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Neurotrophin receptors in select cutaneous malignancies with a propensity for perineural invasion

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NEUROTROPHIN RECEPTORS IN SELECT CUTANEOUS MALIGNANCIES WITH A PROPENSITY FOR PERINEURAL INVASION

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ABSTRACT

Perineural invasion (PNI) in cutaneous squamous cell carcinoma (cSCC) and desmoplastic melanoma (DM) may be a negative prognostic finding, and likely contributes to increased rates of local recurrence. The biological mechanisms underlying PNI remain unclear, although several lines of evidence implicate neurotrophins and their receptors. Expression of the high affinity nerve growth factor (NGF) receptor TrkA has been shown to be associated with PNI in numerous malignancies, although literature in cutaneous neoplasms is sparse. Given this, we sought to ascertain the incidence of PNI in a cohort cSCCs using double immunostaining (DIS), and to investigate PNI's relationship with TrkA expression and established histopathologic prognosticators. In DMs we investigated the relationship between TrkA and PNI. In DM we additionally analyzed expression of the low affinity NGF receptor (p75NGFR) and the presence of a functional polymorphism in the glial cell line-derived neurotrophic factor (GDNF) receptor RET (RETp) as they relate to PNI.

In this IRB approved study, cSCCs from the head and neck (H&N) and 53 from non-H&N areas were immunohistochemically analyzed for PNI (DIS with S100 and p63) and TrkA expression. For DM, 43 cases were immunohistochemically evaluated for

TrkA and p75NGFR expression while RETp was detected by direct DNA sequencing. The presence of each was correlated with histologically observed PNI.

In cSCCs, comparing H&N versus non-H&N areas; using hemotoxylin and eosin (H&E) PNI was detected in 11% versus 6% of cases respectively and using DIS, in 23% versus 15% respectively, with significant disagreement between both methods (κ=0.47, p=0.002). There was a 2.33 fold increase in PNI detection with DIS compared to H&E (95%CI: 1.12–4.87; p=0.02). TrkA expression was 2.9 times more frequently observed in cSCCs from the H&N compared to those from non-H&N areas (p=0.01). Regardless of site, TrkA expression was associated with decreased degree of differentiation (OR=6.46, p=0.0006) and high-risk morphologic variants (OR = 6.53, p=0.002). TrkA expression was not significantly associated with PNI (p=0.33).

In DM, PNI was present in 67% of cases. On univariate analysis; p75NGFR was associated with PNI (expression detected in 79% of PNI-positive cases compared to 36% of PNI-negative cases, p=0.005), increased Breslow's depth and greater Clark's Level (p= 0.007 and p= 0.01 respectively). RETp was noted in 28% of cases but was not significantly associated with PNI (p=0.27) or other histopathologic variables. TrkA expression was absent in all cases. PNI was associated with increased Breslow's depth and Clark's Level (p=0.01 and p=0.009 respectively). Controlling for the association between p75NGFR and depth, p75NGFR remained associated with an increased propensity for PNI (OR=4.68, p=0.04).

In conclusion, increased PNI detection with DIS in cSCCs underscores the adjunctive utility of immunohistochemistry in microstaging. Although unlikely to play a role in the development of PNI, TrkA's association with cSCCs from H&N and select histopathologic parameters suggests a role for the NGF-TrKA axis in tumorogenesis while its absent expression in DM suggests that expression is lineage-related. Lastly, In DM, p75NGFR expression is significantly associated with PNI and a more locally aggressive phenotype.

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LIST OF ABREVIATIONS

AJCC	American Joint Committee on Cancer
CI	Confidence Interval
cSCC	Cutaneous Squamous Cell Carcinoma
GDNF	Glial Cell Line Derived Neurotrophic Factor
DIS	Double Immunostaining
DM	Desmoplastic Melanoma
DNM	Desmoplastic Neurotropic Melanoma
H&E	Hematoxylin and Eosin
H&N	Head and Neck
IHC	Immunohistochemistry
NM	Neurotropic Melanoma
OR	Odds Ratio
PNI	Perineural Invasion
RETp	Functional RET polymorphism

INTRODUCTION

1.1 Perineural invasion in cancer

Perineural invasion (PNI) refers to infiltration of the perineural sheath by tumor cells with subsequent tumor proliferation and spread, either proximally or distally along the length of the nerve.[1] Best studied in carcinomas of the pancreas and prostate, PNI has long been understood be an important route of tumor spread.[2] However PNI has been reported in a multitude of lineage-unrelated malignancies. The first recorded observation of PNI in a cutaneous malignancy was reported by Neumann in 1862.[3] Since then, PNI has been reported in multiple cutaneous malignancies including desmoplastic melanoma (DM), basal cell carcinoma, microcystic adnexal carcinoma and cuteanous squamous cell carcinoma (cSCC).[1,4–6] Of these, PNI is best studied in cSCC and DM both of which are known to have a marked propensity for this invasive feature.

1.2 A note on definitions

While the definition of PNI as tumor cells present within the perineural sheath appears straightforward at first glance, in practice there are inconsistencies in the reporting of PNI. In cSCC, the main point of contention centers on differentiating between true PNI and focal abutment of tumor on nerve (when the nerve is surrounded by tumor but not invaded).[7] While originally defined in SCC by Batsakis et al. in 1985 as "Tumor cell invasion in, around and through the nerves",[8] which would include focal abutment, recent definitions have become more stringent. In her 2009 review, Liebig suggested that PNI should be defined as "finding tumor cells within any of the 3 layers of

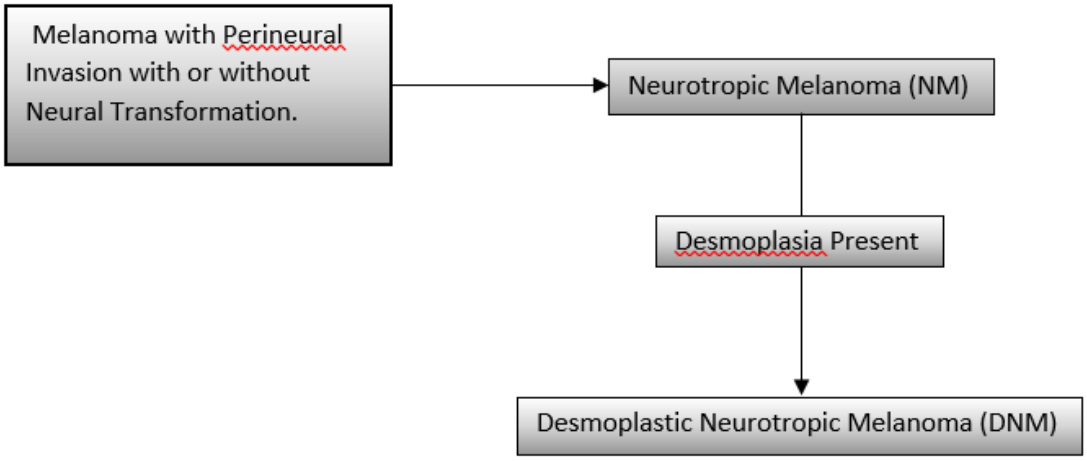
the nerve sheath or at least 33% of the circumferences of the nerve surrounded by tumor cells".[2] Even more stringently, some authors propose that only observing tumor cells within the perineural sheath should be classified as PNI.[9] and for our purposes we have chosen to use this definition when reporting PNI in our cases.

In melanoma a wholly separate problem presents itself when defining PNI. Namely, the conflation of the terms "Neurotropism or Neurotropic" with PNI. The term neurotropism was initially used by Reed and Leonard in 1979 to describe a subset of DMs that displayed prominent "Schwannian differentiation" and/or extensive PNI, which they termed neurotropic melanoma (NM).[10] However, it should be noted that "neurotropic melanoma" may be used to describe any melanomas that exhibit the aforementioned characteristics, and there are reports of both epithelioid and spindle cell melanoma (SCM) without desmoplasia being classified as NM.[9,11]As a result, the label of desmoplastic neurotropic melanoma (DNM) has been used in the case of DMs that present with Schwannian differentiation and/or PNI while NM is reserved for those cases without desmoplasia. Further, because neurotropism can be used to describe both PNI and the distinct cytomorphologic feature of Schwannian differentiation (defined as melanoma cells taking on the cytology of nerve), confusion may result when attempting to determine its prognostic significance.[11,12]

In an effort to reduce the equivocality of the nomenclature, we suggest the use of the term PNI as opposed to neurotropism when describing the histopathological observation invasion of peripheral nerve by melanoma cells. This usage is consistent with the terminology employed in describing other cutaneous malignancies.[1] Hence,

melanomas that display PNI are termed NM, and more specifically DMs that display the same are termed DNMs (Figure 1). In the rare instances where PNI is not present but the melanoma displays a clear neural cytomorphology we agree with the recent suggestions of Su et al. in their case report of such a lesion, that these tumors should be classified as distinct from NM/DNMs, the authors suggested the use of the term "malignant neurotization".[12] Alternatively, the term neural transformation has been used to describe such lesions as well.[13] The importance of properly distinguishing between NM and DNM so-named for prominent PNI vs. those named for neural transformation is more than just semantic. Neurotropism as PNI is likely to have a different prognostic implication than neurotropism as neural transformation, and at least one study has demonstrated this.[13] A clearer understanding of the prognostic relevance of neurotropism would result from separating PNI from neural transformation in diagnosing NM and DNM.

Figure 1. Classification of neurotropic, and desmoplastic neurotropic melanoma.



1.3 Incidence of PNI in cutaneous malignancies

The incidence of PNI varies substantially across cutaneous malignancies. Within a given malignancy there may be further variations in incidence, dependent on histopatholiogic subtype. As a result, reported rates of PNI in the literature span a broad range. In cSCC the reported incidence of PNI varies substantially, with a range of 2-14%, though most studies report rates between 2% and 5%.[4,14,15] Furthermore, It has been suggested that rates of PNI are higher in cSCCs of the head and neck (H&N) compared to other anatomic sites which may confound reports past reports.[16] Comparisons of PNI incidence are complicated by multiple factors, that include the challenges associated with detection of PNI using H&E staining (obscured nerve and morphologically imperceptible tumor cells), lack of a standardized histological definition of PNI (delineating true PNI from focal abutment secondary to impingement of nerve by tumor), differences in detection rates between cryostat and formalin-fixed paraffin embedded tissue sections (Mohs micrographic surgery versus traditional histopathologic examination) and histological mimics such as Renault bodies, perineural fibrosis, reactive neuroepithelial aggregates and reparative perineural hyperplasia. [7,15,17–21] Lastly, in select cutaneous and non-cutaneous malignancies it has been shown that the use of immunohistochemistry (IHC) can aid in the detection of PNI, especially in the tumor bulk where nerve can be obscured by tumor (intratumoral PNI).[9,17,18,22] Such observations indicate that the true incidence of PNI in cSCC may be greater than what has been reported using H&E alone.

