Quantitative Analysis of Tumor-derived Methylated *p16INK4a* Sequences in Plasma, Serum, and Blood Cells of Hepatocellular Carcinoma Patients[1]

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ABSTRACT

Purpose and Experimental Design: **Using real-time quantitative methylation-specific PCR (RTQ-MSP), we quantified methylated *p16INK4a* sequences and determined the fractional concentrations of circulating tumor DNA in plasma, serum, and peripheral blood cells collected preoperatively, intraoperatively, and postoperatively from 49 patients with hepatocellular carcinoma (HCC).**

Results: **RTQ-MSP was sufficiently sensitive to detect down to 10 genome-equivalents of methylated *p16INK4a* sequences. Quantitative MSP data were expressed in terms of the methylation index, which was the percentage of bisulfite converted unmethylated and methylated *p16INK4a* sequences that consisted of methylated *p16INK4a* sequences. Quantities of methylated *p16INK4a* sequences were detected in peripheral circulation of 80% (23 of 29) of HCC patients. No significant difference was seen in the detectability and concentrations of methylated *p16INK4a* sequences (range: 10–4046 genome-equivalents/ml) between preoperative plasma and serum samples from HCC patients. Preoperatively, the *p16INK4a* methylation indices ranged from 0.2 to 100% and from 0.012 to 0.075% in the patients' plasma and buffy coat samples, respectively. After surgical resection, the median *p16INK4a* methylation indices in plasma and buffy coat concordantly decreased 12- and 15-fold, respectively. These results demonstrated the clinical usefulness and effectiveness of peripheral blood RTQ-MSP for detecting and monitoring HCC after treatment. Furthermore, none of the intraoperative plasma samples and only two of the intraoperative buffy coat samples were *p16INK4a* methylation positive.**

Conclusions: **Quantification of epigenetic changes in peripheral blood by RTQ-MSP is useful for the detection and monitoring of HCC.**

INTRODUCTION

Tumor-derived epigenetic changes in plasma and serum are potential molecular markers for numerous cancers (1–5). We have previously demonstrated frequent *p16INK4a* and *p15INK4b* promoter hypermethylation in plasma and serum from patients with HCC[3] (2, 4–6). The biological significance of promoter hypermethylation in tumor progression involves the transcriptional repression of tumor suppressor genes, DNA repair genes, and metastasis inhibitor genes (7, 8). Detection of aberrant DNA methylation has implications for understanding the fundamental mechanisms of oncogenesis (7–9) and may form a novel molecular basis for cancer diagnosis and monitoring (1–5, 10, 11).

Circulating tumor-derived nucleic acids detected in plasma or serum of HCC patients comprise epigenetic and genetic alterations such as promoter hypermethylation of cell cycle regulator genes, *p53* mutations, and mitochondrial DNA mutations (2, 4–6, 12–14). Regarding the concentration of circulating tumor DNA in plasma and serum, we have developed RTQ-MSP for the analysis of CpG sites in the promoter region critical for transcriptional silencing by hypermethylation (15). RTQ-MSP data were completely concordant with conventional MSP results and Southern blotting data using methylation-sensitive restriction enzymes (2, 15, 16). However, RTQ-MSP is much more rapid, accurate, and amenable to large-scale cancer screening. This method has the combined advantages of MSP (high specificity and sensitivity) and real-time PCR (rapidity, large dynamic range, and anticontamination properties). With RTQ-MSP, we can additionally maximize the diagnostic potential of circulating tumor DNA and better our understanding of the biology of this phenomenon.

RTQ-MSP provides a quantitative dimension for the widespread use of methylation analysis in clinical practice and may help assess the effect of cancer treatment. In this investigation,

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; RTQ-MSP, real-time quantitative methylation-specific PCR; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine; PBNC, peripheral blood nucleated cell; LOH, loss of heterozygosity.

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we measured concentrations of tumor-derived methylated *p16INK4a* sequences in plasma, serum, and buffy coat collected from HCC patients before, during, and after surgical resection.

