Allelic Imbalance on 12q22-23 in Serum Circulating DNA of Melanoma Patients Predicts Disease Outcome

Akihide Fujimoto, 1 Steven J. O’Day, 2 Bret Taback, 1 David Elashoff, 3 and Dave S. B. Hoon 1

1 Department of Molecular Oncology, John Wayne Cancer Institute, Santa Monica, California; 2 Cancer Institute Medical Group, Santa Monica, California; and 3 Department of Biomathematics, University of California at Los Angeles School of Medicine, Los Angeles, California

Abstract

Allelic imbalance (AI) encompassing the apoptotic protease-activating factor 1 (APAF-1) locus (12q22-23) is found frequently in metastatic melanoma. Circulating DNA with AI on 12q22-23 in serum was evaluated as a surrogate marker to predict biochemotherapy (BC) treatment response in melanoma patients. Sera were collected from 49 American Joint Committee on Cancer stage IV melanoma patients treated with BC. Serum AI of the 12q22-23 region was demonstrated to be present before and/or after BC. BC responders showed a significantly lower frequency of AI (5 of 24, 21%) compared with nonresponders (11 of 25, 44%; Fisher’s exact test, P < 0.029). Serum AI on 12q22-23 was associated with worse prognosis (log-rank test, P < 0.046). These findings indicate that serial serum genetic analysis of tumor-related AI on 12q22-23 may have clinical use in predicting tumor response to therapy.

Introduction

Apoptotic protease-activating factor 1 (APAF-1) is an essential downstream target of p53 in the intrinsic apoptotic pathway (1–3). Loss of APAF-1 can aid tumor cells to evade immune attack-induced death, circumvent inherent programmed cell death, and resist cancer chemo-, immuno-, and radiotherapy (4, 5). APAF-1 gene is located at the 12q22-23 locus. Soenagas et al. (1) demonstrated that loss of heterozygosity on 12q22-23 was frequent and correlated with decreased APAF-1 mRNA expression in metastatic melanoma. Recently, we have demonstrated that the presence of allelic imbalance (AI) on 12q22-23 region in metastatic melanoma tumors is associated with poor disease outcome (6).

Concurrent administration of biochemotherapy (BC) has shown improvements in response in American Joint Committee on Cancer stage IV melanoma patients (7–11). However, as with most treatment regimens, patient response is difficult to predict. Identification of molecular predictors of therapeutic response may permit more efficient use of resources and improve stratification design strategies.

It is suggested that circulating DNA in the serum/plasma of cancer patients has clinical use as potential markers for disease surveillance (12–17). Previously, we identified circulating tumor microsatellites with AI in the acellular serum/plasma of melanoma (16–18). AI in blood correlated with AI in the respective melanoma tumors and was associated with poorer disease outcome (19). Identifying surrogate serum tumor genetic determinants would be of novel clinical use for assessing therapeutic efficacy and follow-up. On the hypothesis that AI on APAF-1 locus in serum can predict response to BC treatment, we assessed the detection of circulating DNA microsatellites in acellular serum from melanoma patients receiving BC. To determine whether detection of AI on 12q22-23 in circulating DNA could be a surrogate predictor of response to treatment and disease progression, serial serum genetic analysis on 49 American Joint Committee on Cancer stage IV melanoma patients before administration of BC (pre-BC serum) and after its completion (post-BC serum) was conducted.

Materials and Methods

Serum DNA Collection and Preparation. Forty-nine American Joint Committee on Cancer stage IV melanoma patients treated with a concurrent BC regimen of dacarbazine, cisplatin, vinblastin, IFN α-2b, interleukin 2, and tamoxifen as previously reported (7, 8) were selected based on availability of serum and follow-up data (Table 1). Institutional Review Board approval and histopathological confirmation from Saint John’s Health Center and John Wayne Cancer Institute joint committee were obtained before study initiation. Blood was drawn for serum before administration of BC (pre-BC serum) and after completion of BC cycles (post-BC serum). Patients were divided into two groups (responders and nonresponders) based on response criteria (8). Patients who showed complete response (n = 13), partial response (n = 10), or stable disease (n = 3) were included in the responder group (n = 26), whereas patients demonstrating progressive disease were deemed nonresponders (n = 23). Median completed cycles of BC were six for the responder group and three for the nonresponder group.