The reported incidence of PNI in melanoma varies substantially across studies. While it is well understood that the greatest rates of PNI are seen in the DM variant, the

conflation of PNI with neural transformation in the reporting of both NM and DNM makes it difficult to get a precise figure. In their 1998 study in 280 cases of desmoplastic melanoma, Quinn et al. identified 30% of cases as the DNM variant.[23] Although, what proportion of cases were diagnosed as DNM for PNI as compared to exclusively neural transformation was not specified. Busam et al. reported that of 98 cases of DM, 53% displayed nerve involvement.[5] The highest reported rate of 82%, occurred in a study of 28 DMs of the head and neck, suggesting that anatomic location may be of relevance in determining incidence.[24] There may be further variation in incidence within DM subtypes (pure vs mixed), with one study reporting that rates of neurotropism varied significantly, with a higher incidence in the pure subtype (43.9%) vs the mixed subtype (27.1%).[25] The incidence of PNI in non-desmoplastic melanoma is substantially less. Scanlon et al. report that a query of 1,984 primary melanoma cases from the Interdisciplinary Melanoma Cooperative Group at NYU found an overall incidence of 3%.[9] However, this would include melanomas of all subtypes (both DMs and non-DMs). It was noted that cases of DM and acral lentiginous melanoma (ALM) were overrepresented in the PNI positive group.

1.4 Prognostic Impact of PNI in cSCC and DM

The prognostic relevance of PNI in both cSCC and melanoma remains equivocal. In most malignancies in which it is observed (both cutaneous and non-cutaneous) PNI is associated with a more aggressive phenotype, and increased risk of recurrence.[2] In select

malignancies (pancreatic and prostate carcinomas) PNI may be associated with increased mortality.[26,27]

In cutaneous malignancies, the prognostic impact of PNI has best been studied in cutaneous squamous cell carcinoma (cSCC). In 2010, based on a confluence of evidence from numerous prospective cohort studies, the American Joint Committee on Cancer (AJCC) added PNI as a high-risk tumor characteristic in tumor staging.[28] The rationale for inclusion was drawn from Geopfert et al.'s findings in a retrospective review of 967 cSCCs that PNI was linked to metastasis and reduced survival,[14] as well as a study from Leibovitch et al. of 1,177 cSCCs demonstrating an association with recurrence, decreased degree of differentiation, and larger tumor diameter.[4] Further support for the AJCC's use of PNI in tumor staging comes from Cherpelis et al.'s investigation of 200 cSCCs in which a correlation with metastasis was found,[29] Clayman et al.'s analysis of 277 cases linking PNI to decreased disease-specific survival[30] and Moore et al.'s finding in 193 patients with cSCC of the H&N area that PNI is associated with lymph node metastases.[31] The above, combined with the prevalence of cSCC as the second most common cutaneous malignancy underscore the clinical relevance of PNI identification.[32]

Recently, subtleties that have emerged from investing PNI's utility as a prognosticator in cSCC. Namely, the importance of nerve diameter and presence of multiple foci of nerve involvement in determining outcomes of patients with PNI in cSCC.[33] At least two studies have reported that in cSCC, PNI in nerves with a diameter >0.1mm is associated with worse outcomes (metastasis and recurrence) while involvement of nerves <0.1mm was not associated with negative outcomes.[33,34]

However, Lin et al. report that the diameter of the involved nerve had no impact on outcomes in cSCC.[35] Additionally, Carter et al. also report that the involvement of multiple nerves is associated with risk factors that portend worse outcomes when compared to solitary foci of PNI.[34] While, Lin et al. similarly found that patients with focal PNI had better outcomes than those who had diffuse PNI with multiple foci of invasion.[35]

A definitive understanding of PNI's prognostic relevance in melanoma remains to be established. As mentioned previously, discordant definitions of neurotropism serve as a barrier to developing a comprehensive understanding of PNI as a potential prognostic indicator in its own right. Initially neurotropism was understood to be a negative prognostic finding, with Baer *et al*. finding a significant decrease in 8 year overall survival when comparing both NM and DNM to their non-neurotropic counterparts.[11] Soon after, in the largest study to date (280 cases), neurotropism was correlated with a two fold increase in local recurrences but was not associated with survival.[23] In both of these studies, the potential for "skip lesions" in which the tumor extension along the nerves results in falsely negative margins in biopsies was hypothesized to be the cause for the increase in local recurrence. Since then, the majority of studies have reported that neurotropism is not of significant prognostic relevance.[36,37,24,25] (summarized in chronologic order in Table 1), although recently Scanlon *et al*. reported that PNI is associated with increased rates of local recurrence in both DM and non-DM cases.[9] Future studies that attempt to determine the prognostic relevance of PNI would benefit

greatly by laying out an explicit definition of neurotropism as PNI. Additionally, To our knowledge, the prognostic effects of nerve diameter and multiple foci of nerve involvement have not been investigated in melanoma. Future research into these variables would significantly enhance our understanding of the role PNI plays in affecting outcomes.

While the prognostic value of PNI remains equivocal, special care should be taken with cases of DNM/NM from the head and neck as anatomic location may significantly impact the prognostic influence of PNI. The involvement of cranial nerves may result in significant malignant neuropathies,[13] and potentially increase the risk of metastasis to the central nervous system.[16] In melanomas, perineural spread has been observed to occur beyond the skull base as far as the brain.[16]

Table 1. Chronologic overview of studies reporting prognostic impact of neurotropism in melanoma

Author/ Year	**Samples Studies (n)**	**Findings**	**Conclusion**
Smithers *et al.* 1992	DM (58)	Visceral recurrence in tumors with neurotropism was less frequent than in those neural transformation. Local recurrence rate appeared to be higher in tumors with neural transformation than in tumors displaying neurotropism.	Neural transformation is more predictive on local recurrence than neurotropism.[13]

Baer *et al.* 1995	NM (5) DNM (11)	The 8 year survival rate was 90% in patients without neurotropism and 60% in those with neurotropism. Local recurrence occurred in 36% of DNMs and 20% of NMs.	Neurotropism is a negative prognostic indicator.[11]
Quinn *et al.* 1998	DM (190) DNM (90)	Local recurrence occurred in 45% of DNMs compared to 21% of DMs. There was no difference in survival between DM and DNM patients.	Neurotropism portends local recurrence, but not decreased survival in DM.[23]
Su *et al.* 2003	DNM (14) DM (19)	There was no significant association between sentinel lymph node metastasis and neurotropism.	Neurotropism does not increase the risk for lymph node metastasis in DM.[36]
Busam *et al.* 2004	DM (92)	Neurotropism was not significantly associated with reduced disease free survival (p=0.21).	Neurotropism was only marginally associated with reduced Disease-Free survival.[5]
Kay *et al.* 2004	DNM (23) DM (5)	Neurotropism was not significantly associated with overall survival, local recurrence, or distant metastasis.	Neurotropism is not of prognostic relevance in DM.[24]
Livestro *et al.* 2005	DM (39) DNM (50)	Neurotropism was not significantly associated with survival or local recurrence. Of cases with lymph node metastasis 75% were neurotropic.	Neurotropism is not of prognostic relevance in DM.[38]
Chen *et al.* 2008	DNM (128)	Local recurrence occurred in 6% of DNM cases.	Neurotropism may not predict local recurrence to the extent previously thought.[37]

Murali *et al*. 2010	DM (163) DNM (89)	Neurotropism was not associated with local regional recurrence.	Neurotropism is not a prognostic indicator of increased risk for local regional recurrence.[25]
Scanlon *et al*. 2014	DNM (19) NM (23)	PNI detected associated with local recurrence. PNI detected by H&E was associated reduced rates of ulceration.	Neurotropism portends local recurrence but not decreased survival in multiple melanoma variants.[9]

1.5 The biological underpinnings of PNI – the role of neurotrophins.

A comprehensive understanding of the biological processes that promote PNI has yet to be established in any malignancy with a propensity for PNI. Two potential frameworks to explain the pathogenesis of PNI have been proposed. In one, tumor cells adopt a phenotype that enables them to respond to the distinctive microenvironment of nerves and thus acquire properties of enhanced invasiveness and proliferation. In the other, tumors themselves may develop a microenvironment that promotes neurite outgrowth, resulting in the proliferation of nerve twigs into and around the tumor with resulting PNI. These two mechanisms are not mutually exclusive and it is likely that some level of both occur at the same time through reciprocal signaling between tumor cells and nerve.[2]

To date, attempts to illuminate the molecular players involved in the pathogenesis of PNI have focused primarily on a family of peptides known as 11eurotrophins and their respective receptors. Neurotrophins are principally known for their role in controlling the development and maintenance of the peripheral nervous system but are increasingly understood to play significant roles in behavior of multiple lineage-unrelated cancers.[2] The most well characterized neurotrophin is nerve growth factor (NGF), but there are several other members of the neurotrophin family including brain-drived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5).37,38 Glial cell line-derived neurotrophic factor (GDNF) has a similar functional role to the aforementioned 11eurotrophins and is often grouped together with the NGF-related neutrophins. However, structural differences between GDNF and the NGF family warrant that it be considered in its own separate class of 11eurotrophins (the GDNF family of neurotrophic factors).[39] There are several receptors that interact with varying degrees of specificity with 11eurotrophins. The most studied are the Trk family of receptors (which includes TrkA, TrkB, and TrkC), p75NGFR, and RET. The Trk family and RET are comprised of receptor tyrosine kinases, while p75NGFR is a member of the tumor necrosis factor receptor superfamily.[40,41] The Trk family and RET are known to bind their target 11eurotrophins with relatively high-affinity and selectivity (TrkA binds NGF and NT-3, TrkB binds BDNF, NT-3, and NT 4/5, TrkC binds NT-3, RET binds GDNF) while p75NGFR is more promiscuous, binding a multitude of 11eurotrophins with different affinities. Within the context of PNI in cutaneous malignancies, the TrkA-NGF axis,

RET, and p75NGFR have emerged as the most promising neutrophin/nuerotrophin receptor candidates for having a mechanistic role.

1.5.1 The NGF-TrkA axis and PNI

TrkA serves as the membrane-bound high-affinity receptor for NGF and is expressed in multitude of lineage-unrelated cells. In normal epidermis, TrkA expression is found to be greatest in basal keratinocytes with expression dissipating in the superficial layers of the epidermis. There are conflicting reports of TrkA expression in normal melanocytes.[42,43] In epidermal keratinocytes as well as keratinocytes of the follicular epithelia, TrkA is thought to responsible for mediating NGF-induced stimulation of proliferation and inhibition of apoptosis.[43–46] The pro-proliferative effects of NGF on cells expressing TrkA combined with the NGF rich microenvironment surrounding nerves as a result its secretion by Schwann cells,[47] has led many to hypothesize a connection between TrkA and PNI.

