Automated Quantitative Analysis of Tissue Microarrays Reveals an Association between High Bcl-2 Expression and Improved Outcome in Melanoma

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ABSTRACT

The addition of B-cell lymphoma 2 (Bcl-2) antisense to dacarbazine in the treatment of metastatic melanoma demonstrates improved response rates and progression-free survival when compared with dacarbazine alone. Studies on small cohorts of melanoma patients have shown variability in Bcl-2 expression (60%–96% positive). We performed quantitative analysis of Bcl-2 expression in a large patient cohort to assess the association with outcome.

Tissue microarrays containing intact melanoma specimens representing 402 patients (339 with associated survival data) were analyzed with our AQUA system for automated quantitative analysis. Automated, quantitative analysis uses S100 to define pixels as melanoma (tumor mask) within the array spot and measures intensity of Bcl-2 expression using a Cy5 conjugated antibody within the mask. A continuous index score is generated, which is directly proportional to the number of molecules per unit area. Scores were divided into quartiles and correlated with clinical variables.

High Bcl-2 expression was associated with better outcome in the entire cohort and among metastatic specimens only (P = 0.004 and P = 0.015, respectively). Expression was higher in primary than in metastatic specimens (P < 0.0001). There was no association between Bcl-2 expression and Breslow depth or Clark level.

The diverse results within the literature may be due to use of small cohorts or variability in staining technique. These results suggest studies are needed to evaluate the association between quantitative assessment of Bcl-2 expression and response to Bcl-2 targeting therapy toward the goal of improved response rates to these drugs.

INTRODUCTION

The incidence of cutaneous melanoma in the United States has doubled every 10 years for the past 50 years, with 53,600 new cases in 2002. Currently, melanoma is the malignancy with the highest rise in incidence (1). This increase in incidence, compounded by the lack of effective therapy for advanced melanoma, underscores the dire need for improved methods of stratifying and treating patients with metastatic melanoma.

Melanoma is notoriously chemotherapy and radiation therapy resistant. Various chemotherapeutic agents have activity in metastatic melanoma, albeit with disappointingly low response rates of <25% for any single agent or combination of agents (2–6). No drug has improved overall survival when compared with observation (3).

Dacarbazine, the most commonly used agent in the United States, induces a response in 15% to 25% of patients.

One of the likely reasons for failure in treating metastatic melanoma has been the lack of specificity of the drugs given for malignant melanocytes. A recently completed multicenter Phase III clinical trial conducted by Genta Corporation and Aventis Pharmaceuticals (Bridgewater, NJ) randomized patients to receive either dacarbazine alone or dacarbazine and Bcl-2 antisense (G3139, oblimersen sodium, Genta Corporation, Berkeley Heights, NJ). Superior response rates (P = 0.019), progression-free survival rates (P = 0.001), and 12-month survival rates (P = 0.035) were seen for patients treated with G3139 and dacarbazine, with no significant additional toxicity. The median survival rates on the two arms of the study were not significantly different (P = 0.184).3

B-cell lymphoma 2 (Bcl-2) is a protein that was first described in association with a chromosomal translocation in follicular B-cell lymphoma (7). Bcl-2 belongs to a family of proteins that regulates apoptosis. The Bcl-2 protein blocks apoptosis, via the intrinsic pathway, by inhibiting the release of cytochrome C from mitochondria, thus preventing caspase-9 activation (8). The release of cytochrome C into the cytosol triggers a cascade of events that results in compromise of the outer mitochondrial membrane potential, thereby initiating apoptosis (9). Members of the Bcl-2 family are known to be either pro- or antiapoptotic (9). The involvement of Bcl-2 in apoptosis of melanoma cells has been demonstrated through the use of antisense oligonucleotides to Bcl-2 mRNA, which induce apoptosis in melanoma cells derived from different clinical stages (10).

