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WT1 marker is not sufficient for distinguishing between melanoma and melanocytic nevi

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Abstract

Background—The heterogeneous histological features of melanoma may often overlap with melanocytic nevi. For this reason, pathologists have sought after immunohistochemistry to assist with difficult cases. Recently, Wilms' tumor 1 protein (WT1) has been suggested to differentiate between melanoma and melanocytic nevi.

Objective—Our objective was to determine whether immunohistochemistry analysis of WT1 expression is a reliable tool in differentiating cutaneous melanoma from melanocytic nevi.

Methods—Forty-five melanoma and 43 melanocytic nevi were immunostained with anti-WT1 monoclonal antibody (clone 6F-H2).

Results—Forty of the 45 cutaneous melanoma (89%) and 22 of the 43 melanocytic nevi (51%) stained (> 10% cells) for WT1. The highest sensitivity for WT1 was expressed by nodular melanoma (19/20), superficial spreading melanoma (8/10) and Spitz nevi (9/11). At the threshold of above 75% WT1-stained cells, the specificity for melanoma was 95% but the sensitivity was only 31%. At the threshold of 10%, the sensitivity increased to 89% but the specificity decreased to only 49%. Finally, at the threshold of 25% and 50%, the sensitivity and specificity were 71%, 61% and 64%, 77%, respectively.

Conclusions—Our data suggest that melanoma is associated with increased WT1 expression. However, as a single immunostaining marker, WT1 is not sufficient for distinguishing melanoma from melanocytic nevi.

The histological appearance of melanoma may be difficult to differentiate from melanocytic nevi, especially Spitz nevi and dysplastic or 'Clark's' nevi. Pathologists have sought after assistance with difficult cases from immunohistochemistry with limited results. Currently,

none of the frequently used markers for the diagnosis of melanoma show absolute sensitivity and specificity. Therefore, there is an ongoing quest for markers with better features.

Recently, *Wilms' tumor 1 gene* (WT1) has been suggested to differentiate between melanoma and melanocytic nevi.^{1,2} WT1 is located at chromosome 11p13.³ Its product is a transcription factor involved in the development of the hematopoiesis and genitourinary systems, kidney and spleen.^{4–7} WT1 protein may function as either a repressor or an activator in a large variety of neoplasms. Loss of WT1 tumor repressor function promotes Wilms' tumor development, while its wild-type expression promotes a large variety of neoplasms such as leukemia, breast cancer, colorectal cancer, pancreatic cancer, Morbus Kaposi and angiosarcoma.^{8–13} Also, WT1 has been described in six of seven melanoma patients.¹⁴

The first suggestion that WT1 expression may differentiate between malignant and melanocytes was made by Rodeck et al.¹⁵ They showed the presence of WT1 mRNA transcripts in seven of nine melanoma cell lines but in none of the five melanocytic cell lines. Later, Perry et al.¹ claimed that that WT1 is expressed in most melanomas and nearly absent in melanocytic nevi and, therefore, suggested WT1 to be useful in distinguishing melanoma from nevi. They used immunohistochemistry to show WT1-positive staining in up to 84% and 30% of the melanomas and nevi, respectively. Recently, Wagner et al.² claimed that WT1 clearly discriminate between melanoma and acquired melanocytic nevi. They used immunohistochemistry to show WT1-positive staining in 100% and 67% of the melanomas and nevi, respectively, with higher percentage of cells stained in melanoma than in the melanocytic nevi.

Based on the limited number and variety of benign lesions in Perry et al.'s¹ study, 27 and 3, respectively, and on the small number of melanoma patients, 10, in the study of Wagner et al.,² we questioned their conclusion of WT1's proficiency in differentiating between melanoma and melanocytic nevi, thus serving as a reliable immunohistochemical tool in establishing the diagnosis of melanoma.

The aim of this study was to investigate whether immunohistochemical analysis of WT1 expression can be used to reliably differentiate between cutaneous melanoma and melanocytic nevi.

Materials and methods

Patients and specimens

Cases were retrieved from the files of the Pinkus Dermatopathology Laboratory, a private dermatopathology laboratory. The study group consisted of 45 cases of cutaneous melanoma and 43 cases of melanocytic nevi. The melanoma and melanocytic nevi subtypes are listed in Table 1.

