Estrogen Receptor-α Methylation Predicts Melanoma Progression

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Abstract

The role of estrogen receptor α (ER-α) in melanoma is unknown. ER-α expression may be regulated in melanoma via hypermethylation of promoter CpG islands. We assessed ER-α hypermethylation in primary and metastatic melanomas and sera as a potential tumor progression marker. ER-α methylation status in tumor (n = 107) and sera (n = 109) from American Joint Committee on Cancer (AJCC) stage I to IV melanoma patients was examined by methylation-specific PCR. The clinical significance of serum methylated ER-α was assessed among AJCC stage IV melanoma patients receiving biochemotherapy with tamoxifen. Rates of ER-α methylation in AJCC stage I, II, and III primary melanomas were 36% (4 of 11), 26% (5 of 19), and 35% (8 of 23), respectively. Methylated ER-α was detected in 42% (8 of 19) of stage III and 86% (30 of 35) of stage IV metastatic melanomas. ER-α was methylated more frequently in metastatic melanomas than primary melanomas (P = 0.0003). Of 109 melanoma patients’ sera in AJCC stage I, II, III, and IV, methylated ER-α was detected in 10% (2 of 20), 15% (3 of 20), 26% (5 of 19), and 32% (16 of 50), respectively. Serum methylated ER-α was detected more frequently in advanced than localized melanomas (P = 0.03) and was the only factor predicting progression-free [risk ratio (RR), 2.64; 95% confidence interval (95% CI), 1.36-5.13; P = 0.004] and overall survival (RR, 2.31; 95% CI, 1.41-5.58; P = 0.003) in biochemotherapy patients. Hypermethylated ER-α is a significant factor in melanoma progression. Serum methylated ER-α is an unfavorable prognostic factor. (Cancer Res 2006; 66(13): 6692-8)

Introduction

Because it is difficult to predict which primary tumors will progress to regional or distant metastases, cutaneous melanoma remains a challenging disease to manage (1). New strategies for the identification of epigenetic biomarkers may improve the clinical management of melanoma by facilitating earlier disease diagnosis and providing more accurate prognostic information. No major study has examined the epigenetic alterations of hormone receptors in the progression from primary to metastatic melanoma in a large series of patients.

Hypermethylation of gene promoter CpG islands plays a significant role in the development and progression of various cancers, including melanoma (2-6). The identification of hypermethylated genes in tumors has become an accepted approach to assess tumor-related gene inactivation (6-9). We previously reported tumor-related gene hypermethylation in primary and metastatic melanomas (10). Thereafter, we showed the hypermethylation of multiple tumor-related and tumor suppressor genes during progression from primary to metastatic lesions (11). Several genes methylated in primary and metastatic melanomas were also detected in serum as methylated circulating DNA (11). The observation that tumor-related DNA could be detected in circulating serum provided a method of disease surveillance independent of the availability of gross tumor tissue (12-17).

Estrogen receptor α (ER-α) belongs to a superfamily of transcription activators (18, 19) involved in many physiologic processes, including tumor progression (20-22). Loss of ER-α expression has been associated with aberrant CpG island hypermethylation in breast cancer cell lines and tumors (23-27) and shown to modulate breast cancer progression (5). Several studies have reported the presence of ER in melanoma cell lines but analysis of human melanomas has shown variable ER-α expression (28-31). Several in vitro experiments established that tamoxifen is an effective growth inhibitor of melanoma cells (32, 33). Based on the variable presence of ER-α in melanoma cells, as well as anecdotal reports of clinical responses to antiestrogen therapy, several studies of hormonal and chemohormonal treatments were coordinated. Initial trials were encouraging, with improved response rates and median overall survival in patients receiving tamoxifen, particularly women (34, 35). Subsequent trials, however, failed to show significant differences in response rates or overall survival when tamoxifen was used alone or in combination with systemic therapies (36-42). Reasons for the discrepancies in response to antiestrogen therapy between these trials are unknown.