The role of Bcl-2 expression in melanoma and its association with patient outcome has been reviewed recently (9). Studies report a wide range of frequencies of Bcl-2 expression (60%–96%), with inconsistent associations between Bcl-2 expression and clinical outcome. Some studies report that up-regulation of Bcl-2 correlates with advanced stage and poor prognosis (11–13), whereas others report that down-regulation of Bcl-2 is associated with disease progression, advanced stage, and poor prognosis (14–19). It is important to note that other studies have shown that Bcl-2 expression does not change during tumor progression, with equivalent expression levels in melanoma and normal melanocytes (20–22). All of these studies used different methods of measuring Bcl-2 expression, with the majority using immunohistochemistry. Moreover, investigators used different antibodies at differing concentrations, and each of the studies included <100 patients.

Tissue microarrays are a useful tool for simultaneously studying specimens from hundreds of patients (23). This tool carries the inherent advantage of uniform handling of all specimens. Another positive feature associated with the use of tissue microarrays is the recently developed method of automated, quantitative analysis, which provides precise, reproducible, measurement of antigen levels, free of the subjectivity associated with pathologist-based scoring (24). Automated, quantitative analysis provides continuous output scores, as opposed to the arbitrary nominal scores obtained with pathologist-based “by-eye” scoring of 0, 1, 2, or 3 or “positive” and “negative.”
This is particularly important when therapeutic decisions are made based on immunohistochemistry under nonstandardized conditions. In this work we evaluated the expression of Bcl-2 on a large cohort of melanoma specimens using automated, quantitative analysis. We then determined the association between Bcl-2 expression and commonly used clinical and pathological prognostic variables.

**MATERIALS AND METHODS**

**Tissue Microarray Construction.** The tissue microarray was constructed as described previously (25). A total of 570 tissue cores (553 melanomas with duplicate spots for 9 patients and 17 normal skin) measuring 0.6 mm in diameter were spaced 0.8 mm apart on a single glass slide. Of the original cohort, 402 patients had intact tumor cores (devoid of significant necrosis and containing sufficient tumor) for analysis. 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 with a median follow-up time of 60 months. Treatment information was not available for the entire cohort. Some of the stage III patients were treated with interferon-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 of the blocks were reviewed by a pathologist to select representative areas of invasive tumor to be cored. The cores were placed on the tissue microarray using a Tissue Microarrayer (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. Tissue microarray staining was performed twice for this study using two tissue microarrays that included the same cohort of patients, with cores taken from different parts of the tumor (creating 2-fold redundancy).

**Immunohistochemistry.** Staining was performed for automated analysis of melanoma specimens using a newly developed method (24). Tissue microarray slides were deparaffinized by rinsing with xylene, followed by two changes of 100% EtOH and two changes of 95% EtOH. The slides were boiled in a pressure cooker containing 6.5 mmol/L sodium citrate (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked in methanol containing 2.5% hydrogen peroxide for 30 minutes at room temperature. After washing with Tris-buffered saline to reduce nonspecific background staining. To create a tumor mask, rabbit polyclonal antimouse horseradish peroxidase (Envision, Dako Corp.) and goat antirabbit IgG conjugated to Alexa 488 (Molecular Probes, Inc., Eugene, OR) at a dilution of 1:1,000, incubated overnight at 4°C, and rinsed three times in 1× Tris-buffered saline/0.05% Tween-20. The slides were then incubated with the primary antibody, mouse monoclonal anti-Bcl-2 IgG (Dako Corp.), for 1 hour at room temperature, at the recommended dilution of 5.6 μg/mL. Slides were washed again as above and incubated at room temperature for 1 hour with goat antirabbit horseradish peroxidase (Envision, Dako Corp.) and goat antirabbit IgG conjugated to Alexa 488 (Molecular Probes, Inc., Eugene, OR) at a dilution of 1:200. The slides were then washed, and 4,6-diamidine-2-phenyldole, dihydrochloride was added at 1:100 for 1 hour to visualize the nuclei. For primary antibody identification the slides were incubated for 10 minutes with Cy5 directly conjugated to tyramide at a 1:50 dilution (Perkin-Elmer, Boston, MA). The slides were then rinsed in water, and coverslips were mounted.

