R249S	AGG	AGC	
3	HCC030	Qidong	R249S	AGG	AGT	
4	HCC031	Qidong	R249S	AGG	AGT	
5	HCC032	Qidong	R249S	AGG	AGT	
6	HCC033	Qidong	R249S	AGG	AGT	
7	HCC035	Qidong	Y220C	TAT	TGT	
8	HCC039	Qidong	R249S	AGG	AGT	
9	HCC040	Qidong	R249S	AGG	AGC	
10	HCC041	Qidong	R249S	AGG	AGT	
11	HCC043	Qidong	G245C	GGC	GTC	R249S
12	HCC044	Qidong	R249S	AGG	AGT	
13	HCC045	Qidong	R249S	AGG	AGT	
14	HCC047	Qidong	G245V	GGC	GGT	
15	HCC051	Qidong	D184Y	GAT	TAT	
16	HCC054	Qidong	V157F	GTC	TTC	
17	HCC055	Qidong	R249S	AGG	AGT	
18	HCC056	Qidong	294del/fs			
19	HCC062	Qidong	R249S	AGG	AGT	
20	HCC063	Qidong	R273L	CGT	CTT	
21	HCC064	Qidong	R249S	AGG	AGT	
22	HCC066	Qidong	E298X	GAG	TAG	
23	HCC067	Qidong	R249S	AGG	AGT	
24	HCC068	Qidong	R249S	AGG	AGT	
25	HCC069	Qidong	V157F	GTC	TTC	
26	HCC070	Qidong	R213X	CGA	TGA	
27	HCC071	Qidong	R249S	AGG	AGT	
28	HCC073	Qidong	V157F	GTC	TTC	
29	HCC074	Qidong	R249S	AGG	AGT	
30	HCC075	Qidong	R249S	AGG	AGT	
31	HCC076	Qidong	R249S	AGG	AGT	
32	HCC077	Qidong	R249S	AGG	AGT	
33	HCC078	Qidong	R249S	AGG	AGT	
34	HCC079	Qidong	R249S	AGG	AGT	
35	HCC080	Qidong	R249S	AGG	AGT	
36	HCC082	Qidong	R249S	AGG	AGT	
37	HCC084	Qidong	R249S	AGG	AGT	
38	HCC085	Qidong	R249S	AGG	AGT	
39	HCC088	Qidong	A159P	GCC	CCC	
40	HCC089	Qidong	V157F	GTC	TTC	
41	HCC090	Qidong	A159P	GCC	CCC	
42	HCC096	Belgium	R213R	CGA	CGG	
43	HCC107	Belgium	R249S	AGG	AGT	
44	HCC120	United States	V157F	GTC	TTC	
45	HCC122	United States	R213R	CGA	CGG	
46	HCC138	United States	V157F	GTC	TTC	
47	HCC142	Qidong	R249S	AGG	AGT	
48	HCC143	Qidong	R249S	AGG	AGT	
49	HCC145	Qidong	R248W	CGG	TGG	
50	HCC146	Qidong	G245V	GGC	GTC	
51	HCC147	Qidong	R249M	AGG	ATG	
52	HCC148	Qidong	R249S	AGG	AGT	
53	HCC149	Qidong	R249S	AGG	AGT	
54	HCC154	Qidong	R249S	AGG	AGT	
55	HCC157	Qidong	R249S	AGG	AGT	
56	HCC162	Qidong	R249S	AGG	AGT	
57	HCC164	Qidong	V157F	GTC	TTC	
58	HCC165	Qidong	R249S	AGG	AGT	
59	HCC167	Qidong	V157F	GTC	TTC	
60	HCC168	Qidong	R249S	AGG	AGT	
61	HCC169	Qidong	R249S	AGG	AGT	
62	HCC170	Qidong	R249S	AGG	AGT	
63	HCC171	Qidong	R249S	AGG	AGT	

