

Detecting abnormal methylation of tumor suppressor genes *GSTP1*, *P16*, *RIZ1*, and *RASSF1A* in hepatocellular carcinoma and its clinical significance

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Received October 21, 2014; Accepted July 7, 2015

DOI: 10.3892/ol.2015.3536

Abstract. Hepatocellular carcinoma (HCC) has a high rate of mortality. Further studies into epigenetic changes in HCC, particularly the abnormal methylation of tumor suppressor genes (TSGs), are required, since these changes may provide novel biomarkers for early screening and diagnosis of HCC. By using methylation-specific polymerase chain reaction (MSP), the present study detected the methylation status in the promoter region of 4 candidate TSGs, *GSTP1*, *P16*, *RIZ1*, and *RASSF1A*, respectively, in 35 paired HCC and tumor-adjacent liver tissues in addition to 20 normal liver tissues. Their effect on the initiation and progression of HCC was also investigated by analyzing the clinicopathological data. The results of the present study revealed that the methylation level of *RIZ1* and *GSTP1* genes in HCC was significantly increased compared with that in the adjacent tissues ($P < 0.01$) and the normal liver tissues ($P < 0.01$). The methylation frequency of *P16* and *RASSF1A* genes was not significantly increased compared with that observed in the adjacent tissues ($P > 0.05$) but was significantly increased compared with the normal tissues ($P < 0.01$). In HCC tissues, the methylation frequency of the *GSTP1* gene in tumors with capsular invasion was significantly increased compared with that in tumors without capsular invasion ($P < 0.05$). The methylation frequency of *P16* gene in hepatitis B surface antigen (HbsAg)-positive HCC patients was significantly increased compared with that in HbsAg-negative patients ($P < 0.05$). The methylation status of *RIZ1* and *RASSF1A* genes was not significantly correlated with

the clinicopathological data ($P > 0.05$). Previous studies have demonstrated that the methylation status of *RIZ1* and *GSTP1* genes is HCC-specific, and thus may be used as a biomarker to assist the clinical diagnosis of HCC. While the methylation of *GSTP1* gene promoter may associate with the invasiveness of HCC, chronic hepatitis B virus infection may be the cause of methylation-induced *P16* inactivation.

Introduction

HCC is one of the tumors with the highest incidence worldwide, and its incidence and mortality in China have remained high. The occurrence of HCC is results from multiple factors, including the activation of certain oncogenes, inactivation of TSGs and exogenous stimuli. Previous studies have demonstrated that the hypermethylation of CpG islands in tumor suppressor gene (TSG) promoters is closely associated with the formation of HCC, which may transform the spatial structure of chromatin and hence block/silence the transcription of TSGs via recruiting proteins of the methyl CpG binding domain (MBD) family in addition to the associated protein complexes (1,2). Epigenetic silencing is a relatively common mechanism of TSG inactivation (3). The aberrant methylation of tumor-associated genes, particularly TSGs, requires further research.

A high frequency of methylation inactivation of the *GSTP1* gene has been observed in human prostate, kidney, and liver cancers (4-6). Zhang *et al* (7) and Tchou *et al* (8) demonstrated that there is a high frequency of methylation events in the *GSTP1* gene in HCC tumor samples and HCC cell lines, and that the methylation of *GSTP1* in HCC is associated with the action of environmental carcinogens. As a TSG, *P16* gene inactivation may result in excessive cell proliferation, and the promoter methylation on 9p21 in HCC patients represents the most common mechanism of *P16* inactivation (9). Zhong *et al* (10) revealed that the abnormal methylation of the *RASSF1A* gene promoter is present in 95% of HCC tissues; the authors hypothesized that the change in *RASSF1A* gene expression is an early event during hepatitis B virus-induced tumorigenesis of HCC.

