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Neuropilin-2: a novel biomarker for malignant melanoma?

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

[a]The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Johns Hopkins University School of Medicine, 1650 Orleans Street, Baltimore, MD 21231-1000

[b]The Bloomberg School of Public Health, Johns Hopkins University School of Medicine, 1650 Orleans Street, Baltimore, MD 21231-1000

[c]Program in Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, 1650 Orleans Street, Baltimore, MD 21231-1000

[d]Department of Pathology, Johns Hopkins University School of Medicine, 1650 Orleans Street, Baltimore, MD 21231-1000

[e]Department of Dermatology, Johns Hopkins University School of Medicine, 1650 Orleans Street, Baltimore, MD 21231-1000

[f]Ludwig Institute for Cancer Research New York, 1275 York Avenue, New York, NY 10021

[g]Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021

[h]Department of Dermatology, Boston University School of Medicine, 609 Albany Street, Boston, MA 02118

[i]Dermatopathology Section, Department of Dermatology, Boston University School of Medicine, 609 Albany Street, Boston, MA 02118

Abstract

Neuropilin-2 (NRP2), 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 NRP2 function *in vivo* is effective in inhibiting tumor cell metastasis, we sought to determine the clinical relevance of NRP2 expression in cutaneous melanoma. Immunohistochemical analysis of NRP2 expression was evaluated in nevomelanocytic proliferations that included a tissue microarray (TMA) and histologic sections (HS) from samples of primary melanomas (n=42; 40 TMA, 2 HS), metastatic melanomas (n=30; 22TMA, 8 HS) and nevi (n=30; 5 TMA, 25HS), as well as a panel of normal human tissues and select non-melanocytic tumors. Staining for grading and intensity of NRP2 expression was estimated semi-quantitatively as follows for the former: <20%, 20-60% and >60% of tissue present and, for the latter from 0-3 with 3 being the highest and 0 the lowest

*Corresponding Author: Herbert Mescon Professor and Chair, Dermatologist-in-Chief, Boston University School of Medicine, Boston Medical Center, 609 Albany Street, J-507, Boston, Massachusetts 02118-2515, Phone: 617-638-5517, Fax: 617-638-5515, alani@bu.edu.

intensity. In nevomelanocytic proliferations, >20% staining for NRP2 was noted in 36/42 cases (86%) of primary melanoma, in 27/30 cases (90%) of metastatic melanoma and in 9/30 cases (30%) of nevi with differences achieving statistical significance between melanoma (primary and metastatic) and nevi ($p<0.0001$). For staining intensity, an intensity of 2 or more was noted in 36/42 cases (86%) of primary melanoma, in 17/30 cases (57%) of metastatic melanoma and in 7/23 (30%) of nevi with differences achieving statistical significance between melanoma (primary and metastatic) and nevi ($p<0.0001$). In normal human tissue, consistently strong NRP2 staining was noted in kidney (glomerular endothelial cells, collecting tubules and collecting ducts), skin (epidermal keratinocytes) and testes (epithelium of the seminiferous tubules), while 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 co-culture methodology with melanoma and endothelial cells, we have demonstrated successful up-regulation of NRP2 and confirmed the critical role of NRP2 in melanoma-endothelial interactions. Since these co-culture methods were developed to model melanoma metastasis, the significantly increased and enhanced expression of NRP2 staining in primary and metastatic melanoma *versus* nevi in the current study suggests that it is also relevant *in vivo*.

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 co-receptors for the vascular endothelial growth factor receptors and the plexins and bind two known ligands with distinct functions: class 3 semaphorins, which are involved in axonal guidance; and vascular endothelial growth factor (VEGF) family members involved in promoting angiogenesis.(8-10) Neuropilin-2 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 lymphendothelial cell migration and tumor-associated lymphangiogenesis.(11) Neuropilins are expressed in a variety of cancers.(12-16) While 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 (Sema 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 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.