Mechanisms regulating the expression of ER-α in melanoma are poorly defined; to date, no mutation or other gross structural alteration of the ER-α gene has been reported in melanoma. We hypothesized that ER-α gene silencing via gene promoter hypermethylation in primary and metastatic melanoma plays an important role in melanoma progression and may be used as a prognostic molecular biomarker.

Materials and Methods

Melanoma cell line and tumor DNA isolation. DNA was extracted from 11 melanoma cell lines established from metastatic tumors at John Wayne Cancer Institute and one breast cancer cell line (MCF-7) from American Type Culture Collection (Manassas, VA) as previously described (14). Institutional Review Board approval for the use of human tissues was obtained from Saint John’s Health Center and John Wayne Cancer Institute before beginning the study. Patients who underwent surgery for American Joint Committee on Cancer (AJCC) stages I, II, III, and IV melanoma (11 stage I primary tumors; 19 stage II primary tumors; 23 stage III primary...
tumors; 19 stage III metastatic tumors; and 35 stage IV metastatic tumors) were selected consecutively by the database coordinator from our institutional melanoma patient and specimen database (Table 1). Paraffin-embedded tumor specimens from these patients were obtained from the Division of Surgical Pathology at Saint John’s Health Center.

Several 8-μm sections were cut from formalin-fixed, paraffin-embedded blocks as previously described (43). One section from each tumor block was deparaffinized, mounted on a glass slide, and stained with H&E for microscopic analysis. Light microscopy was used to confirm tumor location and assess tissue homogeneity. Additional sections from the tumor block were mounted on glass slides and microdissected under light microscopy. Dissected tissues were digested with 50 μL of proteinase K–containing lysis buffer at 50°C for 12 hours, followed by heat deactivation of proteinase K at 95°C for 10 minutes (5). DNA was extracted as previously described (10).

**Serum DNA isolation.** AJCC stage I (n = 20), stage II (n = 20), stage III (n = 19), and stage IV patients (n = 50) diagnosed with melanoma were assessed for this study (Table 1). Stage I, II, and III patients received no additional adjuvant therapy but stage IV patients received a systemic concurrent biochemotherapy regimen of dacarbazine (DTIC) or temazolamide, cisplatin, vinblastine, IFN-α2b, interleukin-2, and tamoxifen in the setting of one of several phase II trials, as previously reported (40–42).

AJCC stage IV patients (Table 2) were selected and coded by the clinical study coordinator and assessed in laboratory and statistical analyses in a blinded fashion. The selection of stage IV patients was based on patient response or nonresponse to biochemotherapy, availability of clinical follow-up data, completion of the biochemotherapy trial, and specimen availability. Patients were categorized as responders or nonresponders to biochemotherapy based on clinical response criteria (42). Those showing a complete response (n = 13) or partial response (n = 10) were included in the responder group (n = 23) whereas patients showing progressive disease (n = 24) were deemed nonresponders. Patients exhibiting stable disease (n = 3) were considered neither responders nor nonresponders. One patient in the responder group was lost to follow-up and excluded from the survival analysis. Serum drawn from healthy donors (n = 40) served as normal controls.

### Table 1. Clinical characteristics of melanoma patients

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total patients (tissue)</strong></td>
<td>107</td>
</tr>
<tr>
<td><strong>Total patients (serum)</strong></td>
<td>109</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>58 (54%)</td>
</tr>
<tr>
<td>Female</td>
<td>49 (46%)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>11 (10%)</td>
</tr>
<tr>
<td>II</td>
<td>19 (18%)</td>
</tr>
<tr>
<td>III (primary)</td>
<td>23 (21%)</td>
</tr>
<tr>
<td>III (metastasis)</td>
<td>19 (18%)</td>
</tr>
<tr>
<td>IV (metastasis)</td>
<td>35 (33%)</td>
</tr>
</tbody>
</table>

