Association of Circulating Tumor Cells with Serum Tumor-Related Methylated DNA in Peripheral Blood of Melanoma Patients

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Abstract

Although previous studies have separately shown the utility of circulating tumor cells (CTC) or cell-free tumor-related DNA in blood of cancer patients, there has been no investigation of their association and/or the prognostic value of combining these assessments. To date, the true source of tumor-related DNA in serum remains unknown. We hypothesized that CTC is a possible origin of serum tumor-related methylated DNA and their combination can predict disease outcome. To test this hypothesis, we obtained matched pairs of peripheral blood lymphocytes and serum specimens simultaneously from 50 American Joint Committee on Cancer stage IV melanoma patients before administration of biochemotherapy. Peripheral blood leukocytes were analyzed for three mRNA markers of CTC: MAGE-A3, GalNAc-T, and MAGE-A3. Sera were analyzed for two methylated DNA markers: RASSF1A and RAR-β2. CTC were detected in 13 of 15 (86%) patients with serum tumor-related methylated DNA and only in 13 of 35 (37%) patients without methylated DNA (P = 0.001). The number of CTC markers detected significantly correlated with methylated DNA (P = 0.008). CTC and methylated DNA were significantly correlated with chemotherapy-treated patients’ outcome. Patients with both CTC and methylated DNA showed significantly poorer response to biochemotherapy (P = 0.02) and worse time to progression and overall survival (P = 0.009 and 0.02, respectively). The correlation between CTC and serum tumor-related methylated DNA and the significant effect of this correlation on disease outcome indicate that a composite molecular assessment in blood may be a useful determinant of disease status and efficacy of systemic therapy for melanoma. (Cancer Res 2006; 66(12): 6111-7)

Introduction

The metastasis of melanoma to regional lymph nodes and distant sites often portends a poor prognosis (1, 2). Based on the assessment of metastatic disease status, the decision is made on the treatment strategy required. However, the current staging criteria do not accurately take into account various disease progression events and predict treatment response. Because hematogenous dissemination of tumor cells is the main mechanism for distant metastasis, assessment of blood may be a highly desirable approach for detecting systemic tumor cell spreading (3).

Note: K. Koyanagi and T. Mori contributed equally to this work.

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Development of PCR-based techniques has enabled the molecular diagnosis for disseminated tumors in blood of cancer patients (3, 4). Molecular markers in blood can be a useful tool for early detection of subclinical metastatic disease spreading and to monitor treatment response (5, 6).

Investigators have shown the clinical utility of circulating tumor cells (CTC) in blood as surrogate markers for subclinical disease and prognostic factors of disease outcome and treatment response (7–10). CTC in blood are detected by quantitative real-time reverse transcription-PCR (RT-PCR) assay, which allows rapid and reproducible identification of a few tumor cells among millions of peripheral blood leukocytes (PBL; ref. 11). The presence of CTC in blood is associated with poorer disease outcome in melanoma patients (6, 12).

The epigenetic phenomenon of hypermethylation at promoter CpG islands of tumor-related genes in various cancers has been implicated in cancer development and progression (13–16). Hypermethylation of promoter region is a significant epigenetic mechanism that regulates gene transcription. Methylation-specific PCR (MSP) is a sensitive and specific assay for tumor-related DNA methylation in serum and/or plasma (17). A number of studies have evaluated the potential of circulating tumor-related methylated DNA in serum for the molecular diagnosis and prognosis of various types of cancer (5, 18, 19). However, the source of tumor-related cell-free DNA in serum is still unknown and it has been postulated to be from primary/metastatic tumors and/or CTC.

CTC and tumor-related methylated DNA in blood have been separately assessed for their clinical utility; however, no studies have looked into the relation between these two different types of molecular markers. Although the origin and mechanism of cell-free DNA in blood is still debated, death of CTC has been considered as one of the possible sources whereby DNA is released in blood (20, 21). If CTC are the source of cell-free DNA, their physiologic relation would justify coassessment of CTC and serum tumor-related methylated DNA as a potentially powerful composite molecular index of disease outcome in cancer patients.

