# Significance of RASSF1A Hypermethylation in Serum DNA of Hepatocellular Carcinoma

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**Abstract** **Objective** Hypermethylation of Ras association domain family 1A (RASSF1A) is now recognized as an important early event in different classes of carcinogenesis. The objective of the present study was to explore the possible diagnostic value of promoter hypermethylation of RASSF1A in the serum DNA for early detection of hepatocellular carcinoma (HCC). **Methods** Aberrant promoter hypermethylation of RASSF1A was investigated in DNA isolated from the serum of 35 patients with HCC and 10 normal controls by methylation-specific PCR (MSP). **Results** RASSF1A promoter hypermethylation was detected in the serum DNA of 14 cases (40%), no RASSF1A hypermethylation was examined in 10 normal controls. No association was found between serum DNA RASSF1A hypermethylation and the clinicopathological parameters, including age, gender, pathological grade, HBV, para-cirrhosis, AFP, portal vein tumor embolus, tumor capsular and tumor size. **Conclusion** The detection of the promoter hypermethylation of RASSF1A in serum DNA may be a valuable biomarker for early diagnosis in populations with high risk of HCC.

**Key words** Hepatocellular carcinoma; RASSF1A; Promoter hypermethylation

The Ras association domain family 1A (RASSF1A) gene locates in chromosome 3p21.3 [1] and recent studies have shown that inactivation of RASSF1A promoter by methylation was detectable in tumor cell lines and tissues, such as lung[2], gallbladder[3], breast[4], kidney [5], prostate [6], endometrium [7], bladder [8], bile ducts [9], stomach , colon and rectum carcinoma [10]. The hypermethylation of RASSF1A suggests new perspective for the diagnosis of malignant tumor. Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world and the prognosis of patients with HCC is very poor due to the late diagnosis. The methylation of RASSF1A was detected in the serum DNA of HCC patients by Yeo et al[11] and Zhang et al[12]. In order to evaluate the prognostic significance of the hypermethylation of RASSF1A in serum DNA of patients with HCC and explore the value of RASSF1A gene in the hepatocarcinogenesis, promoter, aberrant promoter hypermethylation of RASSF1A was investigated in this study.

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## *MATERIALS AND METHODS*

### Clinical materials

The studied population consists of 35 patients who were diagnosed with operable HCC from February to July 2006 in the First Initiated Hospital to Guangxi Medical University, P.R.China. Informed consent was obtained and the study was approved by the institutional review committee. The preoperative peripheral blood was collected from 35 cases of HCC and 10 cases of healthy donors as controls. Patients' demographics were obtained and included age, gender, pathological grade, HBV, para-cirrhosis, AFP, portal vein tumor embolus, tumor capsular and tumor size.

### DNA extraction

Blood samples were centrifuged at 2000g, and serum was carefully removed from the ethylenediamine tetraacetic acid (EDTA)-containing tubes, transferred to plain polypropylene tubes, and stored at −80℃ until further processing. DNA from serum was extracted using the QIAamp Blood Mini Kit (Qiagen, Germany) according to the manufacturer's recommendations. Serum samples were applied at 400 ml/column and a fi-

nal volume of 100 ml serum DNA was collected.

**Methylated-specific PCR (MSP)**

Methylation of the promoter region was examined by bisulphite DNA modification followed by methylated-specific PCR (MSP). Bisulphite modification using the CpGenome TM DNA Modification Kit (Chmicon, S7820, USA)was performed according to the manufacturer's instructions. Serum DNA amplification was carried out in a GeneAmp PCR System 2400 (PERKIN ELMER, USA) using CpG WIZ RASSF1A Amplification Kit (Chmicon, S7813, USA). A total of 35 cycles was used for serum DNA amplification. The thermal profile consisted of an initial denaturation step of 95℃ for 5 min, followed by repetitions of 95℃ for 45s, 55℃ for 45s, and 72 ℃ for 60s, with a final extension step of 72℃ for 5min. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. A water blank was used as a negative control. Samples were scored as methylated when there was a clearly visible band on the gel with the methylated primers. Triplication was performed for all experiments to ensure the reproducibility of the results.

**Statistical analysis**

Statistical significance was evaluated by $\chi^2$ test for categorical variables with SPSS 13.0, difference was deemed significant when $P<0.05$.