### Table 2. Clinical demographics of stage IV melanoma patients receiving biochemotherapy

<table>
<thead>
<tr>
<th>Patient characteristics (serum donors)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total patients</strong></td>
<td>50</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>38 (76%)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (24%)</td>
</tr>
<tr>
<td><strong>Stage IV</strong></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>13 (26%)</td>
</tr>
<tr>
<td>PR</td>
<td>10 (20%)</td>
</tr>
<tr>
<td>PD</td>
<td>24 (48%)</td>
</tr>
</tbody>
</table>

Stage IV patients’ blood was drawn for serum before administration of biochemotherapy. Ten milliliters of blood were collected in serum separator tubes, centrifuged, run through a 13-mm serum filter (Fisher Scientific, Pittsburgh, PA), aliquoted, and cryopreserved at −30°C. DNA was extracted and processed from serum as previously described (6). DNA quantification was done on all serum specimens using the Picogreen quantification assay (Molecular Probes, Eugene, OR; ref. 44).

### Cell line and tissue DNA sodium bisulfite modification.

Extracted DNA from cell lines and paraffin-embedded melanoma tumors was subjected to sodium bisulfite modification (11). Briefly, 2 μg DNA was denatured in 0.3 mol/L NaOH for 3 minutes at 95°C and then 500 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, Madison, WI) and 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 resuspended in molecular grade H₂O. DNA samples were cryopreserved at −30°C until methylation-specific PCR was done.

### Serum DNA sodium bisulfite modification.

Extracted DNA from serum was subjected to sodium bisulfite modification (44). Briefly, DNA from 500 μL of serum was supplemented with 1 μg salmon sperm DNA (Sigma Chemical Co., St. Louis, MO) and denatured in 0.3 mol/L NaOH for 3 minutes at 95°C and then 500 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 desulfonated in 0.3 mol/L NaOH at 37°C for 15 minutes. Modified serum DNA was prepared and stored identically to tissue samples.

### Detection of methylated ER-α.

ER-α methylation status was assessed using two sets of fluorescent labeled primers specifically designed to amplify methylated or unmethylated DNA sequences of the ER-α promoter region. Primer sequences are provided as methylated sense and antisense sequences, with annealing temperatures and PCR product size: ER-α methylated-specific forward, 5′-TAAATAGGATATATCGGAGTTTGTACG-3′, and reverse, 5′-AACTTAAATAAAGCGAAGAAGAAAG-3′ (61°C, 96 bp); unmethylated-specific forward, 5′-TAAATAGGATATATCGGAGTTTGTACG-3′, and reverse,
5′-AACTTAAAAACACAAAAACAAA-3′ (58°C, 96 bp). Bisulfite-modified DNA was subjected to PCR amplification in a final reaction volume of 20 µL containing PCR buffer, 2.5 mmol/L MgCl₂, deoxy nucleotide triphosphates (dNTP), 0.3 µmol/L primers, and 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). PCR was done with an initial incubation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds, extension at 72°C for 30 seconds, and final hold at 72°C for 7 minutes. DNA from the ER-α-positive breast cancer cell line MCF-7 was used as a control to verify the presence of ER-α DNA from the ER-α-negative melanoma cell line MCA was used as a control to verify the absence of ER-α. A universal unmethylated control was synthesized from normal DNA by bisulfite DNA polymerase and served as a positive unmethylated control (45). Unmodified lymphocyte DNA was used as a negative control for methylated and unmethylated reactions. Lymphocyte DNA treated with SsoI methylase (New England BioLabs, Beverly, MA) was used as a positive methylated control. PCR products were visualized using capillary array electrophoresis (CEQ 8000 XL, Beckman Coulter, Fullerton, CA) in a 96-well microplate format (6). Methylated and unmethylated PCR products from each sample were assessed simultaneously using forward primers labeled with Beckman Coulter WellRED dye-labeled phosphoramidites (Genset oligos, Boulder, CO). Forward methylated-specific primers were labeled with D4pa dye and forward unmethylated-specific primers were labeled with D2a dye. One microliter of methylated PCR product and 1 µL of unmethylated PCR product were mixed with 40-µL loading buffer and a 0.5-µL dye-labeled size standard (Beckman Coulter). Each marker was optimized with methylated and unmethylated controls. Samples showing a peak at the base pair size marker for unmethylated DNA were considered unmethylated whereas those showing a peak at the base pair size marker for methylated DNA were considered methylated.