Materials and Methods

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 non-melanocytic tumors and cutaneous melanomas. Tissue specimens used were not selected for outcomes measurements hence no annotations regarding patient clinical data are included.

Tissue microarrays

For the preparation of TMAs formalin fixed and paraffin-embedded archival tissue blocks were used. 5 -um sections stained with hematoxylin and eosin were obtained to identify different representative areas of interest (e.g. tumor cells of desmoplastic melanoma). From these defined areas, tissue cores were taken with a precision instrument (Beecher Instruments, Sun Prairie, Wis). Samples with a diameter of 0.6 mm from each specimen were punched and arrayed in triplicate on a recipient paraffin block.

Immunohistochemistry

Immunohistochemistry was performed using the EnVision System HRP (DakoCytomation). The slides were deparaffinized and rehydrated using a graded alcohol series. Citrate buffer (pH6.0, 10mM) was used for antigen retrieval. Using the capillary gap method, the sections were incubated overnight with rabbit polyclonal antibodies against NRP-2 (SC-5542, Santa Cruz Biotechnology). A dilution of 1:50 was found to provide the optimum staining results. 3-amino-9-ethyl carbazole (AEC) was used as a chromogen and the sections were counterstained with hematoxylin.

Staining of both TMA slides and slides with regular tissue sections were graded semi-quantitatively as follows: less than 20%, 20-60%, or greater than 60% of the tissue present. The intensity of neuropilin-2 staining was also scored from 0 to 3 with 0 having no NRP-2 staining and 3 having the highest intensity. The TMA slides were scanned and digitized using the Bacus Labs Inc. Slide Scanner (BLISS, Bacus laboratories, Lombard, IL). The images were uploaded into the TMAJ database for evaluation.(19) FRamework for Image Dataset Analysis (FRIDA)(20), a custom open source image analysis software package (available at http://sourceforge.net/projects/fridajhu/), was used for image analysis of TMAs. Hue Saturation and Brightness (HSB) segmentation ranges for red staining and hematoxylin alone (nuclei not staining red) were defined from the tissue microarray image set. Using the specific color pixel definitions for “total tissue”, “positive neuropilin-2 staining tissue”, “stained nuclei”, and “remaining tissue”, the Java software program analyzed images with the selected color pixels to quantify positive staining in the “tissue area”, “nuclei”, and the “NRP-2 area”. Since nuclei were not expected to be stained according to the preliminary testing studies, and nuclei can be very large in tumor cells, the remaining tissue area that was expected to stain for NRP-2 was redefined as tissue that is in the “tissue area” but not in the “nuclei” and subsequently labeled “cytoplasm”. By redefining the total tissue area

without nuclei, a more accurate calculation based on total possible staining area for neuroplin-2 was established. The percentage of staining was calculated by the FRIDA program as the “NRP-2 area”/“cytoplasm” (total tissue area without nuclei).

Statistical Analysis

The results of the FRIDA computer analysis along with the pathologist evaluations were analyzed using the R version 2.6 statistical software program.(21) FRIDA analysis allows for digital analysis of tissue staining. In this case, since NRP2 is a cytoplasmic/cell surface protein, computer analysis of stained tissue specimens was performed after subtracting out the nuclei from all images. The percent staining figure, as determined by FRIDA imaging is the % of tissue which was deemed to be “positive” for the biomarker in question. Since FRIDA is unable to calculate staining intensity independent of % tissue staining, the background level of staining was set to a standard of ++ or greater which is the reason why the % positive staining of tissues by FRIDA analysis is lower than the % staining by reviewer score. For purposes of statistical analyses for grading, lesions with <20% of lesional tissue staining were considered negative and scores of 20% or more were considered positive, while for intensity, those with a sore of 0 or 1 were counted as negative and those with a score of 2 or 3 considered positive. A Welch two sample *t*-test with unequal variances was used to statistically evaluate the FRIDA neuropilin-2 staining differences between melanocytic and non-melanocytic tumors. Overall, the Fisher's exact test was used to determine differences of significance in expression of NRP2 amongst the categories studies. A two-tailed p value of <0.05 was considered to be statistically significant.

