Altered Expression and Splicing of ESRP1 in Malignant Melanoma Correlates with Epithelial–Mesenchymal Status and Tumor-Associated Immune Cytolytic Activity

Jun Yao, Otavia L. Caballero, Ying Huang, Calvin Lin, Donata Rimoldi, Andreas Behren, Jonathan S. Cebron, Mien-Chie Hung, John N. Weinstein, Robert L. Strausberg, and Qi Zhao

Abstract

Melanoma is one of the major cancer types for which new immune-based cancer treatments have achieved promising results. However, anti–PD-1 and anti–CTLA-4 therapies are effective only in some patients. Hence, predictive molecular markers for the development of clinical strategies targeting immune checkpoints are needed. Using The Cancer Genome Atlas (TCGA) RNAseq data, we found that expression of ESRP1, encoding a master splicing regulator in the epithelial–mesenchymal transition (EMT), was inversely correlated with tumor-associated immune cytolytic activity. That association holds up across multiple TCGA tumor types, suggesting a link between tumor EMT status and infiltrating lymphocyte activity. In melanoma, ESRP1 mainly exists in a melanocyte-specific truncated form transcribed from exon 13. This was validated by analyzing CCLE cell line data, public CAGE data, and RT-PCR in primary cultured melanoma cell lines. Based on ESRP1 expression, we divided TCGA melanoma cases into ESRP1-low, -truncated, and -full-length groups. ESRP1-truncated tumors comprise approximately two thirds of melanoma samples and reside in an apparent transitional state between epithelial and mesenchymal phenotypes. ESRP1 full-length tumors express epithelial markers and constitute about 5% of melanoma samples. In contrast, ESRP1-low tumors express mesenchymal markers and are high in immune cytolytic activity as well as PD-L2 and CTLA-4 expression. Those tumors are associated with better patient survival. Results from our study suggest a path toward the use of ESRP1 and other EMT markers as informative biomarkers for immunotherapy.

Cancer Immunol Res; 4(6); 552–61. ©2016 AACR.

Introduction

Melanoma is one of the most invasive malignancies, and patients with melanoma mainly die from tumor metastasis. The reversible phenotype switch between proliferative and invasive cells that drives the metastasis of tumor cells has been proposed as a model for melanoma progression (1). Many transcription factors and signaling pathways induced by changes in the microenvironment have been implicated in tumorigenesis and this phenotypic switch (2, 3). MITF, a master transcriptional regulator involved in melanocyte differentiation and pigment production, has been shown to play a critical role in melanoma development (4, 5). High expression of MITF is associated with transformation of melanocytes and hyperproliferation of the transformed cells. Downregulation of MITF expression leads to greater invasive potential of melanoma cells (3, 6, 7). Changes in the Wnt signaling pathway have also been implicated in the phenotypic switch (8–11). The inversely expressed genes ROR1 and ROR2 are reported to be associated with proliferative and invasive phenotypes in melanoma, respectively (12). However, many of these observations are based on in vitro gain-of-function or loss-of-function experiments and might not reflect the complexity of microenvironment changes and cohesive signaling networks in vivo.

ESRP1 encodes a cell type–specific splicing regulator protein exclusively expressed in epithelial cells such as those comprised of ectoderm-derived tissues and endothelial organ lining. Expression of ESRP1 controls cell type–specific alternative splicing of many genes, such as FGRFR2, CD44, and CTNND1, that are important signaling molecules mediating cell division, growth, and differentiation under specific microenvironment settings (13). For example, two alternative forms of FGRFR2, IIIb and IIIc, which exhibit different affinity with their FGF ligands, are predominantly expressed in epithelial and mesenchymal cells, respectively. The selective expression of the FGRFR2 IIIb or IIIc
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isoforms is under the control of ESRP1 (14). We previously reported that dominance of epithelial or mesenchymal cell types exists in renal cell carcinoma (RCC; ref. 15). Our results support the notion that clear cell renal cell carcinoma (cRCC) originates from mesenchymal-like stem cells embedded in adult kidney, unlike other subtypes of RCC, such as chromophobe, that likely have an epithelial-like stem cell origin. Thus, ESRP1 and status of its target genes might serve as molecular markers of tumor cells with epithelial or mesenchymal properties.