Melanoma cell line 5-aza-2-deoxycytidine and trichostatin a treatment. To confirm down-regulation of ER-α expression by hypermethylation of the ER-α promoter region, we treated cell lines with the DNA-demethylating agent 5-aza-2-deoxycytidine (5-aza-CdR) and the histone deacetylase inhibitor trichostatin A (TSA). In combination with 5-aza-CdR treatment, TSA can up-regulate the mRNA expression of genes silenced due to hypermethylation (26, 27). The MCF-7 cell line was used as a control to verify the absence of ER-α mRNA, whereas MCC, a cell line that has not been shown to express ER-α (28), was used as an ER-α-negative control. Cell lines were maintained in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum, penicillin G, and streptomycin (100 units/mL). Cells were treated with 1,000 nmol/L TSA for 24 hours (Wako Biochemicals, Osaka, Japan) and 1,000 nmol/L 5-aza-CdR for 5 days (Sigma). After treatment with 5-aza-CdR and TSA, melanoma cells were washed with PBS and harvested with 0.25% trypsin-0.53 mmol/L EDTA. DNA from the ER-α-negative control cell line, the MCA melanoma cell line, was used as a RT-PCR control. All reverse transcription reactions were done using Moloney murine leukemia virus reverse transcriptase (Promega) with oligo-dT (GeneLink, Hawthorne, NY) priming as previously described (6). The RNA was quantified and assessed for purity using UV spectrophotometry and the RiboGreen detection assay (Molecular Probes). The expression of mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal reference housekeeping gene, was assessed by RT-PCR on all RNA samples to verify the integrity of RNA and to indicate equal loading of PCR products for gel electrophoresis.

All reverse transcription reactions were done using Moloney murine leukemia virus reverse transcriptase (Promega) with oligo-dT (GeneLink, Hawthorne, NY) priming as previously described (6). cDNA from 250 ng of total RNA was used for each reaction (46). The RT-PCR reaction mixture consisted of 1 µmol/L of each primer, 1 unit of AmpliTaq Gold polymerase (Applied Biosystems), 200 µmol/L of each dNTP, 4.5 mmol/L MgCl₂, and AmpliTaq buffer to a final volume of 25 µL. The primer sequences used were as follows: ER-α, 5′-AGACATGAGAGCTGCCAACC-3′ (forward) and 5′-GCCCAGGACAGGTTGACACCC-3′ (reverse); GAPDH, 5′-GGTGTTGACATGGGGAGGAGT-3′ (forward) and 5′-GACTGGTTCATGAGTTTCC-3′ (reverse). Samples were amplified with 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds for ER-α and GAPDH, respectively.

ER-α-positive (MCF-7 cell line) and ER-α-negative (MCA melanoma cell line) controls and reagent controls for RT-PCR assays were included as previously described (46). All PCR products were separated on 1.5% Tris-borate EDTA (TBE) agarose gels for ER-α and 2% TBE agarose gels for GAPDH and stained with SYBR Gold (Invitrogen Detection Technologies, Eugene, OR). Each assay was repeated in triplicate.

Biostatistical analysis. The correlation between ER-α methylation status of primary and metastatic melanomas with AJCC stage was assessed using the χ² method. Similarly, the correlation between ER-α methylation status of circulating serum DNA with known clinical prognostic factors and biochemotherapy response was assessed by the χ² method. Additionally, a multivariate logistic regression model was developed to correlate clinical prognostic factors and serum circulating ER-α methylation status with response to biochemotherapy.