Results

Normal tissue

Summary of qualitative immunohistochemical analysis of NRP2 staining for TMAs of normal tissues is detailed in Table 1. NRP2 staining was noted in liver, kidney, fallopian tubes, pancreas, placental tissue, testis, prostate, striated muscle cells, specimen specific breast ductal tissue, skin epidermis, spleen, and endometrial tissue (Figure 1). Briefly, mild NRP-2 staining was noted in the normal liver with scattered hepatocyte staining and glandular epithelial cells of the prostate; moderate NRP-2 staining was noted in striated muscle cells; intermittent/selective staining was noted in mucosal lining cells of fallopian tubes, syncytiotrophoblast cells of the placental villi, fetal capillaries within the villous cores, breast duct epithelial cells, endometrial tissue stroma cells and glandular cells; and strong staining was noted in glomerular endothelial cells, collecting tubules and collecting ducts of renal tissue, epidermis of the skin and epithelium of the seminiferous tubules in tissue from testes. All other tissue types were negative for NRP2.

Nevomelanocytic proliferations

Immunohistochemical staining for Neuropilin-2 of TMA and individual histologic sections of primary malignant melanomas, metastatic melanomas and nevi are summarized in Tables 2 and 3.

Primary melanoma

NRP2 staining of >60% or more was noted in 36/42 cases of primary cutaneous malignant melanoma at an intensity of 2 (3/42 cases) and 3 (33/42 cases). Of the 6 cases that exhibited <20% tissue staining with NRP2, 5 were desmoplastic malignant melanoma. In keeping with this, the FRIDA analysis for primary cutaneous malignant melanomas stained for NRP2 showed a mean for all the tissues analyzed of 46.9%, while desmoplastic malignant melanoma had the least percentage stained with an average of 8.5% (Figure 2).

Metastatic melanoma

NRP2 staining of >20% or more was noted in 10/30 cases and >60% in 17/30 cases at an intensity of 2 (6/30 cases) and 3 (11/30 cases). The FRIDA analysis of metastatic melanomas showed a mean of 45.4%, similar to that of primary cutaneous malignant melanoma. In order to confirm that expression of NRP2 was limited to melanoma cells seen in the metastatic setting, tumor specimens were evaluated for NRP2 and Melan-A. Expression of NRP2 matched that of Melan-A in metastatic melanomas suggesting specific expression of NRP2 in these cells (Figure 3).

Nevi

NRP2 staining of >20% or more was noted in 8/30 cases and >60% in 1/30 cases at an intensity of 2 (7/30 cases). Suprabasal keratinocytes stained positively for NRP2 (Figure 4). No NRP2 staining was noted in normal melanocytes within the epidermis.

Non-melanocytic tumors

Immunohistochemical staining for NRP2 was evaluated in a variety of tumors (Table 4, Figure 5). Overall, 7/14 adenocarcinomas exhibited mild/intermittent NRP2 staining (5 breast primary and 2 colonic primaries), 2/3 leiomyosarcomas exhibited NRP2 staining, 2/3 cases of transitional cell carcinoma exhibited mild NRP2 staining and 4/5 cases of renal cell carincoma exhibited strong positive staining with NRP2. All ovarian mucinous, ovarian serous, lung adenocarcinoma, liposarcomas, spindle cell sarcomas, non-small cell lung cancer (squamous cell carcinoma), and malignant fibrous histiocytoma cases were negative for NRP2.

The FRIDA computer analysis of the variety of tumors indicated the mean percentage of all stained tumor tissues was 10.4%. Renal cell carcinoma had the highest mean percentage stained with 49.9%. The computer analysis of the remaining positive neuropilin-2 tumors calculated the average percentage stained as follows: breast carcinoma ductal – 5.1%, breast carcinoma lobular – 2.9%, colon adenocarcinoma – 3.7%, leiomyosarcoma – 9.9%, transitional cell carcinoma – 9.7% (Table 2).