Studies in several non-cutaneous malignancies have shown TrkA expression to be associated with PNI.[48–53] In both salivary adenoid cystic carcinoma and pancreatic ductal adenocarcinoma, TrkA was found to mediate enhanced invasiveness in cell cultures in response to NGF treatment.[50,53] In pancreatic cancer cell lines this pro-invasive response has been linked to an increased production of select matrix metalloproteinases (MMP-2) in response to NGF.[54] It is hypothesized that the expression of TrkA receptors on tumor cells allows them to responds to the NGF- rich microenvironment surrounding nerves,

resulting in increased production of proteolytic and enzymes and subsequent invasion through the perineural sheath. In cutaneous malignancies, evidence for TrkA's association with PNI was first reported in a study by Chen-Tsai *et al.* in which 4 cases of squamous cell carcinoma with PNI and 3 cases without PNI were analyzed immunhistochemically for Trk expression.[6] Notably, the authors used a promiscuous antibody that binds TrkA, B, and C. The authors found staining was strongest in the PNI positive cases suggestive of a relationship between TrkA and PNI but limited sample size and non-specificity of the antibody prevented any conclusive conclusions from being made.

Evidence for TrkA expression in melanomas is conflicting. Data from *in vitro* studies has shown TrkA expression to be absent.[55–57] While an immunohistochemical study in 152 primary and 64 metastatic melanoma biopsies detected significant levels of expression of the activated form of the TrkA receptor (p-TrkA), with expression being observed more frequently in biopsies of primary tumors (55%) compared to metastases (30%).[58] In the same study, the authors reported that membranous p-TrkA expression was significantly associated with decreased overall survival, and elevated markers of proliferation (cyclin-A and Ki-67). Although, p-TrkA expression was seen more often in nodular melanomas than the superficial spreading variant, spindle cell and desmoplastic melanomas were not included in the study.

1.5.2 The GDNF-RET axis and PNI

RET is a pro-oncogene encoding a membrane-bound receptor tyrosine kinase for GDNF. RET acts in concert with the GFRα1 co-receptor to bind GDNF, and like NGF-TrkA signaling, the RET-GDNF axis serves to support the survival and development of the peripheral nervous system.[59] Activating mutations in the RET gene have been associated with numerous cancers including neuroblastoma, papillary thyroid carcinoma, pancreatic cancers, and colon cancer.[59–61] Interestingly, inactivating mutations in RET have been associated with deficiencies in neural crest cell migration during development, most notably in Hirschsprung's disease which is caused by insufficient development of the enteric nervous system.[62]

Most of the research into RET's role in malignancies has been done in pancreatic cancers where RET-GDNF signaling has emerged as an important mediator of PNI.[63–65] Using immunohistochemistry, Zeng et al. have shown strong positive staining for GDNF in 88% of pancreatic ductal adenocarcinomas with PNI compared to 60% of those without PNI.[65] Furthermore, in vitro experiments have shown that treatment of pancreatic cancer cell lines with GDNF results in an increased production of matrix metalopreinase-9 (MMP-9) with a corresponding increase in invasive capacity – an effect that is lost after treatment with an inhibitor of RET.[66] Secretion of proteolytic enzymes like MMP-9 may facilitate degradation of the perineural sheath, essential for PNI. Taken together, the pro-invasive effects of GDNF-RET signaling, combined with the increased expression of GDNF in PNI positive pancreatic cancers, lend credence to the hypothesis that RET-GDNF signaling plays a role in the development of PNI.

Adding another dimension to the RET-PNI connection, a study conducted by Cavel et al. showed nerves invaded by pancreatic cancer had significantly elevated populations of endoneurial macrophages in excisions from both patients and mice.[63] These macrophages were found to secrete high levels of GDNF which when applied to pancreatic cells in culture promoted chemotactic migration of the cancer cells. Furthermore, the migration enhancing effect was lost after the pancreatic cancer cells were transfected with either siRNA against GFRα1 or RET inhibitor treatment, suggesting paracrine signaling between cancer cells expressing GFRα1/RET and GDNF secreting endoneurial macrophages. Most recently, it has been shown in a mouse model for PNI that peripheral nerves are capable of releasing soluble GFRα1 receptor, which enables GDNF binding in pancreatic cancer cells that lack GFRα1 but express RET, resulting in PNI.[64] Conversely, in this same study it was shown that tumor cells that lack RET did not undergo PNI, even in the presence of soluble GFRα1. Taken together, these results suggest that RET's causative relationship with PNI may be dependent on both paracrine signaling involving endoneurial macrophages, and additionally requires the presence of the GFRα1 co-receptor either in it's soluble form or expressed on the tumor cells themselves.

While the literature is lacking regarding the role of RET in cSCC, numerous lines of evidence suggest that RET is of importance in the pathogenesis of melanoma. Transgenic mouse lines with either over-activated RET mutations or overexpression of wild-type RET develop melanosis and benign melanocytic lesions that eventually progress to metastatic melanoma.[67–71] It has been shown that the progression from

benign lesions to melanomas corresponds with increasing levels of RET expression and its activation in tumors from these mouse models.[69] . It is also interesting to note that in RET transgenic mice, repeated UV irradiation results in the activation of RET leading to melanocytic tumor development at an accelerated rate compared to sham irradiated controls.[70] Evidence from human melanoma cell cultures further underscores the potential role of RET-GDNF signaling in melanomagenisis. In select human melanoma cell lines, Mrna transcripts for RET, GFRα1, and GDNF were detected, while human melanocytes were found to only express GFRα1.[71] A functional polymorphism (G691S) in the RET gene (RETp) has been shown to amplify RET's responsiveness to GDNF treatment in melanoma cell culture.[72,73] This mutation is found as a germline mutation in 15-20% of the general population but may also occur as somatic mutation in some instances.[73] Compared to RETwt melanoma cells, those with RETp displayed amplified proliferation, migration, and invasion after the addition of GDNF to culture medium, suggesting that RETp sensitizes the RET receptor to GDNF stimulation.[72] Furthermore, when Narita et al. analyzed 71 non-DM biopsies and 70 DM biopsies, the incidence of RETp was greater in the DM group than in the non-DM group (61% and 31% respectively).[72] Our own experience indicates that within DM, RETp is more frequently found in the pure subtype as compared to the mixed subtype.[74] As DMs are found predominantly in chronically sun exposed areas, the known role of UV light in modifying RET signaling may be of importance. Although RET-GDNF signaling appears to play a role in the development of PNI in pancreatic cancers, and additionally the activating

RETp mutation is found to be more frequently expressed in DM, a firm connection between RET and PNI in melanoma remains to be established.

1.5.3 p75NGFR and PNI

The most studied of all the neurotrophin receptors in melanoma, especially in the spindle cell and desmoplastic variants, is p75NGFR (alternatively called p75NTR, CD271, and the low affinity NGF receptor). The p75NGFR receptor is also the most promiscuous of neurotrophin receptors and is known to bind NGF, NT-3, NT-4/5, and BDNF with somewhat lower affinity than the Trk family of neurotrophin receptors. Unlike the Trk family and RET, p75NGFR is not a receptor tyrosine kinase, belonging instead to the tumor necrosis factor receptor superfamily.[41] In neurons, the role of p75NGFR is complex and poorly understood. It has been suggested that in the presence of TrkA, p75NGFR acts to increase TrkA's binding affinity for NGF and promote survival while, in the absence of TrkA, NGF binding to p75NGFR induces apoptosis.[41] Additionally, the migration of Schwann cells during embryonic development is thought to be regulated by p75NGFR.[75] In cancers, strong expression of p75NGFR is often seen in schwanomas, neuroblastoma, and other tumors of neural crest origin, although it's functional role is still unclear.[76]

Conflicting reports exist regarding the expression of p75NGFR in melanocytes. Numerous in vitro studies have found expression of p75NGFR in human melanocyte cell culture.[77] In addition, it has been shown that treatment of melanocyte cell cultures with

phorbol 12-tetradecanoate 13-acetate, a potent activator of protein kinase-C, induces upregulation of p75NGFR expression and causes the melanocytes to adopt a dendritic cytomorphology.[77] However, ex vivo studies using immunohistochemistry have reported negative p75NGFR staining in melanocytes but positive staining in nevic corpuscles within neurotized nevi.[78] Most immunohistochemical studies on formalin-fixed paraffin-embedded tissue samples report negative p7NGFR staining in epidermal melanocytes.

In 1996, two studies reported that p75NGFR was strongly expressed in melanomas with a spindled phonotype, including DM and SCM, but not in epithelioid melanomas.[79,80] Iwamoto et al. observed that p75NGFR was strongly expressed in all DMs and SCMs studied while only expressed in 1/11 (9%) of epithelioid melanomas.[79] At the same time, Kanik et al. emphasized the diagnostic utility of p75NGFR as an immunohistochemical adjunct for diagnosing DM in S100 negative spindle celled lesions, finding that p75NGFR had superior staining characteristics compared to S100 in both SCM, DM and NM.[80] They also noted that p75NGFR staining had an inverse relationship with HMB-45 staining, as tumors that stained strongly for p75NGFR did not express HMB-45 and vice versa. Since these early reports from 1996, several studies have used p75NGFR immunohistochemical staining in an attempt to enhance diagnostic accuracy in spindle celled melanomas. Usually, p75NGR staining is compared to that of S100, a well-established marker of neural crest-derived cells. S100 is nearly ubiquitously expressed across all melanoma variants, however in rare instances S100 negative SCMs and DMs have been reported, presenting a diagnostic pitfall for the pathologist.[81] In these instances p75NGFR has been shown to be a superior marker for melanoma, and, based on

this, many have recommended inclusion of p75NGFR in an initial immunohistochemical screening panel when dealing with equivocal cutaneous spindle celled lesions.[80,82,83]

Although p75NGFR may indeed be useful in the identification of SCM and DM in S100 negative cutaneous spindle cell lesions, discrepancies in the literature regarding its sensitivity,[84,85] as well as staining in scars,[86,87] present potential diagnostic pitfalls. Immunohistochemical studies have shown variable expression of p75NGFR in SCM and DM, ranging from 17% 85 to 100%.[71,79,82,83] The reasons for these discrepancies are not clear, and may be the result of a combination of factors. Briefly, these may include variability in antibodies, concentrations, and epitope retrieval methods. Our own analysis of studies utilizing p75NGFR staining indicates no unifying reason for the discrepancy in results. Notably, the studies by Kanik et al. and Huttenbach et al. each used the same antibody (p75NGF-R from Boehringer, Manheim) with markedly different results (Kanik et al. reported 100% positivity in SCM/DM while Huttebach et al. reported 33%).[80,84] A second source of variability may be a result of variable scoring rubrics used to establish criteria for p75NGFR positivity. Our own experience underscores the need for establishing rigid, clear, and scientifically grounded cutoffs for positivity when attempting to correlate immunohistochemical staining with histopathological parameters. This is more complicated in the realm of DMs where it has been shown that in the mixed subtype, p75NGFR staining is mostly present in the spindle cell component but not in cells with epithelioid cytomorphology.[82]