MATERIALS AND METHODS

Patients and Control Subjects. With informed consent and ethics approval, 85 peripheral blood samples and 45 surgically removed tumors were collected prospectively from HCC patients. The corresponding nontumor liver tissues were also obtained for methylation analysis. The diagnosis of HCC was confirmed histologically in all cases. A total of 65 preoperative (1 h before surgery; $n = 29$), intraoperative ($n = 8$), and postoperative (1 month after surgery; $n = 28$) plasma samples was obtained from 29 HCC patients. Furthermore, 43 preoperative ($n = 22$) and postoperative ($n = 21$) serum samples were collected from 23 of 29 patients and 20 additional HCC patients. As controls, plasma samples were collected from 50 non-HCC patients with chronic hepatitis/cirrhosis and healthy volunteers. Taken together, 100 buffy coat samples were collected from the 29 HCC patients (65 samples), 15 non-HCC patients with chronic hepatitis/cirrhosis, and 20 healthy volunteers.

DNA Extraction from Tumors, Plasma, Serum, and Buffy Coat Samples. DNA was extracted from HCCs and nontumor liver tissues using the QIAamp Tissue Kit (Qiagen, Hilden, Germany). Peripheral blood samples were centrifuged at $3000 \times g$, and plasma and serum samples were carefully collected from EDTA-containing and plain tubes, respectively. DNA was extracted from 400 μl of plasma/serum using the QIAamp Blood Kit (Qiagen). The buffy coat was isolated from peripheral blood, and DNA was extracted using standard proteinase K treatment and phenol/chloroform/isoamylalcohol extraction.

Bisulfite Conversion of DNA. Bisulfite modification was conducted based on the principle that bisulfite treatment of DNA would convert unmethylated cytosine residues into uracil, whereas methylated cytosine residues would remain unmodified (2–6). Thus, methylated and unmethylated DNA sequences after bisulfite conversion would be distinguishable by sequence-specific primers. Bisulfite treatment was conducted using the CpGenome DNA Modification Kit (Intergen, New York, NY). One μg of tissue/buffy coat DNA or extracted plasma/serum DNA was treated with sodium bisulfite following the manufacturer's recommendations.

Development of RTQ-MSP. RTQ-MSP is based on the continuous optical monitoring during fluorogenic PCR (17). In this system, two amplification primers and a dual-labeled fluorogenic hybridization probe are used (17). One fluorescent dye serves as a reporter (FAM), and its emission spectrum is quenched by a second fluorescent dye (TAMRA). During the extension phase of PCR, the 5′ to 3′ exonuclease activity of the TaqDNA polymerase (18) cleaves the reporter from the probe, thus releasing it from the quencher, resulting in an increase in fluorescence emission at 518 nm.

Three real-time MSP systems were developed for quantifying bisulfite-converted methylated *p16INK4a* sequences (the *p16INK4aM* system), bisulfite-converted unmethylated *p16INK4a* sequences (the *p16INK4aU* system), and unconverted wild-type *p16INK4a* sequences (the *p16INK4aW* system). Bisulfite-modified DNA was amplified using specifically designed primers for the methylated, unmethylated, and wild-type *p16INK4a* sequences (2, 4, 6). The sense and antisense primers for the methylated sequence were *p16*MF (5′-TTATTAGAGGGTGGGGCGGATCGC-3′) and *p16*MR (5′-GACCCCGAACCGCGACCGTAA-3′), respectively. For the *p16INK4aM* system, *p16*MF and *p16*MR were used in conjunction with a fluorogenic probe *p16*MT [5′-(FAM)-AGTAGTATGGAGTCGGCGGCGGG-(TAMRA)-3′]. The fluorogenic probe contained a 3′-blocking phosphate group to prevent probe extension during PCR. All of the fluorogenic probes were custom-synthesized by Perkin-Elmer Applied Biosystems (Foster City, CA). For the *p16INK4aU* system, the sense and antisense primers, *p16*UF (5′-TTATTAGAGGGTGGGGTGGATTGT-3′) and *p16*UR (5′-CAACCCCAAACCACAACCATAA-3′) were used in conjunction with a fluorogenic probe *p16*UT [5′-(FAM)-AGGTAGTGGGTGGTGGGGAGTAGTATGGAGTTG-(TAMRA)-3′]. For the *p16INK4aW* system, the primers *p16*WF (5′-GTGGGGCGGACCGC-3′) and *p16*WR (5′-GCCGCGGCCGTGG-3′) were used in conjunction with a fluorogenic probe *p16*WT [5′-(FAM)-AGCAGCATGGAGCCGGCGG-(TAMRA)-3′].

