2018

The Nervous System And Cancers Of The Head And Neck

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THE NERVOUS SYSTEM AND CANCERS OF THE HEAD AND NECK

by

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Bachelor of Science
Winthrop University, 2008

Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Biomedical Science

School of Medicine

University of South Carolina

2018

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DEDICATION

I dedicate this body of work first and foremost to the many courageous patients and their families who have selflessly contributed to better understanding the complexities of chronic disease. To my late grandmother, Evelyn Graves, who was a light to this world and an inspiration to all and who was a victim of an intractable disease of the nervous system; and to my beautiful girls: your continuous curiosity, support, and zeal for life is a perpetual source of drive to do right in hopes of making the world a better place.

ACKNOWLEDGEMENTS

I would like to thank my colleague, advisor, and friend Dr. Lucia Pirisi-Creek for allowing me the creative latitudes to explore my passion for progress in the frontiers of neuroscience, technology, and chronic disease. It has been an honor to manage and work alongside her and our team members in our laboratory group over these past years. I would also like to thank my clinical mentor Dr. Jim Wells for his surgical mentorship, drive, and commitment to true progress in caring for patients with head and neck cancer.

I thank my committee members, including Dr. Peter Botrous for his perpetual intrigue and support on novel translational projects, Dr. Kim Creek for his critical thought on molecular mechanisms, and Dr. Susan Wood for her leadership and expertise in exploring the intricacies surrounding how chronic stress impacts our lives. I extend my gratitude to our collaborators at NIH, Johns Hopkins, Vanderbilt, MUSC, University of and Pittsburgh. I thank the many friends, mentors, and colleagues who I have met along this path for your enduring interest and support of my work.

Finally, I cannot begin to express enough gratitude to my family for their support through this journey. I thank my mother and father, my mother- and father-in-law, siblings, and grandparents for supporting me in this quest for wisdom.

ABSTRACT

The anatomy of the head and neck is closely associated with the nervous system which plays an important role in the prognosis of head and neck cancer (HNC). However, the molecular interactions between these compartments and HNC remain poorly understood. We present a novel big data approach utilizing clinical data, sequencing, and machine learning to identify and validate potential molecular pathways by which the nervous system affects the development and progression of HNC. Our studies demonstrate across multiple datasets that perineural invasion (PNI) frequently occurs in HPV+ HNC. Furthermore, we show novel activating and missense mutations and pathways that may play important roles in the progression of HNC. We hypothesized that HPV+ cancers might be driven by different neurotrophic-associated genes and programs. We observed that neuroendocrine and neurotrophin signaling through the nerve growth factor receptor (NGFR) can be observed in differentiating HPV+ versus HPV- and PNI+ versus PNI- HNC. These observations may provide significant therapeutic targets for HNC.

PREFACE

All the work presented herein was conducted by Christian A. Graves and colleagues while in the laboratory of Lucia A. Pirisi-Creek at the University of South Carolina School of Medicine and under the clinical guidance of James R. Wells at the WJB Dorn VAMC located in Columbia, SC. All translational research involving human specimens and associated methods were reviewed and conducted under approval by the Institutional Review Boards at the University of South Carolina, the Dorn VAMC, the Medical University of South Carolina, and the Vanderbilt University Medical Center.

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n	The number of samples in a particular study or cohort.
p	The p-value is the statistical level of calculated significance.
χ^2	Chi-squared distribution of a sum of squares of independent variables.