Iwamota et al. first hypothesized that p75NGFR may play a role in mediating PNI in melanomas after observing that p75NGFR was expressed in a significantly greater

proportion of DMs than epithelioid melanomas.[79,88] The authors suggested that the expression of p75NGFR in melanoma cells may allow them to respond with increased invasiveness and proliferation to the NGF rich microenvironment surrounding nerves. It was known at the time that p75NGFR expressed in melanoma cell lines mediated NGF induced extracellular matrix invasion, most likely through increased production of heparanase (a protease capable of digesting key components of the extracellular matrix).[56–58] Additionally, Iwomota et al. reported that p75NGFR mediated NGF's survival promoting effects in melanoma cell cultures.[88] Taken together, these observations supported Iwamoto et al.'s hypothesis. However, since they did not report directly on rates of PNI, they could only conclude that p75NGFR was associated with the SCM and DM variants and not functionally involved in PNI. The first study to attempt to directly correlate p75NGFR expression and PNI came from Chan and Tahan in 2009, in which the authors used immunohistochemistry to compare p75NGFR expression in a cohort of PNI positive vs. PNI negative melanomas of diverse histopathologic subtypes (DM, SCM, and epitheliod melanomas).[85] They observed p75NGFR expression in 8/10 PNI positive melanomas compared to 1/9 PNI negative melanomas, and that "all invasive melanomas with PNI showed some degree of spindle cell differentiation in the vertical growth phase".[85]

Outside of its connection to PNI, additional perspectives on the role of p75NGFR in melanomagenisis have emerged in recent years. In 2010, Boiko et al. published a report of a subpopulation of "melanoma initiating cells" that to behaved as cancer stem cells.[89] When transplanted into mice with engrafted human skin, these cells consistently

resulted in the development of melanoma tumors and in some cases metastasis to lungs and liver. Using fluorescence activated cell sorting in freshly resected melanomas, it was determined that CD271 (p75NGFR) was a reliable marker for these melanoma initiating cells. Notably, when taken from the same tumors CD271+ but not CD271- cells resulted in melanoma development after transplantation into mice. Of the resected tumors expressing CD271+, expression was found in between 2.5% and 41% (average of 16.7%) of the tumor cell population, and similar proportions were observed in the melanomas that developed in mice after transplantation of CD271+ cells. These results suggest that that CD271+ cells are capable of regenerating the more differentiated CD271- cells in vivo. The authors also report that CD271+ cells lack expression of traditional melanocyte (Tyrosinase/MART/HMB-45). These finding were replicated by Civenni et al. with the additional finding that transplanted CD271- cells were at times capable of forming tumors but only in natural killer cell depleted and SCID mice.[90] Interestingly, in the same study the authors report that staining a microarray of 200 primary and metastatic melanomas taken from patients showed that co-expression of CD271 and SOX10 was associated with metastasis and worse prognosis. The inverse correlation between p75NGFR expression and melanocyte markers in vitro was further elucidated in two recent studies.[91,92] Furuta et al. observed that cytotoxic T-lymphocytes secrete NGF which, upon binding to p75NGFR on melanoma cells, results in the down-regulation of melanocyte markers.[91] Additionally p75NGFR was found to be frequently co-expressed with programmed cell death ligand 1 (PDL-1), a potent inducer of cytotoxic T-lymphocyte apoptosis and that both p75NGFR and PDL-1 may be upregulated in

response to interferon gamma. These results have important implications in explaining the frequent failures of targeted T-cell therapy towards traditional melanoma antigens. In the second study, Redmer et al. showed that knockdown of CD271 in melanoma cell lines results in a loss of other neural crest stem cell markers such as SOX10 and furthermore that expression of melanocyte markers and CD271 was mutually exclusive.[92] Of potential relevance to DM, this change was associated with an alteration in cytomorphology from spindled to an epithelioid variant.

From a review of the literature, it appears that pathologists and basic scientists have distinct schools of thought regarding the role of p75NGFR. Pathologists have been focused on employing staining for p75NGFR to improve diagnostic accuracy in spindle cell lesions as well as understanding its potential role in mediating PNI, while investigators seeking to understand the underlying fundamental biology of melanomas have focused on p75NGFRs role as a potential marker for melanoma stem cells. It is difficult to reconcile the observations that p75NGFR is associated with tumor initiating cells and suppression of T-lymphocytes while at the same time pathologists know it to be associated with DM which as discussed previously, has a relatively good prognosis. To reconcile these perspectives, significant gains in our understanding of the complex behavior of the p75NGFR receptor are required. The potentially varying effects of its numerous ligands, the consequences of its interactions with the Trk family of neurotrophin receptors, and its newly appreciated role as a marker for tumor initiating cells are all areas ripe for future investigation.

1.6**Aims/Objectives**

Given the unresolved questions regarding the biological underpinnings of PNI, and it's somewhat equivocal nature as a histopathologic prognosticator, we sought to further investigate PNI and the expression of select neurotrophin receptors in cSCC and DM.

In the current study we sought to answer the following questions:

- How does PNI correlate with established histopathologic prognosticators in cSCC and DM?
- Does the use of p63/S100 double immunostaining (DIS) improve the detection of PNI in cSCC?
- Does TrkA (in DM and cSCC), RETp (in DM), or p75NGFR (in DM), correlate with propensity for PNI or other histopathologic prognosticators?

Cutaneous Squamous Cell Carcinoma – Materials and Methods

2.1 Sample Selection

This study was approved by the institutional review board of Boston University School of Medicine (IRB Docket #s H-32816, H-32952). Archival tissue samples with a diagnosis of cSCC from H&N (n=57) and non-H&N areas (n=53) were retrieved from the pathology files of the Skin Pathology Laboratory, Boston University School of Medicine, Boston, MA, USA. Inclusion criteria were randomly selected cases of primary cSCC with adequate tumoral tissue to allow for immunohistochemical analyses. Histologic sections of all cases were reviewed by two board-certified dermatopathologists (initial sign-out on all by a dermatopathologist; cases were then re-reviewed, and the diagnoses confirmed). All patient data were de-identified.

2.2 Immunohistochemical analyses

For all stains, formalin-fixed, paraffin-embedded tissue of primary cSCC (n=110) were baked at 75°C for 30 min. Sections were deparaffinized with xylene and rehydrated in a series of decreasing concentrations of ethanol solution. Heat-induced antigen retrieval was carried out in DAKO retrieval solution (DAKO, Carpenteria, CA, USA) with a Ph of 6.1 in a 98°C bath for 20 minutes. All immunostained slides were reviewed and scored in a blinded fashion.

2.2.1 Double staining for PNI detection

Surface cuts from the same block (on which H&E staining was done) were double stained with p63 and S100 to evaluate PNI. Briefly, slides were treated with dual endogenous enzyme block (DAKO) then incubated with anti-p63 rabbit monoclonal antibody (BioCare, Concord, CA, USA) 1:100 dilution for 40 min at room temperature, followed by treatment with polymer HRP (DAKO) for 20 minutes. Color was developed with DAB (DAKO). Slides were then incubated with Doublestain Block (DAKO) for 10 minutes followed by a 40 minute incubation with anti-S100 rabbit monoclonal antibody at a dilution of 1:500. After treatment with the second antibody, slides were incubated with Rabbit/Mouse Link (DAKO) for 20 minutes, followed by polymer/AP (DAKO) for 20 minutes. Color and contrast were developed with permanent red solution (DAKO) and hematoxylin respectively. For p63, nuclear staining of epidermal basal and suprabasal cells, outer root sheath of hair follicles, and the germinitive layer of sebaceous glands served as positive internal controls in each case where they could be visualized. For S100, cytoplasmic staining of Langerhans cells, basal melanocytes, and uninvolved nerve fibers served as positive internal controls in each case where they could be visualized.

Original H&E slides as well as DIS slides were reviewed in a blinded fashion for presence of PNI. PNI positive status was assigned to samples in which the presence of neoplastic cells within the perineural space was observed. Only unequivocal cases of PNI were recorded as positive.

2.2.2 Assessment of TrkA expression

The slides were treated with dual endogenous enzyme block (DAKO) then incubated with the anti-TrkA (14G6) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:200 for 50 minutes at room temperature, followed by treatment with polymer HRP (DAKO) for 20 minutes. Color development and contrast were achieved using DAB and hematoxylin respectively.

Given TrkA's role as a membrane bound receptor tyrosine kinase, positive TrkA expression was defined as either membranous or mixed membranous/cytoplasmic staining observed in tumor cells. Tumor cells displaying only cytoplasmic staining were considered negative. Membranous staining of basal epidermal cells, eccrine epithelial cells, mature sebocytes, and the outer root sheath of hair follicles served as positive internal controls where they could be visualized. A semi-quantitative scoring system was utilized with scoring cutoffs as follows: 0=0%, 1=1-5%, 2=6-25%, 3 = 26-50%, 4 = >50%. For purposes of statistical analysis, cases with a score of 3 or higher were considered positive.

2.3 Statistical Analysis

A chi-square test of independence was used to examine differences in the distribution of demographic and prognostic variables between the following groups: H&N versus non-H&N; PNI-positive versus PNI-negative; and TrkA-positive versus

TrkA-negative cases. In ascertaining the association of PNI with histopathological variables, cSCC cases were pooled regardless of where expected frequency counts were less than 5, Fisher's exact test was used to generate $\chi 2$ and p-values. DIS was compared to H&E using a Cohen's Kappa coefficient of agreement. The association between TrkA and PNI staining was explored using Spearman's Rank Correlation, and cases of cSCC were pooled regardless of their location (H&N or non-H&N). Stuarts' Tau-C was utilized to verify statistical results from correlation of TrkA and PNI. All statistical tests were done using SAS® 9.3.

Cutaneous Squamous Cell Carcinoma – Results

3.1 Comparison of histopathologic characteristics of cases from the H&N and non-H&N areas

Of cases from the H&N, 42/57 (74%) were from males compared to 24/53 (45%) of non-H&N cases (p=0.002) and 42/57 (74%) of cases from the H&N were well differentiated compared to 48/53 (91%) of those from non-H&N areas (p=0.003, Table 2). While cases from the H&N displayed on average a shallower depth of invasion and lower Clark Level than cases from non-H&N areas, differences did not achieve statistical significance (p=0.28 and p=0.13 respectively). High-risk morphologic variants (acantholytic/pesudoglandular, desmoplastic, or spindle cell subtypes) were more common in cases of the H&N than those from non-H&N areas (19% versus 9.4% respectively) although this difference was not significant (p=0.14).