Previous studies have suggested the benefit of biochemotherapy in advanced-stage melanoma (22–28). Identifying surrogate markers of treatment response would be of tremendous clinical value to manage the patients receiving systemic therapy, such as biochemotherapy. Monitoring of surrogate molecular markers may be informative on whether to continue or alternate the treatment, and prediction of treatment efficacy can bring an improvement on the patients’ quality of life. Because melanoma is a heterogeneous disease, multiple molecular marker assays may be more favorable compared with single-marker assays to detect tumor spreading in blood (4, 9, 19, 29–31). Recently, we showed that sequential monitoring of CTC using a multimarker quantitative real-time RT-PCR assay could predict the treatment outcome in American Joint...
Committee on Cancer (AJCC) stage III melanoma patients receiving neoadjuvant biochemotherapy (6). We have also shown that serum tumor-related hypermethylated DNA at tumor-related gene promoter regions was frequently found in patients with metastatic tumors (31).

We hypothesized that CTC levels in blood may be correlated with tumor-related methylated DNA in serum and that the combination of these molecular variables may allow more accurate prognostic assessment in melanoma patients. In this study, we assessed a physiologic and prognostic correlation between CTC and tumor-related methylated DNA in the blood of 50 patients receiving biochemotherapy for AJCC stage IV melanoma.

Materials and Methods

Patients and blood specimens. After approval from the Institutional Review Board of the John Wayne Cancer Institute and the Saint John's Health Center (Santa Monica, CA), this study was carried out. All patients signed informed consent for the use of their blood specimens. All patients were diagnosed with AJCC stage IV melanoma and treated with a concurrent biochemotherapy regimen composed of dacarbazine, cisplatin, vinblastine, IFN-α2b, interleukin 2, and tamoxifen, and clinical results of the trial were described as previously reported (22, 32). Fifty patients from this single-institute trial were retrospectively selected by the database coordinator based on availability of cryopreserved specimens, completion of the biochemotherapy treatment, and their clinical follow-up (38 males, and 12 females; median age, 45 years; range, 18-70 years; Table 1). Patients were categorized by their response to biochemotherapy (22): complete response (CR; n = 13), partial response (PR; n = 10), stable disease (SD; n = 3), and progressive disease (PD; n = 24; median follow-up time, 12.5 months; range, 1-79 months).

We obtained matched paired nucleated cell fraction of blood and serum samples from our cryopreserved banks. All blood specimens were collected before administration of biochemotherapy and were coded by a computer-generated number before processing the blood. Blood specimens for the nuclear cell fraction and serum for respective assays were drawn simultaneously. The study was conducted in a double-blinded fashion; the patients' disease status was not known to individuals who did the blood assay or analyzed the data and the assay results were not known to the individual who recorded disease status. The CTC and serum DNA methylation assay were done independently by two investigators (K.K. and T.M., respectively) who were blinded to the other assay results. Statistical analyses for the combined results of two assays were done by a biostatistician (J.L.W.) independently.

CTC assay. Peripheral blood specimens were collected in sodium citrate–containing tubes and the first several milliliters were discarded to eliminate skin-plug contamination (29). Nucleated cell fractions were isolated from blood and cryopreserved in liquid nitrogen until thawed for the study as previously described (10). Briefly, the nucleated cells from blood were isolated using a gradient and Purescript RBC lysis solution (Gentra, Minneapolis, MN).

Tri-Reagent (Molecular Research Center, Cincinnati, OH) was used to isolate total cellular RNA from PBL (29). RNA was quantified and assessed for purity by UV spectrophotometry. Reverse transcription reactions were done using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) with oligo-dT primer (33).

The multimarker quantitative real-time RT-PCR assay was done to assess the presence of melanoma antigen recognized by T cells 1 (MART-1), β1→4-N-acetylglucosaminyltransferase (GalNAc-T), and melanoma antigen gene A3 family (MAGE-A3) mRNAs. The sensitivity and specificity of the quantitative real-time RT-PCR assay using three markers for detection of CTC have been validated and could be used as a surrogate of CTC in blood as previously described (6, 11). Multimarker quantitative real-time RT-PCR assay was done with the use of the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primer and probe sequences were designed for quantitative real-time RT-PCR assay and sequences used have previously been reported (11). We transferred 4 μL of cDNA from 200 ng of total RNA to individual wells of a 384-well PCR plate, in which 0.5 μmol/L of each primer, 0.3 μmol/L probe, and 5 μL of iTaq custom supermix with ROX (Bio-Rad Laboratories, Hercules, CA) were added to a final volume of 10 μL. Samples were amplified with a precentering hold at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing/extension at 55°C for 1 minute for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; at 59°C for MAGE-A3, at 62°C for GalNAc-T, and at 58°C for MAGE-A3). The standard curve was generated with the threshold cycle (Ct) of seven serial dilutions of plasmid templates (10^−10^-10 copies). The Ct of each sample was interpolated from the standard curve and the number of mRNA copies was calculated. PCR efficiency, assessed from the slopes of standard curves, was between 90% and 100%. The correlation coefficients for all standard curves (Ct versus log copy number) in the study were ≥0.99.