Ten ml of blood were collected in serum separator tubes, and serum was immediately separated from cells by centrifugation (3000 rpm, 15 min) and filtered through a 13-mm serum filter (Fisher Scientific, Pittsburgh, PA). Serum was aliquoted and cryopreserved at −30°C. DNA was extracted from 800 μl of serum using QIAamp extraction kit (Qiagen, Valencia, CA) as described previously (18). Control DNA from each melanoma patient was obtained from the respective peripheral blood lymphocytes.

Microsatellite Analysis. Four microsatellite markers (D12S1657, D12S393, D12S1706, D12S1346) encompassing the APAF-1 gene locus (12q22-23), which were frequent for identifying loss of heterozygosity in primary and metastatic melanomas (6), were used for this analysis. The locations of microsatellite markers and APAF-1 gene were verified using the current updated National Center for Biotechnology Information database (February 2004). PCR primer sets for specific allele loci were obtained from Research Genetics, Inc. (Huntsville, AL). Forward primers were labeled with WellRed phosphoramidite-linked dye or active ester-labeled dye. The PCR amplification was performed in a 10-μl reaction volume with 1 μl of template for 40 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by a 7-min final extension at 72°C. PCR product separation was performed using capillary array electrophoresis (CAE CEQ 8000XL; Beckman Coulter, Inc., Fullerton, CA). Peak signal intensity and relative size were generated by a fragment analysis system software (Beckman Coulter, Inc.). AI was defined when one allele showed ≥40% reduction of peak intensity for serum DNA as compared with the corresponding allele identified in the control DNA. The markers showing homozygosity, microsatellite instabilities, and insufficient PCR amplification were scored as noninformative. Five sera in which ≤1 marker was informative were excluded from clinical correlation analysis because of difficulty to define AI status on this locus by one or less marker. All AI were confirmed by repeating the assay.
Statistical Analysis. Correlation between AI on 12q22-23 and BC response was assessed using the Fisher’s exact test. Survival length was determined from the first day of BC treatment, to death, or the date of last clinical follow-up. Survival curves were drawn by Kaplan-Meier method, and differences between curves were analyzed using the log-rank test. Cox’s proportional hazards regression model was used for multivariate analysis and calculation of the risk ratio (19). Stepwise variable selection was adopted with a selection rule of $P < 0.1$ for variables.

Results

Frequency of AI on 12q22-23. In the analysis of all 49 patients sera, the frequencies of AI for microsatellite markers D12S1657, D12S393, D12S1706, and D12S346 in informative cases were 22% (6 of 27), 15% (5 of 34), 11% (4 of 38), and 20% (8 of 41) in pre-BC serum and 19% (5 of 26), 22% (7 of 32), 13% (5 of 38), and 17% (7 of 41) in post-BC serum, respectively (Fig. 1). Five patients’ (2 responders, 3 nonresponders) sera in which ≤1 marker was informative were excluded from clinical correlation analysis because of the difficulty to define AI status using one or less informative marker. Samples AI positive for at least one marker was found in 16 of 44 (36%) pre-BC serum and 16 of 44 (36%) post-BC serum. Representative examples are shown in Fig. 2.

Correlation with BC Response. Loss of APAF-1 gene may account for cellular resistance to chemo- and immunotherapy. To evaluate the use of 12q22-23 AI as a predictor of response to systemic therapy of advanced stage melanoma patients, we assessed BC-treated patients in a retrospective analysis, whereby patients were divided into responders and nonresponders. AI on 12q22-23 status in pre-BC serum was assessed to predict patients who were likely to respond to BC. The frequency of AI on 12q22-23 in pre-BC serum was significantly lower in the responder group (5 of 24, 21%) than in the nonresponder group (11 of 20, 55%; Fisher’s exact test, $P = 0.029$). There were no significant differences in the frequency of AI on