Table 2. Histopathologic characteristics of cutaneous SCCs from the H&N and non-H&N areas

Variable	H&N cases, No. (%) n=57	Non-H&N cases, No. (%) n=53	*p-values*
Sex			
Male	42 (73.7)	24 (45.3)	0.002*
Female	15 (26.3)	29 (54.7)	
Depth (mm)			
0.01-2.00	21 (36.9)	12 (22.7)	0.280
2.01-3.99	30 (52.6)	35 (66.0)	
> 4.00	6 (10.5)	6 (11.3)	
Clark Level			
I	0 (0.0)	0 (0.0)	0.130
II	0 (0.0)	0 (0.0)	
III	13 (22.8)	6 (11.3)	
IV	44 (77.2)	47 (88.7)	
V	0 (0.0)	0 (0.0)	
Differentiation			
Well differentiated	42 (73.7)	48 (90.6)	0.003*
Moderately differentiated	15 (26.3)	5 (9.4)	
Poorly differentiated	0 (0.0)	0 (0.0)	
High-risk Morphologic Variant[a]			
Yes	11 (19.3)	5 (9.4)	0.140
No	46 (80.7)	48 (90.6)	

[a]High-risk morphologic variants include acantholytic, desmoplastic, or spindle cell subtypes

3.2 PNI in cSCC

Review of H&E stained sections, revealed PNI in 6/57 (11%) of cases from the H&N and 3/53 (6%) of cases from non-H&N areas (p=0.49). Using DIS, PNI was detected in 13/57 (23%) of cases from the H&N and 8/53 (15%) of cases from non-H&N areas (p=0.30). Thirteen additional cases of PNI were detected with DIS (Fig. 2), representing an increase of 2.33 times compared to H&E (95%CI: 1.12–4.87; p=0.02). Of these, 7 involved nerves where located intratumorally while 6 involved nerves where located peritumorally (Fig. 3). In the H&N, DIS increased the rate of PNI pickup by 2.17 times compared to H&E (13/57 versus 6/57; 95%CI: 0.89–5.30; p=0.08) while in non-H&N areas, DIS increased the rate of PNI pickup by 2.67 times compared to H&E (3/53 versus 8/53; p=0.20). There was discordant detection of PNI determined by H&E compared to DIS (κ=0.47, p=0.002). Of histopathologic prognosticators evaluated, only tumor depth was found to be significantly associated with PNI (p=0.002) (Table 3). Furthermore, a linear trend was observed showing increasing depth correlates with increased incidence of PNI (p=0.008)

Table 3. PNI detected by double immunostaining and histopathologic prognosticators

Variable	PNI positive cases[a] No. (%) n = 21	PNI negative cases[a] No. (%) n = 89	*p-values*
Sex			
Male	15 (71.4)	51 (57.3)	0.320
Female	6 (28.6)	38 (42.7)	
Depth (mm)			
0.01-2.00	0 (4.8)	32 (36.0)	0.006[*]
2.01-3.99	16 (76.2)	49 (55.0)	
> 4.00	4 (19.0)	8 (9.0)	
Clark Level			
I	0 (0.0)	0 (0.0)	
II	0 (0.0)	0 (0.0)	1.00[b]
III	3 (14.3)	16 (18.0)	
IV	18 (85.7)	73 (82.0)	
V	0 (0.0)	0 (0.0)	
Differentiation			
Well differentiated	17 (80.9)	73 (82.0)	1.00[b]
Moderately differentiated	4 (19.1)	16 (18.0)	
Poorly differentiated	0 (0.0)	0 (0.0_	
High-risk Morphologic Variant[c]			
Yes	3 (14.3)	13 (14.6)	1.00[b]
No	18 (85.7)	76 (85.4)	

[*]Unadjusted p-values were calculated using chi-squared test of independence or Fisher's test when expected frequencies were less than 5; unadjusted p-values were considered significant when <.05

[a]cases were pooled from H&N and non-H&N regions to examine bivariate associations with PNI.

[b]There was insufficient power to resolve an approximate p-value using Fisher method.

[c]High Risk Variants include acantholytic, desmoplastic, or spindle cell subtypes.

Figure 2. Bar chart demonstrating PNI incidence by H&E and double immunostaining

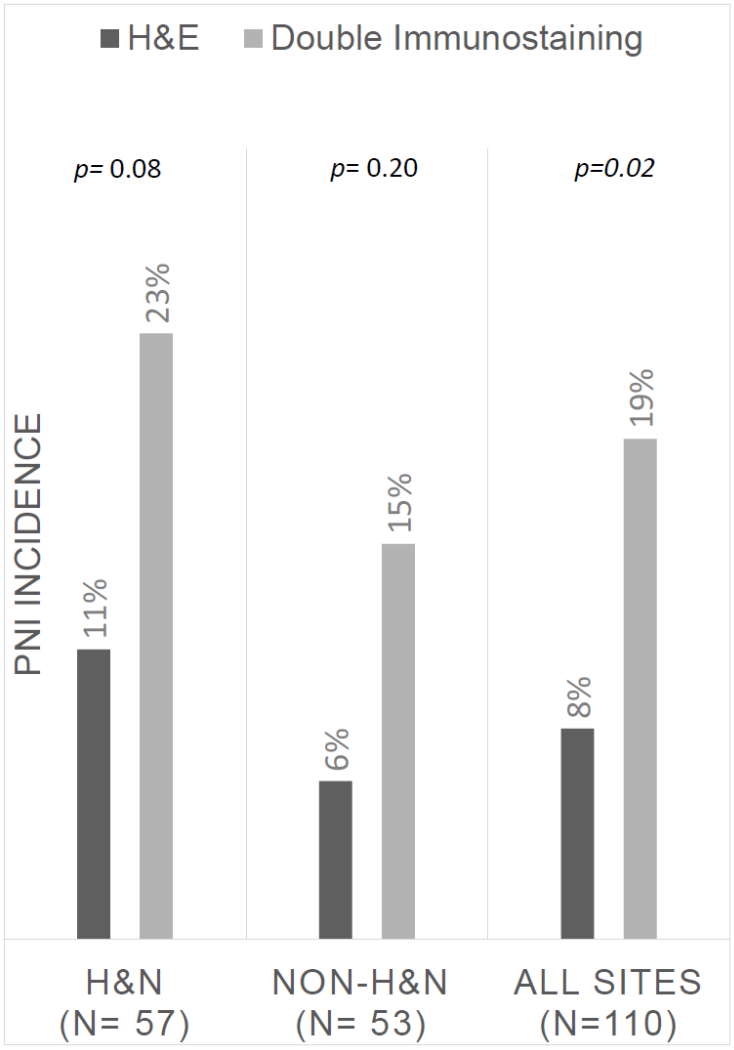
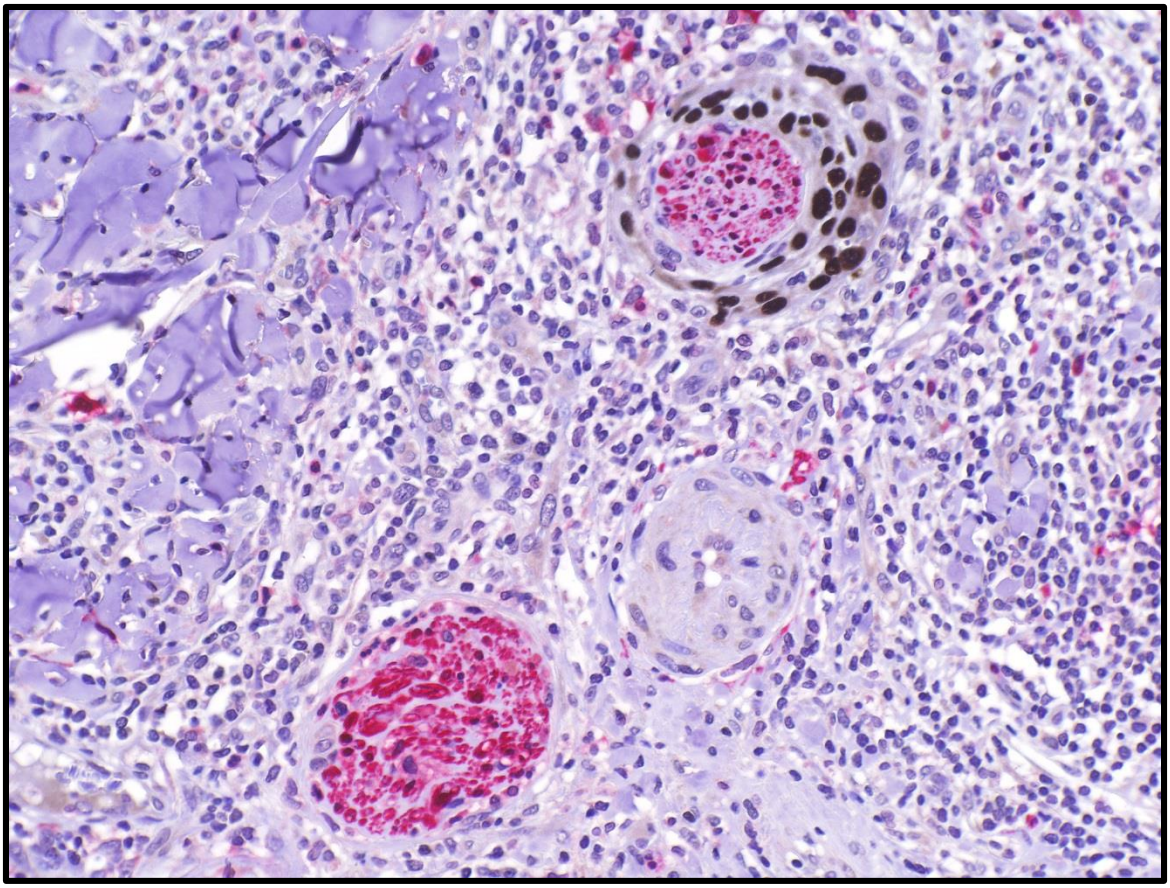


Figure 3. Representative examples of double immunostaining with p63 (for tumor, nuclear staining, brown chromogen) and S100 (for nerve, cytoplasmic staining, red chromogen)



3.3 TrkA in cSCC

TrkA expression was 2.90 times more likely to be observed in cases from the H&N compared to non-H&N areas (28/57 versus 12/48, p=0.01). Regardless of site, the TrkA positive group had a lower proportion of well differentiated and a higher proportion of moderately differentiated SCCs compared to the TrKA negative group (65% and 35% versus 92% and 8% respectively, p=0.0006). A significantly greater proportion of TrkA positive cases were high-risk morphologic variants (12/40, 30% TrKA positive versus 4/65, 6.2% TrKA negative; p=0.002) (Table 4, Fig. 4). TrkA was expressed in 25% of PNI positive cSCCs as compared to 16% of PNI negative cSCCs, although this difference was not significant (p=0.33).