A Welch two sample *t*-test with unequal variances comparing the FRIDA results for melanocytic and non-melanocytic neuropilin-2 expression was performed using the R statistical software package.(21) The melanocytic tumors had a mean percentage NRP2 staining of 40% versus the non-melanocytic tumor mean of only 10%. The difference in the means was 30%, and the 95% confidence interval for the difference in percentage stained was (23.6, 35.5). The difference in the means was found to be statistically significant ($p<0.0001$).

Discussion

Neuropilin-2 has been demonstrated to play a major role in the development of the normal lymphatic vasculature(22) and recent studies suggest that blocking of NRP2 binding to VEGF-C inhibits tumor cell metastasis.(11) Since melanoma progression and metastasis are intimately linked to the process of lymphangiogenesis(23), we evaluated the expression of the lymphangiogenesis-associated receptor, Neuropilin-2, in primary cutaneous malignant melanoma. In these studies we have found elevated NRP2 expression in melanomas *versus* other tumor types (P<.0001). Previous studies have suggested an association between NRP2 expression and tumor prognosis in bladder and lung cancers, with elevated expression of NRP2 associated with a worse prognosis(17,24,25). Our current data suggest that NRP2 may also serve as a prognostic indicator in patients with melanoma given the relative lack of expression in nevi. Favoring this further is the low expression of NRP2 in desmoplastic

melanomas which generally confer a more favorable prognosis versus other histologic subtypes of melanoma.(26) Our tissue microarray panel of normal tissue confirmed previous studies that showed that NRP2 receptors are found in glomeruli, islet cells of the pancreas, and skin.(27-29) We also found unexpected staining patterns in the primary human tissues evaluated in these studies, including a notably high expression in testis as well as staining in striated muscle, liver, subrabasal epidermal keratinocytes, placenta, fallopian tubes, endometrium, breast, spleen and prostate. This associated elevated NRP2 expression in both melanomas and testis is particularly notable as melanomas are notorious for their expression of testis antigens, and NRP2 provides an additional link between these two cell types.(30)

Of all the tumors studied, only renal cell carcinoma (clear cell) stained strongly positive for NRP2 with greater than 20% of the tissue staining, which is not surprising as normal renal tissue stains strongly positive for NRP2 in renal glomeruli and tubules.(28) All other tumors that stained NRP2 positive were of low intensity, with <20% stained, which is significantly lower than the majority of melanomas evaluated.

More recently, using a heterotypic co-culture methodology with melanoma and endothelial cells, we have demonstrated successful up-regulation of NRP2 and confirmed the critical role of NRP2 in coordinated cell patterning and growth. (32). Thus, NRP2 represents an important mediator of melanoma-endothelial interactions. (32) Since these co-culture methods were developed to model melanoma metastasis, the significantly increased and enhanced expression of NRP2 staining in primary and metastatic melanoma *versus* nevi in the current study suggests that it is also relevant *in vivo*. In addition, given the differential expression of NRP2 in benign and malignant melanocytic tumors, we suggest that NRP2 may be a useful prognostic biomarker in melanoma. Given that NRP2 can be expressed in a secreted form (31), detection of this secreted protein may also be useful as a surrogate melanoma marker for the identification of patients with occult metastatic disease.

Diagnosis of melanoma can be difficult as there is histologic overlap between benign and malignant lesions which can lead to both over- and under-diagnosis. In addition, determining the prognosis for a particular patient using current clinical criteria may be imprecise. The most useful prognostic indicators of primary cutaneous melanomas are Breslow depth and presence or absence of ulceration. However, many patients with thick melanomas are free of metastasis, while others with thin tumors die early from their disease. Despite numerous investigations to date, there are currently no adequate methods to accurately identify which melanomas will progress to vertical growth and metastasis, and the need for useful prognostic biomarkers in melanoma is great. In this current manuscript we demonstrate significant expression of the cell surface receptor, NRP2, in human melanomas with minimal expression of NRP2 in benign melanocytic nevi and absent expression in normal human melanocytes. Large-scale studies of NRP2 in melanocytic tumors with outcome analyses will need to be undertaken to determine the prognostic significance of this biomarker in melanocytic tumors.

