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**Original contribution**

# Neuropilin-2: a novel biomarker for malignant melanoma?☆

**Erica C. Rushing MD, MPH[a,b], Megan J. Stine PhD[a,c], Sarah J. Hahn BS[a], Sofia Shea MD[e], Mark S. Eller PhD[h], Alaa Naif MD[h], Sarika Khanna MD[a], William H. Westra MD[d], Achim A. Jungbluth MD[f], Klaus J. Busam MD[g], Meera Mahalingam MD[i], Rhoda M. Alani MD[a,c,e,h,*]**

[a]*The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Johns Hopkins University School of Medicine, Baltimore, MD 21231-1000, USA*
[b]*The Bloomberg School of Public Health, Johns Hopkins University School of Medicine, Baltimore, MD 21231-1000, USA*
[c]*Program in Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21231-1000, USA*
[d]*Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21231-1000, USA*
[e]*Department of Dermatology, Johns Hopkins University School of Medicine, Baltimore, MD 21231-1000, USA*
[f]*Ludwig Institute for Cancer Research New York, NY 10017, USA*
[g]*Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA*
[h]*Department of Dermatology, Boston University School of Medicine, Boston, MA 02118, USA*
[i]*Dermatopathology Section, Department of Dermatology, Boston University School of Medicine, Boston, MA 02118, USA*

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**Summary** Neuropilin-2, a cell surface receptor involved in angiogenesis and axonal guidance, has recently been shown to be a critical mediator of tumor-associated lymphangiogenesis. Given that lymphangiogenesis is a major conduit of metastasis in melanomas and that blocking neuropilin-2 function in vivo is effective in inhibiting tumor cell metastasis, we sought to determine the clinical relevance of neuropilin-2 expression in cutaneous melanoma. Immunohistochemical analysis of neuropilin-2 expression was evaluated in nevomelanocytic proliferations that included a tissue microarray and histologic sections from samples of primary melanomas (n = 42; 40 for tissue microarray, 2 for histologic sections), metastatic melanomas (n = 30; 22 for tissue microarray, 8 for histologic sections), and nevi (n = 30; 5 for tissue microarray, 25 for histologic sections), as well as a panel of normal human tissues and select nonmelanocytic tumors. Staining for grading and intensity of neuropilin-2 expression was estimated semiquantitatively as follows for the former: less than 20%, 20% to 60%, and more than 60% of tissue present, and for the latter from 0 to 3, with 3 being the highest and 0 the lowest intensity. In nevomelanocytic proliferations, more than 20% staining for neuropilin-2 was noted in 36 (86%) of 42 cases of primary melanoma, in 27 (90%) of 30 cases of metastatic melanoma, and in 9 (30%) of 30 cases of nevi with differences achieving statistical significance between melanoma (primary and metastatic) and nevi ($P < .0001$). For staining intensity, an intensity of 2 or more was noted in 36 (86%) of 42 cases of primary melanoma, in 17 (57%) of 30 cases of metastatic melanoma and in 7 (30%) of 23 cases of nevi, with differences achieving statistical significance between melanoma

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* Corresponding author. Boston Medical Center, Boston University School of Medicine, Boston, MA 02118-2515, USA.
*E-mail address:* alani@bu.edu (R. M. Alani).


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(primary and metastatic) and nevi ($P < .0001$). In normal human tissue, consistently strong neuropilin-2 staining was noted in kidney (glomerular endothelial cells, collecting tubules, and collecting ducts), skin (epidermal keratinocytes), and testes (epithelium of the seminiferous tubules), whereas in tumoral tissue, consistently strong staining was noted only in renal cell carcinoma but not in any of the other tumors studied. More recently, using a heterotypic coculture methodology with melanoma and endothelial cells, we have demonstrated successful up-regulation of neuropilin-2 and confirmed the critical role of neuropilin-2 in melanoma-endothelial interactions. Because these coculture methods were developed to model melanoma metastasis, the significantly increased and enhanced expression of neuropilin-2 staining in primary and metastatic melanoma versus nevi in the current study suggests that it is also relevant in vivo.