Immunostaining procedure

Five-micrometer sections were deparaffinized in xylene and rehydrated in graded ethanols. For antigen retrieval, the sections were microwaved in citrate buffer pH 6.0 (BioGenex, San Ramon, CA, USA) for 12 min at 95°C and then cooled for 30 min in buffer before immunostaining. After treating the sections with 3% hydrogen peroxide for 15 min, they were incubated with anti-Wilms' tumor 1 (WT1, clone 6F-H2 mouse IgG monoclonal antibody; code M3561; DakoCytomation, Carpinteria, CA, USA) primary antibody at a dilution of 1 : 50 for 60 min. The Bio-Genex detection system was used on an automated I 6000 immunostainer utilizing Multilink biotinylated anti-Ig for 20 min, horseradish peroxidase-conjugated secondary antibody for 20 min followed by development with 3-

amino-9-ethyl-carbazole for 10 min (BioGenex). The specimens were then counterstained with hematoxylin. All incubations were performed at room temperature, and between incubations, the sections were washed with Tris-buffered saline.

Controls

Sections from a nodular melanoma were used as positive controls, while identical specimens incubated without the primary monoclonal antibody served as negative controls.

Cell enumeration

Two dermatopathologists (D. R. M. and D. A. M.), who were blinded to the patients' medical records, independently reviewed the specimens. The positively stained cells were evaluated by light microscopy at $\times 400$ magnification in a semiquantitative fashion (0–10%, 11–25%, 26–50%, 51–75% and $> 75\%$). The mean value of the evaluated cell profiles of three sequential horizontal fields gave the relative density of the cells. A specimen was considered positive for WT1 if more than 10% of the cells were immunostained, similar to Perry et al.¹

Statistical analysis

Differences in demographic characteristics between individuals with melanoma and those with melanocytic nevi were compared using a two-sample *t* test for age and a chi-square test for gender. To assess whether WT1 is independently associated with diagnosis, we evaluated its association with age and gender using a rank correlation for the association with age and a chi-square test for gender. Logistic regression models were used to further assess the association of WT1, age and gender with diagnosis. WT1 was parameterized with indicator variables, and we tested for the significance of differences between increasing categories of WT1 immunostaining. Holm's procedure was used to adjust for multiple comparisons.

Results

Individuals with melanocytic nevi ranged in age from 46 days to 82 years, with a median age of 31 years, while individuals with melanoma range in age from 44 to 88 years with a median age of 59 years (Table 2). There is a statistically significant association of age with percent of cells stained (Spearman's $\rho = 0.22$, $p = 0.04$) and of age with diagnosis ($p < 0.0001$). Because these associations indicate that age may be a confounding factor, two analyses were conducted, one for all individuals ($n = 88$) and one restricted to individuals of 40 years or older ($n = 62$).

WT1 immunostaining of melanoma subtypes

Forty of the 45 melanoma specimens (89%) stained positively for WT1 (Fig. 1 & 2). Melanoma showed no clustering at any of the range groups of positive cells. Approximately one third of the specimens had more than 75% of their cells stained for WT1, one third of the specimens had 51–75% cells stained and a quarter of the specimens had 11–50% cells stained (Fig. 2). With no adjustment for difference in age, melanoma specimens stained more intensively for WT1 than did melanocytic nevi (all participants $p < 0.0001$; age 40 years or older, $p = 0.0004$). The statistical significance of the overall test may be attributed to the large differences between the two highest levels (51–75% and 76–100%) and the lowest level (0–10%; Fig. 2). When a logistic model is used to adjust for differences in age, the overall association between melanoma and increasing percent of cells staining for WT1 is reduced but is still significant ($p = 0.01$). We find, however, that the odds of melanoma do not increase with increasing levels of WT1 staining ($p = 0.14$). A greater proportion of

melanoma specimens (89%) than melanocytic nevi specimens (51%) stained positively (> 10%) for WT1. Moreover, 64% of melanoma specimens had more than 50% of their cells stained compared with only 24% of the melanocytic nevi (Fig. 2).

WT1 immunostaining of melanocytic nevi subtypes

Twenty-two of the 43 melanocytic nevi (51%) stained positively for WT1 (Fig. 2). Specimens positive for the WT1 marker (> 10% cells) were present in all the examined nevus subtypes. Only two melanocytic nevi specimens (5%), both compound nevi, had more than 75% of their cells stained (Table 1, Fig. 2). Spitz nevus showed the highest proportion of specimens, approximately 50% (5/11) having more than 50% positive stained cells (Table 1).