LIST OF ABBREVIATIONS

AR ..... Alveolar Ridge

ATR ..... Ataxia Telangiectasia Radp-6-related

AURKβ .....Aurora-Kinase β

BCL2L2 ..... Bcl-2-like Protein 2

BDNF ..... Brain Derived Neurotrophic Factor

BM ..... Basal Mucosae

BOT ..... Base of Tongue

CCP .....Comprehensive Cancer Panel

CDKN2A .....Cyclin Dependent Kinase Inhibitor N2

CGrA ..... Chromogranin A

CK20 ..... Cytokeratin 20

COL1A1 .....Collagen Type-1, Alpha-1

DAB ..... 3,3'-Diaminobenzidine

EBV ..... Epstein-Barr Virus

ERCC5.....Excision Repair 5 Endonuclease

EMT .....Epithelial-Mesenchymal Transition

EP300 ..... E1A Binding Protein P300

FFPE..... Formalin Fixed Paraffin Embedded

FM..... Floor of Mouth

GABRP .....GABA Receptor Pi Subunit

GAD1 ..... Glutamate Decarboxylase 1

GDNF ..... Glial Cell Derived Neurotrophic Factor

GSEA ..... Gene Set Enrichment Analysis

GO ..... Gene Ontology

GRIN2C ..... Glutamate Receptor Inotropic 2C

GRM8 ..... Glutamate Receptor Metabotrophic-8

GWAS ..... Genome Wide Association Study

HNC ..... Head and Neck Cancer

HP ..... Hard Palate

HPV ..... Human Papillomavirus

HPx ..... Hypopharynx

HRP ..... Horseradish Peroxidase

IMRT ..... Intensity Modulated Radiation Therapy

ISP ..... Ion Sphere Particle

ISL1 ..... Islet-1

L ..... Lip

LIM Lim11 ISLI-1 Mec-3

Lx ..... Larynx

MAF ..... Mutant Allele Frequency

MCC ..... Merkel Cell Carcinoma

MCPyV ..... Merkel Cell Polyomavirus

MLL3 ..... Mixed-lineage Leukemia Protein 3

NER ..... Nucleotide Excision Repair

NEFH ..... Neurofilament Heavy Chain

NGF ..... Nerve Growth Factor

NGFR.....Nerve Growth Factor Receptor

NSM.....Non-silent Somatic Mutations

OC .....Oral Cavity

OPx.....Oropharynx

OS..... Overall Survival

OT.....Oral Tongue

PDE4DIP ..... Phosophodiesterase 4D Interacting Protein

PDGFRA..... Platelet Derived Growth Factor Alpha

PNI.....Perineural Invasion

POU4F1..... POU Class 4, Homeobox 1

PRKDC .....Protein Kinase, DNA-activated, Catalytic Peptide

SIM2 ..... Single-minded Homolog 2

Syn.....Synaptophysin

SNP .....Single Nucleotide Polymorphism

T..... Tongue

TCGA.....The Cancer Genome Atlas

TP53 .....Tumor Protein p53

TrkA/B.....Tropomyosin receptor Kinase A/B

TRPV6 .....Transient Receptor Potential Cation Channel Subfamily V 6

UV.....Ultraviolet

UNC-5..... Netrin Receptor

CHAPTER 1

INTRODUCTION

The Head and Neck anatomy includes intricately interconnected neurologic, immunologic, and endocrine tissues. As the terminus of the aerodigestive tract, this region is also continually exposed to pathogens. As a result, many chronic diseases of the head and neck - including neoplasia - pathologically involve diverse neurological, endocrine, and immunological components. Despite seminal historical discoveries suggesting these relationships, diagnostic and scientific understanding of the molecular underpinnings and drivers of head and neck neoplasia remain poorly understood.

Head and neck cancers (HNC) constitute approximately 3% of all new cancer diagnoses per year (Leemans et al. 2011). HNC risk factors include stress-related behaviors such as tobacco use and alcohol abuse, and infection with oncogenic viruses like human papilloma virus (HPV), Merkel Cell Polyomavirus (MCPyV), and Epstein-Barr Virus (EBV) (Kang et al. 2016). Thus a mosaic of drivers exist in HNC. Despite the discovery of interactions between HNC and the nervous system through early anatomical studies of perineural invasion (PNI) in HNC, accurate pathologic diagnosis and staging of PNI remains a confounding factor in management of the disease (Cruveilhier 1835). It is estimated that PNI is present but misdiagnosed in up to 70% of cases and that

involvement with nervous tissues occurs between 40-95% of all patients with HNC (Chi et al. 2016; Johnston et al. 2012). The anatomical involvement of neuroendocrine and the nervous system structures has recently become an area of translational inquiry.

