Large-scale analysis of the genetic and epigenetic alterations in hepatocellular carcinoma from Southeast China

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Abstract

Our knowledge about molecular alterations during hepatocarcinogenesis is still fragmentary, due to lack of comprehensive genetic and epigenetic analyses in the same set of hepatocellular carcinomas (HCCs). In this study, we conducted a large-scale analysis, including mutation screening in 50 genes and methylation assays in three genes in 54 pairs of HCCs and their neighboring non-cancerous tissues. All samples were collected from the residents in Southeast China. We found HBV infection and chronic hepatitis/cirrhosis in 83.3% and 98.1% of the cases, respectively. Mutations were identified in 18 out of 54 (33.3%) samples, with p53 alterations in 14 cases and β-catenin mutations in four tumors. No mutations were identified in the neighboring tissues. Interestingly, 9 out of 14 (64.3%) tumors carrying p53 mutations displayed substitution of serine by arginine at codon 249, a characteristic change believed to be induced by aflatoxin-B1. Furthermore, p53 mutation was significantly associated with shorter recurrence-free survival (P = 0.004). The results also revealed aberrant methylation in two or more genes in as high as 90% of tumors and 40% of adjacent tissues. The frequency of RASSF1A hypermethylation was much higher than that of p16INK4a and HAI2 in both HCC and neighboring tissues, indicating that deregulation of RASSF1A may precede the other two genes. These data suggest that aberrant methylation occurs before mutation and is an early event in the development of this set of HCC. Our findings highlight p53 as a prognostic factor of HCC and RASSF1A as a potential target in preventing malignant transformation of hepatocytes.

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1. Introduction

Hepatocellular carcinoma (HCC), one of the most common malignancies worldwide, is seeing a striking increase in its incidence [1,2]. The prominent agents associated with HCC include chronic hepatitis B (HBV) and C (HCV) virus infection, chronic alcohol consumption, dietary exposure to aflatoxin-B1 (AFB1) and virtually all cirrhosis-inducing conditions [3]. HCC is one of the most lethal and prevalent cancers in China, especially in the southeast region, where HBV and AFB1 are main attributable risk factors [3,4].

As in other solid tumors, multiple genetic and epigenetic changes have been described in HCC. The signaling pathways, including the p53, WNT/β-catenin and TGF-β pathways, have been outlined as common targets deregulated during hepatocarcinogenesis [3–7]. In addition, many other HCC-associated genes have also been documented in different studies [3,8]. However, despite the rapid expansion of the information, most published studies have analyzed only few genes in individual HCCs [8–10]. Furthermore, studies from different laboratories show heterogeneity of genomic aberrations, which may result from the distinct etiological factors and diverse host genetic
background in different cohorts [8,9]. To date, our knowledge about molecular alterations during the development of HCC is still fragmentary and incomplete, due to lack of comprehensive detection for both genetic and epigenetic lesions in the same set of HCCs [9,10]. It is believed that simultaneous evaluation of multiple genes and regulatory pathways in HCC should help to identify the causative factors, the critical regulatory pathways involved in hepatocarcinogenesis, the markers for early detection and prognosis prediction, as well as the new therapeutic targets [3,8,9].

Although HCC is highly prevalent in Guangdong, a province in Southeast China, the information about the genetic/epigenetic pattern of genetic/epigenetic alterations in HCC from this region is rather limited [11,12]. In the present study, we have conducted a large-scale analysis, including mutation screen in 50 genes and methylation assays in three genes in the same set of HCCs and their neighboring non-cancerous tissues. All samples were collected from the residents in Guangdong. The goal of our study is to reveal the pattern of genetic/epigenetic alterations in HCC from this region, and to explore the correlations of the molecular changes with clinicopathological characteristics.