Supplementary Table 2. Continued

No.	ID	Geographic region	Mutation	Wild type	Mutation type	Double mutation
64	HCC175	Qidong	R249S	AGG	AGT	R249S
65	HCC176	Qidong	R249S	AGG	AGC	
66	HCC181	Qidong	I255S	ATC	AGC	
67	HCC190	Qidong	R249S	AGG	AGT	
68	HCC191	Qidong	V157F	GTC	TTC	
69	HCC193	Qidong	R249S	AGG	AGT	
70	HCC194	Qidong	R249S	AGG	AGT	
71	HCC196	Qidong	R249S	AGG	AGT	
72	HCC199	Qidong	R249S	AGG	AGT	
73	02-285A	Shanghai	R249S	AGG	AGT	
74	02-314A	Shanghai	H193R	CAT	CGT	
75	02-345A	Shanghai	R249S	AGG	AGC	
76	02-346A	Shanghai	G199V	GGA	GTA	
77	02-354A	Shanghai	G266R	GGA	AGA	
78	02-355A	Shanghai	I195F	ATC	TTC	
79	02-361A	Shanghai	R249S	AGG	AGT	
80	02-366A	Shanghai	S127P	TCC	CCC	
81	02-407A	Shanghai	H193R	CAT	CGT	
82	02-416A	Shanghai	R249S	AGG	AGT	
83	02-423A	Shanghai	R249S	AGG	AGT	
84	02-424A	Shanghai	R249S	AGG	AGT	
85	02-427A	Shanghai	R249S	AGG	AGT	
86	02-432A	Shanghai	R249S	AGG	AGT	
87	02-454A	Shanghai	C135Y	TGC	TAC	
88	02-458A	Shanghai	R249S	AGG	AGT	
89	02-460A	Shanghai	R249S	AGG	AGT	
90	02-461A	Shanghai	A159P	GCC	CCC	
91	02-466A	Shanghai	R249S	AGG	AGT	
92	03-017A	Shanghai	R174W	AGG	TGG	N268D
93	03-025A	Shanghai	R249S	AGG	AGT	
94	03-047A	Shanghai	R267P	CGG	CCT	
95	03-057A	Shanghai	T253S	ACC	TCC	
96	03-058A	Shanghai	R249S	AGG	AGT	
97	03-062A	Shanghai	R306X	CGA	TGA	
98	03-093A	Shanghai	R249S	AGG	AGT	
99	03-099A	Shanghai	R249S	AGG	AGT	
100	03-102A	Shanghai	G244D	GGC	GAC	
101	03-147A	Shanghai	R249S	AGG	AGT	
102	03-148A	Shanghai	S127P	TCC	CCC	
103	03-164A	Shanghai	R249S	AGG	AGT	
104	03-187A	Shanghai	N239N			
105	03-197A	Shanghai	R175L	CGC	CTC	
106	03-199A	Shanghai	C176S	TGC	TCC	
107	03-210A	Shanghai	R249S	AGG	AGT	
108	03-211A	Shanghai	G266V	GGA	GTA	
109	03-213A	Shanghai	Y236C	TAC	TGC	
110	03-219A	Shanghai	R280G	AGA	GGA	
111	03-228A	Shanghai	I232T	ATC	ACC	
112	03-231A	Shanghai	K132N	AAG	AAT	
113	03-233A	Shanghai	R249S	AGG	AGT	
114	03-235A	Shanghai	R249S	AGG	AGC	
115	03-248A	Shanghai	C182X	TGC	TGA	
116	03-268A	Shanghai	V157F	GTC	TTC	
117	03-273A	Shanghai	R249S	AGG	AGT	
118	03-277A	Shanghai	R249S	AGG	AGT	
119	03-278A	Shanghai	I162F	ATC	TTC	
120	03-280A	Shanghai	R249S	AGG	AGT	
121	03-305A	Shanghai	R249S	AGG	AGT	
122	03-313A	Shanghai	R249S	AGG	AGT	
123	03-314A	Shanghai	C277F	TGT	TTT	
124	03-316A	Shanghai	R249S	AGG	AGT	
125	03-326A	Shanghai	R249S	AGG	AGT	
126	03-333A	Shanghai	R249S	AGG	AGT	