Survival length was determined from the first day of biochemotherapy treatment to death or the date of last clinical follow-up. Survival curves were derived using the Kaplan-Meier method and the differences between curves were analyzed using the log-rank test. Cox’s proportional hazards regression model was used for multivariate analyses. Age, gender, Eastern Cooperative Oncology Group (ECOG) status, lactate dehydrogenase (LDH) level, number of metastasis sites, and ER-α methylation status were included in the multivariate model using a stepwise method for variable selection.

Results

Detection of methylated ER-α DNA in cell lines. Initially, we assessed ER-α in established metastatic melanoma cell lines. The frequency of hypermethylated ER-α in metastatic melanoma cell lines was 91% (10 of 11). Among these lines, six had only a methylated-specific peak whereas four cell lines showed both methylated- and unmethylated-specific peaks. These experiments optimized our methylation-specific PCR assay for ER-α and showed the high frequency of hypermethylated ER-α in metastatic melanoma cells cultured in vitro.

ER-α reexpression with 5-aza-CdR and TSA treatment. To determine if cells with hypermethylated ER-α can be induced to reexpress ER-α mRNA, we treated cell lines with 5-aza-CdR and TSA. In untreated cell lines, ER-α mRNA was detected in MCB and MCC, but not in MCA (Fig. 1). ER-α mRNA expression was restored to a detectable level in MCA after 5-aza-CdR and TSA treatment (Fig. 1). After treatment with 5-aza-CdR for 5 days followed by treatment with TSA for 24 hours, the MCA showed an unmethylated-specific DNA peak when assessed by methylation-specific PCR (Fig. 2). To further verify hypermethylation of the ER-α gene promoter region in melanoma, purified PCR products after sodium bisulfite modification were directly sequenced using a CEQ DYE Terminator Cycle Sequencing Kit (Beckman Coulter). Promoter region CpG islands were fully methylated in the MCA cell line, which does not express ER-α, whereas MCC, a cell line that
expresses ER-α, showed no evidence of primer region CpG island hypermethylation. With an optimized assay for the detection of methylated ER-α and the demonstration that reversal of methylation leads to reexpression of ER-α mRNA, we then approached the detection of methylated ER-α in paraffin-embedded melanoma specimens.

Detection of methylated ER-α in melanomas. We evaluated 53 paraffin-embedded primary melanomas (stage I, n = 11; stage II, n = 19; stage III, n = 23) using methylation-specific PCR. Overall, the frequency of methylated ER-α in primary melanomas was 32% (17 of 53). Similar rates of methylated ER-α were detected in primary tumors among the patients assessed regardless of stage. The frequency of ER-α methylation in AJCC stage I, II, and III primary melanoma tumors was 36% (4 of 11), 26% (5 of 19), and 35% (8 of 23), respectively (Fig. 3A).

Additionally, 54 paraffin-embedded metastatic melanomas were assessed, including stage III lymph node metastases (n = 19) and stage IV distant metastases (n = 35; 14 s.c., 9 lymph nodes, 6 lung, 5 colorectal, and 1 liver). Methylated ER-α was detected in 42% (8 of 19) of stage III and 86% (30 of 35) of IV metastatic melanomas (Fig. 3A). The frequency of methylated ER-α detected in stage IV metastatic tumors was significantly higher than in stage III metastatic tumors (P = 0.0003). Overall, ER-α was methylated in 70% (38 of 54) of metastatic tumors, a 2.3-fold increase in frequency compared with primary melanomas.