Fluorogenic PCR was set up in a reaction volume of 50 μl with components supplied in the TaqMan PCR Core Reagent Kit (Perkin-Elmer Applied Biosystems). Each reaction contained 5 μl of 10× buffer A; 300 nM each amplification primer; 25 nM corresponding fluorogenic probe; 200 μM each dATP, dCTP, and dGTP; 400 μM dUTP; and 1.25 units of AmpliTaq Gold polymerase. The $MgCl_2$ concentrations were 2 and 1.75 mM for *p16INK4aM/p16INK4aW* and *p16INK4aU* systems, respectively. DMSO (Merck, Darmstadt, Germany) was added at final concentrations of 5% for *p16INK4aM* and *p16INK4aU* systems and 10% for the *p16INK4aW* system. One or 5 μl of bisulfite-converted DNA from plasma, serum, buffy coat, or tissue was used per RTQ-MSP assay.

DNA amplification was carried out in a 96-well reaction plate format in an Applied Biosystems 7700 Sequence Detector. Thermal cycling was initiated with a denaturation step of 12 min at 95°C. The subsequent thermal profile for *p16INK4aM* and *p16INK4aU* RTQ-MSP was 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min. For the *p16INK4aW* system, the thermal profile was 95°C for 30 s, 50°C for 1 min, and 72°C for 1 min. Data obtained after 40 and 55 cycles of amplification were analyzed for tissue/buffy coat and plasma/serum samples, respectively. Multiple negative water blanks were included in each analysis. Amplification data, collected by the 7700 Sequence Detector and stored in a Macintosh computer (Apple Computer, Cupertino, CA), were then analyzed using the Sequence Detection System software (Version 1.6.3) developed by Applied Biosystems.

A calibration curve was run in parallel with each analysis. A human cancer cell line, HS-Sultan (American Type Culture Collection CRL-1484), previously shown to have *p16INK4a* methylation by Southern blotting analysis using methylation-sensitive restriction enzymes and conventional MSP (2, 16), was used for constructing the calibration curve for *p16INK4aM* RTQ-MSP. Similarly, normal PBNC DNA with unmethylated *p16INK4a* sequences was used for constructing the calibration curve for *p16INK4aW* (before bisulfite conversion) and *p16INK4aU* (after bisulfite conversion) systems. A conversion

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factor of 6.6 pg of genomic DNA/diploid cell was used for expressing quantitative results in genome-equivalents (19). One genome-equivalent was defined as the amount of a particular target sequence in a single reference cell.

To determine the dynamic range of RTQ-MSP, we analyzed samples containing serial dilutions of HS-Sultan DNA using the *p16INK4aM* system (15). The system was sufficiently sensitive to detect down to 10 genome-equivalents of methylated *p16INK4a* sequences. Using wild-type or bisulfite-converted normal PBNC DNA, the detection limits of the *p16INK4aU* and *p16INK4aW* systems were 10 genome-equivalents and 1 genome-equivalent, respectively.

The threshold cycle of *p16INK4aM* RTQ-MSP, set at 10 SDs above the mean baseline fluorescence calculated from cycles 1 to 15, was inversely proportional to the input target quantity for PCR (on a logarithmic scale). The reproducibility of bisulfite conversion followed by RTQ-MSP was tested by performing replicate bisulfite conversions of HS-Sultan DNA (100 pg), followed by *p16INK4aM* RTQ-MSP. For the *p16INK4aU* system, multiple bisulfite conversions of normal PBNC DNA were performed for the analysis.

Circulating DNA concentration in plasma/serum (genome-equivalents/ml) was calculated using the equation, $C = Q \times V_{post-bis}/V_{PCR} \times V_{elu}/(V_{pre-bis}) \times 1/V_{ext}$. C = target (*p16INK4aM*, *p16INK4aU*, or *p16INK4aW*) concentration in plasma/serum (genome-equivalents/ml); Q = target quantity (copies equivalent to the HS-Sultan genome or normal PBNC genome) determined by the sequence detector in PCR; $V_{post-bis}$ = volume of water for resuspending bisulfite-converted DNA (10 μl); V_{PCR} = volume of bisulfite-converted DNA used for PCR (1 or 5 μl); V_{elu} = total volume of DNA eluted (50 μl/Qiagen column); $V_{pre-bis}$ = volume of DNA used for bisulfite-conversion (45 μl); and V_{ext} = volume of plasma/serum extracted (0.4 ml).