The present study comparatively analyzed the changes in methylation level of 4 TSGs in samples from HCC tumors, tumor-adjacent tissues, and normal liver tissues, and

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Key words: hepatocellular carcinoma, suppressor gene, methylation

investigated their correlation with the occurrence and progression of HCC by consulting the clinicopathological data, in order to provide a novel way for the early screening and gene diagnosis and therapy of liver cancer.

Materials and methods

Written informed consent was obtained from the families of all patients, and the Human Research Ethics Committee of the Affiliated Nanjing University Drum Tower Hospital (Nanjing, China) approved the use of all samples under a protocol that conforms to the provisions of the Declaration of Helsinki (as revised in Seoul, 2008).

Specimens. The tumor specimens were collected from HCC patients who had undergone surgical treatment in the Department of Hepatobiliary Surgery of the Affiliated Drum Tower Hospital, School of Medicine, Nanjing University, in the Changzhou First People's Hospital, and in the Third Affiliated Hospital of Soochow University, during the period between January 2013 and January 2014. The patients did not receive any anticancer treatment and had no other endocrine, immune, and metabolic diseases prior to the surgery. Any hemorrhagic and necrotic regions were avoided during the tumor sample collection. The tumor-adjacent liver tissues were obtained from the area within 1.5 cm distance from the edge of HCC, and histologically confirmed for no infiltration of cancer cells. The normal control group contained 20 cases of pathologically confirmed normal liver tissue. All the specimens were frozen in liquid N₂ immediately following the resection, then transported, and stored at -80°C.

Methylation-specific polymerase chain reaction (MSP) and result interpretation. The DNA samples were extracted from the liver specimens using a DNA extraction kit (Sangon Biotech Shanghai Co., Ltd., Shanghai, China), following the manufacturer's instructions. The concentration and purity of the extracted DNA were measured on a UV spectrophotometer (UV-240; Shimadzu Corp., Kyoto, Japan), and suitable DNA samples were stored at -80°C.

Bisulfite modification of the DNA samples was conducted using anEZ DNA Methylation-Direct™ Kit (Zymo Research, Irvine, CA, USA) according to the kit instructions. The DNA samples were then amplified by MSP and analyzed for methylation status based on the differential amplification pattern.

The primer sequences, annealing temperature, and product sizes are presented in Table I. The PCR system contained PCR Mixture 2X Mix 15 µl, U or M-Primer F 0.5 µl, U or M-Primer R 0.5 µl, Modified DNA 1-5 µl; the total volume was adjusted to 30 µl with deionized H₂O. The PCR conditions were as follows: 94°C denaturation, 3 min; 94°C denaturation 30 sec, annealing 30 sec, and 72°C extension 30 sec, 40 cycles; 72°C extension 7 min. The amplification product was stored at 4°C. The PCR products (10 µl each) were separated by 2% agarose gel electrophoresis, and the images were captured on a gel imaging and analysis system (Yu Long Co., Ltd., Kunming, China).

Samples that amplified from the primer pair for methylated DNA sequence were considered methylation-positive, whereas samples that amplified from the primer pair for unmethylated sequence were considered methylation-negative. Samples with

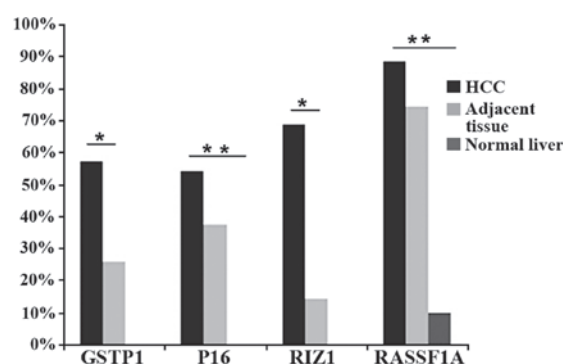


Figure 1. The status of methylation of GSTP1, P16, RIZ1, and RASSF1A genes in the 3 groups of tissues. *P<0.05. **P<0.01.