Characteristics of WT1 as a biomarker for melanoma

There was an increase in the melanoma:melanocytic nevi ratio with progressive increase in the number of positively stained cells, from 1 : 5 (5 : 21 specimens) at 0–10%, 1 : 2 (8 : 8 specimens) at 11–25%, 1 : 2 (3 : 4 specimens) at 26–50% and 2 : 3 (15 : 8 specimens) at 51–75% to 7 : 8 (14 : 2 specimens) at > 75% (Fig. 2). Sixty-three percent of individuals were correctly classified for the > 75% threshold, and approximately 70% of individuals are correctly classified no matter whether > 10%, > 25% or > 50% is used as the threshold for melanoma. If > 10% is selected as the threshold, sensitivity is quite high, 89%, but specificity is low, 49% (Fig. 3). If either > 25% or > 50% is chosen, both sensitivity and specificity are mediocre. If > 75% is selected, sensitivity is very low (31%) but specificity is exceedingly high, 95%. The test characteristics and conclusions are essentially the same if the analysis is restricted to individuals older than 40 years.

WT1 as a biomarker for melanoma adjusting for age and gender

Because the overall association between WT1 and melanoma is statistically significant ($p < 0.0001$) and because melanoma increases significantly with age, we tested whether WT1 is a marker of melanoma and not merely a marker of aging. We analyzed WT1 staining of melanocytic nevi and melanoma only in 40 years or older individuals. Again, WT1 staining is significantly associated with increased odds of melanoma ($p = 0.01$). In the model with age and gender are added, age significantly increases the odds of melanoma, but being female significantly decreases the odds. All the estimated odds ratios lack precision, as indicated by the width of the 95% confidence intervals.

Discussion

The expression of WT1 is not confined to melanocytic neoplasms. WT1 has been reported in a wide range of nonmelanocytic neoplasms, that is hematogenic,^{16–19} angiogenic,¹³ kidney^{20,21} and neural neoplasms,¹⁴ as well as in other types of thyroid, ovarian, testicular and breast carcinomas.^{22–25}

WT1 is not nearly absent in melanocytic nevi as previously suggested.¹ We found that 51% of the melanocytic nevi expressed the WT1 marker, thus WT1 is not a unique feature of melanoma cells. In support of our finding are the data of Perry et al.¹ and Wagner et al.² of 30% and 37% of the melanocytic nevi samples expressing the WT1 marker, respectively. The higher percentage of positive WT1 melanocytic nevi in our study compared with Perry et al. may reflect our use of a wider spectrum of melanocytic nevi subtypes. This included the addition of Spitz nevi, blue nevi, congenital nevi and solar lentigo. Also, it is conceivable that the actual proportion of positive WT1 melanocytic nevi in Wagner et al.'s² study is higher than the reported 37% because it included only atypical nevi and Spitz nevi with more than 50% positive cells. Moreover, while Perry et al. and we used 10% WT1-

stained cells as a threshold for marking a specimen as positive, Wagner et al.² used a threshold of 20% for 21 of the 27 specimens that contained WT1-positive cells.^{1,2} The detection of WT1 protein in 30–51% of the melanocytic nevi specimens in the above three immunohistochemical studies is in apparent contrast to the lack of WT1 mRNA in melanocyte cell cultures.¹⁵ This discrepancy may reflect a WT1-negative property of the five selected cell lines, as is also the case in approximately 50% of the melanocytic nevi sections in our study.