The methylation index (percentage) in a sample was calculated according to the equation, $M/(M + U) \times 100\%$. M is the concentration of methylated *p16INK4a* sequences measured by *p16INK4aM* RTQ-MSP, and U is the concentration of unmethylated *p16INK4a* sequences measured by *p16INK4aU* RTQ-MSP after bisulfite conversion.

The completeness of bisulfite conversion was measured by calculating the percentage of *p16INK4a* sequences remained as the unconverted wild-type sequence (%W) using the equation, $W/(W + M + U) \times 100\%$. W is the concentration of wild-type *p16INK4a* sequences measured by the *p16INK4aW* system.

Statistical Analyses. Statistical analyses were carried out using SigmaStat 2.03 and SPSS 7.5 software.

RESULTS

***p16INK4a* Promoter Methylation in Tumor, Plasma, and Serum from HCC Patients.** Aberrant *p16INK4a* promoter methylation was found in 67% (30 of 45) of surgically resected HCCs by using *p16INK4aM* RTQ-MSP. None of the corresponding nontumor liver tissues had methylated *p16INK4a* sequences. Unmethylated *p16INK4a* sequences were detected in all tumors and nontumor tissues by using *p16INK4aU* RTQ-MSP. Quantities of methylated *p16INK4a* sequences were detected in plasma or serum from 47% (14 of 30) of the HCC patients with tumoral *p16INK4a* methylation. Among the 15 HCC patients without tumoral *p16INK4a* methylation, methylated *p16INK4a* sequences were not found in plasma or serum.

Table 1 Quantification of methylated *p16INK4a* sequences in preoperative serum and plasma samples from HCC patients

Concentration of methylated *p16INK4a* sequences (genome-equivalents/ml)	No. of HCC patients	
	Serum samples (n = 22)	Plasma samples (n = 29)
1000–10000	0	1
100–1000	1	3
10–100	3	2
1–10	0	3
<1	18	20

Quantification of Methylated *p16INK4a* Sequences in Preoperative and Postoperative Serum Samples. Forty-three serum samples from 43 HCC patients were analyzed by using *p16INK4aM* RTQ-MSP. Four of 22 (18%) preoperative serum samples contained methylated *p16INK4a* sequences, corresponding to 29, 36, 54, and 103 genome-equivalents/ml (median quantity = 45 genome-equivalents/ml, n = 4; Table 1). However, none of 21 postoperative serum samples were *p16INK4a* methylation positive. All 43 serum samples had unmethylated *p16INK4a* sequences, corresponding to 10–11961 genome-equivalents/ml (median quantity = 343 genome-equivalents/ml) as analyzed by using the *p16INK4aU* system.

***p16INK4a* Methylation Indices of Preoperative, Intraoperative, and Postoperative Plasma Samples.** Sixty-five preoperative, intraoperative, and postoperative plasma samples collected from 29 HCC patients were analyzed by using *p16INK4aM*, *p16INK4aU*, and *p16INK4aW* RTQ-MSP systems. Representative amplification plots of *p16INK4aM* RTQ-MSP are shown in Fig. 1. The amplification curves shift toward the right with a lower input target quantity. The linearity of the graph demonstrates the large dynamic range and the accuracy of RTQ-MSP. Similar results were obtained for the *p16INK4aU* and *p16INK4aW* systems.

Nine of 29 (31%) preoperative plasma samples had methylated *p16INK4a* sequences, corresponding to 10, 10, 10, 41, 46, 296, 334, 943, and 4046 genome-equivalents/ml (median quantity = 46 genome-equivalents/ml, n = 9; Table 1). No significant difference was observed in the concentrations of methylated *p16INK4a* sequences between plasma samples (n = 29) and serum samples (n = 22) collected preoperatively from the HCC patients (P = 0.286; Mann-Whitney rank-sum test).