Table 1  Clinical characteristics of BC patients*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>$n$</th>
<th>Pre-BC serum</th>
<th>Post-BC serum</th>
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<tbody>
<tr>
<td>Total patients</td>
<td>49</td>
<td>16/44 (36%)</td>
<td>16/44 (36%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
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<td></td>
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<tr>
<td>Male</td>
<td>38</td>
<td>14/35 (40%)</td>
<td>13/35 (37%)</td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>2/9 (22%)</td>
<td>3/9 (33%)</td>
</tr>
<tr>
<td>Age in yrs (median, 45 yrs)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;50</td>
<td>33</td>
<td>12/30 (40%)</td>
<td>10/30 (33%)</td>
</tr>
<tr>
<td>≥50</td>
<td>16</td>
<td>4/14 (29%)</td>
<td>6/14 (43%)</td>
</tr>
<tr>
<td>BC response</td>
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</tr>
<tr>
<td>Responder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>13</td>
<td>1/2 (8%)</td>
<td>4/12 (33%)</td>
</tr>
<tr>
<td>PR</td>
<td>10</td>
<td>3/9 (33%)</td>
<td>4/9 (44%)</td>
</tr>
<tr>
<td>Nonresponder</td>
<td>3</td>
<td>1/3 (33%)</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>LDH (IU/liter)</td>
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<td></td>
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<tr>
<td>≤190</td>
<td>22</td>
<td>7/19 (37%)</td>
<td>6/19 (32%)</td>
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<tr>
<td>&gt;190</td>
<td>27</td>
<td>9/25 (36%)</td>
<td>10/25 (40%)</td>
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<tr>
<td>No. of metastasis sites</td>
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<tr>
<td>≤2</td>
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<td>10/25 (40%)</td>
<td>7/25 (28%)</td>
</tr>
<tr>
<td>&gt;2</td>
<td>21</td>
<td>6/19 (32%)</td>
<td>9/19 (47%)</td>
</tr>
</tbody>
</table>

*BC, biochemotherapy; AI, allelic imbalance; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; LDH, lactate dehydrogenase.

![Fig. 1. Allelic imbalance (AI) on 12q22-23 for prebiochemotherapy (BC) sera. Res, responder; nonR, non responder; ●, retention of heterozygosity; ○, AI, (●), noninformative; L, allelic loss at 12q22-23; R, allele retained at 12q22-23; and ND, allele status not determined.](image-url)
12q22-23 in post-BC serum between the responder group (9 of 24, 38%) and the nonresponder group (7 of 20, 35%). No other known melanoma prognostic factors were associated with BC response (post-BC serum, gender, age lactate dehydrogenase, and metastasis site; not significant).

AI-positive group in pre-BC serum had significantly worse survival than the AI-negative group (log-rank test, $P = 0.046$; Fig. 3A). Response to BC had a significant effect on overall survival (log-rank test, $P < 0.0001$; Fig. 3B). Using a Cox’s proportional hazards regression model, AI in pre-BC serum and elevated lactate dehydrogenase ($\geq 190$ IU/liter) significantly correlated with survival (AI in pre-BC serum, risk ratio 2.33, 95% confidence interval 1.08–5.03, $P = 0.032$; lactate dehydrogenase, risk ratio 2.82, 95% confidence interval 1.23–6.54, $P = 0.015$). Other prognostic factors in the model such as sex, age, and number of metastatic disease sites were not significant. Because of the significant correlation of AI with BC response, BC response was excluded from variables.

Discussion

Since the discovery of circulating tumor-derived DNA in serum/plasma, investigators have sought to determine the clinical use of these findings in cancer patients. The purpose of the study was to determine whether circulating DNA for AI could identify response in a well-controlled clinical trial for a particular therapeutic intervention. We recently demonstrated that 12q22-23 AI of metastatic melanoma tumors was associated with poorer disease outcome. The study also demonstrated that APAF-1 loss increased during tumor progression from primary to systemic metastatic (6). Because tumor cells susceptible to apoptosis may be an important determining factor for BC response in melanoma patients, we sought to determine whether circulating tumor DNA containing these genetic alterations could serve as a predictive surrogate of treatment response. In the present study, we demonstrated that 12q22-23 AI can be detected in pre-BC patients’ sera and was associated with overall response. Our results provide evidence for APAF-1 loss as a potential surrogate in the (immuno-)chemoresistance of systemic metastatic melanoma. One major problem in the evaluation of systemic therapy is predicting tumor response and assessing tumor genetic changes during treatment. Current imaging approaches are highly subjective and provide limited information. More importantly, it is not feasible to perform repetitive informative tumor sampling to assess genetic changes during treatment of systemic disease. In this study, we demonstrate a new approach for assessing a tumor genetic marker associated with apoptosis.