ER-α methylation status was also determined for 10 paraffin-embedded normal tissues from various organ sites (pancreas, n = 2; liver, n = 2; thymus, n = 2; lung, n = 2; and skin, n = 2). Methylated ER-α was detected in 90% (9 of 10) of normal tissues, indicating that ER-α is usually methylated and silenced in normal tissue. Because we frequently detected methylated ER-α in paraffin-embedded primary and metastatic melanomas, we assessed the detection of methylated ER-α in the serum of AJCC stage I to IV melanoma patients to evaluate its role as a blood marker for disease detection.

Detection of circulating methylated ER-α DNA in serum. Previously, we showed that circulating methylated DNA markers can be valuable surrogates of tumor progression (11, 44). Hence, we developed an optimized assay to detect the presence of free circulating methylated ER-α DNA in serum. The frequency with which methylated ER-α was detected in serum increased with tumor progression and according to AJCC stage. In the analysis of 109 melanoma patients’ sera, the frequency of circulating methylated ER-α in AJCC stage I, II, III, and IV sera was 10% (2 of 20), 15% (3 of 20), 26% (5 of 19), and 32% (16 of 50), respectively (Fig. 3B). The frequency of serum methylated ER-α was increased in patients with more advanced disease; methylated ER-α was detected in stage III/IV more frequently than in stage I/II (P = 0.034). Methylated ER-α was detected in the sera of only 1 of 40 healthy normal donors, an 82-year-old female. Representative methylation peaks from normal donor sera, normal liver tissue, melanoma patient sera, and melanoma tumors are provided in Fig. 4. Healthy normal donors ranged in age from 20 to 84 years (mean, 56 years); the gender distribution of normal volunteers was comparable to that of melanoma patients assessed. Having established that methylated ER-α can be reliably detected in the sera of melanoma patients but not in normal volunteers and is a marker of disease progression, we focused our attention on assessing the clinical utility of methylated ER-α as a predictor of disease outcome.

Clinical utility of circulating methylated ER-α. Before receiving systemic concurrent biochemotherapy, blood from AJCC stage IV melanoma patients was obtained and retrospectively assayed for the detection of circulating methylated ER-α DNA.

Figure 2. Representative methylation-specific PCR results of melanoma cell line (MCA) with and without 5-aza-CdR plus TSA treatment. M, methylated-specific product; U, unmethylated-specific product. Only a methylated peak was initially observed (untreated). An unmethylated peak appeared after treatment with 5-aza-CdR plus TSA (treated).

Figure 3. A, frequency of methylated ER-α DNA in melanoma tumors according to AJCC stage. Prim, primary melanoma tumor; Met, metastatic melanoma tumor. B, frequency of methylated ER-α DNA in melanoma patients’ sera according to AJCC stage. Norm <50, normal healthy volunteers younger than 50 years. Norm ≥60, normal healthy volunteers ages 60 years or older.
Serum ER-α methylation from stage IV patients was assessed to predict the patients most likely to respond to biochemotherapy. The median time of clinical follow-up after the initial blood draw was 12.5 months. The frequency of circulating methylated ER-α for responders (4 of 23, 17%) was significantly lower (P = 0.018) than nonresponders (12 of 24, 50%). In a multivariate logistic regression model that included known clinical prognostic factors for melanoma, the presence of circulating serum methylated ER-α DNA was the only factor that significantly correlated with response to biochemotherapy [risk ratio (RR), 0.21; 95% confidence interval (95% CI), 0.06-0.81; P = 0.023]. Patients categorized as biochemotherapy responders had significantly better overall survival compared with patients deemed biochemotherapy nonresponders (log-rank, P < 0.0001).

Regardless of response to biochemotherapy, patients with serum methylated ER-α had significantly worse progression-free survival compared with patients in whom methylated ER-α was not detected (log-rank, P = 0.002). Serum methylated ER-α, LDH >190 IU/L, and age <50 years were significantly correlated with progression-free survival in a univariate analysis (log-rank; methylated ER-α, P = 0.002; LDH >190 IU/L, P = 0.013; age <50 years, P = 0.028).