2. Materials and methods

2.1. Tumor samples and patients

HCC and corresponding neighboring non-cancerous liver tissues were collected from 54 patients undergoing tumor resection at the Cancer Center, Sun Yat-sen University (Guangzhou, China). Both tumor and non-cancerous samples were histologically confirmed. The histological grade of tumor differentiation was assigned according to the Edmondson and Steiner grading system. The tissue samples were snap frozen in liquid nitrogen immediately after surgical resection and then stored at −80 °C until analysis. Total genomic DNA was isolated using a standard protocol with proteinase digestion, phenol–chloroform extraction and ethanol precipitation.

Clinicopathological details and follow-up information were obtained through hospital records. The studied subjects are residents in Guangdong Province, PR China. The median age of the patients was 46.6 years (range, 27–70 years) and the male/female ratio was 5.8:1. HBV or HCV infection was diagnosed when HBV surface antigen (HBsAg) or HCV antibody (HCV-Ab) was detected by ELISA in the serum isolated from peripheral blood. As shown in Fig. 1A, HBV and HCV infection were identified in 45 (83.3%) and 3 (5.6%) cases, respectively. Two patients showed evidence of infection for both HBV and HCV. All tumors, except for one, originated from the background of chronic hepatitis and/or cirrhosis (Fig. 1A). Informed consent was obtained from each patient. This study was approved by the Institute Research Ethics Committee at Cancer Center, Sun Yat-sen University.

2.2. Mutation analysis

Fifty genes (Table 1) involved in multiple pathways that control different cellular processes were analyzed. Gene mutations were screened by polymerase chain reaction-based single-strand conformation analysis (PCR-SSCA) and confirmed by direct DNA sequencing. The PCR primers were designed with primer3 (http://www.urogene.org/methprimer/index1.html): MF (5′-GGT TTA TTG GTT TTG GC-3′) and MR (5′-ACC TAA ATC TAC TCC TCA GC-3′) to amplify the methylated promoter; UF (5′-GTA GTT TGG TAT TGG TTT TGG-3′) and UR (5′-AAT ACC TAA ATC TCC TCA CTC ACA-3′) for the unmethylated promoter. It had been reported that the promoters of p16INK4a and HA12 were methylated in Huh7 [15,16] and the promoter of RASSF1A was methylated in Hep3B [17]. Therefore, DNA from HCC cell lines, Huh7 or Hep3B, was used as a positive control for methylation of the respective gene promoter. DNA from normal lymphocytes was used as a negative control for methylation.

2.3. Methylation analysis

The methylation status of the promoters of the p16INK4a, RASSF1A and HAI2 genes were determined by sodium bisulfite modification and methylation-specific PCR (MSP), as described previously [13]. The primer sets and PCR conditions used to assess the promoters of p16INK4a and RASSF1A were derived from previous reports [13,14]. HA12 gene was examined using the following primers, which were designed with Methprimer (www.urogene.org/methprimer/index1.html): MF (5′-GGT TTA TTG GTT TTG GC-3′) and MR (5′-ACC TAA ATC TAC TCC TCA GC-3′) to amplify the methylated promoter; UF (5′-GTA GTT TGG TAT TGG TTT TGG-3′) and UR (5′-AAT ACC TAA ATC TCC TCA CTC ACA-3′) for the unmethylated promoter. It had been reported that the promoters of p16INK4a and HAI2 were methylated in Huh7 [15,16] and the promoter of RASSF1A was methylated in Hep3B [17]. Therefore, DNA from HCC cell lines, Huh7 or Hep3B, was used as a positive control for methylation of the respective gene promoter. DNA from normal lymphocytes was used as a negative control for methylation.