Cytolytic activity is calculated as the geometric mean of GZMA and PRF1 using log2 expression values. We used normal tissue gene expression data obtained from the GTEx portal (http://www.gtexportal.org/home/) to identify two skin-specific genes (KRT2 and LOR). With the highest KRT2/LOR expression ratio, its median expression of KRT2/LOR plus 3 × standard deviation is lower than RPKM (reads per kilobase of transcript per million mapped reads) 16. We therefore call melanoma samples with KRT2 or LOR expression above RPKM 16 as keratinocyte contaminated.

Cloning of full-length ESRP1 and transfection into SKMEL28

PCR was undertaken with High Fidelity Taq polymerase (Invitrogen) using cDNA from the BT20 cell line: Forward 5'-ATGACGGCCCTCTCCGGATTACCTGTC-3' and reverse 5'-AATAAACCACCATTCITGGG-3'. The PCR product was recovered and cloned in pCDNA pcDNA3.1/V5-His using the pcDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen). The correct full-length ESRP1 and transfection into SKMEL28.
length clone was determined by Sanger sequencing using the T7 promoter and V5 reverse primer.

Transfection was performed using Effectene (Qiagen) according to the manufacturer’s instructions. Western blotting was performed as previously described (15). The antibodies used included: mouse monoclonal anti-V5 (clone 2F11F7; Life Technologies) and anti–ESRP-1/2 (clone 23A7.C9; Rockland Immunocoulmics) and rabbit polyclonal anti-actin (20-33; Sigma-Aldrich).

Statistical tests
The Krustal–Wallis test (nonparametric ANOVA) was used for multigroup significance tests. Breslow thickness was categorized in four levels as I, <1 mm; II, <2 mm; III, <4 mm; and IV, 4+ mm. The Fisher exact test is used for testing sample distribution. The Kaplan–Meier log-ranked test was performed for survival benefits. All tests were done in R, Graphpad, SSPSv12 or ArrayStudio.

Results
Inverse correlation of ESRP1 expression to tumor-associated immune cytolytic activity
The use of antibodies to CTLA-4 or PD-1 has induced remarkable and durable antitumor responses in patients with melanoma and other types of cancers (20). However, only a minority of patients treated achieve complete tumor regression (21, 22). Therefore, identifying predictive immunotherapy biomarkers is critical for both selecting appropriate patients for immunotherapy and for our complete understanding of the mechanism of action of these agents. It has been reported that PD-L1 expression is regulated by microRNA-200/ZEB1 in lung cancer (23), suggesting that tumor EMT status may be associated with immunotherapy response. To examine the relationship between EMT and tumor immune cytolytic activity, we interrogated multiple cancer types in the TCGA database.

We used ESRP1 as a biomarker of EMT status because its expression is highly restricted to epithelial cells and its level is tightly linked to the selective expression of epithelial or mesenchymal-specific alternative splice forms of multiple genes such as FGFR2 (15, 24). We also applied a two-gene expression signature (PRF1 and GZMA) of immune cytolytic activity (25). These genes encode perforin and granzyme A proteins, which are specifically expressed by CD8+ cytotoxic T cells upon activation. The two-gene signature significantly correlates with T-cell markers, including cytotoxic T lymphocyte (CTL) markers, as well as with pan-cancer survival benefits. As shown in Fig. 1, ESRP1 expression was inversely correlated to this signature of cytolytic activity in many cancer types, including lung, colon, prostate, breast, bladder, and thyroid, with significant P values (< 1 × 10−10; Supplementary Table S1). Although a trend of inverse correlation exists in other
cancer types, the Pearson correlation coefficient has shown less statistical significance (Supplementary Table S1).

**Tissue-specific expression of truncated ESRP1 transcript in melanoma and normal melanocyte**

Intriguingly, melanoma, a tumor of neural crest origin (26), had high ESRP1 expression compared with other neural crest–derived tumors, such as glioblastoma and low-grade glioma (Supplementary Fig. S1A). This was also observed in cancer cell lines [Cancer Cell Line Encyclopedia (CCLE) dataset], where ESRP1 was highly expressed in cells of epithelial origin, as is the case in most carcinoma cell lines, but not expressed in cells of mesenchymal origin, such as sarcomas and lymphomas, nor in other neuronal tumor cell lines (Supplementary Fig. S1B). ESRP1 expression was high in cell lines of “skin” melanoma origin (61/62 CCLE skin cell lines are from melanoma).