Taken together, 17 of 65 (26%) plasma samples, including 9 of 29 (31%) preoperative and 8 of 28 (29%) postoperative samples, were *p16INK4a* methylation positive. None of the 8 intraoperative plasma samples had methylated *p16INK4a* sequences. All 65 plasma samples contained unmethylated *p16INK4a* sequences, corresponding to 10–1243 genome-equivalents/ml (median quantity = 65 genome-equivalents/ml) as analyzed by using the *p16INK4aU* system. None of the 65 samples had detectable wild-type *p16INK4a* sequences, showing the completeness of bisulfite modification.

The fractional concentration of circulating tumor-derived DNA in plasma, the proportion of bisulfite converted unmeth-

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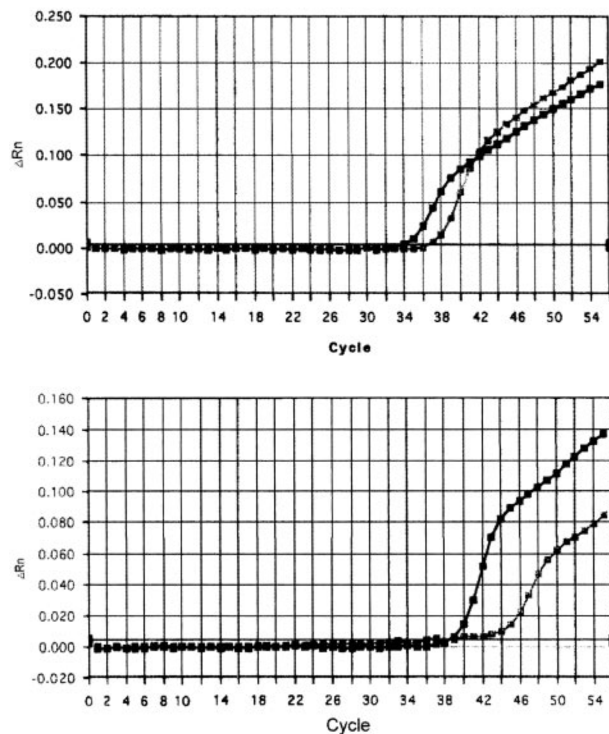

Fig. 1 Amplification plots for the *p16INK4aM* RTQ-MSP analysis of four plasma DNA samples. X axis, the cycle number of quantitative PCR; Y axis, ΔRn, the fluorescence intensity over the background.

Table 2 The *p16INK4a* methylation indices of preoperative, intraoperative, and postoperative plasma samples from HCC patients

	No. of HCC patients		
p16INK4a methylation index (%)	Preoperative samples (n = 29)	Intraoperative samples (n = 8)	Postoperative samples (n = 28)
50–100	3	0	1
10–50	3	0	2
1–10	0	0	2
0.1–1	3	0	3
<0.1	20	8	20

Table 3 The *p16INK4a* methylation indices of preoperative, intraoperative, and postoperative buffy coat samples from HCC patients

	No. of HCC patients		
p16INK4a methylation index (%)	Preoperative samples (n = 29)	Intraoperative samples (n = 8)	Postoperative samples (n = 28)
0.06–0.10	3	0	1
0.03–0.06	7	0	2
0.01–0.03	7	2	3
<0.01	12	6	22

ylated and methylated *p16INK4a* sequences that consisted of tumor-derived methylated *p16INK4a* sequences, was calculated for each methylation-positive plasma sample. For the 9 preoperative plasma samples, the *p16INK4a* methylation indices were 0.2, 0.4, 0.6, 21.3, 35, 46.7, 73.1, 85.3, and 100% (median methylation index = 35%; n = 9; Table 2). On the other hand, the *p16INK4a* methylation indices on the 8 postoperative plasma samples were 0.3, 0.3, 0.4, 1.6, 4.5, 11.2, 28, and 71.5% (median methylation index = 3.05%; n = 8; Table 2), corresponding to 10, 13, 32, 34, 34, 60, 77, and 103 genome-equivalents of methylated sequences/ml (median quantity = 34 genome-equivalents/ml). The median methylation index in postoperative plasma was 12-fold lower than the preoperative median methylation index among the HCC patients studied. In the prospective longitudinal study of 8 patients possessing *p16INK4a* methylation in plasma samples, which were collected both preoperatively and postoperatively, the *p16INK4a* methylation indices demonstrated a decreasing trend after surgical resection in 63% of the HCC patients (P = 0.07; Wilcoxon test).