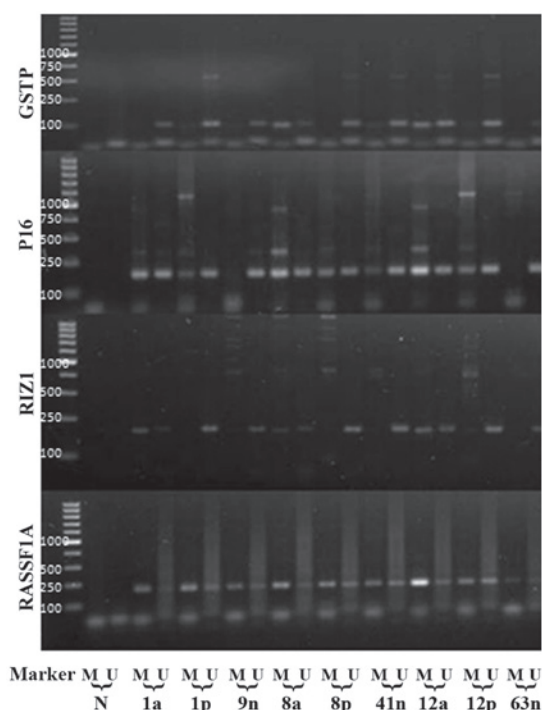


Figure 2. The MSP products of GSTP1, P16, RIZ1, and RASSF1A genes in part of the HCC, paired tumor-adjacent, and normal liver tissues. The lowest marker band represents 100 bp. N, PCR negative control; M, methylated; and U, unmethylated. a, cancerous tissue; p, adjacent liver tissue; and n, normal liver tissue.

PCR products from both primer pairs were considered partially methylated, which also represents a methylation-positive status.

Statistical analysis. The data were statistically analyzed using SPSS statistical software, version 17.0 (SPSS, Inc., Chicago, IL, USA). The count and measurement data were compared by χ^2 test and *t*-test respectively. The association between the gene methylation in HCC tissues and the clinical data was analyzed using a Fisher's exact probability test. P<0.05 was used to indicate a statistically significant difference.

Results

The frequency of methylation of *GSTP1* and *RIZ1* genes in HCC tissues were 57.1% (20/35) and 68.6% (24/35) respectively,

Table I. Primers for methylation-specific polymerase chain reaction.

Gene	M/U	Primer sequence (5'-3')	Annealing temperature	Product length
<i>GSTP1</i>	M	(F)-TTCGGGGTGTAGCGGTCGTC (R)-GCCCCAATACTAAATCACGACG	61	91
	U	(F)-GATGTTTGGGGTGTAGTGGTTGTT (R)-CCACCCCAATACTAAATCACAACA	55	91
<i>P16</i>	M	(F)-TTATTAGAGGGTGGGGCGGATCGC (R)-CAACCCCAAAACCACAACCATAA	60	150
	U	(F)-TTATTAGAGGGTGGGGTGGATTGT (R)-CAACCCCAAAACCACAACCATAA	55	151
<i>RIZ1</i>	M	(F)-GGATTTCGCGGTGATTTAC (R)-AACTCCAATCGAAAAAAACG	69	161
	U	(F)-ATGGGATTTGTGGTGATTTAT (R)-CTTAACTCCAATCAAAAAAACA	58	161
<i>RASSF1A</i>	M	(F)-GGGTTTTGCGAGAGCGC (R)-CGCTAACAAACGCGAACCG	52	169
	U	(F)-GGTTTTGTGAGAGTGTGTTTAG (R)-CACTAACAAACACAAACCAAC	52	169

M, methylated, U, unmethylated.

Table II. The methylation status of *GSTP1*, *P16*, *RIZ1*, and *RASSF1A* genes.