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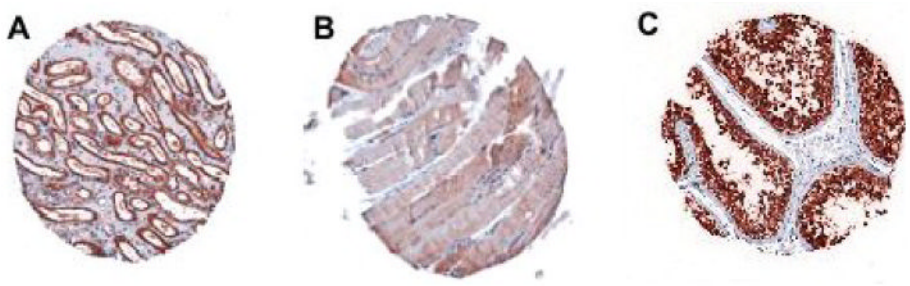

Figure 1. Representative staining for Neuropilin-2 in normal human tissues

A. Normal Kidney

B. Striated Muscle

C. Testis

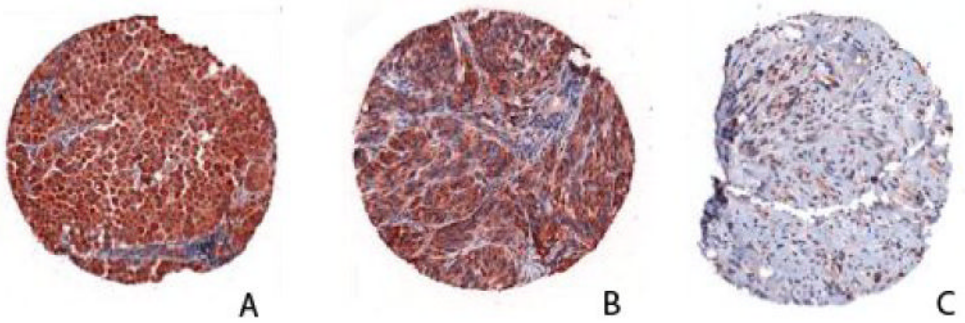

Figure 2. Representative staining for Neuropilin-2 in melanoma (primary and metastatic)