Each quantitative real-time RT-PCR assay was done at least twice and included marker-positive and marker-negative controls and reagent controls (reagent alone without RNA or cDNA). GAPDH gene was used as a housekeeping gene. Any specimen with inadequate GAPDH mRNA copies (<10,000) was excluded from the study. No sample was excluded from the study. The mean mRNA copy number was used for analysis. All healthy normal donors' blood was processed and prepared as with the melanoma patients.

Table 1. Clinical characteristics of biochemotherapy patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients, n = 50</th>
<th>n [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>38</td>
<td>76</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>34</td>
<td>68</td>
</tr>
<tr>
<td>≥50</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>ECOG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>216</td>
<td>(range, 108-4056)</td>
</tr>
<tr>
<td>≤190</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td>&gt;190</td>
<td>28</td>
<td>56</td>
</tr>
<tr>
<td>No. metastatic sites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2</td>
<td>29</td>
<td>58</td>
</tr>
<tr>
<td>&gt;2</td>
<td>21</td>
<td>42</td>
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<td>Prior chemotherapy</td>
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<td>8</td>
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<tr>
<td>Prior IFN</td>
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<td>14</td>
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<tr>
<td>Biochemotherapy response</td>
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<tr>
<td>CR</td>
<td>13</td>
<td>26</td>
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<tr>
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<td>SD</td>
<td>3</td>
<td>6</td>
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<tr>
<td>PD</td>
<td>24</td>
<td>48</td>
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sera. Controls were matched as closely as possible in gender and age range. The ratio of males and females was 67% and 33%, respectively, similar to the melanoma patients. The age range was 24 to 65 years, with a mean of 41 years.

Extracted DNA was subjected to sodium bisulfite modification (31). Briefly, DNA from 500 μL of serum was supplemented with 1 μg salmon sperm DNA (Sigma, St. Louis, MO) and denatured in 0.3 mol/L NaOH for 3 minutes at 95°C. Overall, 530 μL of a 2.5 mol/L sodium bisulfite/125 mmol/L hydroquinone solution were added. Samples were incubated under mineral oil in the dark for 3 hours at 60°C. Salts were removed using the Wizard DNA Clean-Up System (Promega) and samples were desulfonated in 0.3 mol/L NaOH at 37°C for 15 minutes. Modified DNA was precipitated with ethanol using Pellet Paint NF (Novagen, Madison, WI) as a carrier and then resuspended in molecular grade water.

We assessed the methylation status of Ras association domain family protein 1A (RASSF1A) and retinoic acid receptor-β2 (RAR-β2) hypermethylation of the promoter region CpG islands was frequently detected in metastatic melanoma tumors (31). Each methylation status of the bisulfite-treated DNA was determined using two sets of fluorescently labeled primers designed to amplify methylated or unmethylated DNA sequences. The primer sets for MSP were as follows: RASSF1A, 5’-GTGTTAACGCGTTGCGTATC-3’ (forward) and 5’-AACCCCCGCAAATAAAAACGA-3’ (reverse); RAR-β2, 5’-GAACGCCGACC-GATTCCAGT-3’ (forward) and 5’-GACCAATCCAACCGAAGC-3’ (reverse). The unmethylated-specific primer sets were as follows: RASSF1A, 5’-TTTGGTTGAGTGTTGTAATG-3’ (forward) and 5’-CAAACCCCCCAAAC-TAAAAACA-3’ (reverse); RAR-β2, 5’-GGATTGGGATGTTGAGAATGT-3’ (forward) and 5’-CAACCCATCCCAACAAAACA-3’ (reverse). Forward methylated sequence-specific primers were labeled with D2a dye and forward unmethylated sequence-specific primers were labeled with D2a dye. Optimized PCR assay condition and assessment of PCR product using capillary array electrophoresis (CAE, CEQ 8000XL, Beckman Coulter, Inc., Fullerton, CA) were previously described (31).

Lymphocyte DNA obtained from healthy donors underwent sodium bisulfite modification and a universal unmethylated control synthesized by New England Biolabs, Beverly, MA) lymphocyte DNA was used as a positive methylated control. Blood processing, RNA and DNA extraction, PCR assay set-up, and post RT-PCR product analysis were carried out in separate designated rooms to prevent cross-contamination; RNA and DNA extraction and PCR studies were done by separate individuals.