Similarly, patients with circulating methylated ER-α had significantly worse overall survival compared with patients in whom methylated ER-α was not detected (log-rank, P = 0.002). Circulating methylated ER-α and serum LDH >190 IU/L significantly correlated with overall survival (log-rank; methylated ER-α, P = 0.002; LDH >190 IU/L, P = 0.015). Other prognostic factors (gender, age, ECOG, and number of metastasis sites) were not significant.

A multivariate Cox’s proportional hazard regression model was developed to correlate clinical factors and ER-α methylation status with progression-free and overall survival. Age, gender, ECOG status, LDH level, number of metastasis sites, and ER-α methylation status were included in the model using a stepwise method for variable selection. Serum methylated ER-α was the only independent factor predicting progression-free (Fig. 5A; RR, 2.64; 95% CI, 1.36-5.13; P = 0.004) and overall survival (Fig. 5B; RR, 2.31; 95% CI, 1.41-5.58; P = 0.003).

**Methylated ER-α gender and age.** Because ER-α hypermethylation is influenced by both age and gender in other cancers, we assessed the relation of these factors to methylated ER-α status in primary and metastatic melanomas and serum. There was no significant difference in the frequency of methylated ER-α in paraffin-embedded tumors or sera between male and female patients, nor was there any significant difference in the frequency of methylated ER-α in tumors between patients ≥60 years old and patients <50 years old.

**Discussion**

Methylated ER-α has been detected in neoplasia of the colorectum, lung, and breast (21, 22, 24, 26, 27). The reported expression level of ER-α in melanoma has been variable, with several studies failing to show the presence of ER-α using monoclonal antibodies (28–31). Tamoxifen has been used in chemotherapy and biochemotherapy regimens for over a decade (36–39). Although improved response rates have been reported with its use, tamoxifen has not been shown to significantly improve overall survival in advanced melanoma (40, 41). This is the first study reporting a potential mechanism for the failure of tamoxifen in the treatment of melanoma. We have shown that the variable down-regulation of ER in melanoma is due to epigenetic control of its expression via gene promoter region hypermethylation.

Our studies show that methylated ER-α can be detected in melanoma cell lines and ER-α mRNA expression can be reestablished after demethylation with 5-aza-CdR and TSA. Additionally, methylated ER-α can be detected in paraffin-embedded primary and metastatic melanoma tumors, showing its value as a biomarker of tumor progression. Methylated ER-α DNA was detected in the serum of melanoma patients with AJCC stage I to IV disease and was a biomarker of disease progression. Furthermore, serum circulating hypermethylated ER-α in AJCC stage IV melanoma patients predicted response to biochemotherapy, progression-free survival, and overall survival.

Our *in vitro* experiments showed that all but one of the 11 metastatic melanoma cell lines assayed had methylated ER-α. This suggests that *in vitro* culturing may promote the epigenetic silencing of ER-α or select for a subpopulation of cells with methylated ER-α. 5-aza-CdR alone did not significantly increase ER-α mRNA expression (data not shown); the histone deacetylase
biochemotherapy trial. We attempted to predict the response to the serum of stage IV melanoma patients enrolled in a concurrent chemotherapeutic regimen. We assessed the predictive utility of this marker in a select population of patients with advanced melanoma. The frequency of ER-α methylation served as a marker of tumor progression from primary to metastatic disease and from regional nodal metastasis to distant visceral metastasis. As with breast cancer, the expression of ER-α mRNA as regulated by ER-α methylation is directly or indirectly related to the development of metastasis.

Because we were able to detect methylated ER-α in primary and metastatic melanomas, we assessed whether or not methylated ER-α could function as a blood-based biomarker for diagnosis and disease surveillance. In the current study, methylated ER-α was detected in the serum of AJCC stage I to IV melanoma patients in a pattern related to disease progression. In a subset of matched melanoma tumor and serum sample pairs, all patients with methylated DNA detected in serum had primary or metastatic tumors with methylated ER-α as well (data not shown).