A. Metastatic melanoma

B. Primary cutaneous malignant melanoma

C. Desmoplastic malignant melanoma

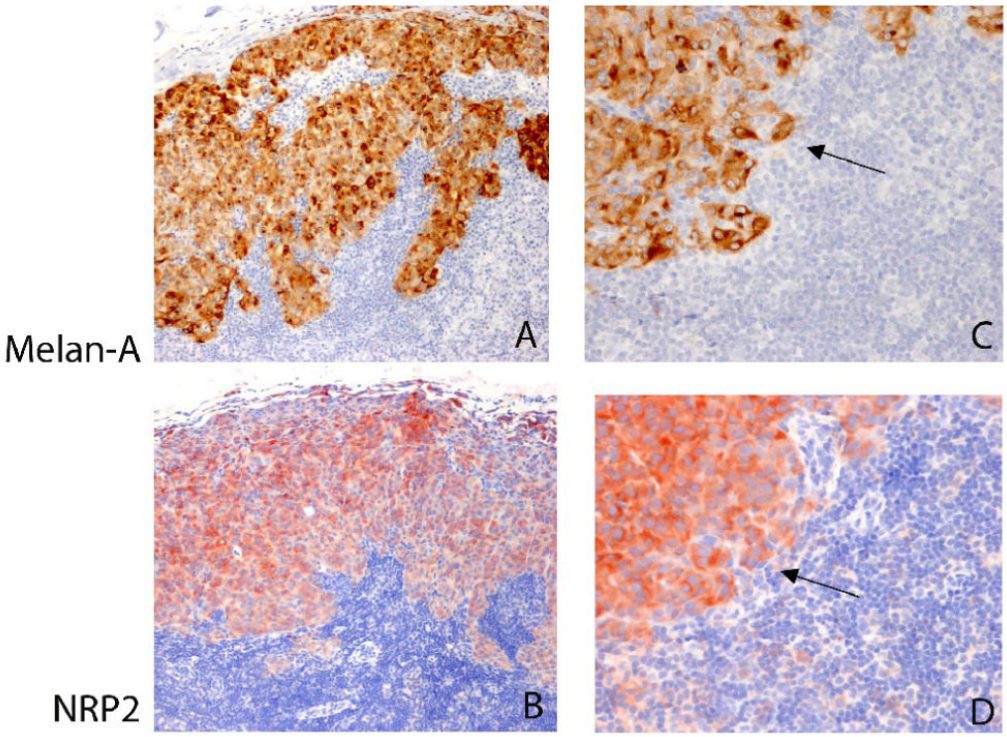

Figure 3. NRP2 expression is limited to metastatic melanoma cells

A, C. Low- (A) and high- (C) power of Melan-A stained metastatic melanoma cells within a lymph node.

B, D. Low- (A) and high- (D) power of NRP2 stained metastatic melanoma cells within a lymph node (arrow highlighting matched populations stained by Melan-A and NRP2).

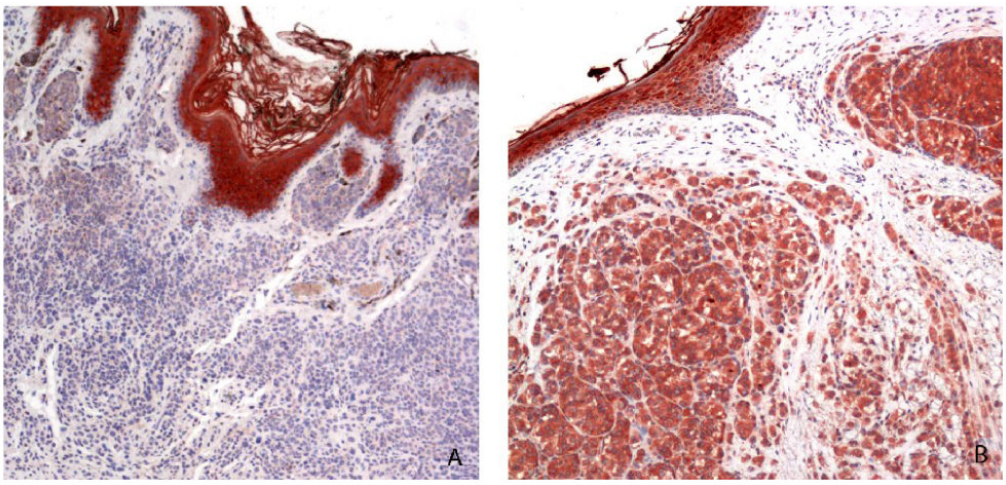

Figure 4. NRP2 staining in nevi and melanoma

A. Nevus, Note suprabasal expression of NRP2 (Red) in the epidermis and lack of staining of normal melanocytes.

B. Melanoma.

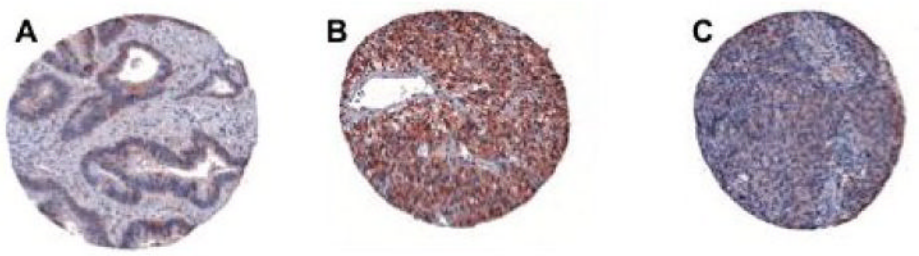

Figure 5. Representative staining for Neuropilin-2 in non-melanocytic tumors

A. Colon adenocarcinoma

B. Renal cell carcinoma (Clear Cell)

C. Ductal breast carcinoma

Table 1
Tissue Microarray Immunohistochemical Analysis of Normal Tissues with NRP2