2.4. Statistical analysis

Two-tailed Chi-square and Fisher exact tests were used for the analysis of categorical data. The recurrence-free survival was calculated from the date of tumor resection to the time of first recurrence or death. Patients without recurrence at the time of last follow-up were treated as censored events. Survival curves were constructed according to Kaplan–Meier method and the differences between groups were analyzed using log rank test. Association between the molecular changes or clinical characteristics of patients and the recurrence-free survival was first analyzed by univariate Cox regression analysis. Significant prognostic factors found by univariate analysis were further evaluated by multivariate Cox regression analysis. The influence of each factor, described as the hazards ratio (HR), was assessed by Cox proportional hazard model. A P-value of less than 0.05 was used as the criterion of statistical significance, and all statistical tests were two-sided. All analyses were performed using SPSS software (Version 13.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Mutation analysis in 50 protein-coding genes

To identify the genetic alterations involved in the development of HCC, a large-scale mutation analysis in the coding sequences and/or the promoter regions of 50 protein-coding genes was performed using PCR-SSCA and direct DNA sequencing. The information on all studied genes, including the regions subjected to mutation analysis and their potential functions, are listed in Table 1. We chose those genes that had shown genetic alterations or deregulated expression in human cancers, or had been mapped to the chromosomal regions that were frequently lost in HCC, or had displayed tumor suppressor functions in animal models. Furthermore, the selected genes are all involved in the regulatory pathways that control the growth, proliferation, apoptosis, differentiation, motility or adhesion of cell (Table 1). HCC tissues included in this study were collected from 54 patients, among which 45 (83.3%) displayed HBV infection and 53 (98.1%) originated from the background of chronic hepatitis and/or cirrhosis (Fig. 1A). In total, 21 muta-
Fig. 1. Summary of the identified genetic and epigenetic alterations. (A) Clinicopathological characteristics of patients. The contents in each column are as follows: (a) HBV infection; (b) HCV infection; (c) chronic hepatitis; (d) degree of cirrhosis; (e) tumor thrombus in portal vein (PVTT); (+) indicates presence; (−) for absence. Arabic numbers in the column d indicate the degree of cirrhosis: 0, no cirrhosis; 1, mild cirrhosis; 2, moderate cirrhosis; 3, severe cirrhosis. (B) The somatic mutations detected. (C) The methylation patterns in each pair of samples. T: tumor tissue; N: neighboring non-cancerous tissue. The empty circle: unmethylation; the grey circle: partial methylation; the filled circle: complete methylation; Ni: non-informative.

tions were found in 18 out of 54 (33.3%) HCCs (Fig. 1B). The genomic fragments displaying mutations in the tumor tissues were then further analyzed in the corresponding neighboring non-cancerous liver tissues. Interestingly, none of mutations were observed in the adjacent tissues. Furthermore, all identified mutations distributed in the p53 and β-catenin genes, with mutation rates of 25.9% (14/54) and 7.4% (4/54), respectively, while no mutations were detected in the other 48 genes.

Seventeen out of 21 alterations observed in this set of HCC were allocated to the p53 gene, comprising 15 transversions, one transition and one deletion (Fig. 1B). We identified a novel mutation c.536delA, which was predicted to result in frame-shift and
thereby a pre-mature product of 245 amino acids. Although the whole coding region of the p53 gene was examined, all identified mutations lay in the highly conserved DNA-binding domain of the p53 protein, with eight of them in exon 5 and nine in exon 7 (Fig. 1B). Notably, mutations in exon 7 were all located at the third nucleotide of codon 249, resulting in the substitution of serine for arginine (R249S, Fig. 1B).

The four mutations observed in the β-catenin gene were all missense mutations, with three transitions and one transversion. All mutations caused the amino acid substitutions at the potential GSK-3β phosphorylation sites, including codons 37, 41 and 45 (Fig. 1B). Interestingly, none of the tumors carrying β-catenin mutations had concomitant p53 mutations (Fig. 1B), implying different mechanisms underlying the inactivation of p53 pathway and the activation of β-catenin pathway.

