Subcellular Localization of Activating Transcription Factor 2 in Melanoma Specimens Predicts Patient Survival

Aaron J. Berger,1 Harriet M. Kluger,2 Ning Li,1 Eric Kielhorn,3 Ruth Halaban,3 Ze’ev Ronai,4 and David L. Rimm1
Departments of 1Pathology, 2Medicine, and 3Dermatology, Yale University School of Medicine, New Haven, Connecticut, and 4The Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, New York

Abstract

The transcription factor activating transcription factor 2 (ATF2) has been shown to be associated with melanocytic oncogenesis and melanoma tumor proliferation in preclinical models. The clinical significance of ATF2 expression is unknown. To determine the prognostic value of ATF2 in melanoma, we evaluated the pattern and level of ATF2 expression in a large cohort of melanoma specimens. Immunohistochemical staining was performed on a tissue microarray representing 544 patients with a mean follow-up time of 60 months. Expression was evaluated semiquantitatively and correlated with overall survival and other clinicopathological data. Strong cytoplasmic ATF2 expression was associated with primary specimens rather than metastases (P < 0.0001) and with better survival (P = 0.0003). Strong nuclear ATF2 expression was associated with metastatic specimens (P < 0.0001) and with poor survival (P = 0.0008). Patients who had both weak cytoplasmic and strong nuclear ATF2 staining had the worst outcome, both among the full cohort of patients (P < 0.0001) and among the patients with localized disease (n = 269; P < 0.0001). On multivariate analysis of the primary cutaneous specimens, weak cytoplasmic staining and strong nuclear staining was an independent predictor of poor outcome, as was Clark level. Nuclear ATF2 is likely to be transcriptionally active, whereas cytoplasmic ATF2 probably represents an inactive form. These findings support other preclinical findings in which transcriptionally active ATF2 is involved in tumor progression-proliferation in melanoma. Moreover, our findings suggest that ATF2 might be a useful prognostic marker in early-stage melanoma.

Introduction

The only clinically reliable markers used to predict outcome in malignant melanoma are Breslow depth, Clark level of invasion, presence of ulceration, and lymph node involvement (1). These parameters form the basis of the new American Joint Committee on Cancer Staging System. However, within each stage, there is significant variability in outcome, and although the staging is helpful in determining prognosis and therapy at the time of diagnosis, more accurate measures based on tumor biology are needed (1). Better predictors of poor outcome in resected melanoma could enhance our ability to appropriately select patients in need of adjuvant therapy.

Numerous tissue and serological markers have been studied, as reviewed by Li et al. (2). Among them, a number of transcription factors, including ATF2,5 have been shown to be associated with tumor growth and metastasis (3, 4) in cell culture and animal models, but their prognostic role in melanoma has not been determined. ATF2 is among the primary transcription factors regulated by p38, mitogen-activated protein kinase, and JNK signaling pathways (5, 6). Phosphorylation of ATF2 by these kinases is central in determining its stability (7) and transcriptional activity, which is dependent on its heterodimerization with other members of the bZIP family, c-Jun, cAMP-responsive element binding protein, nuclear factor κB, and retinoblastoma (5, 8–10). ATF2 plays an important role in acquisition of resistance of melanoma cells to chemotherapeutic drugs and radiation (11–13). Inhibition of ATF2 activity efficiently inhibits melanoma growth and ability to metastasize and sensitizes melanoma to a range of chemotherapeutic agents and radiation (14, 15).

In this study, we sought to evaluate the prognostic role of ATF2 by studying the pattern and levels of ATF2 expression using a large cohort melanoma tissue microarray and associating expression with clinical and pathological data.

Materials and Methods

Tissue Microarray Construction. The tissue microarray was constructed as described previously (16). A total of 570 tissue cores (553 melanomas with duplicate spots for 9 patients and 17 normal skin samples) measuring 0.6 mm were spaced 0.8 mm apart on a single glass slide. The cohort was constructed from paraffin-embedded formalin-fixed tissue blocks obtained from the Yale University Department of Pathology archives. The specimens were resected between 1959 and 1994, with a follow-up range between 2 months and 38 years (median follow-up time, 60 months). Treatment information was not available for the entire cohort. Some of the stage III patients were treated with IFN-based therapy, and the stage IV patients were treated with a range of therapies including chemotherapy, biological therapy, vaccine therapy, and supportive care. Slides from all blocks were reviewed by a pathologist (N. L.) to select representative areas of invasive tumor to be cored. The cores were placed on the tissue microarray using a Tissue Micorarrayer (Beecher Instruments, Silver Spring, MD). The tissue microarrays were then cut to 5-μm sections and placed on glass slides using an adhesive tape transfer system (Instumedics, Inc., Hackensack, NJ) with UV cross-linking.