Supplementary Table 2. Continued

No.	ID	Geographic region	Mutation	Wild type	Mutation type	Double mutation
127	03-335A	Shanghai	R249S	AGG	AGT	
128	03-359A	Shanghai	R249S	AGG	AGT	
129	03-366A	Shanghai	G245S	GGC	AGC	
130	03-381A	Shanghai	A159P	GCC	CCC	
131	03-387A	Shanghai	V272M	GTG	ATG	
132	03-425A	Shanghai	R249S	AGG	AGT	
133	03-450A	Shanghai	K164X	AAG	TAG	
134	03-468A	Shanghai	del126-133			

Supplementary Table 3. Univariate Test for the Structural Classification of *TP53* Mutations in Chinese Cohort

Structural classification	Number of mutations	Hazard ratio	95% CI lower	95% CI upper	<i>P</i> value
L2	6	1.80	0.66	4.90	.247
L3	82	2.04	1.45	2.85	<.001
Non-L3	43	1.56	0.99	2.48	.053
S4	13	3.38	1.74	6.57	<.001
Non-S4	112	1.76	1.28	2.41	<.001
Disruptive	90	1.85	1.32	2.58	<.001
Nondisruptive	36	1.62	1.00	2.64	.048

Supplementary Table 4. Fisher Exact Test for the Selected Clinical Features and *TP53* Mutations in Chinese Cohort

	Mutation type	Wild type	Odds ratio	95% CI lower	95% CI upper	<i>P</i> value
Mutation, any type						
Sex (male/female)	112/13	170/35	1.77	0.87	3.82	.109
Age (<50/≥50, y)	73/52	100/105	1.47	0.92	2.37	.112
AFP (≥300/<300 ng/mL)	74/48	96/104	1.67	1.03	2.71	.029
Size (≥5/<5 cm)	73/51	104/101	1.39	0.86	2.24	.171
Presence of cirrhosis (yes/no)	86/38	163/42	0.58	0.34	1.01	.046
Grade (III, IV/I, II)	71/42	113/66	0.99	0.59	1.66	1.000
HBV (+/−)	106/19	190/19	0.56	0.27	1.17	.109
HCV (+/−)	2/123	3/206	1.12	0.09	9.89	1.000
Hot spot mutations						
Sex (male/female)	76/8	170/35	1.95	0.84	5.11	.144
Age (<50/≥50, y)	54/30	100/105	1.86	1.09	3.32	.019
AFP (≥300/<300 ng/mL)	47/34	96/104	1.50	0.86	2.61	.148
Size (≥5/<5 cm)	48/35	104/101	1.33	0.77	2.31	.299
Presence of cirrhosis (yes/no)	53/30	163/42	0.46	0.25	0.83	.007
Grade (III, IV/I, II)	53/26	113/66	1.19	0.66	2.18	.575
HBV (+/−)	71/13	190/19	0.55	0.24	1.27	.146
HCV (+/−)	0/84	3/206	0.00	0.00	6.04	.560

Supplementary Figure 1. Prognostic values of *TP53* mutations in patients who have several clinical features. (A–D) Kaplan–Meier plot for survival between patients with hot spot mutated and wild-type *TP53* in the subpopulations of patients who have low tumor grade (I, II; A), low serum AFP level (<300 ng/mL; B), large tumor size (>5 cm; C), or cirrhosis (D). WT indicates wild-type *TP53*.

Supplementary Figure 2. Gene set enrichment of *TP53* mutated and wild-type HCC. (A) The functional enrichment scores in the Gene Ontology (GO) hierarchy for each sample were calculated using hypergeometric test with threshold of $P < .01$ in the 366 HCC data set. A total of 80 GO terms were significantly enriched at least in the mutation-type (MT) or wild-type (WT) group ($P < .01$, hypergeometric test) in the samples, Chinese, or white groups (right bars). (B) In the Chinese cohort ($n = 238$), the enrichment scores in each group of hot spot, non-hot spot, and wild-type were calculated. A total of 73 GO terms were significantly enriched at least in 1 group (right). The GO terms with less than 10 genes or the GO terms enriched with less than 10% of samples were excluded in each analysis. The GO terms of proliferation/cell cycle- and metabolism-related functions were indicated with different colors (right bar).