**Automated Image Acquisition.** Images were acquired using automated, quantitative analysis, as described previously (24, 26). This method has been validated in other tumors. When compared with traditional immunohistochemistry, this method is as good or better at predicting outcome based on expression of prognostic markers. In previous works it has been validated for expression of β-catenin in colon cancer, Her-2/neu in breast cancer, and α-methylacyl-CoA racemase in prostate cancer (24, 26, 27). Briefly, areas of tumor were distinguished from stroma and non-melanoma tissue by creating a mask with the S100 signal tagged with Alexa 488 (Cy2). Coalescence of S100 at the cell surface was used to identify the membrane/cytoplasm compartment within the tumor mask, whereas 4,6-diamidino-2-phenylindole was used to identify the nuclear compartment within the tumor mask. The target marker, Bcl-2, was visualized with Cy5 (red). Cy5 was used, because its emission peak is outside the color spectrum of tissue autofluorescence (24). Fig. 1 demonstrates tumor masking, subcellular compartmentalization, and marker identification.

Multiple monochromatic, high resolution (1024 × 1024 pixel 0.5-μm) grayscale images were obtained for each histospot, using the 10X objective of an Olympus AX-51 epifluorescence microscope (Olympus, Melville, NY) set up with an automated Prior microscope stage and video and digital image acquisition driven by custom program and macro-based interfaces with IPLabs (Scanalytics Inc., Fairfax, VA) software.

**Algorithmic Image Analysis.** Images were analyzed using algorithms that have been extensively described previously (24, 26, 27). Formalin-fixed tissues can exhibit autofluorescence, causing background peaks. Two images (one in-focus and one out-of-focus) were taken of the compartment-specific tags and the target marker. A percentage of the out-of-focus image was subtracted from the in-focus image for each pixel, representing the signal to noise ratio of the image. An algorithm described as rapid exponential subtraction algorithm was used to subtract out the noise (out-of-focus information) in a uniform fashion for the entire microarray, as described in detail (24, 26). Subsequently, the pixel locale assignment for compartmentalization of expression algorithm...
was used to assign each pixel in the image to a specific subcellular compartment and the signal in each location was calculated. Pixels that cannot accurately be assigned to a compartment were discarded. The data were saved and subsequently expressed as the average signal intensity per compartment area. For Bcl-2, no signal was detected in the nuclear compartment. The Bcl-2 signal from the membrane/cytoplasmic compartment of cells within the S100 tumor mask was measured on a scale of 0 to 255 and expressed as target signal intensity relative to membrane/cytoplasmic compartment area.

**Statistical Analysis.** The JMP v.5 (SAS Institute Inc., Cary, NC) software was used for data analyses. Continuous automated, quantitative analysis scores of Bcl-2 expression were divided into quartiles, and associations with clinical and pathological parameters were completed using the χ² 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 generated using the Kaplan-Meier method, with significance evaluated using the Mantel-Cox log-rank test.

**RESULTS**

**Immunohistochemical Staining of Melanoma Tissue Microarrays.** Two separate tissue microarrays that included the same cohort of patients but with different cores were used for this study. Of the 402 melanoma patients represented on the tissue microarrays, 317 (78%) specimens on the first array had good staining of the S100 mask and were interpretable for Bcl-2 staining, whereas 310 (76%) of the spots on the second array fulfilled all of these criteria. Histospots that were deemed uninterpretable had insufficient tumor cells in the spot, loss of tissue in the spot, or an abundance of necrotic tissue. Fig. 1 demonstrates the automated, quantitative analysis system for Bcl-2 staining, with red representing the Bcl-2 within the S100 mask and blue representing the nuclei within the S100 mask. Continuous AQUA scores were then divided into quartiles, reflecting the use of routine statistical divisions in the absence of an underlying justification for division of expression levels. Quartiles were chosen rather than tertiles, quintiles, or other divisions, based on the size of our cohort and the absence of an underlying biological hypothesis that would suggest other cut-points.