A close examination of ESRP1 expression at the exon level in melanoma revealed that a majority of tumors do not express full-length ESRP1 transcript. Instead, only the last four exons (exons 13–16) were highly expressed and presumably would not generate ESRP1 protein that carries normal function. Based on the absolute ESRP1 expression level and the relative exon 13–16 expression ratio (see Materials and Methods), we classified melanomas in three subgroups (Fig. 2A). In approximately 20% of melanomas, designated ESRP1-low, ESRP1 was expressed at low levels (RSEM value of less than 125). In approximately two thirds of melanomas, the ESRP1 truncated form was prominent (exon ratio above 0.65), whereas about 10% of all tumors express full-length ESRP1 transcripts in substantial amounts (Table 1). Notably, the one normal control sample in the TCGA melanoma dataset fell into the ESRP1-truncated group, suggesting that the truncated transcript form was not tumor specific, but rather produced during normal melanocyte differentiation. We did not observe any aberrant change in the ESRP1 homolog, ESRP2, which also has epithelial-specific expression, although higher ESRP2 expression was associated with the ESRP1 full-length group, as expected (Fig. 2A).

When tumor sites were aligned to ESRP1 groups, we observed enrichment of primary tumors inside the ESRP1 full-length group (Fig. 2A and Table 1). This raised a concern about whether full-length expression was from contaminating keratinocytes, which are often found in primary melanomas. Using gene expression data from normal tissue, obtained from the GTEx portal (http://www.gtexportal.org/home/), we identified two skin-specific genes (KRT2 and LOR) with strong expression (Supplementary Fig. S2A), and used these two genes to estimate keratinocyte contamination in TCGA samples. Indeed, in two skin melanoma cell lines (COLO-679 and G-361), most CAGE reads for ESRP1 were seen to be aligned at the 5′ end promoter region. Thus, the truncated ESRP1 resulted from alternative TSS usage at an approximate location of chr8:95690440 (hg19) at the start of exon 13 in melanoma. The two known functional domains (Rnasel1 and RRM domains) are missing from the truncated ESRP1, so the product from this new isoform is unlikely to retain its normal function (Fig. 3B).

Upstream sequence analysis identified a consensus E-box motif (CACGTCG) located ~57-bp upstream of ESRP1 exon 13 (Fig. 3B), which is a known binding site for MITF, a master regulator of melanocyte development and a known melanoma oncogene (4, 5). Several lines of evidence support the idea that MITF regulates the expression of truncated ESRP1 through binding to the E-box motifs inside intron 12 and initiated new transcription from within exon 13. First, correlation of global gene expression to truncated ESRP1 expression resulted in MITF as the most correlated gene, with a Pearson coefficient of 0.77 (Fig. 3C), and with many of the remaining top correlated genes being MITF target.
Figure 2.
Detection of truncated ESRP1 transcripts in melanoma. A, ESRP1 expression at gene and exon levels in melanoma (tumor samples, n = 47; normal melanocyte, n = 1). Melanoma samples are organized into three groups (see Materials and Methods) and ordered by ESRP1 expression. ESRP1 exon expression heatmap is shown at top, followed by ESRP1 expression values in a barplot. A truncation ratio of 0.65 was used to call full-length ESRP1 tumors (see Materials and Methods). Position of the normal sample is marked by a vertical yellow line. Tumor sites for primary, regional subcutaneous, lymph node, and distant metastasis tumors are marked by dark-blue lines. The “keratin” lane marks potential contamination of keratinocytes (see Materials and Methods). B, expression of ESRP1 at exon level in CCLE cancer cell lines. C, detection of low, truncated, and full-length ESRP1 expression in melanoma cell lines by real-time PCR. Two probe sets amplify regions flanking exon 11–12 and exon 13–14, respectively. Top, relative signal intensity of the two probe sets normalized by normal kidney. Bottom, percentage of exon 13–14 amplicon out of the total signal. HEM, Hermes cell line.
genes. Second, the consensus E-box found in humans is conserved in monkeys and apes but not in mice and rats (Fig. 3B and Supplementary Fig. S3). Consistently, we detected little mouse Esrp1 expression using mouse nevi and melanoma RNA-Seq samples (see Materials and Methods). Third, knockdown of MITF by shRNA in 501MEL and Hermes cell lines leads to reduced expression of MITF target genes (e.g. CDK2) and of truncated Esrp1 (Fig. 3D). Together, these results suggest that MITF regulates truncated Esrp1 expression in melanomas.

