Wilms Tumor 1 Expression Present in Most Melanomas but Nearly Absent in Nevi

Betsy N. Perry, MD; Cynthia Cohen, MD; Baskaran Govindarajan, PhD; George Cotsonis, MA; Jack L. Arbiser, MD, PhD

Background: Pigmented lesions are common, yet they present diagnostic and therapeutic challenges. They range from nevi, which are clinically stable, to melanomas, which are notorious for distant metastasis and death. Both nevi and melanomas arise from melanocytes, which are neural crest derivatives, and melanocyte precursors migrate from the paraspinal area to their eventual location at the dermoepidermal junction. Atypical nevi have been clinically considered to be precursors of melanoma, and recently, biochemical abnormalities have been found that are present in both nevi and melanomas, including inactivation of the p16INK4a tumor suppressor gene and mutations in B-raf. These mutations suggest not only that nevi and melanomas share a common origin but also that additional events are required for transformation to malignant melanoma.

Observations: We performed a Panomics protein array comparing a radial growth melanoma cell line with a vertical growth melanoma cell line and found that the transcription factor Wilms tumor 1 is highly expressed in the vertical growth cell line compared with the radial growth cell line. Using immunohistochemical analysis, we compared expression of archival nevi and melanomas in a tissue microarray.

Conclusion: We found that Wilms tumor 1 is expressed in most melanomas but is nearly absent in nevi. Immunohistochemical analysis for Wilms tumor 1 may be clinically useful in distinguishing nevi from melanoma.

Arch Dermatol. 2006;142:1031-1034

WE HAVE PREVIOUSLY observed that common signaling abnormalities may be present in neoplasms that arise from distinct genetic mechanisms. For example, we have demonstrated that mitogen-activated protein (MAP) kinase is universally activated in hemangiomas of infancy but not in malignant endothelial neoplasms. In melanomas, which can arise through mutations in N-ras and B-raf, or loss of phosphatase and tensin homologue (PTEN) and p16INK4a, MAP kinase activation is a common feature of malignant transformation. Wilms tumor 1 (WT1) is a transcription factor (TF) initially isolated through a reverse genetic approach in hereditary Wilms tumor, but it can also be activated by translocation in desmoplastic small round cell tumors or transcriptionally elevated in leukemias. Using a Panomics array (Panomics Inc, Fremont, Calif), we found WT1 to be highly expressed in melanoma cells. Given its diverse activities, we examined WT1 as a potential signaling molecule in malignant melanomas. We found WT1 to be highly expressed in primary melanomas but not in nevi. Wilms tumor 1 immunohistochemical analysis, which is available in most hospital laboratories, may be a useful tool in distinguishing melanoma from nevi.

METHODS

SIGNAL TRANSDUCTION ARRAY

The human TF complementary DNA (cDNA) array was done to compare the TFs involved in the human melanoma cell lines PMWK and MMAN, which are derived from radial growth and vertical growth melanomas, respectively. Total RNA was isolated from these cells using TRI reagent (Sigma-Aldrich, St Louis, Mo), and the array was performed using TransSignal Human TF cDNA array (Panomics).

In brief, biotin-labeled cDNA was synthesized using TF cDNA primer mixture and biotin-deoxyuridine triphosphate with avian myeloblastosis virus reverse transcriptase (Roche Applied Science, Indianapolis, Ind), and the reverse-transcriptase polymerase chain reaction was carried out according to the manufacturer’s instructions. The labeled cDNA probe was hybridized with the array membrane at 42°C overnight. Detection was performed after adding 20 mL of 1X blocking buffer to each membrane and incubation at room temperature for 15 minutes with gentle shaking. Then, 20 µL of streptavidin–horseradish peroxidase...
conjugate was added directly to 20 mL of 1X blocking buffer, and shaking was continued at room temperature for 15 minutes. Each membrane was washed 3 times for 8 minutes per wash at room temperature with 20 mL of 1X wash buffer, followed by 20 mL of 1X detection buffer per membrane and incubation at room temperature for 5 minutes. Next, 2 mL of substrate working solution containing equal volumes of the luminol enhancer and peroxide solution was added and incubated at room temperature for 5 minutes, and membranes were exposed using BioMax film (Kodak, Rochester, NY).

IMMUNOHISTOCHEMICAL ANALYSIS

Sections of formalin-fixed, paraffin-embedded tissue (5 µm) were stained for the presence of WT1 using a monoclonal WT1 antibody (1:40) (6F-H2, code M3561; Dako Corp, Carpinteria, Calif) with an avidin-biotin complex technique and heat-induced antigen retrieval. An avidin-biotinylated enzyme complex kit (Dako LSAB2, Dako Corp) was used in combination with the automated Dako Autostainer (Dako Corp). Hematoxylin was the counterstain. Negative controls had primary antibody replaced by buffer. Mesothelioma sections were used as positive controls.