Statistical analysis. χ² analysis was used to assess the correlation between any two markers. Cochran-Armitage trend test was used to examine the significance of association between marker detection and treatment response to biochemotherapy, and also between CTC and serum methylated DNA level. χ² test was used to assess the correlation between CTC/serum DNA methylation marker and prognostic factors [gender, age, Eastern Cooperative Oncology Group (ECOG) status, lactate dehydrogenase, number of metastatic sites, prior treatment, and biochemotherapy response]. Time to progression and overall survival from the start of biochemotherapy were used for outcome measurement. The correlation of marker detection with recurrence-free survival and overall survival was examined using the log-rank test. Kaplan-Meier survival curves were plotted. Two-sided P ≤ 0.05 was considered to be statistically significant.

Results

Detection of CTC in blood. MART-1, GalNAc-T, and MAGE-A3 mRNAs were frequently detected in melanoma cell lines and not detected in PBL from 49 healthy donors; sensitivity and specificity of the quantitative real-time RT-PCR assay using three markers have previously been described (11). Individual melanoma mRNA markers were detected in 18 (36%), 12 (24%), and 8 (16%) patients for MART-1, GalNAc-T, and MAGE-A3, respectively, in 50 blood specimens from stage IV melanoma patients obtained before biochemotherapy treatment. Seventeen (34%) patients had one CTC marker, 6 (12%) had two markers, and 3 (6%) had three markers. Twenty-six patients (52%) had at least one CTC marker detection. There was no significant association between any two individual markers.

Next, we assessed the correlation between CTC markers in blood and disease outcome. In patients with CR and PR (n = 23), individual CTC marker was detected in 3 (13%), 5 (22%), and 3 (13%) patients for MART-1, GalNAc-T, and MAGE-A3, respectively, and 9 (39%) patients had at least one CTC marker (Table 2A). By contrast, in patients with PD (n = 24), individual markers were detected in 14 (58%), 6 (25%), and 5 (21%) patients, respectively, and 16 (67%) patients had at least one CTC marker. The number of

<table>
<thead>
<tr>
<th>Table 2.</th>
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<tbody>
<tr>
<td>(A) Correlation of CTC markers with disease outcome</td>
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<tr>
<td>Response</td>
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<tr>
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<tr>
<td>CR/PR (n = 23)</td>
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<tr>
<td>(B) Correlation of serum methylated DNA markers with disease outcome</td>
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<tr>
<td>Response</td>
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<tr>
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</tr>
<tr>
<td>CR/PR (n = 23)</td>
</tr>
<tr>
<td>PD (n = 24)</td>
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</table>

*Cochran-Armitage trend test.
CTC markers detected was significantly correlated with disease outcome ($P = 0.03$).

We then evaluated the CTC markers as prognostic factors of survival. Patients with at least one CTC marker had significantly lower time to progression ($P = 0.03$) and overall survival ($P = 0.04$) than patients without CTC marker (Fig. 1). For patients without CTC markers, the estimated overall survival was 78% [95% confidence interval (95% CI), 67-87%] at 12 months and 61% (95% CI, 46-75%) at 24 months. For patients with at least one CTC marker, the estimated overall survival was 43% (95% CI, 26-62%) at 12 months and 19% (95% CI, 7-40%) at 24 months.

Association between CTC and serum methylated DNA. In patients with serum methylated DNA ($n = 15$), MART-1, GaINAc-T, and MAGE-A3 mRNAs were detected in 9 (60%), 5 (33%), and 4 (27%) patients, respectively. In patients without any serum methylated DNA ($n = 35$), MART-1, GaINAc-T, and MAGE-A3 mRNAs were detected in 9 (27%), 7 (23%), and 4 (11%) patients, respectively. Detection of MART-1 was significantly correlated with detection of serum methylated DNA ($P = 0.003$). Of 15 patients with serum methylated DNA, 13 (87%) patients had at least one CTC marker and 2 (13%) had no CTC marker detected (Table 3A). By contrast, 13 of 35 (37%) patients without serum methylated DNA had at least one CTC marker and 22 (63%) had no CTC marker detected. The presence of CTC markers was significantly correlated with the presence of serum methylated markers ($P = 0.001$). The number of CTC markers detected was also significantly correlated with detection of serum methylated DNA in paired specimens ($P = 0.008$). These results suggested that the presence of CTC in blood has a significant association with circulating methylated DNA in serum.