Six responder patients with AI negative in pre-BC serum became AI positive in post-BC serum. One possibility is that there is increasing tumor-derived serum DNA caused by continued BC-induced apoptosis. An alternative hypothesis is that continual treatment of these patients selected for AI-positive dominant tumor cell clones. There is also a possibility of long-term circulating DNA from BC responses. There is continual debate as to the half-life of circulating DNA. Recent studies measuring fetal DNA from maternal plasma (20) indicated that circulating DNA is cleared rapidly and that the estimated half-life is <1 h. One of the objectives of BC therapy is to attack through multiple targets on tumor cells. However, if the agents’ mechanism of death all rely on the APAF-1-induced pathway, then there is limited benefit. BC treatment likely will influence the clonal selection of specific melanoma cells resistant to treatment. In responder cases, BC therapy could kill the majority of APAF-1-express-
ing tumor cells, thus leaving behind minimal disease. Long-term BC therapy and other systemic therapies may promote selection of APAF-1-negative clones that eventually become a dominant metastatic phenotype. This may explain why long-term remissions are rare and why melanoma patients with systemic metastasis are generally poor and why there are nonresponders to immuno-, chemo-, and radiotherapy.

One of the major problems in assessing tumor genetic markers is the availability of melanoma tumor specimens from distant metastasis. The ability to assess serial blood for tumor genetic markers provides the availability of melanoma tumor specimens from distant metastasis. Previously, we identified circulating tumor DNA microsatellites with AI in the acellular plasma of patients with melanoma (16–17). The circulating DNA AI correlated with genetic alterations present in the respective melanoma tumors and with poorer disease outcome. Identifying serum circulating tumor genetic determinants as surrogates particularly relevant to apoptosis resistance would be of significant clinical use for therapeutic design. Most approaches in patient treatment focus on the target gene(s) instead of the susceptibility of the tumor to undergo apoptosis. This information may prove vital in predicting individual patient treatment responses.

Melanoma progression is associated with continued selection of clones that resist apoptosis. Systemic melanoma metastasis is a product of genotypic selection of clones that can proliferate and overcome apoptosis. Systemic metastatic tumors are usually highly genetically unstable and heterogeneous. Serum DNA is likely to represent the genotype of the most dominant tumor clone present at the time of analysis. BC may additionally induce clonal selection, whereby resistant tumor cells survive and become more dominant after therapy. Therefore, to address this problem, it may be more efficacious to create treatment regimens that target multiple different critical cell regulatory pathways not related to each other. The study was focused on American Joint Committee on Cancer stage IV melanoma patients with poor prognosis. The purpose of the study was to develop a method to predict patients likely to respond to BC. To date, the BC treatment is still controversial and needs validating. Identification of patients likely to respond would have a significant improvement efficacy. One of the limitations in the study is that all responder patients are not identified; development of other potential informative DNA markers relating to key apoptosis factors need to be assessed to improve predictive sensitivity. A limitation to the assay at this time that needs to be additionally worked out is obtaining sufficient DNA from serum to perform a more comprehensive and quantitative analysis.

The retention of heterozygosity in the serum DNA analysis is demonstrated, and three plausible explanations can be provided: (a) the tumor cell does not carry AI at the locus; (b) homozygous deletion at the locus has occurred in tumor cells; and/or (c) tumor-derived DNA in serum can be underdetected because of low abundance or interference of cell-derived DNA. These factors may effect the interpretations of the results. Additional refinement of the technology and the addition of informative markers may improve the assay efficacy. Our results suggest that AI on 12q22-23 is an important determining factor for response to BC and becomes a dominant functional genotypic aberration with disease progression. Advanced melanomas are more likely to be resistant to therapy that requires the activation of the APAF-1-intrinsic apoptotic pathway. Development of therapeutics to supplement APAF-1 function in the apoptosis pathway may be needed to improve treatment efficiency in melanoma patients. This study demonstrates that detecting loss of a key apoptotic gene locus in serum can be used as a surrogate genetic determinant in cancer patients to predict response to therapy. To our knowledge, this is the first study to evaluate the association between a circulating tumor DNA of a specific apoptosis gene locus and patient’s disease outcome in response to therapy. Circulating AI of 12q22-23 may be used as a potential prognostic marker of melanoma progression during therapy, whereby serial serum genetic analysis monitoring can be accomplished. The study needs to be validated with other forms of therapy to determine their universal efficacy in predicting treatment response. Serial serum genetic analysis offers a new approach of monitoring tumor genetic changes in patients with systemic disease in which a tumor is not available.

References

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