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Quantitative assessment of *neuropilin-2* as a simple and sensitive diagnostic assay for spitzoid melanocytic lesions

Anna Eisenstein^{a,*}, Izabela P. Panova^{a,*}, Hye J. Chung^a, Lynne J. Goldberg^a, Qing Zhang^b, Rossitza Lazova^{c,e}, Jag Bhawan^a, Klaus J. Busam^d, James T. Symanowski^b, Rhoda M. Alani^a, and Byungwoo Ryu^a

^aDepartment of Dermatology, Boston University School of Medicine, Boston, Massachusetts

^bDepartment of Cancer Biostatistics, Levine Cancer Institute, Carolinas Health Care System, Charlotte, North Carolina

^cDepartment of Dermatology, Yale University School of Medicine, New Haven, Connecticut

^dDepartment of Pathology, Memorial Sloan Kettering Cancer Center, New York City, New York

^eDepartment of Pathology, California Skin Institute, San Jose, California, USA

Abstract

There is a significant need for the development of diagnostic tools that can precisely distinguish Spitz nevi and spitzoid melanomas. Here, we report the development of a PCR-based quantitative diagnostic assay for spitzoid melanocytic lesions utilizing the expression ratio of *neuropilin-2* and *melan-A* genes in primary tumor specimens. We find that the expression ratio of *neuropilin-2*/*melan-A* is significantly increased in spitzoid melanomas compared with Spitz nevi. The diagnostic potential of this quantitative assay was validated in two independent sets of patient samples as demonstrated in a receiver operating characteristic curve analysis showing an area under the curve value of 91.8%. Furthermore, the assay was found to quantitatively distinguish the clinical nature of atypical spitzoid melanocytic lesions that were diagnostically undetermined using histopathologic criteria alone. Our data indicate that this quantitative assay may be used as a tool in determining the diagnostic classification of histologically challenging spitzoid tumors.

Keywords

atypical spitzoid melanocytic lesions; diagnostic assay; *melan-A*; *neuropilin-2*

Correspondence to Byungwoo Ryu, PhD, Department of Dermatology, Boston University School of Medicine, 609 Albany Street, J-502, Boston, MA 02118, USA Tel: + 1 617 638 5535; ryub@bu.edu. Correspondence to Rhoda M. Alani, MD, Department of Dermatology, Boston University School of Medicine, 609 Albany Street, J-507, Boston, MA 02118, USA Tel: + 1 617 638 5517; alani@bu.edu.

*Anna Eisenstein and Izabela P. Panova contributed equally to the writing of this article.

Conflicts of interest

There are no conflicts of interest.

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Introduction

Spitz nevi (SN) and spitzoid melanoma (SM) may resemble each other clinically and histologically making the diagnosis difficult and complicating the evaluation of a benign versus malignant lesion [1-3]. In fact, many cases of SM were misclassified as SN until the tumors progressed leading to recurrence, metastasis, or even death. SN generally occur in childhood, whereas SM mostly occur later in life; however, this paradigm is not always true making management of these lesions difficult [4]. Attempts to identify molecular features, such as p16 expression and DNA copy number changes that distinguish SNs from SMs, have been made with limited success [5-7]. As such, there is a need for a diagnostic tool to accurately and consistently differentiate SNs from SMs.

A recent study of neuropilin-2 (NRP2) expression by immunohistochemistry (IHC) in spitzoid melanocytic lesions (SNLs) suggests the potential use of NRP2 as a histopathological diagnostic marker for differentiating SN from malignant SMs [8]. NRP2 is a transmembrane glycoprotein acting as receptors for semaphorins, axon guidance molecules crucial for nervous system development, and for the vascular endothelial growth factor family of angiogenesis factors [9,10]. NRP2 is known to be expressed in human neoplasms and considered involving in malignant progression and metastasis [11,12]. Furthermore, NRP2 expression in human tissue specimens has been shown to differentiate benign nevi from malignant melanomas [13]. Given these findings, we sought to develop a simple diagnostic assay based on quantitative measurement of *NRP2* transcript levels using a PCR technique that may be able to distinguish SN from SMs.

Patients and methods

Patient samples

After obtaining approval for these studies by the Institutional Review Board of Boston University School of Medicine, two independent patient sample sets were assembled as test and validation sets. Formalin-fixed paraffin-embedded patient tissues (FFPE) of SN ($n = 8$) and SM ($n = 7$) obtained from Boston University Skin Pathology Laboratory and the Department of Pathology at Memorial Sloan Kettering Cancer Center, respectively were assembled as a test sample set. FFPE patient tissues of SN ($n = 22$) and SM ($n = 16$) obtained from the Skin Pathology Laboratory at Boston University, Department of Dermatology and the Yale University Spitzoid Neoplasm Repository, respectively, were assembled as a validation sample set.

Quantitative RT-PCR

Absolute quantification of *NRP2* and *melan-A* transcript levels was performed using reverse transcription PCR technique as reported in our previous study [13]. The expression ratio of *NRP2* to *melan-A* was computed to normalize the *NRP2* expression to the total number of melanocytes in the specimen, and the resulting data point was used as a representation of the level of *NRP2* gene expression in the tissue sample.

Statistical analysis

The NRP2/melan-A expression ratio data were z-score transformed for statistical analysis. Welch's *t*-test was used to compare differences in NRP2/melan-A expression ratio between SN and SM. For comparisons between the three groups, one-way analysis of variance and Tukey's post-hoc test were used. *P* values less than 0.05 were considered statistically significant. Receiver operating characteristics curve analysis was used to evaluate the predictive performance of the NRP2/melan-A expression ratio and the optimal cut-point was determined using Youden's *J*-statistic. All analyses were performed using R, version 3.3.1.