### 3.2. Methylation analysis in the p16INK4a, RASSF1A and HAI2 genes

Growing evidences have suggested that abnormal methylation is an early event during carcinogenic process [18,19]. p16INK4a, RASSF1A and HAI2 are negative regulators of cell growth [15,20,21]. Hypermethylations in the p16INK4a, RASSF1A and HAI2 genes have been shown to result in silencing of the gene expression [14–17]. To investigate whether aberrant methylation is an alternative mechanism responsible for the development of our set of HCC, MSP was employed to assess the methylation status of the promoters of these three genes in both HCC tissues and the corresponding neighboring non-cancerous liver tissues. We classified the methylation status into three groups based on the following criteria (Fig. 2A): unmethylation group, PCR product was got only with primers specific for unmethylated allele but not with primers specific for methylated one; complete methylation group, PCR product was observed only with primers specific for methylated allele but not with primers specific for unmethylated one; partial methylation group, PCR product was obtained not only with primers specific for methylated allele but also with primers specific for unmethylated one. The results showed that RASSF1A was methylated at the highest frequency, followed by HAI2 and p16INK4a (Figs. 1C and 2B). Furthermore, the extent of hypermethylation in the HCC tissues was significantly higher than that in the neighboring non-cancerous tissues (Figs. 1C and 2B). Surprisingly, 90% of tumors and 56% of adjacent tissues carried complete methylation in at least one of the three genes analyzed (Fig. 1C). Furthermore, complete methylation in two or more genes were identified in 30% of HCCs but none of adjacent tissues (Fig. 1C). If the cases with partial and complete methylation status were combined, we found that all tumor and adjacent tissues displayed aberrant methylation in at least one gene, while 90% of tumor samples and 40% of neighboring tissues showed abnormal methylation in two or more genes (Fig. 1C). Interestingly, the adjacent liver tissues never concurrently showed hypermethylation in both p16INK4a and HAI2 genes (Fig. 1C).

### 3.3. Association analysis between the identified molecular changes and the recurrence-free survival of HCC patients

We next investigated whether the identified molecular changes were associated with the recurrence-free survival of HCC patients. The recurrence-free survival was analyzed using Kaplan–Meier curve, log-rank test and Cox regression. It is known that clinicopathological parameters can affect the prognosis of cancer patients. Therefore, association between the clinical variables and the recurrence-free survival were first studied. The results showed that patients with tumor thrombus in portal vein (PVTT) or higher degree of cirrhosis had significantly poorer recurrence-free survival in our study cohort (Fig. 3A and B and Table 2), while other parameters were not the influential factors (Table 2).

We then evaluated the effect of genetic and epigenetic changes on the recurrence-free survival of patients. We found that p53 mutations were significantly associated with worse recurrence-free survival (Table 2, HR = 2.83, 95%
Fig. 2. Methylation analysis of the promoters of the p16INK4a, HAI2 and RASSF1A genes. (A) Representative examples of methylation-specific PCR (MSP) for p16INK4a, HAI2 and RASSF1A. DNAs from HCC and neighboring non-cancerous tissues were subjected to bisulfite treatment and subsequent MSP analysis with primers specific to the methylated (M) or unmethylated (U) allele. The circles under each sample indicate different extent of methylation: empty circle, indicates unmethylation, where PCR product is only present in the lane marked U; filled circle, for complete methylation, where PCR product is only visible in the lane marked M; grey circle, indicates partial methylation, where PCR product can be seen in both M and U lanes. HCC cell line, Huh7 (harboring methylated p16INK4a and HAI2) or Hep3B (carrying methylated RASSF1A), was used as a positive control (PC), while normal lymphocyte was used as a negative control (NC) for methylation of the respective gene promoter. The H2O, instead of bisulfite-treated DNA, was added to the PCR reaction mixture as blank control (BC) for PCR contamination. (B) The frequency distribution of the methylation events in the promoters of the p16INK4a, HAI2 and RASSF1A genes. The black wedges reflect the percentage of cases with complete methylation, the grey ones indicate the percentum of patients with partial methylation, and the white wedges for the percentage of unmethylated ones. T: tumor tissue; N: neighboring non-cancerous tissue.