Interactions between these tissues and the presence of PNI in diseases of the head and neck offer compelling potential to better understand HNC. To this end, we hypothesize that unique neural, endocrine, and immunological pathways are present in HNC. Our projects explore the molecular relationships between PNI, neuroendocrine tissues, oncogenic viruses and outcomes in HNC. Through the use of machine learning and big data analytic techniques conducted in clinical HNC samples, we have successfully identified molecular targets including those involved with the peripheral nervous system and neurotrophic factor signaling. We have deployed clinical trial evaluation in retrospective and prospective trials to better elucidate the importance of this triad of neurological, endocrine, and immunologic interactions. Taken together, these big data findings provide compelling translational targets and potential strategies for the palliation and treatment of HNC.

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CHAPTER 2

MACHINE LEARNING AND MOLECULAR PATHWAY ANALYSIS OF PNI IN HNC

Perineural invasion (PNI) in cancers of the head and neck (HNC) is universally associated with poor progression-free survival and decreased mortality (Johnston, Yu, and Kim, 2012). Patients present with increased locoregional recurrence, metastasis, and treatment failure. Despite the clear association with a more severe clinical picture, and historic observation of this aggressive sub-type of HNC, diagnosis and treatment remain technically limited with few validated markers of perineural involvement (Soo et al., 1986; Brandwein-Gensler et al., 2005; Vural et al., 2000; Carter et al., 1983). Pathologic diagnosis of perineural involvement is often only revealed by serial sectioning by experienced pathologists and is often an incidental finding (Brandwein-Gensler et al., 2005; Fagan et al., 1998; Kurtz et al., 2005). Early recurrence also drives a more extensive work-up and often indicates repeated cycles of chemoirradiation. Current standard of care recommends salvage intensity modulated radiotherapy and RTOG trials investigating adjuvant cetuximab are underway, however these studies are not designed specifically to address PNI (Johnston, Yu, and Kim, 2012). Control rates of post-operative elective intensity modulated radiation therapy (IMRT) versus patients who were not treated with IMRT were recently reported at 100% and 82%, respectively

(Kropp et al., 2013). Despite long-term appreciation of this clinical entity the molecular underpinnings differentiating neurotrophic cancers in the head and neck from non-neurologically invasive disease remain poorly understood. Soo and colleagues demonstrated early treatment failure, diminished three-year survival and increased locoregional recurrence in perineural disease (Soo et al., 1986). Several other retrospective studies have evaluated the proximity of lesions to nerves < 1mm in diameter demonstrating the importance of this metric in the pathologic work-up of these tumors (Brandwein-Gensler et al., 2005; Fagan et al., 1998).

To better assess molecular drivers of PNI we retrospectively analyzed the recently published The Cancer Genome Atlas (TCGA) pan-cancer dataset for clinical, molecular, and survival trends significant in pathologically diagnosed PNI versus non-neurotrophic HNC (Kandoth et al., 2013). TCGA is a National Cancer Institute supported genomic consortium designed to enhance community and clinical knowledge of genomic drivers for the most malignant cancers.

We herein confirm previous reports of a diminished overall survival in patients with PNI+ HNC and this is correlated with commonly described mutations in HNC in both PNI+ and PNI- disease. Lesions with PNI also occurred more frequently within the oral tongue and appeared to be driven by neurogenic pathways while PNI- lesions were significantly associated with alterations of SMAD2/4 signaling. Importantly, this analysis revealed several significantly up-regulated pathways related to neurogenesis and neural signaling that have yet to be described in HNC. Taken together, these neurogenic-related

targets provide a rational explanation for the decreased survival and aggressive disease in PNI+ HNC, and suggest alternative mechanisms which may be exploited to benefit patients with aggressive disease.