Table 4. TrkA and histopathologic prognosticators

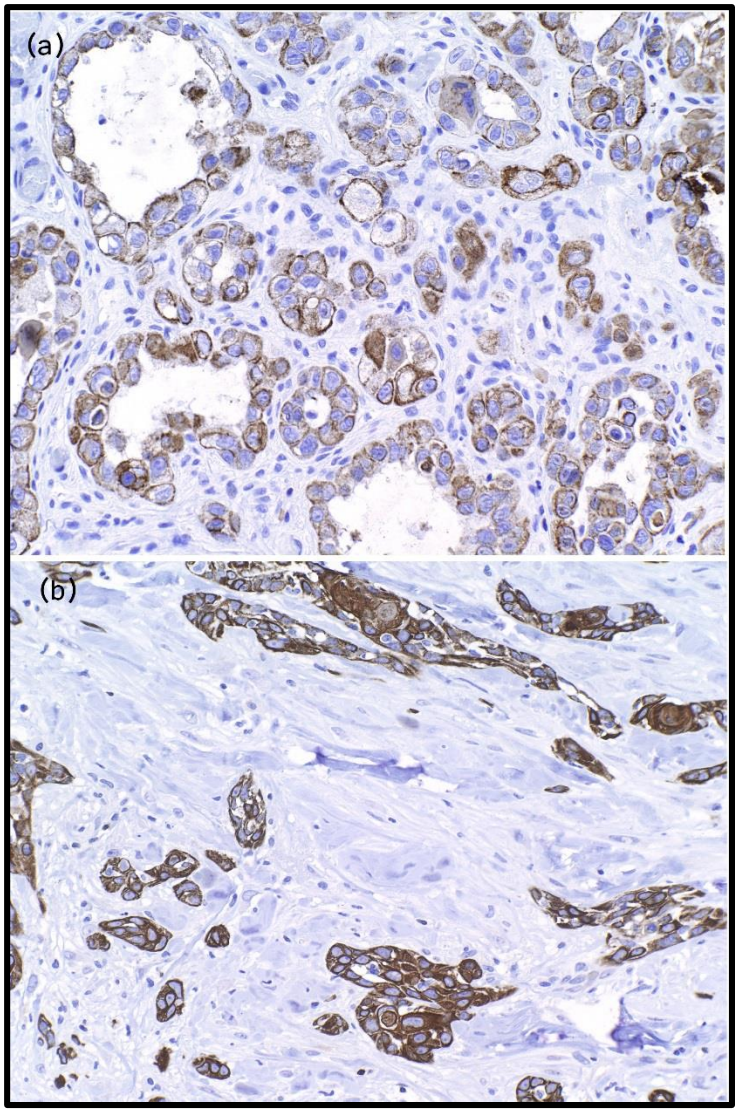
Variable	TrkA Positive Cases[a] No. (%) n = 40	TrkA Negative Cases[a] No. (%) n = 65	*p-values*
Sex			
Male	28 (70.0)	36 (55.4)	0.14
Female	12 (30.0)	29 (44.6)	
Site			
H&N	28 (70.0)	29 (44.6)	0.01*
Non-H&N	12 (30.0)	36 (55.4)	
Depth (mm)			
0.01-2.00	15 (37.5)	18 (27.7)	0.57
2.01-3.99	21 (52.5)	40 (61.5)	
> 4.00	4 (10.0)	7 (10.8)	
Clark Level			
I	0 (0.0)	0 (0.0)	0.79
II	0 (0.0)	0 (0.0)	
III	8 (20.0)	11 (16.9)	
IV	32 (80.0)	54 (83.1)	
V	0 (0.0)	0 (0.0)	
Differentiation			
Well differentiated	26 (65.0)	60 (92.3)	0.0006*
Moderately differentiated	14 (35.0)	5 (7.7)	
Poorly differentiated	0 (0.0)	0 (0.0)	
High-risk morphologic Variant[b]			
Yes	12 (30.0)	4 (6.2)	0.002*
No	28 (70.0)	61 (93.9)	
Perineural Invasion			
Present	10 (25.0)	11 (16.9)	0.33
Absent	30 (75.0)	54 (83.1)	

* Unadjusted p-values were calculated using chi-squared test of independence or Fisher's test when expected frequencies were less than 5; unadjusted p-values were considered significant when <.05

[a]TrkA data was unavailable for 5 cases due to tissue loss during antigen retrieval.

[b]High-risk morphologic variants include acantholytic, desmoplastic, or spindle cell subtypes

Figure 4: Representative examples of immunohistochemical staining with TrkA in high-risk morphologic SCC variants



a SCC, acantholytic/pseudoglandular variant, case 98

b. SCC, desmoplastic variant, case 99

Desmoplastic Melanoma – Materials and Methods

4.1 Sample Selection

This study was approved by the institutional review board of Boston University School of Medicine (IRB Docket #s H-32816, H-32952). Archival annotated tissue samples with a diagnosis of DM (n=43) were retrieved from the pathology files of the Skin Pathology Laboratory, Boston University School of Medicine, Boston, MA, USA. Inclusion criteria were randomly selected cases of primary melanoma with adequate tumoral tissue to allow for analyses. Histologic sections of all cases were reviewed by two board-certified dermatopathologists (initial sign-out on all by a dermatopathologist; cases were then re-reviewed, and the diagnoses confirmed). All patient data were de-identified.

4.2 Immunohistochemical Analyses of TrkA Expression

Formalin-fixed, paraffin-embedded tissue of primary melanomas (n=43) were baked at 75°C for 30 min. Sections were deparaffinized with xylene and rehydrated in a series of decreasing concentrations of ethanol solution. Heat-induced antigen retrieval was carried out in Dako retrieval solution (Dako, Carpenteria, CA, USA) with a Ph of 6.1 in a 98°C bath for 20 minutes. All immunostained slides were reviewed in a blinded fashion. The slides were treated with dual endogenous enzyme block (Dako) then incubated with anti-TrkA (14G6) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:200 for 50 minutes at room

temperature, followed by treatment with polymer HRP (DAKO) for 20 minutes. Color development and contrast were achieved using DAB and hematoxylin respectively.

Positive TrkA expression was defined as either membranous or mixed membranous/cytoplasmic staining observed in tumor cells, those displaying only cytoplasmic staining were considered negative Membranous staining of basal epidermal cells, eccrine epithelial cells, mature sebocytes, and the outer root sheath of hair follicles served as positive internal controls where they could be visualized.

4.3 Analysis of p75NGFR expression and RET Polymorphism

Sections from the same formalin-fixed paraffin-embedded tissue blocks of DM cases used in the present study were analyzed previously for p75NGFR expression and RET G619S polymorphism.[74] Briefly, p75NGFR expression was determined via IHC analysis using an anti-p75NGFR antibody (1:100 Biocare Medical, LLC, Concord, CA, USA), slides were scored using cut-offs based on the methodology reported by Iwamoto et al.,17 for statistical purposes cases with 11% or more of tumor cells staining were considered positive. RET polymorphisms were detected by direct DNA sequencing in the RET coding region on exon 11 with the forwarding primer, more detailed methodology can be found in Miller et al (2012).

4.4 Statistical Analyses

The associations between PNI and p75NGFR with potential confounding factors were separately assessed using univariate analysis. A chi-squared test of independence was utilized for categorical demographics (gender) and the following histopathologic prognosticators: Breslow's depth; Clark's Level; pure vs mixed subtype; host response; ulceration; and regression. Fisher's exact test was used if the expected frequencies were less than 5, and a student's t-test was used to examine the association of age with PNI and p75NGFR. Factors associated with both PNI and p75NGFR, but not believed to be on the causal pathway, were controlled for in a multivariable logistic regression model. Although underpowered, additional logistic models were examined utilizing factors associated with either PNI or p75NGFR (but not both), as well as a stratified analysis of mixed versus pure type DM. All statistical tests were done using SAS® 9.3.

DESMOPLASTIC MELANOMA – RESULTS

5.1 Clinical Data

Of the 43 cases of DM studied, 24 were male and 19 were female. The median age at the time of diagnosis was 72 years (range: 16–92), and tumors presented in predominantly sun-exposed sites. A total of 21 of 43 were located on the head and neck, 14 of 43 on the extremities, and 7 of 43 on the trunk. Clinically apparent pigmentation was reported in 18 of 41 cases (data not available for two cases).

5.2 PNI in DM

PNI was observed in 29/43 (67%) of cases of DM. Of histopathologic variables analyzed, both greater Clark's Level and increased Breslow's depth were significantly associated with PNI ($p=0.002$ and $p=0.01$ respectively). Notably, 31% of PNI-positive cases exhibited Clark's Level V invasion compared to 7% of PNI negative cases. Presence of mitosis, host response, ulceration, and regression were not significantly associated with PNI. No difference was noted in the propensity of the pure and mixed variants of DM for PNI (Table 5).

Table 5. PNI and Histopathologic Variables in Desmoplastic Melanoma

Variable	PNI Positive Cases, No. (%) n=29	PNI negative cases, No. (%) n=14	*p-values**
Sex			
Male	16 (57.1)	8 (57.1)	0.68
Female	12 (42.9)	6 (35.3)	
Breslow's Depth (mm)			
0.01-1.00	2 (6.9)	7 (50.0)	**0.01**
1.01-2.00	6 (20.7)	3 (21.4)	
2.01-4.00	14 (48.3)	3 (21.4)	
> 4.00	7 (24.1)	1 (7.1)	
Clark's Level			
I	0 (0.0)	99 (0.0)	
II	0 (0.0)	99 (0.0)	**0.002**
III	0 (0.0)	5 (35.7)	
IV	20 (69.0)	8 (57.1)	
V	9 (31.0)	1 (7.1)	
Desmoplastic Variant[#]			
Pure	15 (51.7)	9 (64.3)	0.44
Mixed	14 (48.3)	5 (35.7)	
Mitosis			
Present	26 (89.7)	10 (71.4)	0.19
Absent	3 (10.3)	4 (28.6)	
Host Response			
Present	13 (44.8)	4 (28.6)	0.34
Absent	16 (55.2)	10 (71.4)	
Ulceration			
Present	2 (6.9)	1 (7.1)	1.00**
Absent	27 (93.1)	13 (92.9)	
Regression			
Present	1 (3.4)	2 (14.3)	0.24
Absent	28 (96.6)	12 (85.7)	

*: Unadjusted p-values were calculated using chi-squared test of independence or Fisher's test when expected frequencies were less than 5; unadjusted *p*-values were considered significant when <.05

**: There was insufficient power to resolve an approximate p-value using Fisher method.

[#]: Tumors were classified as mixed desmoplastic melanoma per previously established criteria[24] if two morphologically distinct cell populations were present; with at least 10% of cells representing conventional melanoma. Pure desmoplastic melanomas were defined as those with >90% of cells displaying typical desmoplastic melanoma morphology.

Note: 1 case missing gender data, 1 case missing age data

5.3 TrkA in DM

TrkA expression was negative in all cases of DM. Negative expression was confirmed by validation of positive staining of internal controls (Figure 5D-F).