CI = 1.28–6.25; Fig. 3C, log rank \( P = 0.004 \). Recurrence within the first post-operative year occurred in all patients (11/11, 100%) harboring p53 mutations, but only in 18 out of 34 (52.9%) cases without p53 mutations. Considering that the existence of PVTT and higher grade of cirrhosis were prognostic factors for poorer recurrence-free survival, further stratification for the clinical parameters (Table 2) was performed. Interestingly, the significant association of p53 mutation with poorer recurrence-free survival was also observed in the patients without PVTT (Table 2, HR = 4.76, 95% CI = 1.84–12.3; log rank \( P < 0.001 \)) and in the patients without or with mild cirrhosis (Table 2, HR = 3.85, 95% CI = 1.26–11.8; log rank \( P = 0.008 \)). In addition, we also observed a trend of poorer recurrence-free survival in patients with p16INK4a hypermethylation in tumor tissues, although the statistic analysis only showed borderline significance (Fig. 3D and Table 2). The significant prognostic factors found by univariate analysis, including p53 mutation, PVTT and cirrhosis stage, were further evaluated by multivariate Cox regression analysis. The result confirmed that these factors conferred independent impact on the prognosis of patients (Table 2).

The association between the identified molecular changes and the clinicopathological parameters was also analyzed by Chi-square and Fisher exact tests. The results revealed a significantly higher rate of HAI2 hypermethylation in the neighboring non-cancerous tissues from heavy smokers compared with those from nonsmokers or light smokers (OR = 6.22, 95% CI = 1.29–30.10, \( P = 0.042 \)). However, no significant correlations were found between mutation/methylation status and other clinical variables (data not shown).

4. Discussion

This study provides a large-scale analysis for both genetic and epigenetic alterations and their relations to the clinicopathological characteristics of HCC. All tumors analyzed, except for one, derived from the background of chronic hepatitis and/or cirrhosis. Additionally, HBV infection was present in 83.3% of the samples, indicating that our study cohort is a representative of the subset of HCC arising from cirrhosis and that HBV infection is the most considerable risk factor for HCC in Guangdong.

The 50 genes subjected to mutational analysis (Table 1) are involved in different regulatory pathways that have been implicated in various types of cancers including HCC. Our results disclose that mutations are mainly convergent at the p53 and \( \beta \)-catenin genes, suggesting the involvement of dys-
Fig. 3. Kaplan–Meier recurrence-free survival (RFS) curves for HCC patients. (A) Comparison between the cases with and without PVTT; (B) Comparison between the patients with higher and lower degree of cirrhosis; (C) Comparison between the cases with mutated and wildtype p53; (D) Comparison between the patients with and without p16INK4a hypermethylation. (+) presence; (−) absence. Log-rank P values were indicated. Tick marks represent censored data.

The function of these two molecules in the development of HCC. p53 is a well-known tumor suppressor gene that is involved in cell cycle control, DNA repair, and apoptosis [3,8,22]. It has been demonstrated that the p53 deficient mice are susceptible to both spontaneous and chemical-induced tumors [3,22]. β-catenin plays roles in the cell adhesion and the transmission of proliferating signal through Wnt pathway [3,8,23]. Activation of the Wnt pathway by growth signal stabilizes the β-catenin protein, which is then translocated to the nucleus, where β-catenin acts as a coactivator of the TCF/LEF transcription factor and in turn upregulates the expression of a set of cancer-relevant genes, including MYC, cyclin D1, survivin, COX2 and MMP7 [3,8]. Deregulation in the p53 or β-catenin/Wnt pathway has been implicated as critical steps during carcinogenesis [3–5,8,22–25]. The absence of mutations in the neighboring non-cancerous tissues suggests that these alterations are late events during progression of our set of HCC.