2.1 MATERIALS AND METHODS

Data Source

The TCGA is an initiative to provide publicly available sequencing, mutational, and expression data for 25 selected cancers to generate advances in clinical discovery. These studies were exempt from formal review by the University of South Carolina Institutional Review Board and complied with the TCGA guidelines for data use (Kandoth et al., 2013).

Patient Selection

All data were downloaded from the data portal on the TCGA site and parsed based on patients diagnosed with head and neck cancer. All cases were diagnosed by board-certified pathologists and only patients with complete clinical history were considered. Demographic data included sex, age at diagnosis and race. Clinical data considered included clinical stage, overall survival, treatment, and anatomic site. Clinical data including stage, grade, perineural invasion status, and AJCC TNM classification were also included (AJCC, 2002).

Genomic mutational status and gene expression data were accessed from the TCGA data portal Synapse and downloaded using the R package synapse client. TCGA data are organized in levels based on the extent of analysis and

normalization, with level 1 being raw data and level III data corresponding to published data. All level III HNSC pancancer data (*n*=173) were assessed following normalization with a FDR of 0.03 and FC of 2.0 and *p value* <0.05 was considered significant. Data were queried for perineural (72 cases) versus no evidence perineural disease (101 cases). All sequencing reads were analyzed by two methods: the DESeq2 package and EdgeR in the R package followed by additional Gene Set Enrichment Analysis (GSEA) (AJCC, 2002; Anders & Huber, 2010; Mayburd et al., 2006). Ingenuity pathway analysis (Ingenuity® Systems, http://www.ingenuity.com) a web-delivered application that enables the discovery, visualization, and exploration of molecular interaction networks in gene expression data, was utilized to analyze transcriptomic data (Mayburd et al. 2006).

Statistical Analysis

All Categorical statistical analysis between clinical and demographic variables and Kaplan Meier Survival analysis was carried out in GraphPad PRISM 6.0. A p-value <0.05 was considered significant. Ingenuity pathway analysis (Ingenuity Systems, USA) was utilized to determine significant pathways between PNI+ versus PNI- data downloaded from the Synapse Analyses. All pathway data were analyzed using t-tests *in silico*.

2.2 RESULTS

Clinical Presentation of the TCGA HNC Cohort. The overall incidence of PNI was 34% (*n*=124/366) in patients included in the clinical HNC TCGA pan-cancer

analysis while 66% (*n*=242/366) patients were PNI-negative. The median age and sex distribution of PNI+ vs. PNI- were 62.5 and 60 and 31% vs. 27% female and 69% vs. 73% male, respectively (Table 2.1). Grade at diagnosis distributions were similar between perineural involvement versus unaffected lesions with Grade 2 being the most common (66% vs. 57%, respectively). Interestingly, PNI+ lesions presented with higher staging (Stage IVA; 67% vs. 38%, PNI+ vs. PNI-). Lymphadenopathy was also more common in PNI+ versus PNI- disease (38% versus 14%) although this might have been biased by a more extensive work-up of patients with PNI+ disease. The more aggressive disease was also confirmed in the PNI cohort with an increase in higher tumor stage (≥T4 71% vs. 43%, PNI+ vs. PNI-, respectively) and nodal involvement (49% vs. 21%, PNI+ vs. PNI-, respectively) (Table 2.2).

Lesions of the oral tongue were the most commonly associated with perineural invasion (48% vs. 21%) while lesions of the larynx were most prevalent in the PNI- group (29% vs. 19%) ($p<0.0001$) (Table 2.3) The types diverged when comparing incidence and PNI+ patients presented with diminished median survival of 2.45 years versus 4.36 years ($p=0.0128$; Wilcoxon Log-Rank) upon survival analysis (Figure 2.1).