CTC and serum methylated DNA as a predictor of disease outcome. We combined the results from two different molecular assays, quantitative real-time RT-PCR and MSP, and determined if they could improve the assessment of disease outcome and prognosis. In patients with CR and PR, 13 (57%) patients had neither CTC nor serum methylated DNA and only 3 (13%) had
both blood molecular markers (Table 3B). By contrast, in patients with PD, 7 (29%) patients had neither CTC nor serum methylated DNA and 10 (42%) had both blood molecular markers. Patients with both variables showed the poorer response to biochemotherapy ($P = 0.02$). No other known prognostic factors, such as gender, age, ECOG status, lactate dehydrogenase, number of metastatic sites, or prior treatment, were associated with biochemotherapy response.

Next, we evaluated the correlation of these variables with survival. We divided the patients into three groups: patients without CTC and serum methylated DNA, those with either type of molecular marker, and those with both type of molecular markers. Patients with both blood molecular markers had significantly lower time to progression ($P = 0.009$) and overall survival ($P = 0.02$) among these three groups (Fig. 3). For patients without CTC and serum methylated DNA, the estimated overall survival was 81% (95% CI, 69-88%) at 12 months, 63% (95% CI, 49-78%) at 24 months, 52% (95% CI, 34-70%) at 36 months, and 42% (95% CI, 24-62%) at 48 months. For patients with either marker, the estimated overall survival was 63% (95% CI, 46-77%) at 12 months, 40% (95% CI, 22-60%) at 24 months, 25% (95% CI, 11-48%) at 36 months, and 16% (95% CI, 5-38%) at 48 months. For patients with both blood molecular markers, the estimated overall survival was 50% (95% CI, 27-65%) at 12 months, 21% (95% CI, 8-45%) at 24 months, 9% (95% CI, 2-31%) at 36 months, and 4% (95% CI, 7-22%) at 48 months. These analyses showed that the combination of both blood molecular markers improves the assessment of prognosis in stage IV melanoma patients treated with biochemotherapy.

### Discussion

There is continual growing evidence for various cancers supporting the concept that molecular detection of hematogenous spreading of tumor cells can be used as an indicator of disease progression (3, 4). Assessment of blood for detecting CTC or their nucleic acid, such as DNA, can be of clinical utility. Although PCR-based techniques can identify a few specific target markers, a single-marker assay is limited by the heterogeneous nature of tumors and expression of tumor-related genes. As we previously showed, using the appropriate panel of molecular biomarkers (multimarker) could allow sensitive detection of metastatic cells (11, 29, 31). To date, CTC in blood and tumor-related methylated DNA in serum have been separately investigated, and many studies have suggested the usefulness of CTC or serum methylated DNA as

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### Table 3.

(A) Association between CTC and serum methylated DNA markers

<table>
<thead>
<tr>
<th>Serum DNA methylation</th>
<th>No. CTC markers (%)</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Methylation (n = 15)</td>
<td>2 (13%)</td>
<td>8 (53%)</td>
</tr>
<tr>
<td>Unmethylated (n = 35)</td>
<td>22 (63%)</td>
<td>9 (26%)</td>
</tr>
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(B) Correlation of CTC/serum methylated DNA with disease outcome

<table>
<thead>
<tr>
<th>Response</th>
<th>Blood markers (%)</th>
<th>$P^*$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>CTC or serum DNA</td>
</tr>
<tr>
<td>CR/PR (n = 23)</td>
<td>13 (57)</td>
<td>7 (30)</td>
</tr>
<tr>
<td>PD (n = 24)</td>
<td>7 (29)</td>
<td>7 (29)</td>
</tr>
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*Cochran-Armitage trend test.*

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![Figure 3](image-url)

**Figure 3.** Kaplan-Meier curves of time to progression (left) and overall survival (right) based on CTC and serum methylated DNA detection.
a surrogate marker of subclinical disease spreading for various cancers (7–10, 13–16). A question that is of interest to us is whether there is an association between CTC and serum methylated DNA. In this study, we showed that detection of CTC was correlated with tumor-related methylated DNA. In addition, we showed the clinical utility of these two different molecular variables as prognostic factors of disease outcome in AJCC stage IV melanoma patients.