The AQUA quartiles for the two arrays were highly correlated (χ² = 73.03, P < 0.0001). The intensity of the S100 staining by area for individual patients between the two arrays was similarly highly correlated (χ² = 70.25, P < 0.0001). Of the 234 patients that had two assigned values for Bcl-2 AQUA scores, 6 (2.5%) had discrepancies in their scores that were greater than one quartile between the two arrays, and these cases were excluded from the analysis. Composite scores (n = 402) from the two microarrays were formed that included the mean raw score in cases with small differences (1 quartile).

There was a solid correlation between strong Bcl-2 staining and tumor site, with Bcl-2 staining in the third or fourth quartile being associated with primary lesions (P < 0.0001), as demonstrated in the Box-plot in Fig. 2.

Of the 402 composite scores, 339 (84%) had associated survival information. These cases included 159 primary cutaneous melanoma specimens, 15 specimens from local recurrences, and 165 lymph node and distant metastases.

**Survival Analysis.** Kaplan-Meier survival curves were generated for Bcl-2 expression, as shown in Fig. 3A. These curves show that increased membranous/cytoplasmic Bcl-2 expression was correlated with better outcome (log-rank P = 0.004). Patients were stratified based on the location of their biopsy specimen. Kaplan-Meier survival curves were generated for primary lesions and for nonprimary lesions (including lymph node, visceral, and cutaneous metastases). Fig. 3B shows significantly better overall survival among high Bcl-2 expressers in the nonprimary sites (P = 0.015), whereas we found no significant difference in survival for the primary lesions (P = 0.661), as shown in Fig. 3B and C. We additionally subdivided the nonprimary lesions into lymph node metastases and distant metastases, and the survival curves are shown in Fig. 4A and B, respectively. There was a trend toward better survival with high Bcl-2 expression among the lymph node specimens (P = 0.065), whereas a stronger association with better survival was seen among the high expressers with distant metastases (P = 0.038).

**Clinicopathological Correlations and Multivariate Analyses.** Univariate analysis was performed using the continuous data and the Mantel Cox test. Low Bcl-2 expression was associated with worse overall survival (hazard ratio = 1.94, 95% confidence interval, 1.28–2.93, P = 0.0048).

Although division of the data into quartiles was somewhat arbitrary and based on conventions, the curves in Fig. 3A strongly suggest a splitting of the data to define the first and second quartiles as “low” expression and third and fourth as “high.” The designations of “high” (greater than median) or “low” (less than median) expression are used for the remainder of the analyses.

Association between commonly used clinical and pathological prognostic variables and survival was examined (Table 1). Breslow depth, Clark level, presence of ulceration, biopsy site (primary versus metastasis), and gender were all associated with prognosis on univariate analysis. Age was not associated with survival in this cohort. In addition, the association between Bcl-2 expression and these variables was studied. Bcl-2 expression was more likely to be high in the primary lesions than in nonprimary lesions (P < 0.0001). No association was found between Bcl-2 expression and any of the other variables mentioned above.

Using the Cox proportional hazards model, we performed multivariate analyses to assess the independent predictive value of Bcl-2 expression in our cohort. For the multivariate analysis we included standard prognostic variables for melanoma: tumor location (primary versus nonprimary site), Breslow depth, Clark level, presence of ulceration on primary lesions, gender, and age at diagnosis. Bcl-2 expression was an independent prognostic indicator, as were stage, Breslow depth, and Clark level. Results of the multivariate analysis are shown in Table 2.