Results and discussion

The expression ratios were significantly elevated in the patient samples from SMs as compared with the SNs ($P = 0.04$) in the test set (Fig. 1a). To validate this finding and the reliability of the assay protocol as a potential diagnostic test, we analyzed an independent sample set (validation set). The diagnostic potential of the assay protocol was confirmed in the validation set. *NRP2/melan-A* expression ratio was significantly elevated in the SMs as compared with the SN ($P = 0.00078$) (Fig. 1b). Receiver operating characteristic curve analysis of the expression ratios showed an area under the curve value of 91.8% (Fig. 1c). An *NRP2/melan-A* expression ratio of -0.4 (z-score transformed) was identified as an optimal cut-point using Youden's *J*-statistic [14] with the specificity (86.4%) and the sensitivity (87.5%) demonstrating the usefulness of the assay as a potential diagnostic test.

In order to critically assess diagnostic accuracy of the assay protocol, we decided to re-evaluate the initial diagnoses of the patient tissues used in this study and reassess their correlations with the *NRP2/melan-A* expression ratios. Available tissue samples from the test and validation sets ($n = 45$, images of remaining eight samples were not available at the time of re-evaluation) were re-evaluated anonymously by five independent dermatopathologists. Diagnoses of each case were scored numerically at a scale of 1–5 (1 = benign, 2 = likely benign, 3 = undetermined, 4 = likely malignant, and 5 = malignant). The average score was used for new accurate diagnosis (SN: 2; undetermined: > 2 and < 4; and SM: 4) (Supplementary Table S1, Supplemental digital content 1, <http://links.lww.com/MR/A19>). This re-evaluation resulted in the identification of a sample group diagnosed as undetermined ($n = 9$) in addition to the anticipated groups of SNs ($n = 19$) and SMs ($n = 17$). This result demonstrated the difficulty of accurate diagnosis of SNLs by histological method alone. Reassessment of the *NRP2/melan-A* expression ratio of the samples also separated the three diagnostic groups (Fig. 2a). Values of the expression ratios for the histologically undetermined group were found to be between those of the SN and SM groups. This data suggests that the PCR-based diagnostic assay is able to accurately reflect the correct diagnoses of complex SNLs in accordance with expert dermatopathologists. To explore whether the *NRP2/melan-A* expression ratio may be able to diagnose histologically undetermined tissues, the expression ratios of the individual samples in this group were compared with those of SNs and SMs. The median *NRP2/melan-A* expression ratio of the undetermined group was 0.542 that included four patient samples (cases 8, 11, 33, and 45) with expression ratios that were lower than the median expression ratio of the benign SN group (0.321), suggesting that these cases could be categorized as likely benign SN.

Whereas, the other four cases (cases 6, 13, 22, and 37) possessed expression ratios that were closely aligned with the SM group (median: 1.132), suggesting these cases may be categorized as likely malignant SMs based on the assay (Supplementary Fig. S1 and Table S1, Supplemental digital content 1, <http://links.lww.com/MR/A19>). Although the prognosis cannot be verified in those cases because of lack of clinical outcome information, these analytical data strongly suggest that a diagnostic test based on the quantitative measurement of the *NRP2/melan-A* expression ratio would be a useful diagnostic tool to differentiate histopathologically ambiguous cases.

The RT-PCR-based assay protocol has been optimized for the absolute quantification of *NRP2* and *melan-A* transcripts in FFPE tissues. Use of the expression ratios allows for a simple assay that eliminates the need for tissue microdissection. An additional benefit of this assay is the sensitivity of the system that requires minimal amounts of histologic specimens for accurate diagnosis. Unlike histopathologic analysis, this diagnostic assay generates quantifiable numerical values by which sample to sample comparisons can be achieved in an objective manner; therefore, diagnostic utility can be objectively assessed between specimens obtained at various local clinics. Furthermore, to increase sensitivity of our assay system for clinical use, a cutoff ratio of 0.46 may be used to identify 94% of SMs biopsied. These study results suggest that this rapid, simple, quantitative diagnostic test may prove clinically useful in challenging cases of this nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Distribution patterns of *NRP2* and *melan-A* gene transcript copy number ratios in groups of tissue specimens obtained from patients with Spitz nevi and spitzoid melanomas. For statistical analysis the expression ratios were z-score transformed. (a) Box-Whisker plot analysis of a test set of patient samples. (b) Box-Whisker plot analysis of a validation set of patient samples. (c) AUC-ROC curve for the *NRP2* and *melan-A* gene expression ratio showing specificity and sensitivity as a diagnostic biomarker distinguishing Spitz nevus and spitzoid melanoma. AUC, area under the curve; ROC, receiver operating characteristic.

Fig. 2. Identification of spitzoid melanocytic lesions and their correlations with values of *NRP2/melan-A* gene expression ratios. (a) Histologic re-examination of tissue specimens by five independent dermatopathologists identifies three groups of samples: Spitz nevi (SN), spitzoid melanoma (SMs), and atypical spitzoid neoplasms (undetermined). Box-Whisker plot analysis of the NRP2/melan-A expression ratio of the three sample groups shows complete segregation between SN and SMs. Analysis was performed in a z-score transformed dataset. (b, c) Two representative cases (cases 13 and 33). Case 33 could be classified as likely benign Spitz nevus based on the diagnostic NRP2/melan-A expression ratio, whereas case 13 could be categorized as likely malignant spitzoid melanoma. (d, e) Hematoxylin and Eosin stained section of Spitz nevus (case 33) and spitzoid melanoma (case 13), respectively.