Group	Methylation status							
	<i>GSTP1</i>		<i>P16</i>		<i>RIZ1</i>		<i>RASSF1A</i>	
	+	-	+	-	+	-	+	-
HCC (n=35)	20	15	19	16	24	11	31	4
Adjacent tissue (n=35)	9	26	13	22	5	30	26	9
Normal liver (n=20)	0	20	0	20	0	20	2	18
P-value	0.008 ^a	0.036 ^b	0.150 ^a	0.005 ^b	<0.01 ^a	0.199 ^b	0.124 ^a	<0.01 ^b

^aComparison between HCC and tumor-adjacent tissues; ^bcomparison between HCC and normal liver tissues.

both of which were significantly increased ($P<0.01$) compared with the levels in adjacent liver tissues (25.7% [9/35] and 14.3% [5/35] respectively). The frequencies of methylation of *P16* and *RASSF1A* genes in HCC tissues were 54.3% (19/35) and 88.6% (31/35), respectively, which did not significantly differ ($P>0.05$) from those in adjacent liver tissues (37.1% [13/35] and 74.3% [26/35]). No methylation of the *GSTP1*, *P16*, and *RIZ1* genes was observed in the 20 cases of normal liver tissue, and the methylation of *RASSF1A* gene was identified in 10% (2/20) normal liver tissues (Table II, Figs. 1 and 2).

The frequency of *GSTP1* gene methylation in HCC with capsular invasion was significantly increased compared with in HCC without capsular invasion ($P<0.05$). Among the HCC samples, the frequency of *P16* gene methylation in HbsAg-positive HCC tissues was significantly increased compared with those in HbsAg-negative HCC tissues ($P<0.05$). The methylation status of *RIZ1* and

RASSF1A genes did not demonstrate significant correlation with the clinicopathological data of patients ($P>0.05$; Tables III and IV).

Discussion

HCC is one of the tumors with highest incidence worldwide. Due to an insidious onset, the majority of HCC patients are diagnosed at an advanced stage, which results in a <20% clinically resectable rate (11,12). With the exception of α -fetoprotein (AFP), at present, there remains a lack of well-recognized effective tumor markers for routine clinical detection of HCC. Therefore, identifying HCC-associated tumor markers and studying the underlying molecular mechanisms is of great importance. Epigenetics refers to a genetic mechanism that enables the occurrence of inheritable alterations in gene expression without changing the DNA sequence (3). Epigenetic

Table III. The correlation between the methylation state of *GSTP1* and *P16* genes with the clinicopathological data of HCC patients.

Clinicopathology	<i>GSTP1</i> methylation			<i>P16</i> methylation		
	-	+	P-value	-	+	P-value
Gender			0.473			0.452
Male	11	16		13	14	
Female	4	4		3	5	
Age (years)			0.281			0.364
≤50	6	5		6	5	
>50	9	15		10	14	
AFP (μg/l)			0.518			0.156
≤20	6	7		4	9	
>20	9	13		12	10	
Tumor size (cm)			0.404			0.210
≤5	9	10		7	12	
>5	6	10		9	7	
Cirrhosis			0.482			0.347
+	10	12		9	13	
-	5	8		7	6	
HbsAg			0.668			0.024
+	12	16		10	18	
-	3	4		6	1	
Capsular invasion			0.017			0.596
+	5	15		9	11	
-	10	5		7	8	
Distal metastasis			0.567			0.481
+	4	6		4	6	
-	11	14		12	13	
Differentiation			0.340			0.602
High to medium	13	15		13	15	
Poor	2	5		3	4	

HCC, hepatocellular carcinoma; AFP, α-fetoprotein; HbsAg, hepatitis B surface antigen.

silencing may result in the inactivation of TSGs, thereby causing the initiation and progression of tumorigenesis (3). The aberrant methylation of tumorigenesis-associated genes, particularly the TSGs, are receiving more attention.