The p53 mutations have been found in about 30% of HCC cases worldwide, ranging from 20% in North America to 67% in Africa [25]. The rate of p53 mutation (25.9%) in our set of tumors is higher than that reported from Europe and USA (15–25%) [7,25], but lower than that observed in Jiangsu and Guangxi Province (over 40%), the regions in China with high incidence of HCC [7,26,27]. The mutation frequency of β-catenin in the GSK-3β phosphorylation sites in HCCs ranges from 0% to 44% [8], with higher mutation rate identified in HCV-associated HCC [3,28]. The rate of β-catenin mutation in our cohort (7.4%) is similar to that in HCCs (8.1%) from Guangxi, the neighboring province of Guangdong [29]. The differences of the mutation spectrums in different regions probably reflect the distinctness in etiological factors and host genetic background.

Although all the coding sequences and the exon-intron junctions of the p53 gene were examined in this study, the mutations found were all located in the DNA binding domain of p53, which has been demonstrated to be the critical region for its function [24,30]. Notably, among seventeen p53 mutations identified, nine were located at nucleotide (nt) 747, three at nt536, two at each of nt490 and nt395, and one at nt475 (Fig. 1B). This intriguing pattern strongly suggests that certain endemic factors are responsible for HCC development in Guangdong. Further detailed analysis revealed that 9 out of 14 (64.3%) HCC with p53 mutations had substitution of serine by arginine at codon 249, a characteristic alteration believed to be induced by AFB1 [24,30,31], suggesting that exposure to AFB1 is another causative agent of HCC in Guangdong. In addition, we also found that the p53 mutation was significantly associated with shorter recurrence-free survival, which is consistent with the results of other studies [32,33]. All β-catenin mutations observed in our set of HCC were mapped to the potential GSK-3β phosphorylation sites. It has been shown that mutations in
therefore may have a greater impact on selection of cell subpopulations to occur at a much higher frequency than gene mutations and chronic hepatitis\[16,37,38\]. Therefore, it is understandable to hypothesize that the epigenetic changes may precede the initial mutations in the neighboring non-cancerous tissues although to a lower extent and occur at the early stage of hepatocarcinogenesis. Aberrant hypermethylation was much higher than that in tumor tissues. Moreover, aberrant methylation of RASSF1A gene may precede the development of the Wnt/β-catenin pathway [8,23].

In contrast to genetic alterations that can lead to immediate disruption of protein function, hypermethylation affects gene expression in a gradual and progressive manner [34]. It is believed that the epigenetic makeup is much more amenable to the environment influences compared with the genetic counterparts [35,36]. Besides, epigenetic lesions have been shown to occur at a much higher frequency than gene mutations and therefore may have a greater impact on selection of cell subpopulation during carcinogenesis [18,34,36]. In the present study, abnormal methylation was found in all tumor samples analyzed. Furthermore, hypermethylation was also observed in the neighboring non-cancerous tissues, although to a lower extent compared with that in tumor tissues (Figs. 1C and 2B). However, gene mutations were identified only in 33.3% of tumor samples, considering that almost all patients in this study developed HCC from the background of chronic hepatitis or cirrhosis. The observed mutually exclusive methylation patterns of p16INK4a and HAI2 genes in neighboring non-cancerous tissues may indicate different environment–epigenetic interactions. Dietary factors such as tobacco, alcohol and folate have been reported to be associated with specific gene methylation pattern [21,39]. Here we also observed an association between cigarette smoking and abnormal methylation of HAI2 in the neighboring non-cancerous tissues.

The ubiquity of the aberrant methylation existing in our set of HCCs was largely attributed to the high methylation rate of HCCs was largely attributed to the high methylation rate of RASSF1A in both HCC and neighboring non-cancerous tissues. Analyzing the neighboring non-cancerous tissues and none of the neighboring tissues. Our findings suggest that the epigenetic changes may precede the initial mutations and occur at the early stage of hepatocarcinogenesis. Aberrant methylation has been observed in liver tissues with cirrhosis or chronic hepatitis [16,37,38]. Therefore, it is understandable to have such high frequency of hypermethylation in our set of samples, considering that almost all patients in this study developed HCC from the background of chronic hepatitis or cirrhosis. The observed mutually exclusive methylation patterns of p16INK4a and HAI2 in neighboring non-cancerous tissues may indicate different environment–epigenetic interactions. Dietary factors such as tobacco, alcohol and folate have been reported to be associated with specific gene methylation pattern [21,39]. Here we also observed an association between cigarette smoking and abnormal methylation of HAI2 in the neighboring non-cancerous tissues.