5.4 RETp in DM

Functional RET polymorphism (RETp) was detected in 12/42 (29%) of DMs (one case, 42, had insufficient tissue for analysis). While a greater percentage of PNI positive cases exhibited RETp (39% in PNI positive versus 13% in PNI negative cases) this difference was not significant on univariate analysis ($p=0.27$). RETp was not associated with any histopathologic variables on univariate analysis (Table 6). Multivariate analysis controlling for the presence of p75NGFR expression did not show a significant association with PNI.

Table 6. RET Polymorphism and Histopathologic Variables

Variable	RET Polymorphism No. (%) n = 12	RET Wild-Type No. (%) n = 30	*p-values**
Sex			
Male	9 (75.0)	14 (48.3)	0.17
Female	3 (25.0)	15 (51.7)	
Depth (mm)	1 (8.3)		
0.01-1.00	4 (33.3)	8 (26.7)	0.20
1.01-2.00	3 (25.0)	5 (16.7)	
2.01-4.00	4 (33.3)	13 (43.3)	
> 4.00		4 (13.3)	
Clark Level			
I	0 (0.0)	0 (0.0)	1.00**
II	0 (0.0)	0 (0.0)	
III	1 (8.3)	4 (13.3)	
IV	8 (66.7)	19 (63.3)	
V	2 (25.0)	7 (23.3)	
Desmoplastic Variant			
Pure	7 (58.3)	16 (53.3)	0.77
Mixed	5 (41.7)	14 (46.7)	
Mitosis			
Present	8 (66.7)	27 (90.0)	0.09
Absent	4 (33.3)	3 (10.0)	
Host Response			
Present	6 (50.0)	11 (36.7)	0.43
Absent	6 (50.0)	19 (63.3)	
Ulceration			
Present	2 (8.3)	2 (6.7)	1.00**
Absent	10 (91.7)	28 (93.3)	
Regression			
Present	1 (8.3)	2 (6.7)	1.00**
Absent	11 (91.7)	28 (93.3)	

Note: 1 case missing gender data, 1 case missing age data.

*: Unadjusted p-values were calculated using chi-squared test of independence or Fisher's test when expected frequencies were less than 5; unadjusted p-values were considered significant when <.05

**: There was insufficient power to resolve an approximate p-value using Fisher method.

5.5 p75NGFR in DM

Overall, p75NGFR expression was detected in 28/43 (65%) of DMs (17 with 10% or less staining, 1 with 11-50% staining, 7 with 51-75% staining and 20 with >75% staining of cells). Staining intensity was uniformly strong wherever present. On univariate statistical analysis, p75NGFR expression was significantly associated with increasing Breslow's depth and greater Clark's Level (p=0.007 and p=0.01 respectively, Table 7). P75NGFR was significantly associated with PNI (Table 8), with expression noted in 79% of PNI positive cases and 36% of PNI negative cases (p=0.005).

After examining potential confounders using univariate analysis, only depth was associated with both PNI and p75NGFR. Although both Breslow's Depth and Clark's Level provided information on the associations of depth with PNI and p75NGFR, we chose to dichotomize the depth as <2mm and ≥2mm to increase statistical power in the multivariable logistic model. Adjusting for the potential confounding effects of depth, we observed that expression of p75NGFR was associated with a 386% increase in odds of PNI (Adjusted Odds Ratio=4.68, 95% CI: 1.03-21.17, p=0.04). Additional multivariable logistic models were then created using histopathologic factors that were marginally associated with PNI or p75NGFR. After step-wise inclusion of additional confounding factors in the multivariate analyses, the magnitude of the association between PNI and p75NGFR, as measured by the odds-ratio, remained relatively stable (Table 9). Although not reaching the 0.05 significance level, the pure variant showed a greater association between PNI and p75NGFR (OR=6.57, 95%CI: 0.84–51.04, p=0.07) compared to mixed DM (OR=4.50, 95%CI: 0.25–80.56, p=0.31).

Table 7. p75NGFR Expression and Histopathologic Variables

Variable	p75NGFR positive cases No. (%) n = 28	p75NGFR negative cases No. (%) n = 15	*p-values**
Sex			
Male	55 (55.6)	9 (60.0)	0.78
Female	12 (44.4)	6 (40.0)	
Depth (mm)			
0.01-1.00	2 (7.1)	7 (46.7)	**0.007**
1.01-2.00	6 (21.4)	3 (20.0)	
2.01-4.00	12 (42.9)	5 (33.3)	
> 4.00	8 (28.6)	0 (0.0)	
Clark's Level			
I	0 (0.0)	0 (0.0)	**0.01**
II	0 (0.0)	0 (0.0)	
III	2 (7.1)	3 (20.0)	
IV	16 (57.1)	12 (80.0)	
V	10 (35.7)	0 (0.0)	
Desmoplastic			
Variant	15 (53.6)	9 (60.0)	0.69
Pure	13 (46.4)	6 (40.0)	
Mixed			
Mitosis			
Present	25 (89.3)	11 (73.3)	0.22
Absent	3 (10.7)	4 (26.7)	
Host Response			
Present	12 (42.9)	5 (33.3)	0.54
Absent	16 (57.1)	10 (66.7)	
Ulceration			
Present	2 (7.1)	1 (6.7)	1.00**
Absent	26 (92.9)	14 (93.3)	
Regression			
Present	2 (7.1)	1 (6.7)	1.00**
Absent	26 (92.9)	14 (93.3)	

*: Unadjusted p-values were calculated using chi-squared test of independence or Fisher's test when expected frequencies were less than 5; unadjusted p-values were considered significant when <.05

**: There was insufficient power to resolve an approximate p-value using Fisher method.

Note: 1 case missing gender, 1 case missing age data.

Table 8. Neurotrophin Receptors and Perineural Invasion in Desmoplastic Melanoma – Univariate analysis

	Positive for TrkA Expression No. (%)	**Positive for p75NGFR Expression No. (%)**	**Positive for RET polymorphism[#] No. (%)**
PNI Positive Cases n=29	0 (0.0)	23 (79.3)	10 (38.5)
PNI Negative Cases n=14	0 (0.0)	5 (35.7)	2 (12.5)
p-values*	1.00	**0.005**	0.27

***:** Unadjusted p-values were calculated using chi-squared test of independence or Fisher's test when expected frequencies were less than 5; unadjusted p-values were considered significant when <.05

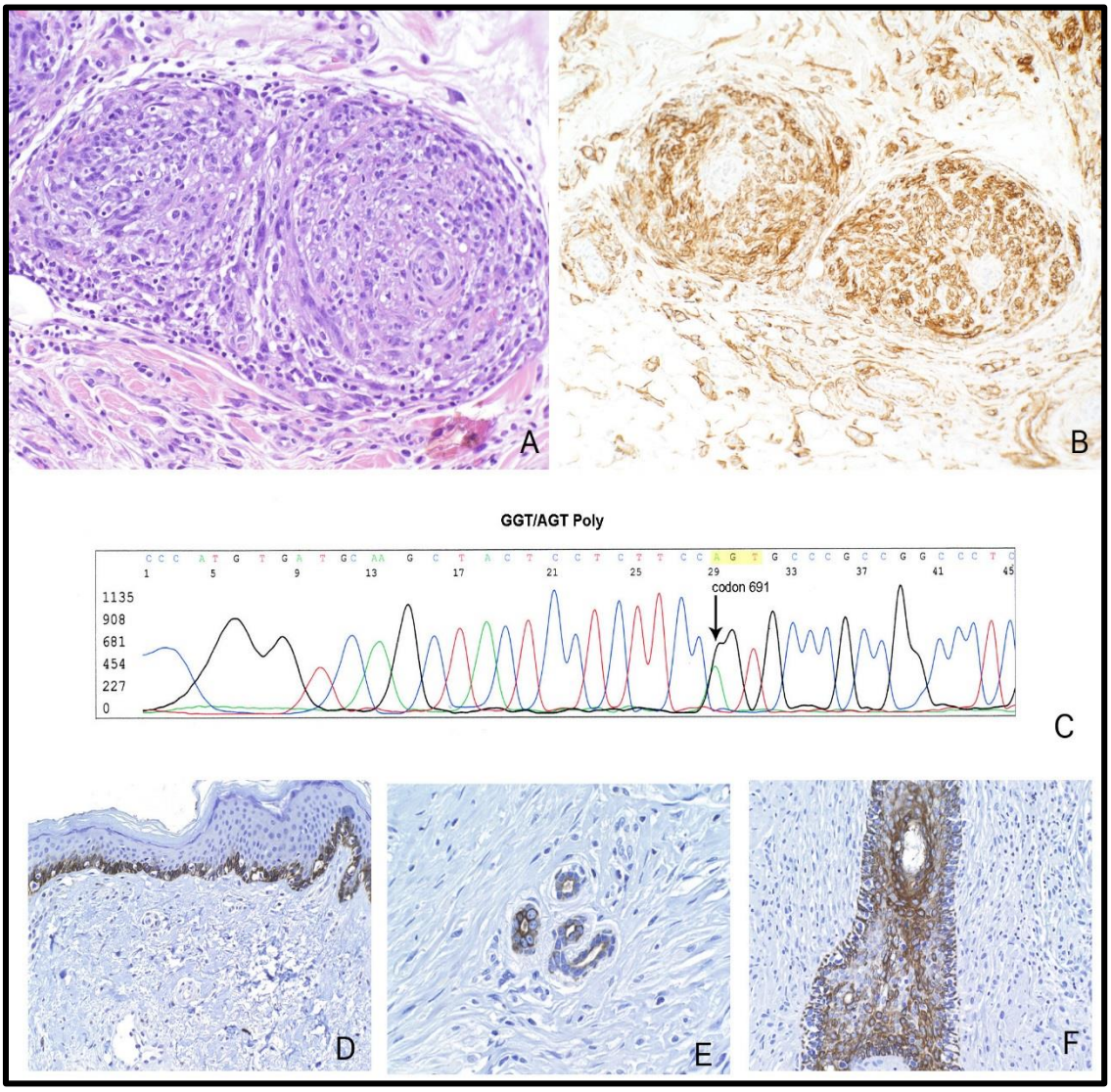
[#]: One case was not analyzed for RET polymorphism (Case #42).

Table 9. Multivariate Analysis of p75NGFR Expression and Propensity for PNI Controlling for Potential Confounding Variables.

Model	**OR[#]**	**95% CI**	***p-values***
p75NGFR + depth	4.68	1.03-21.17	**0.04**
p75NGFR + depth + host response	4.26	0.90-20.09	0.07
p75NGFR + depth +mitosis	4.26	0.92-19.78	0.06
P75NGFR + depth + host response + mitosis	3.74	0.76-18.42	0.10

[#]: odds ratio for PNI in p75NGFR positive vs p75NGFR negative cases, controlling for the other factors in the model.