**Table 1** Relationship between hypermythelation of RASSF1A in serum DNA and clinicopathological parameters of HCC.

| Clinicopathological parameters | | N | Positive | $\chi^2$ | *P* |
|---|---|---|---|---|---|
| Age | ≥50 | 16 | 6 | 0.077 | 0.782 |
| | <50 | 19 | 8 | | |
| Gender | male | 28 | 12 | 0.476 | 0.490 |
| | female | 7 | 2 | | |
| Pathological grade | I~II | 20 | 8 | 0.000 | 1.000 |
| | III~IV | 15 | 6 | | |
| HBV | positive | 30 | 11 | 0.972 | 0.324 |
| | negative | 5 | 3 | | |
| Para-cirrhosis | with | 23 | 10 | 0.338 | 0.561 |
| | without | 12 | 4 | | |
| AFP(ng/ml) | ≥400 | 24 | 9 | 0.199 | 0.656 |
| | <400 | 11 | 5 | | |
| Portal vein tumor embolus | with | 6 | 2 | 0.134 | 0.714 |
| | without | 29 | 12 | | |
| Tumor capsular | with | 14 | 5 | 0.179 | 0.673 |
| | without | 21 | 9 | | |
| Tumor size(cm) | ≥5 | 26 | 11 | 0.224 | 0.636 |
| | <5 | 9 | 3 | | |

## *RESULTS*

Aberrant methylation of the RASSF1A promoter region was detected in the serum DNA of 14 patients from 35 cases with HCC (40%) by MSP. None of the serum samples from 10 healthy blood donors displayed RASSF1A promoter methylation (table 1). The age, gender, pathological grade , HBV, para-cirrhosis, AFP, portal vein tumor embolus, tumor capsular and tumor size of the 35 patients with methylation status of RASSF1A of the serum DNA are illustrated in Table 1. There was no association between serum DNA RASSF1A methylation with these parameters.

## *DISCUSSION*

Promoter hypermethylation has been found to frequently occur in tumor suppressor and cancer genes involved in many different signaling pathways and to be present in a different pattern in different tumor types [13]. The expression of the longer isoform, RASSF1A, is lost in many tumor lines and primary tumors by promoter methylation, while RASSF1C remains unmethylated. It has been suggested that RASSF1A methylation is one of the most common aberrations so far identified in human cancers and that the loss of the functional protein may promote the development of many human tumors. Hypermethylation of the RASSF1A has been detected frequently in the tissue of different cancers [2-10], while in the body fluid, methylation of RASSF1A promoter has also been documented in 50% sputum and in 84% serum of lung cancer [14], in 35% urine of bladder cancer [15],56~60% serum of breast caner [16,17] and 5% serum of undifferented nasopharyngeal carcinoma [18], which pointed out the role of RASSF1A methylation in DNA for the early diagnosis of tumors. Detection of methylated DNA has then been suggested as a potential biomarker for early detection of cancer. In this study, HCC tumor serums demonstrated hypermethylation in RASSF1A gene (40%, 14/35), which is consistent with the findings of other investigators (42.5 ~70% ) [11,12]. Though the exact mechanism how the tumor DNA gets into the systemic circulation is unclear, the present result can still suggest that RASSF1A methylation might be a potential marker of incipient malignancy in the human hepatocarcinogenesis, since, like any ideal biomarker, it appears early in the course of disease and is detectable in biological samples that can be obtained noninvasively.

Although one may expect the presence of promoter methylation in serum DNA to be associated with more advanced disease, previous studies on promoter methylation of RASSF1A in blood and tissues have not shown any correlation with tumor staging, except Yeo et al[11], who reported the promoter methylation of RASSF1A in the serum DNA of the patients with HCC was correlated to the tumor size. The bigger tumor gains the more chance to get hypermethylation. In the present study, different from expectation, no association was found between the HCC serum RASSF1A methylation and any of the clinicopathological parameters. Different clinic samples and different amount of the patients can partly explain this difference.

The ability to detect aberrant promoter methylation in serum DNA may potentially enable screening HCC to be conducted with a noninvasive method. Although the present study has detected no association between the presence of aberrant methylation and the clinical and pathological factors, further studies are required to confirm the role of detecting RASSF1A promoter hypermethylation in serum DNA as a tool for screening and prognosticator for HCC.

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