Immunohistochemistry. The tissue microarray slide was deparaffinized by rinsing with xylene, followed by two changes of 100% ethanol and two changes of 95% ethanol. The slides were then boiled in a pressure cooker containing 1 mM EDTA (pH 7.5) for antigen retrieval. Endogenous peroxidase activity was blocked with 2.5% methanol in hydrogen peroxide for 30 min at room temperature. After washing with TBS, the slides were incubated at room temperature for 30 min in 0.3% BSA/1× TBS to reduce nonspecific background staining. The primary antibody, rabbit polyclonal anti-ATF2 IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was then added at a dilution of 1:50, and the slides were incubated overnight at 4°C in a wet chamber and then rinsed three times in 1× TBS/0.05% Tween 20. Biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was added for 1 h, and the slides were washed in TBS-Tween as described above, incubated for with VECTASTAIN ABC-AP Reagent (Vector Laboratories) for 1 h, washed again

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5 The abbreviations used are: ATF2, activating transcription factor 2; JNK, c-Jun NH2-terminal kinase; TBS, Tris-buffered saline; CI, confidence interval; IFN, interferon.
in TBS-Tween as described above, and incubated for 30 min with an alkaline phosphatase substrate solution (Vector Laboratories). The slides were then rinsed in water, counterstained with hematoxylin, and mounted with Immuno-mount (Shandon, Pittsburgh, PA).

**Evaluation of Immunohistochemical Staining.** The regions of most intense staining were scored by eye for each spot. Cytoplasmic staining and nuclear staining of the melanoma cells were scored separately. Because of the small size of the histospot (0.6 mm in diameter), no area variable was included in the scoring. The staining was graded using the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. Specimens with no invasive melanoma or specimens that were not interpretable were excluded from the analysis. The tissue microarray was scored separately by two independent observers (A. J. B. and H. M. K.), with a very high correlation between scorers \((P < 0.0001)\). A consensus score was determined for spots with discrepant scoring between the observers.

**Statistical Analysis.** JMP 5.0.1 and Statview 5.0.1 (SAS Institute Inc., Cary, NC) software were used for data analyses. The correlation between the consensus score and the relationship of ATF2 expression and clinicopathological parameters was assessed using the \(\chi^2\) test. The prognostic significance of the parameters was assessed for predictive value using the Cox proportional hazards model with overall survival as an end point. Survival curves were calculated using the Kaplan-Meier method, with significance evaluated using the Mantel-Cox long-rank test.

**Results**

**Immunohistochemical Staining of Melanoma Tissue Microarrays.** Of the 553 melanoma tumors on the tissue microarray, 479 (87%) were interpretable for cytoplasmic and nuclear ATF2 staining. Spots that were deemed uninterpretable had insufficient tumor cells in the spot, loss of tissue in the spot, or an abundance of necrotic tissue. Of the 479 interpretable specimens, 400 (84%) also had associated survival information. Fig. 1 demonstrates histospots representative of scores 3 for cytoplasmic and for nuclear staining (Fig. 1, A and B). The cases included 269 primary cutaneous melanoma specimens, 20 local recurrences, 18 melanomas originating in mucous membranes (vulva, anus, nasal mucosa, and rectum), 118 lymph node metastases, 65 cutaneous metastases, and 50 distant metastases. The site was not known for four of the patients. There was a strong correlation between
nuclear ATF2 expression and lymph node or metastatic sites ($P < 0.0001$, $\chi^2$ test), whereas cytoplasmic ATF2 expression correlated with primary lesions ($P < 0.0001$).

**Survival Analysis.** The cytoplasmic and nuclear ATF2 expression levels were each evaluated for association with overall survival. Kaplan-Meier survival curves generated for cytoplasmic and nuclear ATF2 were split by ordinal score, as shown in Fig. 2, A and B. These curves show that increased cytoplasmic expression was correlated with better outcome ($P = 0.0005$). Conversely, increased nuclear ATF2 staining was associated with worse outcome ($P = 0.0013$). Although these data are only semiquantitative, the curves suggest a splitting of the data to define scores of 0 and 1 as “low” or “negative” expression and 2 and 3 as “high” or “positive” for both nuclear and cytoplasmic staining. The curves for scores of 0 and 1 overlap each other, likely secondary to the difficulty in determining the difference between absent or very weak staining by eye, particularly for weak or absent nuclear staining in the presence of strong cytoplasmic staining and vice versa. Therefore the designations of “high” or “low” expression are used for the remainder of the analyses. This result is shown in a Kaplan-Meier plot in Fig. 2, C and D.