Epithelial and mesenchymal cell phenotypes are associated with Esrp1 status in melanoma

We examined the expression of a set of epithelial and mesenchymal markers in the three Esrp1 subgroups after removing keratinocyte-contaminated samples (Fig. 4A). Esrp1-low tumors exhibited a strong expression signature of mesenchymal markers like N-cadherin (CDH2) and TWIST1. About half of melanomas with full-length Esrp1 expression displayed higher expression of an epithelial marker panel including genes such as keratin. This

Table 1. Distribution of melanoma tumors on Esrp1 expression status and tumor site before and after (before/after) removing potentially keratinocyte contaminated samples

<table>
<thead>
<tr>
<th>Esrp1 expression status</th>
<th>Primary tumor</th>
<th>Regional lymph node</th>
<th>Cutaneous and subcutaneous</th>
<th>Distant metastasis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esrp1-low</td>
<td>4/7</td>
<td>56/52</td>
<td>9/19</td>
<td>13/13</td>
<td>92</td>
</tr>
<tr>
<td>Esrp1-trun</td>
<td>69/76</td>
<td>154/153</td>
<td>49/47</td>
<td>49/47</td>
<td>321</td>
</tr>
<tr>
<td>Esrp1 full-length</td>
<td>32/5</td>
<td>12/12</td>
<td>6/5</td>
<td>6/5</td>
<td>56</td>
</tr>
<tr>
<td>Total</td>
<td>105/64</td>
<td>222/217</td>
<td>74/69</td>
<td>68/65</td>
<td>469/415</td>
</tr>
</tbody>
</table>

a, P value < 0.0001 by the Fisher exact test.

Figure 3.
Melanomas express a novel transcriptional isoform of Esrp1. A, alternative TSS of Esrp1. Top, CAGE libraries capture reads mapped to TSS sites at MLANA (top) and Esrp1 (bottom) gene loci. Depth of reads coverage is labeled on the right. Exons in alternative transcripts are drawn in filled boxes. B, Esrp1 coding structure. The E-box location is marked out by a red box. Exon 13 is labeled to show the relative exon positions. Alternative TSS for the truncated Esrp1 within exon 13 is marked by a red arrow. Functional domains encoded by Esrp1, including RnaseH-like domain and RRM (RNA recognition motif) are shown in colored boxes in the gene structure. Sequence surrounding the junction of intron 12 and exon 13 is shown below with comparison among other species. C, correlation between MITF and Esrp1 gene expression. Samples are categorized into Esrp1-low and Esrp1-trun groups. D, shMITF downregulates expression of the truncated Esrp1. CDK2, a known MITF target, and ACTB (Actin beta) are used as positive and negative controls of the experiment, respectively.
was not restricted to primary tumors, but was also found in tumors from regional cutaneous/subcutaneous locations, lymph nodes, and distant metastatic sites. Approximately two thirds of tumors expressing truncated ESRP1 seemed to reside in a state between epithelial and mesenchymal, even though ESRP1 was functionally compromised in these tumors. This suggested that loss of ESRP1 function per se is not sufficient to render a mesenchymal phenotype, at least in melanoma. Surprisingly, E-cadherin (CDH1) had higher expression in both ESRP1 full-length and ESRP1-truncated groups. We also examined the isoform switch in the FGFR2 gene, which showed a clear transition from the epithelial isoform (FGFR2-b) in ESRP1 full-length tumors to the mesenchymal isoform (FGFR2-c) in tumors with truncated and low ESRP1 (Fig. 4B). This finding not only validated our computational prediction of ESRP1 full-length expression, but also confirmed that truncated ESRP1 was unable to exert its normal function to induce isoform switch in the FGFR2 gene.

Previously, several studies defined molecular subtypes of melanoma using gene expression profiling (3, 27–29). Among those, Hoek and colleagues classified melanoma into “proliferative” and “invasive” subtypes based on the microarray profiling of 86 cultured melanomas (3). When we compared the 105-gene signature and MITF expression levels (RSEM) are aligned to ESRP1 subgroups. Samples in all heatmaps are in the same order as in Fig. 2.