As controls, plasma DNA samples from 50 non-HCC patients with chronic hepatitis/cirrhosis and healthy volunteers were subjected to bisulfite conversion and RTQ-MSP. No methylated *p16INK4a* sequences were found in any of these samples. Instead, unmethylated *p16INK4a* sequences were detected in all of these control subjects, corresponding to 10–34 genome-equivalents/ml (median quantity = 24 genome-equivalents/ml).

***p16INK4a* Methylation Indices of Preoperative, Intraoperative, and Postoperative Buffy Coat Samples.** Sixty-five buffy coat samples collected from 29 HCC patients preoperatively, intraoperatively and postoperatively were analyzed by using *p16INK4aM*, *p16INK4aU*, and *p16INK4aW* RTQ-MSP systems. Thirty-eight percent (25 of 65) of buffy coat samples were *p16INK4a* methylation positive. Seventeen of 29 (59%) preoperative samples showed *p16INK4a* methylation indices varying from 0.012 to 0.075% (median methylation index = 0.015%, n = 29; Table 3), containing 50–230 genome-equivalents of methylated sequences/μg buffy coat DNA (median quantity = 57 genome-equivalents/μg DNA) as analyzed by using the *p16INK4aM* system. Only 2 of 8 intraoperative samples showed *p16INK4a* methylation indices of 0.019 and 0.02%, containing 50 and 55 genome-equivalents of methylated sequences/μg DNA. Six of 28 (21%) postoperative samples showed *p16INK4a* methylation indices varying from 0.011 to 0.069% (median methylation index = 0.001%, n = 28; Table 3), containing 50–80 genome-equivalents of methylated sequences/μg DNA (median quantity = 63 genome-equivalents/μg DNA). In concordance with the quantitative profile in plasma, the median methylation index in postoperative buffy coat was 15-fold lower than the preoperative median methylation index among the HCC patients studied.

In 10 HCC patients possessing *p16INK4a* methylation in buffy coat samples, which were collected both preoperatively and postoperatively, the *p16INK4a* methylation indices were reduced significantly after surgical resection, confirming the decreasing trend in plasma samples (P = 0.01; Wilcoxon test). All 65 buffy coat samples from HCC patients contained unmethylated *p16INK4a* sequences, corresponding to 31355–552285 genome-equivalents/μg DNA (median quantity = 235565 genome-equivalents/μg DNA) as analyzed by using the *p16INK4aU* system.

As controls, buffy coat DNA samples from 35 non-HCC

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patients with chronic hepatitis/cirrhosis and healthy volunteers were subjected to bisulfite conversion and RTQ-MSP. No methylated *p16INK4a* sequences were found in any of these samples. Instead, unmethylated *p16INK4a* sequences were detected in all of these control subjects, corresponding to 16873–220457 genome-equivalents/μg DNA (median quantity = 82364 genome-equivalents/μg DNA).

DISCUSSION

The current RTQ-MSP analysis of plasma/serum from HCC patients confirms and extends our previous work indicating that aberrant *p16INK4a* promoter methylation is a common phenomenon in HCC (2, 4–6). Highly sensitive RTQ-MSP was able to measure circulating tumor-derived methylated *p16INK4a* sequences in a significant proportion of HCC patients with tumoral *p16INK4a* methylation. Also, the high specificity of the assay was proved by the absence of methylated *p16INK4a* sequences in plasma/serum from all of the HCC patients without tumoral *p16INK4a* methylation and all of the control subjects.

For the first time, we have determined the fractional concentration of circulating tumor-derived DNA in HCC patients varying from 0.2 to 100%. For the 9 methylation-positive preoperative plasma samples, the top three *p16INK4a* methylation indices were >50%, supporting previous data showing the presence of tumor-associated LOH and microsatellite instability (allele shift) in plasma/serum of cancer patients (20–22). Theoretically, tumoral LOH was detectable only when the fractional concentration of circulating tumor DNA was >50%. Cellular or allelic heterogeneity, normal cell contamination, and differential shedding of tumor DNA from malignant cells can all affect the LOH detection of particular alleles in plasma/serum. The present quantitative data illustrate that the RTQ-MSP system is much more sensitive than the LOH analysis in that *p16INK4a* methylation indices down to 0.2% are readily detectable.