Analysis of Genomic Mutations in Perineural Invasion Sub-typed Patients. Because no studies have yet evaluated PNI disease versus disease without PNI for molecular mutations, we next assessed genomic mutations in patients with PNI+ and without PNI- lesions in the most aggressive disease (OS< 2.5 years).

38/105 (37%) PNI+ and 52/193 (27%) PNI- were found to cluster in poor survivorship of <2.5 years.

Two hundred ninety-eight patients (*n*=298/366; 81%) had sufficient data from deep-sequencing and were considered for both PNI mutational and survival assessment. Of these sequenced patients, 36% (*n*=105/298) were PNI+ and 64% (*n*=193/298) were PNI- (Table II). This incidence falls within the range reported 27-82% (Johnston, Yu, & Kim 2012; Mendenhall et al. 1989). Importantly, these data represent the largest assessed cohort to date with full matched clinical and mutational data sets.

The median number of non-silent somatic mutations (NSM) were similar for each cohort (105 vs. 111, PNI+ vs. PNI-). Furthermore, we observed a lower incidence of NSMs in HPV+ lesions when considering PNI+ (74) and PNI- (66) specimens as previously reported (data not shown). Assessment between PNI+ tumors versus PNI- for non- synonymous gene mutations revealed a similar incidence in total events involving TP53 (87% and 88%), CDKN2A (24% and 27%), NOTCH1 (24% and 25%). Interestingly, PNI- tumors exhibited increased mutation rates for AJUBA (7 vs. 1 mutation) and EP300 (6 vs. 1 mutation) compared to PNI+ tumors while no specific drivers were observed in PNI+ tumors.

Alterations in Gene Ontology Pathways in the Perineural Invasion Sub-Type. HNC is increasingly recognized as a heterogeneous disease with a spectrum of disease sub-types with accompanying mutations and gene

expression profiles, so we next considered variations in the transcriptome of PNI+ versus PNI- disease (Walter et al., 2013). Analysis of raw RNAseq data included 73 PNI+ and 101 PNI- patients with complete raw data. Interestingly, top differentially expressed gene ontology (GO) pathways associated with G-protein coupled receptor signaling (GO:0007186; $p = 2.56E^{-7}$), sensory perception (GO:0007600; $p = 9.22E^{-7}$), dopamine metabolic process (GO:0042417; $p = 4.80E^{-6}$), neurological system process (GO: 0050877; $p = 5.11E^{-6}$), and sensory perception of chemical stimulus (GO: 0007606; $p = 4.37E^{-5}$) were observed in PNI+ cancers (Figure 2.2). To our knowledge, this is the first time these GO pathways have been associated with PNI+ disease.

We next conducted pathway analysis to determine significant genes associated with PNI+ vs. PNI- genes. Interestingly, IPA analysis revealed a significant up-stream regulation by brain-derived neurotrophic factor (BDNF; $p=8.3311E^{-6}$). This trend was complemented by a positive heuristic for NGF signaling (3.667) which was a predicted positive regulator (Löw et al., 2008). Gene expression profiling revealed elevated neurotrophin and diminished inhibitory netrin signaling - two important neurogenic pathways (Figure 2.3). Importantly, these data correlate with the poorly differentiated status observed in the PNI+ versus PNI- (Table 2.2). Taken together, these target pathways represent novel mechanisms underlying the mutational status and gene expression profiles in PNI-associated cancers of the head and neck.

2.3 TABLES AND FIGURES

Table 2.1. Patient demographics extracted from the TCGA data set with respect to the presence or absence of PNI.