GSTP1 gene is located on human chromosome 11q13, encoding an enzyme with detoxification and protein-binding functions (13). Previous studies have demonstrated that the high-frequency of methylation inactivation of *GSTP1* gene is restricted to certain human cancers, including prostate, kidney, breast, and liver cancers (4,5). Another previous study demonstrated that the frequency of *GSTP1* gene methylation in HCC is 41-85%, and that the methylation of *GSTP1* gene in HCC is associated with the effect of environmental carcinogens (7). The frequency of *GSTP1* gene methylation in HCC and its cell lines may be as high as ~85%, which is significantly increased compared with those in abnormal proliferation-induced liver nodules and cirrhosis tissues (8). The present study demonstrated that the frequency of *GSTP1* methylation in

HCC (57.1% [20/35]) was significantly increased ($P<0.01$) compared with that in tumor-adjacent tissues (25.7% [9/35]), while no abnormal methylation was detected in normal liver tissues. The association between *GSTP1* methylation in HCC and the clinicopathological data was investigated, and the results demonstrated that the frequency of *GSTP1* methylation in HCC with capsular invasion was significantly increased compared with that in HCC without capsular invasion ($P<0.05$), while no significant correlation between other clinicopathological features and *GSTP1* methylation was detected ($P>0.05$). Capsular invasion is associated with the metastasis and invasion of tumor. *GSTP1* gene promoter methylation interferes with its normal expression or function, leading to an accumulation of β-catenin in the cells (14). Since the latter is important in mediating the epithelial cell adhesion, such change may therefore facilitate the intrahepatic metastasis of HCC (6). Therefore, the aberrant methylation of *GSTP1* gene may be associated with the invasiveness of HCC.

Table IV. The correlation of the methylation state of *RIZ1* and *RASSF1A* genes with the clinicopathological data of HCC patients.

Clinicopathology	<i>RIZ1</i> methylation			<i>RASSF1A</i> methylation		
	-	+	P-value	-	+	P-value
Gender			0.492			0.665
Male	8	19		3	24	
Female	3	5		1	7	
Age (years)			0.521			0.372
≤50	3	8		2	9	
>50	8	16		2	22	
AFP (μg/l)			0.626			0.522
≤20	4	9		1	12	
>20	7	15		3	19	
Tumor size (cm)			0.352			0.630
≤5	7	12		2	17	
>5	4	12		2	14	
Cirrhosis			0.144			0.478
+	5	17		2	20	
-	6	7		2	11	
HbsAg			0.619			0.609
+	9	19		3	25	
-	2	5		1	6	
Capsular invasion			0.281			0.200
+	5	15		1	19	
-	6	9		3	12	
Distal metastasis			0.309			0.681
+	2	8		1	9	
-	9	16		3	22	
Differentiation			0.619			0.609
High to medium	9	19		3	25	
Poor	2	5		1	6	

HCC, hepatocellular carcinoma; AFP, α-fetoprotein; HbsAg, hepatitis B surface antigen.

As a TSG, the inactivation of *P16* may lead to excessive cell proliferation, accelerated cell cycle, and hence a premature entry into the S phase prior to the completion of DNA repair, resulting in tumorigenesis. Jin *et al* (9) demonstrated that the promoter methylation on 9p21 is the most common mechanism for the inactivation of TSG *P16* in HCC. Narimatsu *et al* (14) studied the methylation status of *P16* in 35 cases of HCC infected by HBV and/or HCV using the MSP method; their results indicated that *P16* methylation may be induced by hepatitis virus in livers with chronic inflammation prior to the tumorigenesis of HCC. The present study demonstrated that the frequency of *P16* gene methylation in HCC (54.3% [19/35]) was increased compared with in the adjacent liver tissues (37.1% [13/35]), though the difference was not statistically significant ($P>0.05$); there was no abnormal methylation of *P16* detected in the normal liver tissues, implying a potential tumorigenic tendency of the tumor-adjacent tissues and a possible involvement of *P16* methylation in the occurrence of HCC. Further statistical analysis of the association between the methylation

results and the clinicopathological data of HCC indicated that the frequency of *P16* methylation in HbsAg-positive patients was significantly increased compared with in HbsAg-negative ones ($P<0.05$), indicating that chronic HBV infection may be the result of methylation inactivation of *P16*.