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<table>
<thead>
<tr>
<th>Variable</th>
<th>Case no.</th>
<th>HR (95% CI)a</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age: ≥45/&lt;45</td>
<td>27/27</td>
<td>0.69 (0.36–1.34)</td>
<td>0.278</td>
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<td>Gender: male/female</td>
<td>46/8</td>
<td>1.07 (0.44–2.58)</td>
<td>0.884</td>
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<td>AFP: ≥20/&lt;20 ng/ml</td>
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<td>0.83 (0.40–1.73)</td>
<td>0.605</td>
</tr>
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<td>HBV infection: +/-b</td>
<td>45/9</td>
<td>2.12 (0.74–6.04)</td>
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<tr>
<td>Cirrhosis: moderate or severe/no or mild</td>
<td>24/30</td>
<td>2.13 (1.07–4.24)</td>
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<tr>
<td>Tumor size: ≥5/&lt;5 cm</td>
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<td>Tumor number: &gt;1/&lt;1</td>
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<tr>
<td>Tumor capsule: +/-</td>
<td>21/33</td>
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<tr>
<td>PVTT: +/-b</td>
<td>10/44</td>
<td>3.91 (1.70–8.93)</td>
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<td>Edmondson grade: ≥III/I–III</td>
<td>25/28</td>
<td>1.66 (0.85–3.25)</td>
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<td>Alcohol: ≥50/&lt;50 g/day</td>
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<tr>
<td>Tobacco: ≥0.5/&lt;0.5 packs/day</td>
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<tr>
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<td>p53: mutant/wild type</td>
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<td>3.85 (1.26–11.8)</td>
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<td>HAI2 methylation: +/-b</td>
<td>36/14</td>
<td>0.93 (0.45–1.91)</td>
<td>0.840</td>
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</tbody>
</table>

| Multivariate analysis                              |          |               |               |
| PVTT: +/-b                                         | 10/44    | 3.77 (1.66–8.55) | 0.001         |
| p53: mutant/wild type                              | 14/40    | 2.47 (1.12–5.43) | 0.025         |
| Cirrhosis: moderate or severe/no or mild           | 24/30    | 2.11 (1.05–4.20) | 0.036         |

P value under 0.05 was considered as statistical significance, and was highlighted in bold.
a HR and P values were calculated using univariate or multivariate Cox regression, as indicated.
b (+) presence; (−) absence.
c Tumor capsule: (+) with complete encapsulation; (−) with incomplete or without encapsulation.
d Analysis was performed in all patients.
e Analysis was performed only in the patients without PVTT.
f Analysis was performed only in the patients with mild or without cirrhosis.

Table 2
Univariate and multivariate analysis of recurrence-free survival in HCC patients
genetic alteration, can be reversed by eliminating the toxic agents or with the treatment of therapeutic interventions and chemical agents, such as 5-aza-deoxycytidine [44]. Therefore, those genes with abnormal methylation occurring in the early stage of hepatocarcinogenesis, such as RASSF1A, may serve as potential molecular targets for prevention of malignant transformation. In addition, we observed a trend of poorer recurrence-free survival with the treatment of therapeutic interventions and chemical agents, such as 5-aza-deoxycytidine [44]. Therefore, those}

In summary, we conducted a large-scale mutation/methylation analysis and identified the patterns of genetic/epigenetic alterations in HCC from Guangdong. Our findings suggest p53 and \( \beta \)-catenin mutations as late events and epigenetic changes such as methylation at the RASSF1A promoter as early events during the development of HCC from Southeast China. The results also highlight p53 as a prognostic factor and RASSF1A as a potential target for the prevention of HCC.

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