We selected three CTC markers and two serum methylated DNA markers for this study based on our previous findings (11, 31). All the markers were frequently found in blood from metastatic melanoma patients but not in blood from healthy donors. As expected, detection rates of multimarker assay were higher than any individual marker assay and these findings were consistent with previous studies (29, 36, 37). Detection rate of individual CTC marker was higher in patients with serum methylated DNA marker and the number of CTC markers significantly increased in patients with serum methylated DNA. These findings strongly suggest that CTC is a potential source of circulating methylated DNA.

To our knowledge, this is the first report that shows the correlation between two different molecular marker types in cancer patients’ blood. Recently, DNA methylation status of tumor-related gene promoter region has turned out to be one of the most common tumor-related molecular alterations in various cancers, and many studies have shown tumor-related methylated DNA recovered from plasma or serum of cancer patients. Several sources of the cell-free DNA are suggested (i.e., lysis of CTC or micro-metastases in organs, DNA leakage from cell as the result of tumor necrosis or apoptosis, or spontaneous release of DNA into circulation from primary and metastatic tumors; refs. 20, 38). However, to date, there is no agreement on the origin and mechanism that produces cell-free DNA in blood. This issue is important and still remains enigmatic. Although it is difficult in human patients to directly elucidate the origin of cell-free DNA in circulation, the significant correlation between CTC and serum methylated DNA implicates CTC as a possible candidate of serum methylated DNA. DNA degradation occurs in normal and tumor cells by apoptosis, producing small fragmented DNA, which is rapidly cleared out (20, 39). However, other mechanisms of tumor cell destruction may involve nonapoptotic process, and it has been suggested that circulating DNA may be produced by cells undergoing nonapoptotic death in blood, resulting in larger fragments of DNA in serum. Lysis of CTC due to physiologic forces or nonapoptotic factors in the blood circulation may contribute to an increase in circulating methylated DNA. This would yield more circulating DNA if more CTC were in circulation. Our findings indicated that the number of CTC markers correlated with detection of serum tumor-related methylated DNA; thus, greater number of CTC in blood is likely to release larger amount of DNA. The issues about spontaneous release of DNA from CTC in blood and half-life of circulating cell-free tumor-related DNA have been poorly understood. The destruction of CTC in blood and the immediate release of DNA may account for greater frequency of cell-free DNA in advanced-stage melanoma patients.

The numbers of CTC markers and serum methylated DNA markers detected were significantly correlated with treatment response. Combination assessment clearly showed that status of CTC and serum methylated DNA could predict tumor progression and survival. These findings suggested the high risk and aggressiveness of tumors in patients with large amounts of CTC and tumor-related DNA in bloodstream, resulting in systemic tumor spreading and failure of the treatment. Assessment of CTC and/or circulating tumor-related DNA can provide useful information on ongoing tumor spreading and treatment response in advanced-staged patients receiving adjuvant therapy, as well as early detection of tumor metastasis.

Although CTC and/or serum tumor-related methylated DNA predicts the disease outcome, the particular role of resistance to drugs might be considered because investigators have reported that resistance to cisplatin and tamoxifen, both of which were components of this biochemotherapy regimen, has been associated with RASSF1A hypermethylation in other malignancies (16, 40). The association between RASSF1A serum hypermethylation and poor clinical response to biochemotherapy may in part be mediated by melanoma resistance to the tamoxifen and cisplatin components of the regimen. These findings suggested that appropriate selection of specific markers based on functional role of the genes was important to assess the response to adjuvant therapy and to predict disease outcome.

Molecular detection of real-time tumor spreading is highly important for predicting the treatment efficacy and the management of melanoma patients. Patients with CTC or tumor-related methylated DNA showed poorer disease outcome compared with the patients without blood markers, and patients with both markers showed worst disease outcome. Thus, the composite assay system based on assessment of two different variables, CTC and serum tumor-related methylated DNA, enabled a more informative assessment as “lethal molecular biomarkers” and has greater advantage for predicting the outcome. Although we simultaneously assessed the blood specimens obtained before biochemotherapy treatment and showed the significance of blood molecular markers as a prognostic marker, serial monitoring of surrogate markers will provide more useful information for deciding on therapeutic strategy. As treatment regimens become multimodal and multiphasic, there will be an urgent need for clinically relevant surrogate markers that can be used to determine treatment response. To verify the clinical utility of CTC and serum methylated DNA, we are currently investigating the association between these two molecular markers and their significance as a potential prognostic factor using sequential blood specimens in a multicenter trial.

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ment by real-time reverse transcriptase-polymerase 
blood as surrogate prognostic indicators of melanoma 
tumor markers in the blood: early prediction of disease 
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