**DISCUSSION**

Our goal was to quantitatively assess expression of Bcl-2 on a large cohort of melanoma specimens in an objective, automated fashion and to evaluate the association between Bcl-2 expression and clinical outcome. The values are divided into quartiles and halves based on
quantitative assessment of expression rather than arbitrary pathologist-based divisions of staining into nominal scores of 0, 1, 2, and 3 or “positive/negative.” Bcl-2 expression was significantly higher in the primary specimens than in the nonprimary specimens. High Bcl-2 expression was tightly associated with improved survival in the entire cohort and in the subset of nonprimary lesions, as well as in the smaller subset of distant metastases. Our results were reproducible when using a second array with different cores from the same patients.

Bcl-2 in early stage malignant melanoma may play a role in tumor development by sparing the cells from apoptotic death or through cooperation with other oncogenes. Our data suggest that the increase in Bcl-2 expression is an early event in melanoma oncogenesis. The loss of Bcl-2 expression in metastatic melanoma, particularly the more aggressive metastatic melanomas, suggests that mechanisms other than Bcl-2 are involved in the survival and growth of these highly aggressive malignant melanocytes. Moreover, our data imply that Bcl-2 expression decreases as loss of differentiation of aggressive malignant melanocytes occurs.

From a clinical perspective, our findings need to be interpreted within the context of use of Bcl-2 antisense for stage IV melanomas. While specific, targeted therapy was used in the G3139 trial described in the introduction, there was no stratification of patients based on Bcl-2 expression within their tumors, and patient specimens were not evaluated for Bcl-2 expression before enrollment in the trial. Other targeted therapies, such as trastuzumab in breast cancer (28), rituximab in non-Hodgkin’s lymphoma (29), and STI571 in gastrointestinal stromal tumors (30) have selectively been given to patients whose tumors express HER-2/neu, CD-20, and c-kit, respectively. HER-2/neu is expressed in 25% to 30% of breast cancers, and had trastuzumab been given to all of the metastatic breast cancer patients, the response rates would have been <3% rather than the 12% reported in the original trials (28).

The association of Bcl-2 expression with improved survival in metastatic lesions in this study and the apparent increase in response rates with the addition of G3139 to dacarbazine in the clinical trials can be explained in one of three possible ways. First, Bcl-2 expression in melanoma might be similar to estrogen receptor in breast cancer, in that estrogen receptor promotes tumor growth, yet is associated with improved overall survival. Pharmaceutical targeting of estrogen receptor results in additional improved survival (31). Similarly, targeting Bcl-2 might additionally improve survival in patients that are already in a better prognostic group. Moreover, Bcl-2 is expressed in follicular lymphoma, a relatively indolent malignancy, in which responses to therapy with G3139 have been reported (32). Second, Bcl-2 antisense might be effective only when there are lower levels of Bcl-2 mRNA in the melanoma cells, and the high expressers might be
saturating the cytoplasmic G3139. It is thought that oligonucleotides are taken up primarily through endocytosis. After internalization, confocal and electron microscopy studies have indicated that the bulk of the oligonucleotides enter the endosome/lysosome compartment where most of the material either becomes trapped or degraded. Nevertheless, some intact oligonucleotides can escape from the vesicles, enter the cytoplasm, and then diffuse into the nucleus, where they presumably bind their target mRNA and assert their effect (33).

Third, response to G3139 might be totally independent of Bcl-2 protein levels. One Phase I study of this drug examined Bcl-2 levels in the tumors. Nevertheless, some intact oligonucleotides can escape from the vesicles, enter the cytoplasm, and then diffuse into the nucleus, where they presumably bind their target mRNA and assert their effect (33).

Additional studies are needed to elucidate the association between levels of Bcl-2 expression and other commonly used clinical markers of prognosis.

### Table 2

<table>
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<th>Marker</th>
<th>P value</th>
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<th>95% confidence interval</th>
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<td>Age</td>
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<td>2.015</td>
<td>1.09–3.71</td>
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<td>0.33</td>
<td>0.13–0.86</td>
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<td>Absence of ulceration</td>
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<td>0.351–1.005</td>
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<tr>
<td>High Bcl-2</td>
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**REFERENCES**

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