Figure 5. Desmoplastic melanoma, representative examples of neurotrophin receptors analyzed in current study



(A)PNI, H&E 20X **(B)** Corresponding section demonstrating PNI with p75NGFR immunohistochemicial staining, 20x **(C)** GGT/AGT functional RET polymorphism (RETp) at codon 691 of exon 11 **(D – F)** Immunohistochemical staining with TrkA demonstrating positive staining of only internal controls. **(D)** Positive staining of basal epidermal keratinocytes, negative staining of atypical lentiginous melanocytic proliferation, 20X, (**I**)Positive staining of eccrine glands, negative staining of adjacent DM cells, 40X, **(F)** Positive staining of follicular keratinocytes, negative staining of atypical lentiginous melanocytic proliferation extending along follicular epithelium, 20X.

DISCUSION

In this study PNI was investigated in both cSCC and DM, two cutaneous malignancies with a known propensity for PNI. Further, the expression of TrkA was investigated in both DM and cSCC while p75NGFR and RETp were additionally investigated in DM.

As mentioned previously, select studies have shown that cSCCs located on the on the H&N have a higher propensity for PNI.[14,93–95] Findings from our study, which to the best of our knowledge is the first to specifically compare PNI in SCCs from the H&N versus non-H&N areas indicate that there may be an increased incidence of PNI in the former. While differences between both groups did not achieve statistical significance, we observed a trend towards greater rates in the H&N comparing the incidence in H&E versus DIS ($p=0.49$ & $p= 0.30$ respectively). Due to the relative rarity of PNI detection, larger sample sizes are required to confirm our findings

The difficulty of detecting PNI using H&E alone has resulted in attempts to enhance detection with the use of immunohistochemistry in a spectrum of lineage-unrelated cutaneous as well as non-cutaneous malignancies, with most studies detection [incorporating a single immunostain, typically for enhanced nerve.[9,17,18,22] In cutaneous malignancies, immunohistochemistry has been used for PNI detection in melanoma, basal cell carcinoma (BCC) and cSCC.[9,17,22,96] In melanomas, Scanlon et al. using antibodies to neurofilament found an enhanced detection of PNI (25% with H&E versus 47% with

immunostaining), while in SCCs from the oral mucosa Kurtz et al. using an antibody to S100 also found an increased incidence (62% with H&E versus 82% with immunostaining).[9,17] In a recent study of 34 cases of cSCC, mostly from the H&N, Kelso et al. reported an increase in the incidence of PNI from 18% with H&E staining to 35% with S100 and 38% with p75NGFR (the low-affinity nerve growth factor receptor) immunostaining.[96] Zhou et al. concluded that DIS is superior to using a single imunnostaining in the identification of PNI in gastric adenocarcinoma.[18] Briefly, using anti-S100 antbodies for nerve detection and AE1/3 antibodies to label tumor cells, Zhou et al. observed an increased incidence of PNI from 57% to 65% (comparing S100 staining alone versus DIS) in gastric adenocarcinomas, arguing in favor of an enhanced sensitivity for PNI detection with DIS. Lastly in a recent study of both BCCs and SCCs Berlingeri-Ramos et al. compared PNI detection with S100 and AE1/3 DIS to H&E and reported a 33% increase in PNI detection, noting that DIS "was particularly useful to resolve the diagnostic dilemma of perineural proliferation and peritumormal fibrosis".[22] We too observed a statistically significant increased detection rate of PNI with the use of DIS and additionally found a correlation of DIS detected PNI with tumor depth, a known prognosticator of high-risk cSCC, underscoring the clinical significance of using DIS as an adjunct in tumor microstaging.

TrkA serves as the membrane bound high-affinity receptor tyrosine kinase for the neurotrophin NGF and is known to play a role in the development and maintenance of the peripheral nervous system.[97]Additionally, TrkA expression in normal human keratinocytes along with NGF, has been shown to play a role in proliferation and growth

of keratinocytes in vitro.[46] In keeping with this observation, we noted TrkA expression to be strongest in keratinocytes in the basal layer of the epidermis in uninvolved skin. Of note, this gradient appeared to be lost in epidermis over cSCC tumors with cytoplasmic staining typically observed pan-epidermally suggesting aberrant TrkA expression.

Immunohistochemical studies have shown that TrkA expression correlates with PNI in multiple non-cutaneous malignancies, including salivary adenoid cystic carcinoma, oral SCC, prostate, and pancreatic cancer.[50–52,98] As mentioned previously, Chen-Tsai et al. noted an association between TrkA expression and PNI in cSCC, albeit in a restricted number of samples (n=8), and with their scoring done as a subjective measurement of intensity rather than in a semi-quantitative manner. Furthermore, they did not differentiate between membranous and cytoplasmic expression. In the present study, TrkA positivity was restricted to cases displaying membranous staining and graded in a semi-quantitative manner. Using this stringent criterion, we found TrkA expression was not associated with PNI in cSCC, and not expressed at all in DM. The discrepancies between our findings and those of Chen-Tsai et al. may be explained by the use of different scoring systems or additionally by the fact that in their study, Chen-Tsai et al. used an antibody that labels the entire Trk family and not exclusively TrkA. It is possible that in cSCC PNI is mediated by a different Trk receptor (TrkB or TrkC) although future research is needed.

For DM, in one study by Flørenes et al. using IHC to investigate expression of activated TrkA (p-Trka),[58] positive expression was noted in 55% of primary melanomas (none of which were DMs or of spindled morphologies) and 30% of metastasis. Flørenes

et al. also noted that membranous p-TrkA expression was associated with decreased overall survival, and elevated markers of proliferation (cyclin A and Ki-67). Our finding that TrkA was not expressed in any cases of DM, indicates that TrkA is unlikely to play a role in its pathogensis. Likely explanations for the discordancy between our findings and those of Flørenes et al. include differential TrkA expression in non-DM variants and/or, the use of antibodies targeting different epitopes (for example phosphorylated vs the non-phosphorylated form of the TrKA receptor). Arguing against the former is our own experience using the present antibody in over 40 non-DMs which consistently demonstrated negative staining in all cases (data not included).

In cSCC, despite its lack of correlation with PNI, we observed other noteworthy correlations between TrkA and histopathologic prognosticators. Of Note, our finding of more frequent TrkA expression in cSCCs from the H&N, may relate to previous findings that ultraviolet radiation modulates TrkA expression in human keratinocytes. Ultraviolet radiation is a known potent inducer of apoptosis and there is in vitro evidence to indicate that TrkA and NGF expression are reduced in UV-irradiated human keratinocytes in culture.[46] In the same study, transfection of normal human keratinocytes with NGF resulted in resistance to UV-induced apoptosis, suggesting that shut-down of the TrkA-NGF axis promotes apoptosis. In areas chronically sun exposed, a failure to down-regulate TrkA expression may cause a loss of this protective apoptotic response leading to an increased mutational burden and tumor formation. Additionally, we found TrkA was more commonly expressed in high-risk morphologic variants and less well

differentiated cSCCs, suggesting an association between TrkA expression and changes in cytomorphology.

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Polymorphism in the RET gene (RETp) have been shown to be more common in DMs as compared to conventional melanomas,[73] and in vitro studies of melanoma cell lines have shown that RETp results in enhanced proliferation, migration, and invasion upon treatment with GDNF compared to cells with wild-type RET.[72] In pancreatic cancers RET-GDNF signaling has emerged as a potential mediator of PNI, with paracrine signaling between nerve and tumor cells believed to occur.[63,64] Given this, we hypothesized that RETp would be observed in a greater proportion of PNI positive cases. However, we observed a non-significant 3-fold increase in the proportion of RETp among PNI positive samples compared to PNI negative samples (39% versus 13%). While not significant, our findings suggest an association between RETp and PNI, and a larger study may be better powered to detect a significant correlation. Additionally, for cases in which RETp data was available, 5 displayed PNI but were p75NGFR negative. Of these, 3 (60%) had RETp, suggesting that in the absence of p75NGFR expression RETp may be of relevance in PNI. Our observation that only 7% of PNI positive cases lacked both RETp and p75NGFR, as compared to 57% of PNI negative cases (p=0.0008), underscores the potential role played by each.

While expression of p75NGFR has long been associated with the spindled cytomorphology in melanomas, much of the previous research has focused on the utility

of staining for p75NGFR as an adjunct to help identify S100-negative spindle cell melanomas and, to differentiate spindle cell melanomas from other lineage-unrelated spindle cell lesions of the skin.[80,82,83] Though some have demonstrated staining for p75NGFR in 100% of cases of DM,[80,82,88] we observed positive staining in 65% of our cases. However, select studies have also noted variable expression of p75NGFR in DM, corroborating our findings.[84,85] These discrepancies do not appear to be simply the result of variable scoring rubrics as, using similar cut-offs, studies reporting 100% of DMs staining for p75NGFR did not observe any cases with 10% or less tumoral staining.[80,82,88] Hence, we hypothesize that these differences may be the result of differing anti-bodies used and the relatively small samples sizes (<15) of previous studies.

The fact that p75NGFR expression is found more often in DM than in other subtypes, when taken together with DM's significant propensity for PNI has led some to hypothesize that p75NGFR may play a functional role in PNI.[85,88] Our findings show that beyond p75NGFR's previously known association with this variant, it is further associated with PNI, even after controlling for depth. In vitro studies on melanoma cell lines have shown that p75NGFR mediates the induction of protease expression in response to NGF treatment, resulting in an enhanced invasive capacity.[57] Furthermore, NGF has been shown to exert chemotactic effects on melanoma cell cultures, with p75NGFR mediating the response.[99] Taken together, from a scientific perspective it is reasonable to hypothesize that p75NGFR is associated with a greater propensity for PNI by allowing for both an enhanced invasiveness in response to NGF (permitting proteolytic digestion of the perineurium) and a chemotactic attraction to the NGF-rich

microenvironment of nerve as NGF is known to be secreted by Schwann cells.[47] Lastly, p75NGFR is a promiscuous receptor, and binds other Neurotrophins besides NGF (such as neurotrophin-3 and Brain-derived neurotrophic factor) each of which may have an additional role to play in affecting the behavior of melanomas expressing p75NGFR.

In conclusion, findings from this study indicate that in cSCCs, the increased detection of PNI using DIS and its correlation with select histopathologic prognosticators, underscore the utility of immunohistochemistry as an adjunct in microstaging. The association between TrkA expression and select adverse histopathologic parameters suggests a role for the NGF-TrKA axis in tumorogenesis of cSCC. Our observation that TrkA expression is significantly more common in cSCCs of the H&N than in non-H&N areas is indicative of the heterogeneity amongst SCCs from different anatomic sites. TrkA expression in cSCCs alone and not in DMs indicates that expression may be lineage-related. Expression of p75NGFR in DMs was associated with a greater propensity for PNI. While we did not observe a significant correlation between RETp and PNI, the 3 fold increase in RETp incidence observed in samples displaying PNI suggests that a larger study may be better powered to detect an association. Lastly, our observations in cases of DM with PNI that only a limited number (7%) were negative for both RETp and p75NGFR highlights the potential role played by each.

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