WT1 is not a useful marker in distinguishing between melanoma and melanocytic nevi as previously suggested.^{1,2} We found a higher expression of WT1 in melanoma than in melanocytic nevi, 40 of 45 (89%) vs. 22 of 43 (49%), respectively (Fig. 2 $p < 0.0001$). The association between WT1 and melanoma could not be attributed to gender because the melanocytic nevi and melanoma populations did not differ on this aspect nor could it be attributed to the age difference between the melanocytic nevi and melanoma groups since we confirmed the association by analysis of individuals of 40 years or older. However, to be added to our repertoire of immunohistochemical diagnostic tools, WT1 has to display sensitivity and specificity for melanoma, which are above or at least correspond to those of other currently used diagnostic markers. In most studies, S100, human melanoma black-45 (HMB-45), melanoma antigen recognized by T-cells-1 (MART-1), tyrosinase and microphthalmia-associated transcription factor (MITF) markers show sensitivity and specificity above 75% (reviewed in Sheffield et al.²⁶ and Ohsie et al.²⁷). In our study, the specificity for melanoma was 95% (Fig. 3); however, this was at the cost of a high false negative rate, 69%. Lowering the threshold to 50% WT1-stained cells decreased the specificity for melanoma to 77% and left a relatively high false negative rate, 29%. The sensitivity could be increased to 89% by setting the threshold at 10% WT1-stained cells; however, this decreased the specificity to only 49% (Fig. 3), much below the specificity of other diagnostic markers such as MART-1 (> 94%), tyrosinase (> 96%) or MITF (> 87%; reviewed in Ohsie et al.²⁷). Two factors contributed to the inability of WT1 to reach levels of more than 80% for both specificity and sensitivity, simultaneously. First, the almost uniform distribution of melanoma specimens across levels of WT1 staining (approximately 1/3 in each of the '> 75%', '51–75%' and '50%' WT1-stained cell groups), and second, the inclusion of Spitz nevi, which were WT1 positive in 82% (9/11) of the specimens compared with only 51% (22/43) in the general melanocytic nevi population. The lack of simultaneous high sensitivity and specificity does not support WT1 to be a potent marker in distinguishing melanoma from melanocytic nevi. Moreover, to be considered a good melanoma marker, WT1 should be expected to have a growing association with melanoma along the categories with increasing number of WT1-stained cells; however, this is not the case ($p = 0.14$; Fig. 2). Our findings contradict previous assessment by Perry et al.¹ The differences in results between the studies can be attributed to differences in the size and composition of the melanocytic nevi population and to possible differences in the composition of the melanoma population. In our study, the melanocytic nevi population was larger by 60% and included a wider spectrum of melanocytic nevi subtypes. The melanoma population of the two studies is harder to compare because approximately one third of the melanoma population (15/47) in Perry et al.'s study was titled as 'unclassified' or 'others'. Nevertheless, a large discrepancy between sensitivity and specificity appears in Perry et al. own data, that is at the threshold of more than 75%, the sensitivity and specificity of WT1 positively stained cells were 92.6% and 51%, respectively.¹ Moreover, also in Perry et al.'s study, at no staining range did the sensitivity and specificity levels reach 80% to match currently used immunohistochemical markers for melanoma diagnosis. Differences in melanoma patient population could also account for the differences between our findings and those of Wagner et al.,² who stated that WT1 clearly discriminates between melanoma and acquired melanocytic nevi.²

In contrast to our melanoma population of 45 specimens of six subtypes, melanoma population of Wagner et al.'s² study was limited to 10 specimens of superficial spreading melanoma and nodular melanoma only.² In conclusion, among melanocytic neoplasms WT1 is not confined to melanoma because it is expressed in up to half of the melanocytic nevi tested. Also, as a single immunostaining marker, WT1 usefulness in distinguishing between melanoma and melanocytic nevi is limited because its sensitivity and specificity are substantially lower compared with other currently widely used markers.

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Fig. 1. Wilms' tumor 1 marker (WT1) expression in melanoma (A and B) and melanocytic nevi (C and D). A) Nodular melanoma, 75–100%, (B) nodular melanoma, 0–10% (tailed arrows point at WT1 positively stained cells) and (C and D) junction Spitz nevi, 50–75% (original magnification $\times 400$).

Fig. 2.

Total numbers and percentages of melanocytic nevi and malignant melanoma subtypes at different categories of Wilms' tumor 1 (WT1) positively stained cells ($p < 0.0001$; adjusted to age and gender $p < 0.01$).

Fig. 3. Characteristics of Wilms' tumor 1 (WT1) as a biomarker: sensitivity and specificity for malignant melanoma (MM) at >10%, >25%, >50% and >75% thresholds of WT1 positively stained cells.

Table 1

Melanocytic nevi and melanoma subtypes; patients grouped by percentages of WT1-stained cells within the tumors

Tumor type	Percentages of WT1-stained cells					
	0-10	11-25	26-50	51-75	> 75	Total
Blue nevus	2	1			3	
Junctional nevus	1		1	1		3
Compound nevus	10	2	2	2	2	18
Congenital nevus	3	1				4
Spitz nevus	2	3	1	5		11
Solar lentigo	3	1				4
Total						43
Melanoma <i>in situ</i> /LM	1	4		4	1	10
Metastatic melanoma		1			2	3
Nodular melanoma	1	4		9	6	20
Spindle cell melanoma					1	1
SSMM	2		2	2	4	10
Lentigo malignant melanoma	1					1
Total						45

LM, lentigo maligna; SSMM, superficial spreading malignant melanoma; WT1, Wilms' tumor 1.

Table 2

Association of melanocytic nevi and melanoma with demographic characteristics and percentages of WT1-stained cells

	Melanoma n = 45	Melanocytic nevi n43	Significance	
Age				
Mean	(95% CI)	62 (59–66)	33 (25–40)	< 0.0001
Gender				
Male	20 (44%)	39 (44%)	0.98	
Female	25 (56%)	24 (56%)		

CI, confidence interval; WT1, Wilms' tumor 1.