Knowing that we could detect methylated ER-α in serum, we assessed the predictive utility of this marker in a select population of stage IV melanoma patients enrolled in a concurrent biochemotherapy trial. We attempted to predict the response to therapy based on the methylation status of circulating ER-α. Response rates for systemic therapies in advanced metastatic melanoma are alarmingly low. Biochemotherapy, the use of chemotherapy in conjunction with immune modulators, has produced better response rates (40–42) but outcomes differ greatly between responders and nonresponders. It has been difficult to predict tumor response before or in the early phases of biochemotherapy. Identifying molecular predictors of therapeutic response may permit physicians to treat those patients most likely to respond to therapy while sparing nonresponsive patients unnecessary treatment and its associated morbidity. Methylated ER-α was more commonly detected in the serum of patients who failed to respond to biochemotherapy and was the only factor predictive of response to biochemotherapy. Serum methylated ER-α was the only independent predictor of progression-free and overall survival in a multivariate analysis, surpassing even known clinical prognostic factors.

There are several possible explanations for these findings. First, tamoxifen, a member of the selective ER modulator family, was used in the biochemotherapy regimen of 44 of 50 patients. Patients without serum methylated ER-α, who therefore express ER-α, may be more likely to respond to the antitumor effects of tamoxifen. Conversely, the failure of patients to respond to biochemotherapy may be partially explained by the inability of tamoxifen to exert its antitumor effects when ER-α expression is silenced due to promoter region hypermethylation. This is akin to the clinical situation seen in breast cancer in which tumors not expressing ER-α do not respond to hormone therapy and carry a poorer prognosis (23). ER-α methylation could also reflect a pathophysiologic event that includes a more global hypermethylation of tumor-related genes, thereby providing tumor cells with a growth advantage (8).

Methylated ER-α is present in normal cells of different histology (47, 48). In our serum analysis, however, ER-α was not detected in the serum from normal healthy donors. Normal cells containing methylated ER-α would be expected to release this DNA into the bloodstream. Why, then, was methylated ER-α not detected in normal healthy donors? We believe that methylated ER-α from tumors is cleared less efficiently than methylated ER-α from normal cells. The destruction of normal cells is primarily through apoptosis-related events, resulting in the release of small, characteristic enzyme-degraded fragments of DNA. As a result, the DNA released from normal cells is cleared rapidly and not readily detected in blood. The contrary, tumor cells disrupted by physical trauma or cell necrosis release intact large fragments of DNA (49). Melanoma patients release both free DNA and tumor cells into the bloodstream. Circulating tumor cells may release large fragments of DNA due to nonapoptotic death mechanisms. 5

The detection of methylated ER-α in melanoma patients strongly suggests that the circulating DNA is tumor related.

Age-dependent methylation of ER-α has previously been implicated in other studies (50). In this study, we did not find age differences in ER-α methylation. Among 40 healthy volunteers, methylated ER-α was detected only in one 82-year-old donor, which may be due to factors unrelated to aging, including subclinical cancer. Further detailed studies will validate the presence and significance of ER-α methylation in healthy elderly volunteers.

This is the first study showing the detection of methylated ER-α in both melanoma patients’ tumor tissues and sera. The detection of methylated ER-α in tumors or sera correlates with tumor

5 Unpublished results.
prolongation and is therefore prognostically important. Our findings indicate that detection of methylated ER-α in serum may identify a population of patients with poor melanoma outcome and poor response to systemic therapy in whom alternative treatment management should be considered. Furthermore, our data support the initiation of a prospective biochemistry therapy trial for stage IV melanoma based on serum ER-α methylation status. Such a trial would provide valuable information about the clinical value of tamoxifen in the treatment of melanoma and further test the ability of the ER-α methylation assay to predict response to biochemistry.

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