RIZ1 is a TSG that simultaneously regulates cell proliferation and inhibits oncogenesis (15). The abnormal expression of *RIZ1* is associated with the tumorigenesis of a variety of human tumors, including nasopharyngeal, breast, liver and cervical among other cancers (16,17). Nomoto *et al* (18) reported that the frequency of *RIZ1* gene methylation was 45.2% among patients with HCC in Japan. The results of the present study demonstrated that the frequency of *RIZ1* methylation in HCC (68.6% [24/35]) was significantly increased ($P<0.01$) compared with in the paired adjacent liver tissues (14.3% [5/35]), while no methylation of *RIZ1* was detected in the normal liver tissues. The frequency of *RIZ1* methylation demonstrated a significant gradient among the 3 groups of samples; the rare presence of *RIZ1* in the adjacent

liver tissues indicated highly tumor-specific epigenetic changes in *RIZ1* with the occurrence of HCC, indicating that the methylation of *RIZ1* may be a frequent event during the tumorigenesis of HCC, and that the appearance of *RIZ1* methylation may indicate an immediate formation of HCC. Therefore, *RIZ1* gene methylation may be of great significance for the risk assessment and early diagnosis of HCC. However, further analysis indicated that there was no correlation between *RIZ1* methylation and the clinicopathological data of HCC patients. It should be noted that since the exact role of *RIZ1* promoter methylation in the process HCC carcinogenesis remains unclear, further study with an expanded sample size is required.

RASSF1A is a TSG cloned from lung cancer and reported by Damman *et al* in 2000 (19). In certain cases *RASSF1A* is inactivated via abnormal promoter methylation in primary HCC. Zhong *et al* (10) demonstrated that the abnormal hypermethylation of the *RASSF1A* promoter sequence exists in 95% of HCC tissues, and proposed that *RASSF1A* methylation is a relatively early event during the HBV-induced tumorigenesis of HCC. Previous studies have demonstrated that the frequency of *RASSF1A* gene methylation in HCC is 66.7-100%, and the variation may be associated with ethnic and geographical variabilities (20,21). In the present study, 31/35 (88.6%) of HCC specimens presented with aberrant methylation, as did 26/35 (74.3%) of the corresponding tumor-adjacent tissues; no statistically significant difference in aberrant methylation was observed between the above 2 groups ($P > 0.05$). Taking into account that cancer is a systemic disease, the genetic and epigenetic anomalies occurring in the early stage of carcinogenesis may have already existed in the tumor-adjacent tissues. These results indicated that *RASSF1A* gene methylation may be involved during the occurrence and development of HCC, as an early event and most likely representing an early change in HCC. But given that a significant difference was not observed in the frequency of *RASSF1A* methylation in HCC and adjacent liver tissues, *RASSF1A* was not considered as one of the candidate auxiliary biomarkers for HCC diagnosis.

In summary, by analysis of the methylation frequency of 4 TSGs in HCC, tumor-adjacent, and normal liver tissues, the present study revealed a progressive epigenetic change during the formation of HCC, which reflected the molecular mechanism of the multi-step and multi-stage origin of HCC. In addition, the results demonstrated that the status of *RIZ1* and *GSTP1* gene methylation has good specificity for HCC, may better distinguish between HCC and non-cancerous tissues, and may therefore be used as novel candidate biomarkers to assist the early screening and diagnosis of HCC. With further study on TSG methylation and larger sample sizes in the future, a more thorough insight may be gained into the effect of abnormal methylation of TSGs on the occurrence and development of HCC.

Acknowledgements

The authors wish to acknowledge the excellent technical support of Dr Guang-Hua Luo and Dr Bao-Qiang Wu.

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