## 1. Introduction

Increased tumor vascularity and lymphatic invasion have been shown to contribute to malignant melanoma migration and metastasis [1]. In the transition to vertical growth phase, melanoma progression is heralded by the expression and release of vascular endothelial growth factor (VEGF), which facilitates the growth of both new blood vessels and the tumor itself [2]. In addition, increased lymphangiogenesis has been shown to occur with the transition to melanoma invasion and may even precede development of sentinel lymph node metastases [3-5]. Two subtypes of VEGF (VEGF-A and VEGF-C) produced by melanomas are critical for the reorganization and proliferation of endothelial cells, leading to the development of both blood and lymphatic vasculatures, which generate a route for metastatic dissemination [6,7]. Neuropilins, transmembrane glycoproteins that modulate the development of the nervous and vascular systems, function as coreceptors for the VEGF receptors and the plexins and bind 2 known ligands with distinct functions: class 3 semaphorins, which are involved in axonal guidance, and VEGF family members involved in promoting angiogenesis [8-10]. Neuropilin-2 (NRP2) is expressed by venous and lymphatic endothelial cells and can bind the lymphangiogenesis-associated ligand, VEGF-C. Blocking NRP2 function has recently been shown to inhibit tumor metastasis through effects on lymph endothelial cell migration and tumor-associated lymphangiogenesis [11]. Neuropilins are expressed in a variety of cancers [12-16]. Although NRP1 is generally expressed strongly in epithelial tumors, NRP2 is more highly expressed in tumor cells of neural origin including glioblastomas, melanomas, and neuroblastomas, in addition to osteosarcomas, bladder, pancreatic, and lung tumors [9,17], based on studies in tumor cell lines. In melanoma, exogenous expression of the NRP2 ligand semaphorin 3F in tumor xenografts has been shown to inhibit tumor cell migration and metastasis to lymph nodes and lung without significant effects on tumor cell growth, leading to poorly vascularized tumors [18].

Given the relevance of NRP2 to tumor-associated lymphangiogenesis and tumor metastasis [11] and given the critical role of lymphangiogenesis to melanoma development and progression, we sought to ascertain the potential clinical relevance of NRP2 expression in cutaneous melanoma with a view to determining its utility as a biomarker.

## 2. Materials and methods

### 2.1. Sample selection

This study was approved by the Boston University School of Medicine, Johns Hopkins School of Medicine, and Memorial Sloan-Kettering Cancer Center. Archival materials were retrieved from the pathology files of the Department of Pathology of Memorial Sloan-Kettering Cancer Center and Skin Pathology Laboratory, Boston University School of Medicine, MA. Tissues evaluated included specimen microarrays (primary cutaneous malignant melanoma n = 40, metastatic melanoma n = 22, nevi without architectural disorder, and/or atypia n = 5) as well as regular tissue sections from formalin-fixed, paraffin-embedded archival material (primary cutaneous malignant melanoma n = 2, metastatic melanoma n = 8 and nevi without architectural disorder, and/or atypia n = 25). Of the cases of primary cutaneous malignant melanoma, 37 were conventional and 5 fit into the desmoplastic melanoma category. Tissue microarrays (TMAs) also included normal tissues, various types of nonmelanocytic tumors, and cutaneous melanomas. Tissue specimens used were not selected for outcomes measurements; hence, no annotations regarding patient clinical data are included.

### 2.2. Tissue microarrays

For the preparation of TMAs, formalin-fixed and paraffin-embedded archival tissue blocks were used. Five-micrometer sections stained with hematoxylin and eosin were obtained to identify different representative areas of interest (eg, tumor cells of desmoplastic melanoma). From these defined areas, tissue cores were taken with a precision instrument (Beecher Instruments, Sun Prairie, WI). Samples with a diameter of