Figure 4. ESRP1 subgroups are associated with epithelial–mesenchymal cellular properties. A, expression heatmap of molecular markers for epithelial and mesenchymal cells. B, FGFR2 expression at exon level shows that the mesenchymal and epithelial forms are dominantly expressed in non–ESRP1 full-length and ESRP1 full-length subgroups, respectively. C, heatmap of the 105-gene signature and MITF expression levels (RSEM) are aligned to ESRP1 subgroups. Samples in all heatmaps are in the same order as in Fig. 2.
samples with both proliferative and invasive signatures. MITF is apparently a dominant factor in the proliferative subtype because many MITF target genes were in the 105-gene list and highly expressed in the proliferative subtype, whereas in the ESRP1-low/invasive group, MITF expression was low (Fig. 4C). This implies that increased MITF level may actively block a mesenchymal phenotype, in agreement with previous findings (3).

ESRP1-low melanomas are associated with increased immune cytotoxicity and favorable patient survival

We examined immune cytolytic activity using cytotoxic T-cell marker genes and the two-gene signature in three ESRP1-based melanoma subgroups after removing all primary melanoma samples, because these are not particularly relevant to immune checkpoint therapy. This showed a statistically significant increase of cytolytic activity in the ESRP1-low melanomas (Fig. 5A), which was consistent with our findings in other cancer types (Fig. 1). Predicted cytolytic activity between the ESRP1 full-length and the ESRP1-truncated groups was not significantly different, suggesting the correlation of cytolytic activity was more related to the mesenchymal phenotype rather than to the epithelial phenotype.

We next examined the expression of the PD-1, PD-L1, PD-L2, and CTLA-4 genes in ESRP1 subgroups (Fig. 5A and B). All these molecules, which are targeted by immune checkpoint blockage antibodies, had increased expression in the ESRP1-low tumors. We also examined the possible relationship of ESRP1 grouping to mutational status and clinical parameters. No particular association was found between BRAF and NRAS mutation and ESRP1 groups, although KIT mutations seem to be absent in ESRP1-low tumors (Fig. 5A). The ESRP1 groups were also not associated with the total number of somatic mutations. In the TCGA melanoma dataset, the ESRP1 grouping had a moderate association to Breslow depth, with ESRP1-low tumors having the thinnest Breslow depth (Fig. 5B). Kaplan–Meier survival analysis on TCGA melanoma cases stratified by ESRP1 status showed a trend of more favorable survival of ESRP1-low tumors (P = 0.057, Fig. 5C), which is consistent with the lower Breslow depth values and higher active immune signatures found within this group.

In summary, our data show an inverse correlation between ESRP1 expression and tumor-associated immune cytolytic activity in multiple tumor types. Through the study of altered ESRP1 transcription in melanoma, we gained further insights on the
complex heterogeneity of melanoma cell properties during tumor progression, and strengthened a link between tumor-intrinsic EMT status and tumor microenvironment in melanoma. Results from our study highlight the potential utility of ESRP1 status in predicting response to checkpoint blockade immunotherapy. The conceptual link between tumor EMT status and activated infiltrating lymphocytes may have further implications in the immunotherapy field and will need further elucidation.

Discussion

Cancer immunotherapy by immune checkpoint inhibitors, adoptive T-cell therapy, and therapeutic vaccines has advanced to the first line of treatments for many metastatic cancers, especially melanoma (20, 30, 31). Contributing to this opportunity is the rich stromal cell–orchestrated tumor microenvironment, particularly those tumors with high numbers of tumor-infiltrating lymphocytes (TIL), as is the case in melanoma. Here, we show that melanoma mesenchymal-like tumors were associated with enhanced immune cytolytic activity. Moreover, this phenomenon was corroborated by a negative correlation between immune cytolytic activity and epithelial phenotypes, and especially with the fact that ESRP1 expression was inversely associated with higher cytolytic activity in many other cancer types (Fig. 1). RCC is another cancer type that has shown relatively high sensitivity to immunotherapy (32). Previously, we have demonstrated that clear cell RCC has a more mesenchymal-like phenotype (15). Our current results suggest that tumor cells of mesenchymal nature are able to acquire TILs and are well-suited to immunotherapy. With the success of immune checkpoint blockade therapy (anti–PD-1/PD-L1 and/or anti–CTLA-4) in some patients, an important issue is why some patients are either not responsive or later develop resistance. Among the many factors that contribute to tumor immunity, such as HLA abundance, neoantigen load, and intrinsic properties, our findings suggest that the cellular state also has an impact on immune response susceptibility.