Supplementary Figure 3. Kaplan–Meier plot analysis of survival with *TP53* mutation status and stemness-related signatures. (A and B). Based on the status of expression of embryonic stem (ES) 1 (A) and hepatoblast (HB) (B) signatures and *TP53* mutations, the 366 patients were stratified into 4 groups. ES+ represent the tumors that were enriched with ES1 signature ($P < .01$), whereas other tumors were indicated as ES-. HB+ represents the tumors that were positively enriched with HB_UP and negatively enriched with HB_DOWN signatures ($P < .01$), whereas other tumors were indicated as HB-.

Supplementary Table 5. Gene Set Enrichment Analysis of Embryonic Stem Cell-Related Genes in *TP53* Mutated Breast and Lung Cancers

Signatures	Size (n)	Enrichment score	Normalized enrichment score	Nominal <i>P</i> value	False discovery rate
Breast cancer					
ES1	336	0.566	2.650	0	0
ES2	38	0.652	2.181	0	0
MYC2	749	0.381	1.911	0	1.83×10^{-4}
MYC1	218	0.397	1.784	0	4.47×10^{-4}
SOX2	607	0.278	1.370	1.20×10^{-3}	2.27×10^{-2}
NANOG	810	0.272	1.358	1.14×10^{-3}	2.19×10^{-2}
Oct4	247	0.200	0.911	6.96×10^{-1}	8.07×10^{-1}
NOS	147	0.207	0.886	7.29×10^{-1}	8.03×10^{-1}
Lung cancer					
ES1	356	0.585	2.889	0	0
ES2	37	0.623	2.225	0	0
MYC2	757	0.416	2.150	0	0
NANOG	911	0.349	1.838	0	0
SOX2	677	0.353	1.820	0	9.82×10^{-5}
Oct4	275	0.330	1.590	0	1.99×10^{-3}
MYC1	219	0.293	1.378	1.67×10^{-2}	1.64×10^{-2}
NOS	171	0.287	1.306	4.82×10^{-2}	2.91×10^{-2}

Supplementary Table 6. Differentially Overexpressed Genes in *TP53* Mutated Tumors Compared With Wild-Type Tumors

Gene ID	Symbol	<i>P</i> value (<i>t</i> test)	<i>P</i> value (permuted <i>t</i> test)	False discovery rate	Average (MT)	Average (WT)	Fold difference (unlogged)
934	CD24	.0005	.0016	0.0522	2.18	−0.71	7.44
157273	LOC157273	.0079	.0006	0.0296	1.49	−0.19	3.19
23089	PEG10	.0006	.0008	0.0353	1.18	−0.35	2.89
6690	SPINK1	.0013	.0031	0.0777	1.17	−0.35	2.87
154754	TRY6	.0011	.0001	0.0126	1.21	−0.10	2.48
6066	RNU2	.0160	.0014	0.0491	0.99	−0.22	2.31
174	AFP	.0026	.0020	0.0606	0.77	−0.34	2.16
4100	MAGEA1	.0042	.0029	0.0753	0.94	−0.17	2.16
6364	CCL20	.0003	.0007	0.0341	0.79	−0.32	2.16
1047	CLGN	.0036	.0030	0.0760	0.80	−0.25	2.07
388677	NOTCH2NL	.0022	.0016	0.0526	0.85	−0.18	2.04
8364	HIST1H4C	.0000	.0000	0.0000	0.71	−0.31	2.02
259266	ASPM	.0000	.0008	0.0348	0.76	−0.25	2.01
3248	HPGD	.0016	.0047	0.0994	0.86	−0.14	2.00

MT, mutant type; WT, wild type.