None of the 8 intraoperative plasma samples studied had methylated *p16INK4a* sequences. This was completely concordant with the absence of circulating tumor cells in intraoperative peripheral blood of HCC patients as measured by quantitative reverse transcription-PCR for α-fetoprotein mRNA (23–25). However, a larger cohort of HCC patients is required for consolidating this finding. On the other hand, the *p16INK4a* methylation indices on the 8 methylation-positive postoperative plasma samples were generally lower than those detected preoperatively in the majority of our HCC patients with plasma methylation changes. Five of 8 postoperative plasma samples showed extremely low *p16INK4a* methylation indices of <5%. The majority (71%) of the postoperative plasma samples was *p16INK4a* methylation negative and only 3 of 28 samples showed *p16INK4a* methylation indices of >10%. The *p16INK4a*-methylated sequences detected postoperatively might possibly be derived from apoptotic or necrotic tumor cells, as supported by the absence of metastasis or tumor recurrence in these patients with a median disease-free interval of 18 months.

No significant difference was seen in the detectability and concentrations of methylated *p16INK4a* sequences between preoperative plasma and serum samples of HCC patients, suggesting the usefulness of both plasma and serum for methylation analysis. However, we still chose to express our quantitative results as fractional concentrations of circulating tumor DNA (*p16INK4a* methylation indices) in plasma instead of those in serum. It was because recent data have demonstrated that DNA was released from normal PBNCs into serum during the clotting process (26, 27). This could possibly reduce the fractional concentration of tumor DNA in serum because of a much larger proportion of unmethylated *p16INK4a* sequences derived from normal PBNCs. In this regard, plasma DNA should be used to reflect more closely the *in vivo* conditions, as has recently been demonstrated in an animal model (28).

The mechanism of DNA release from the tumor into plasma/serum may most likely be related to cellular turnover, necrosis, or apoptosis as demonstrated previously *in vitro* and *in vivo* (28). In particular, a spectrum of multiples of 180-bp fragments in plasma may represent the oligonucleosomal DNA of chromatin degraded by caspase-activated DNase, specifying cellular apoptosis. Conversely, DNA fragments of >10 kb could possibly originate from cells dying via necrosis (28). The biological characteristics of a tumor such as the growth rate, the histological grade, and rates of apoptosis and necrosis may affect the amount of tumor DNA released into the bloodstream (29). However, the possibility of active DNA release from tumor cells cannot be ruled out, as has been implicated in lymphocytes and whole organs (30, 31). The RTQ-MSP analysis of peripheral blood may prove valuable for studying the pathophysiological basis of cell-free tumor DNA liberation and tumor cell dissemination into patients' circulation.

Overall, 38% of buffy coat samples from HCC patients showed *p16INK4a* methylation indices varying from 0.011 to 0.075%. Only 2 of 8 intraoperative samples were *p16INK4a* methylation positive in large concordance with the absence of circulating HCC cells inferred intraoperatively by normal α-fetoprotein mRNA levels (23–25, 32). Both the methylation detection rate and *p16INK4a* methylation indices in postoperative samples were significantly lower than those in preoperative buffy coat samples in agreement with the quantitative profiles found in plasma samples. Owing to a greater amount of unmethylated *p16INK4a* sequences derived from numerous normal PBNCs, the *p16INK4a* methylation indices in buffy coat were lower than those in plasma from the same HCC patients. However, the overall *p16INK4a* methylation detectability was higher in buffy coat than in plasma. Regardless of the tumor size, we found methylation abnormalities in peripheral circulation (plasma and/or buffy coat) of a significant proportion (80%, 23 of 29) of HCC patients.

The principles involved in the development of *p16INK4a* RTQ-MSP can also be used to develop similar RTQ-MSP systems for other tumor suppressor genes, DNA repair genes, apoptosis genes, and metastasis-associated genes. In addition, RTQ-MSP can accommodate multiple fluorescent labels for analyzing multiple cancer-associated genes in a time-efficient multiplex format (33, 34). As diagnostic and prognostic markers for HCC, the epigenetic alterations can also be used for a wide variety of other cancers with aberrant genome-wide methylation (35, 36).

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