There were a relatively large number of patients that had high cytoplasmic ATF2 expression with low nuclear expression and vice versa. Therefore, to further stratify the patients, they were divided into three risk groups: (a) high cytoplasmic expression and low nuclear expression; (b) low cytoplasmic expression and high nuclear expression; and (c) high cytoplasmic and nuclear expression or low cytoplasmic and nuclear expression. The Kaplan-Meier survival curves for these three groups of patients are shown in Fig. 3A.

For clinicians treating melanoma patients, perhaps the greatest need for determining prognosis occurs at initial presentation, when patients have a biopsy performed on a changing skin lesion. Therefore, further survival analysis was performed using the primary skin lesions only. When dividing the primary skin lesions into the three ATF2 prognostic groups mentioned above, patients with high cytoplasmic and low nuclear ATF2 expression did better than those with low cytoplasmic and high nuclear expression, and the group with low cytoplasmic and low nuclear or high cytoplasmic and high nuclear expression demonstrated intermediate overall survival ($P < 0.0001$), as can be seen in Fig. 3B.

Univariate analysis was performed using the Mantel Cox test. High cytoplasmic ATF2 expression was associated with good overall survival; (hazard ratio = 0.618; 95% CI, 0.474–0.806; $P = 0.0003$). High nuclear ATF2 expression was associated with poor overall survival (hazard ratio = 1.25; 95% CI, 1.1–1.4; $P = 0.0008$), and the combination of low cytoplasmic ATF2 and high nuclear ATF2 was associated with worse survival (hazard ratio = 1.33; 95% CI, 1.15–1.53; $P < 0.0001$). The pattern of ATF2 expression for primary and regional/metastatic disease and its association with survival are shown in Table 1. The ATF2 expression pattern was only predictive of

### Table 1 Univariate analysis of ATF2 expression by tumor site and subcellular localization

<table>
<thead>
<tr>
<th>Location of lesion</th>
<th>Cytoplasmic ATF2</th>
<th>Median survival (mos)</th>
<th>Survival time Range (mos)</th>
<th>Hazard ratio (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary lesions</td>
<td>Low</td>
<td>70</td>
<td>47</td>
<td>1–463</td>
<td>0.66 (0.52–0.85)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>157</td>
<td>97</td>
<td>0–444</td>
<td>1.33; 95% CI, 1.15–1.33</td>
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<tr>
<td>Metastatic lesions</td>
<td>Low</td>
<td>138</td>
<td>47</td>
<td>1–305</td>
<td>1.02 (0.86–1.2)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>93</td>
<td>39</td>
<td>1–415</td>
<td>1.02 (0.86–1.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Location of lesion</th>
<th>Nuclear ATF2</th>
<th>Median survival (mos)</th>
<th>Survival time Range (mos)</th>
<th>Hazard ratio (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary lesions</td>
<td>Low</td>
<td>153</td>
<td>83</td>
<td>0–463</td>
<td>1.37 (1.08–1.74)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>74</td>
<td>77</td>
<td>1–377</td>
<td>1.03 (0.88–1.23)</td>
</tr>
<tr>
<td>Metastatic lesions</td>
<td>Low</td>
<td>98</td>
<td>44</td>
<td>4–415</td>
<td>1.03 (0.88–1.23)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>133</td>
<td>42</td>
<td>1–387</td>
<td>1.03 (0.88–1.23)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Location of lesion</th>
<th>ATF2 risk group</th>
<th>Median survival (mos)</th>
<th>Survival time Range (mos)</th>
<th>Hazard ratio (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary lesions</td>
<td>Low</td>
<td>111</td>
<td>93</td>
<td>0–444</td>
<td>0.81 (0.57–1.13)</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>88</td>
<td>77</td>
<td>1–463</td>
<td>1.309</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>28</td>
<td>52</td>
<td>1–309</td>
<td>2.14 (1.46–3.04)</td>
</tr>
<tr>
<td>Metastatic lesions</td>
<td>Low</td>
<td>38</td>
<td>41</td>
<td>6–415</td>
<td>0.90 (0.71–1.13)</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>115</td>
<td>46</td>
<td>1–387</td>
<td>1.04 (0.81–1.33)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>78</td>
<td>43</td>
<td>1–305</td>
<td>1.04 (0.81–1.33)</td>
</tr>
</tbody>
</table>
risk pattern was further examined. The high-risk ATF2 pattern was grouped with low cytoplasmic and high nuclear ATF2 staining pre-prognostic variables for melanoma: Breslow depth; Clark level; ulceration among the primary cutaneous lesions. We included the following analyses to assess the independent predictive value of ATF2 expression in static specimens. 

Survival among the primary specimens, and not the regional/metastatic specimens.