Sections were deparaffinized and rehydrated. Antigen retrieval was performed in citrate buffer (pH 6) using an electric pressure cooker for 3 minutes at 120°C and then cooled for 10 minutes before immunostaining. The sections were next exposed to 3% hydrogen peroxide for 5 minutes, primary antibody for 30 minutes, biotinylated secondary linking antibody for 25 minutes, streptavidin enzyme complex for 25 minutes, diaminobenzidine as chromogen for 5 minutes, and Dako autostainer for 15 minutes. These incubations were performed at room temperature; between incubations, sections were washed with Tris-buffered saline. Cover slipping was performed using the Tissue-Tek SCA (Sakura Finetek USA Inc, Torrence, Calif).

Paraffin sections for WT1 antigen staining of melanocytic lesions were obtained from the Department of Pathology, Emory University, Atlanta, Ga, following institutional review board approval. These included 49 primary melanomas (melanoma group) and 27 nevi (nevi group), which were independently reviewed by a pathologist who was blinded to the patients' medical records, demographics, and our working hypothesis.

STATISTICAL ANALYSIS

Baseline features of the melanoma group vs the nevi group were compared using either an independent groups t test (for means) or χ² test (for percentages). Survival probabilities were calculated using Kaplan-Meier methods and compared using log-rank tests. Sensitivity, specificity, positive predictive value, and negative predictive values were calculated using contingency tables with various WT1 cutoffs. Logistic regression was used to model WT1 score, and odds ratios were calculated.

RESULTS

Panomics assay comparing PMWK radial growth melanoma cells and MMAN vertical growth melanoma cells showed that WT1 protein was diminished in radial growth cells compared with vertical growth cells. Because immunohistochemical analysis of WT1 is a standard part of hospital immunohistochemistry panels, we focused on WT1 as a potential marker of melanoma. Western blot analysis of WT1 in both cell lines also showed that WT1 was highly expressed in MMAN vertical growth melanoma cells but poorly expressed in PMWK radial growth cells (Figure 1). This finding prompted us to study its expression in human pigmented lesions.

The primary malignant melanomas studied encompassed the entire clinical spectrum, with slightly more than 50% coming from patients with stage 0 (9 [21.4%]) and stage I (13 [31.0%]) tumors, and 4 (9.5%), 14 (13.3%), and 2 (4.8%) from those with stage II, III, and IV disease, respectively (MIB1 antibody; mean [SD], 10.4 [8.4]).

Superficial spreading malignant melanomas were the most common type of melanoma (n=18 [38.3%]), and 12 (25.5%) of the samples were lentigo maligna. Nodular melanomas were infrequently observed (n=2 [4.2%]), but a substantial portion of samples were deemed “other” or unclassified (3 [6.4%] and 12 [25.5%], respectively). Most of the nevi studied were compound (n=19 [70.4%]), whereas junctional and intradermal nevi were almost equally represented (3 [11.1%] and 4 [14.8%], respectively). Only 1 nevi (3.7%) was labeled “other.”

Immunopositivity for WT1 was more commonly seen in primary malignant melanomas than in nevi (Figure 2). Primary malignant melanomas stained positively for WT1 in 51.0% to 83.7% of cases, but only 7.4% to 29.6% of nevi were positive for WT1 staining, depending on the cutoff value for positivity chosen (Table 1 and Table 2). Specifically, 25 (51.0%) of 49 of malignant melanomas had at least 75% WT1 staining, but only 2 (7.4%) of the 27 nevi studied had such high levels of WT1 positivity. More than 70% of nevi had negligible amounts of WT1, defined as less than 10% WT1 staining, and 85% of nevi had less than 50% WT1 positivity. Conversely, 11 (22.4%) of 49 melanoma lesions showed a paucity of WT1 staining and tended to be advanced melanomas in which confusion with nevi is unlikely.

COMMENT

Melanoma is a malignancy of melanocytes, neural crest derivatives that migrate from the neural crest to peripheral locations, notably the skin, eye, and ear. Once melanocytes have migrated to their final position, they can exist in their normal distribution as 1 of every 10 cells at the dermoeipidermal junction. In addition, they may be found as collections of melanocytes in a variety of lesions, including both congenital nevi and atypical nevi. Although the exact origins of all melanomas are not fully understood, atypical nevi are thought to be precursors of melanoma in some cases. Subsets of nevi share certain biochemical features with melanoma, including decreased expression of the tumor suppressor gene p16INK4a and activating mutations in B-raf.

(Reprinted) Arch Dermatol/Vol 142, Aug 2006 www.archdermatol.com

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The exact transition point of a nevus to a melanoma is not well characterized but is of obvious clinical significance because the treatment and follow-up of these disorders are quite different. One major difference between nevi and early melanomas is the latter’s ability to migrate and proliferate. We previously noted that MAP kinase is a major determinant in melanoma proliferation, and activated MAP kinase is expressed in early melanomas but not in atypical nevi.

In an effort to determine differences between radial growth and vertical growth melanomas, we compared 2 human melanoma cell lines: PMWK, derived from a radial growth melanoma, and MMAN, derived from a vertical growth melanoma. Among the factors differentially expressed in a protein array, WT1 was highly expressed in vertical growth melanomas but much less so in the radial growth melanomas.