Normal Tissues	Average NRP-2 percent staining	Intensity	Cases Positive/Cases Examined
Esophagus	-		0/2
Stomach	-		0/1
Small Bowel	-		0/2
Appendix	-		0/4
Colon	-		0/2
Gallbladder	-		0/1
Lung	-		0/7
Parotid	-		0/2
Omentum	-		0/2
Thymus	-		0/2
Adrenal	-		0/4
Lymph node	-		0/1
Bladder	-		0/3
Vaginal tissue	-		0/1
Thyroid	-		0/3
Amnion	-		0/2
Tonsil	-		0/2
Endometrial	+	low	3/3
Pancreas	+	low	2/2
Prostate	+	moderate	2/2
Spleen	+	moderate	3/4
Breast	+	moderate	2/3
Muscle	++	moderate	3/3
Fallopian tube	++	moderate	2/2
Liver	++	moderate	2/2
Skin	++	high	2/3
Placenta	++	high	2/2
Kidney	++	high	3/3
Testes	+++	high	3/3

- Negative; +, <20% of tissue positive; ++, 20 to 60% of tissue positive; +++, >60% of tissue positive

Table 2

Tissue Microarray immunohistochemical Analysis of Malignant Melanomas and Metastatic Melanomas with NRP-2

Diagnosis	NRP2 staining	Intensity	Proportion Positive	Computer mean (%)
Primary cutaneous melanoma	+++	moderate/high	34/40	46.9
Primary cutaneous melanoma	+	low	6/40	8.5
Metastatic melanoma	+++	moderate/high	13/13	57.1
Metastatic melanoma	++	low	8/9	22.2

- Negative; +, <20% of tissue positive; ++, 20 to 60% of tissue positive; +++, >60% of tissue positive

Table 3

Immunohistochemical Analysis of Primary Melanomas, Metastatic Melanomas, and Nevi (TMA and tissue samples)

Tumor Type	Average Staining		Cases	
	area	intensity	positive	total
Primary melanoma	+++	+++	36	42
Metastatic melanoma	+++	++	27	30
Nevi	++	++	9	30

Staining (area): +, <20%; ++ 20-60%; +++, > 60% staining (intensity): +, low; ++, moderate; +++, high. A case was considered positive if the staining area was ≥ 20%. Average staining area and intensity were calculated only from cases considered positive based on ≥ 20% area stained.

Table 4

Tissue Microarray Immunohistochemical Analysis for Neuropilin-2 Staining of Various Non-Melanocytic Tumors

DiagnosisNRP2 staining	Proportion positive	Computer Mean + (%)	
Breast carcinoma, lobular	+	2/5	2.9
Breast carcinoma, ductal	+	3/5	5.1
Leiomyosarcoma	+	2/3	9.9
Ovarian mucinous	-	0/1	-
Ovarian serous	-	0/4	-
Colon adenocarcinoma	+	2/4	3.7
Transitional cell carcinoma	+	2/3	9.7
Lung adenocarcinoma	-	0/1	-
Liposarcoma	-	0/4	-
Spindle cell sarcoma	-	0/1	-
Malignant fibrous histiocytoma	-	0/4	-
Non-small cell lung ca.(squamous)	-	0/1	-
Renal cell carcinoma (clear cell)	+++	4/5	49.9

- Negative; +, <20% of tissue positive; ++, 20 to 60% of tissue positive; +++, >60% of tissue positive