Clinicopathological Correlations and Multivariate Analyses. Using the Cox proportional hazards model, we performed multivariate analyses to assess the independent predictive value of ATF2 expression among the primary cutaneous lesions. We included the following prognostic variables for melanoma: Breslow depth; Clark level; ulceration; microscopic satellites; and tumor-infiltrating lymphocytes at diagnosis. We used a combined cytoplasmic and nuclear ATF2 risk grouping with low cytoplasmic and high nuclear ATF2 staining presenting the highest risk group. Our high-risk ATF2 pattern remained an independent prognostic indicator in primary lesions, as did Clark level. Results of the multivariate analysis are shown in Table 2.

Correlation between clinicopathological variables and the ATF2 risk pattern was further examined. The high-risk ATF2 pattern was most strongly associated with thick lesions (P < 0.0001) and had a weaker association with Clark level (P = 0.01). There was no significant association between the pattern of ATF2 expression and the site of primary lesion, age, or gender.

Discussion

Numerous preclinical studies have shown that ATF2 contributes to melanoma growth and metastasis, tumor cell survival, and resistance to chemotherapy and radiation. Here we demonstrate, for the first time, that the ATF2 expression pattern is associated with patient survival.

Our data show that strong nuclear ATF2 expression was more frequent in metastatic sites (lymph nodes, bone metastases, or visceral metastases) than in primary cutaneous specimens (P < 0.0001). Furthermore, our data show that nuclear ATF2 expression is a strong predictor of poor survival and that cytoplasmic ATF2 expression is a strong predictor of good outcome in melanoma patients. Among the primary cutaneous specimens, patients who had weak cytoplasmic and strong nuclear staining had poor survival (P < 0.0001), and vice versa for strong cytoplasmic and weak nuclear staining. The expression pattern of ATF2 retained independent prognostic value on multivariate analysis, as did Clark level. Breslow depth was predictive of outcome on univariate analysis. However, when evaluated in multivariate analyses with ATF2, it was not an independent predictor of survival. This is likely due to the correlation between Breslow depth and ATF2 expression.

Our data suggest that subcellular ATF2 localization might be a very useful prognostic marker in primary cutaneous melanoma and might affect decisions regarding patient management, such as the appropriateness of staging evaluation and adjuvant therapy for patients with early stage melanoma.

ATF2 has been primarily implicated as a transcription factor that heterodimerizes with c-Jun and mediates transcriptional activities by Jun-responsive elements (5, 17). ATF2 is among the early response gene products that are activated after stress and DNA damage, depending on its phosphorylation by upstream kinases, including JNK and p38. ATF2 has been identified as one of the primary transcription factors that bind to Jun2-like elements in human melanoma cells (11). Earlier studies that elucidated the possible role of ATF2 in human melanoma have established that it plays an important role in the acquisition of resistance to chemotherapy and radiation therapy (11–13). ATF2 alters melanoma susceptibility to undergo apoptosis, in part, due to its ability to alter the balance between tumor necrosis factor and Fas signaling (13). Inhibition of ATF2 activities, either by coexpression with its dominant negative forms or via short peptides that outcompete the endogenous protein, was found to be an efficient mechanism for sensitizing human and mouse melanoma cells to radiation-induced apoptosis. Furthermore, expression of a small peptide that abolishes ATF2 transcriptional activities inhibited melanoma growth in several mouse tumor models (14, 15). Collectively, these studies suggest a critical role for ATF2 in melanoma progression and resistance to therapy.

The current findings are compatible with the preclinical data and provide insight into the importance of this transcription factor in the development of melanoma. Of particular interest is the finding that nuclear localization of ATF2 correlates with poor prognosis. As a transcription factor, ATF2 is active within the nuclear compartment, where it elicits its transcriptional activities. Strong nuclear staining of ATF2 therefore suggests that ATF2 is constitutively active in these tumors. These findings are consistent with the notion that it is necessary to inhibit ATF2 activities to sensitize these tumors to treatment.

ATF2 phosphorylation is a prerequisite for its stability and transcriptional activities (7, 10). ATF2 kinases are often active in melanoma cells (18), thereby enabling its constitutive phosphorylation and activity. Interestingly, extracellular signal-regulated kinase has been shown to be capable of phosphorylating ATF2 on the same residues as JNK (18), thereby establishing a possible link between B-RAF, which is constitutively active in a large percentage of human melanoma tumors (19), and the activation of ATF2. Nevertheless, the possible existence of another kinase that may be activated in the course of melanoma progression and that would trigger the nuclear localization of ATF2 cannot be excluded.

In summary, our data suggest that the level and localization of ATF2 expression may be clinically useful for assessing prognosis, particularly in primary cutaneous melanoma specimens, warranting prospective confirmation. Furthermore, our findings suggest that ATF2 might be a useful target for new drug development.

References


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