In our studies of human pigmented lesions, we found WT1 to be expressed far more frequently in melanomas when compared with nevi, which show a paucity of WT1 staining in most cases. A retrospective review of the pathology reports of 1 of the 2 nevi in our database that stained strongly for WT1 revealed that the lesion had “features suggestive of an early melanoma” and the recommendation was to treat it as such. Lesions that stained strongly positively for WT1 had 29.7 times greater odds of being a melanoma (95% confidence interval, 5.6-156.2) than those with WT1 scores that were less than 10%, as the following tabulation shows.

Table 1. Statistical Summary of Wilms Tumor 1 (WT1) Staining in Nevi and Melanomas

<table>
<thead>
<tr>
<th>Cutoff Point, %</th>
<th>Specificity, %</th>
<th>Sensitivity, %</th>
<th>PPV, %</th>
<th>NPV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1 ≥ 75</td>
<td>92.6</td>
<td>51.0</td>
<td>92.6</td>
<td>51.0</td>
</tr>
<tr>
<td>WT1 ≥ 50</td>
<td>85.2</td>
<td>61.2</td>
<td>88.2</td>
<td>54.8</td>
</tr>
<tr>
<td>WT1 ≥ 25</td>
<td>77.8</td>
<td>73.5</td>
<td>85.7</td>
<td>61.8</td>
</tr>
<tr>
<td>WT1 ≥ 10</td>
<td>70.4</td>
<td>83.7</td>
<td>83.7</td>
<td>70.4</td>
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</table>

Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

WT1 staining has a high degree of specificity and positive predictive value in delineating primary melanomas from nevi using high cutoff points, whereas sensitivity is increased with cutoffs as low as 10%.

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<table>
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<tr>
<th>WT1 Staining Comparison, %</th>
<th>Odds Ratio (95% Confidence Interval)</th>
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<tbody>
<tr>
<td>≥75 vs &lt;10</td>
<td>29.7 (5.6-156.2)</td>
</tr>
<tr>
<td>≥50 vs &lt;10</td>
<td>5.9 (0.9-37.2)</td>
</tr>
<tr>
<td>≥25 vs &lt;10</td>
<td>7.1 (1.2-43.1)</td>
</tr>
<tr>
<td>≥10 vs &lt;10</td>
<td>5.9 (0.9-37.2)</td>
</tr>
</tbody>
</table>

Even when the cutoff point was set as low as 10%, those lesions that stained more than 10% had 5.9 times more likelihood of being a melanoma compared with those that stained less than 10% (95% confidence interval, 0.9-37.2). Another important parameter for a screening tool is a high positive predictive value, meaning that given a positive test, it is probable that the person actually has the disease. Wilms tumor 1 staining has a high positive predictive value, as 92.6% of those lesions that had WT1 staining of 75% or more were actually melanomas (Table 1).

Although melanomas were much more likely to stain positively for WT1, it was not found to be an independent prognostic factor and did not correlate with clinical stage, disease-free rate, or overall survival rate (data not shown). Interestingly, many of the primary melanomas that did not stain at all for WT1 were some of the most advanced, with 5 (62.5%) of 8 showing evidence of metastasis or locally...
aggressive behavior at the time of diagnosis. Although this is not readily explained by our study, it is of practical importance that WT1 staining would not presumably be required to differentiate these lesions from nevi. We have observed a similar pattern in advanced melanoma with respect to MAP kinase activation, with MAP kinase activation being strongest in early melanoma but decreased with deeper disease. One possible interpretation is that MAP kinase and WT1 may be major effectors in nonmetastatic melanomas, but metastatic melanomas may use other signaling pathways to regulate growth. Even though our initial gene array studies did not show much expression in radial growth melanoma cells, the possibility exists that WT1 may be upregulated in early melanoma by its proximity to the extracellular matrix, which is not present in tissue culture. Our study suggests that immunohistochemical analysis for WT1 may be a useful and readily available application for differentiating dysplastic nevi from malignant melanomas, particularly among early melanomas. Wilms tumor 1 immunohistochemical analysis is readily available in most hospital-based pathology laboratories. We have previously shown that immunohistochemical analysis for WT1 is useful in distinguishing hemangiommas, which express this gene, from vascular malformations, which do not. The positivity of hemangiommas and melanoma may indicate the ability of cells to remodel and move, whereas the lack of positivity in nevi and vascular malformations may indicate that a cell is static and unchanging. Further studies are under way to ascertain the role of WT1 as a therapeutic target in melanomas.

Accepted for Publication: February 15, 2006.
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Financial Disclosure: None reported.

Funding/Support: This study was funded by National Institutes of Health grant RO1 AR47901 and the Veterans Administration Hospital support to Dr Arbiser. Dr Perry is supported by a T32 NIH NRSA Institutional grant. Dr Govindarajan is supported by the Research Career Development Award from the Dermatology Foundation.

Acknowledgment: We thank Suephy C. Chen, MD, MS, for her helpful discussions.

REFERENCES