In this study, we have described the identification of a melanocyte-specific ESRP1 mRNA transcript. This highly expressed isoform is a result of de novo transcription starting from exon 13, leading to a shorter mRNA (truncated ESRP1) that comprises only the last four exons and loses the region coding for the two functional domains of the protein. Although the TCGA melanoma dataset contains only one normal control sample, which showed truncated ESRP1 expression, we did confirm this finding by RT-PCR in a normal melanocyte cell line (HEM; Fig. 2C). Additionally, publicly accessible RNA-Seq data of independently derived melanocyte specimens (NCBI GEO GSE46805 and GSE33092) all show truncated ESRP1 expression in melanocytes (33, 34). The TCGA melanoma dataset is a heterogeneous collection of samples from primary tumors, regional, lymph nodes, and distant metastases. Our initial findings that primary tumors were significantly enriched in ESRP1 full-length tumors (Fig. 2A) raised the concern of keratinocyte contamination as the cause of ESRP1 full-length expression. Using a two-gene signature (KRT2/LOR), we were able to predict and remove a majority if not all keratinocyte contaminated samples from our study. KRT2 encodes keratin 2E, an epidermal keratin expressed largely in the upper spinous layer of epidermal keratinocytes. LOR encodes loricrin, which is a major protein component of the cornified cell envelope found in terminally differentiated epidermal cells. We show that expression of KRT2 and LOR is correlated and highly skin specific (Supplementary Fig. S2A), and the function of these genes suggests they are authentic keratinocyte genes. Even after removal of contaminated samples, a small portion of melanomas still expressed full-length ESRP1, which suggests that expression of full-length ESRP1 could be an intrinsic tumor property.

Based on ESRP1 status, we divided melanomas into three subgroups. Approximately two thirds of melanomas expressed the truncated ESRP1 form that was also observed in normal controls, so this likely represents the default ESRP1 status. Based on the expression of EMT markers, the state of these samples is neither distinctly mesenchymal nor epithelial. Compared with loss of expression of other epithelial genes, the E-cadherin (CDH1) gene is still highly expressed in this subgroup. We have observed that some tumors lose ESRP1 expression and are mesenchymal, and we show that this may be linked to the loss of MITF expression. For other tumors, which have epithelial characteristics, full-length ESRP1 is expressed. The underlying basis and mechanisms for these alterations will need further investigation. However, our preliminary results revealed loss of ESRP1 promoter methylation in the ESRP1 full-length group, indicating the involvement of epigenetic regulation. Also, a few samples that expressed full-length ESRP1 did not highly express epithelial markers. A closer examination of these individual samples identified at least one sample that expressed another novel truncated form, starting from exon 3 of ESRP1, and therefore had escaped our initial computational screen. Consequently, other factors may influence the functional status of ESRP1. The ESRP1-low subgroup was associated with better TILs and overall survival compared with the other two subgroups.

The identification of effective markers for diagnosis and prediction of treatment response in cancer immunotherapy is important to help guide effective intervention strategies for individual patients. Our study indicates that ESRP1-low melanomas with greater immune cytolytic activity have a higher probability of responding to immune checkpoint blockade therapy and suggests a path toward the use of ESRP1 and other EMT markers as informative biomarkers for immunotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O.L. Caballero, D. Rimbaldi, A. Behren, J.S. Cebon, Q. Zhao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Yao, Y. Huang, J.S. Cebon, Q. Zhao
Writing, review, and/or revision of the manuscript: J. Yao, O.L. Caballero, C. Lin, A. Behren, J.S. Cebon, R.L. Straussberg, Q. Zhao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Behren, M.-C. Hung, J.N. Weinstein
Study supervision: J.S. Cebon, Q. Zhao

Grant Support

This work is funded by Ludwig Cancer Research and Regeneron Pharmaceuticals Inc.

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Received October 14, 2015; revised February 29, 2016; accepted March 3, 2016; published OnlineFirst April 4, 2016.
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References

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