		PNI+ *n* = 124		**PNI-** *n= 242*	
	Age	62.5 (19-90)		60 (24-87)	
		n	%	n	%
Sex	Female	39	31%	66	27%
	Male	85	69%	176	73%
Alcohol	Neg	41	33%	74	31%
	Pos	81	65%	163	67%
	ND	2	2%	5	2%
Tobacco	Reformed > 15y	14	11%	42	17%
	Reformed ≤ 15y	38	31%	61	25%
	Current	44	35%	82	34%
	Lifelong	24	19%	50	21%
	ND	4	3%	7	3%
Lymphadenopathy	Neg	59	48%	108	45%
	Pos	47	38%	33	14%
	ND	18	15%	101	42%

Table 2.2. Tumor Stage and Nodal status demonstrates more aggressive disease in PNI+ patients versus PNI- lesions.

	PNI+ *n=124*		**PNI-** *n*=242	
Tumor Stage (T)				
≤ T3	36	29%	128	53%
≥ T4	88	71%	104	43%
Node Stage (N)				
≤N2a	63	51%	180	74%
≥N2b	61	49%	51	21%
ND	0	0%	11	5%

Table 2.3. Frequency of PNI in tumors at various anatomic sites reveals increased PNI in Oral Tongue Lesions. P<0.0001, χ^2 .

Anatomic Site	**PNI+** *n=124*		**PNI-** *n*=242	
AR	2	2%	8	3%
BOT	2	2%	19	8%
BM	6	5%	7	3%
FM	16	13%	18	7%
HP	2	2%	4	2%
HPx	2	2%	2	1%
L	0	0%	2	1%
Lx	20	16%	69	**29%**
OC	23	19%	33	14%
Opx	0	0%	2	1%
OT	50	**40%**	52	21%
T	1	1%	26	11%

Abbreviations: AR: Alveolar Ridge; BOT: Base of Tongue; BM: Buccal Mucosae; FM: Floor of Mouth; HP: Hard Palate; HPx: Hypopharynx; L: Lip; Lx: Larynx; OC: Oral Cavity; OPx: Oropharynx; OT: Oral Tongue; T: Tonsil. N=343 patients.

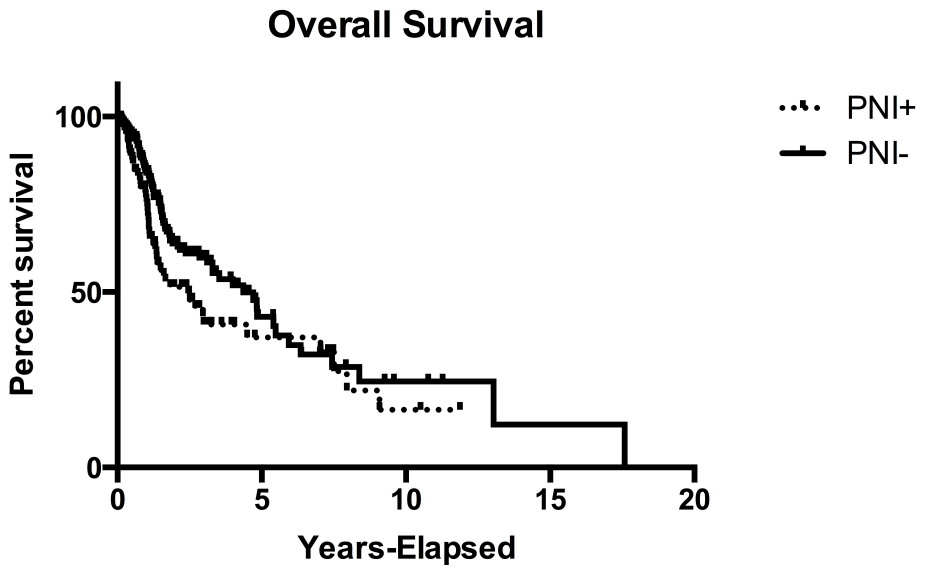

Figure 2.1. Overall Survival is shorter in PNI-associated disease. Patient survival was analyzed by Wilcoxon-Log-Rank analysis. PNI-positive had a median overall survival of 2.46 (95% CI: 0.3913 to 0.8105) versus 4.36 (95% CI: 1.234 to 2.555).

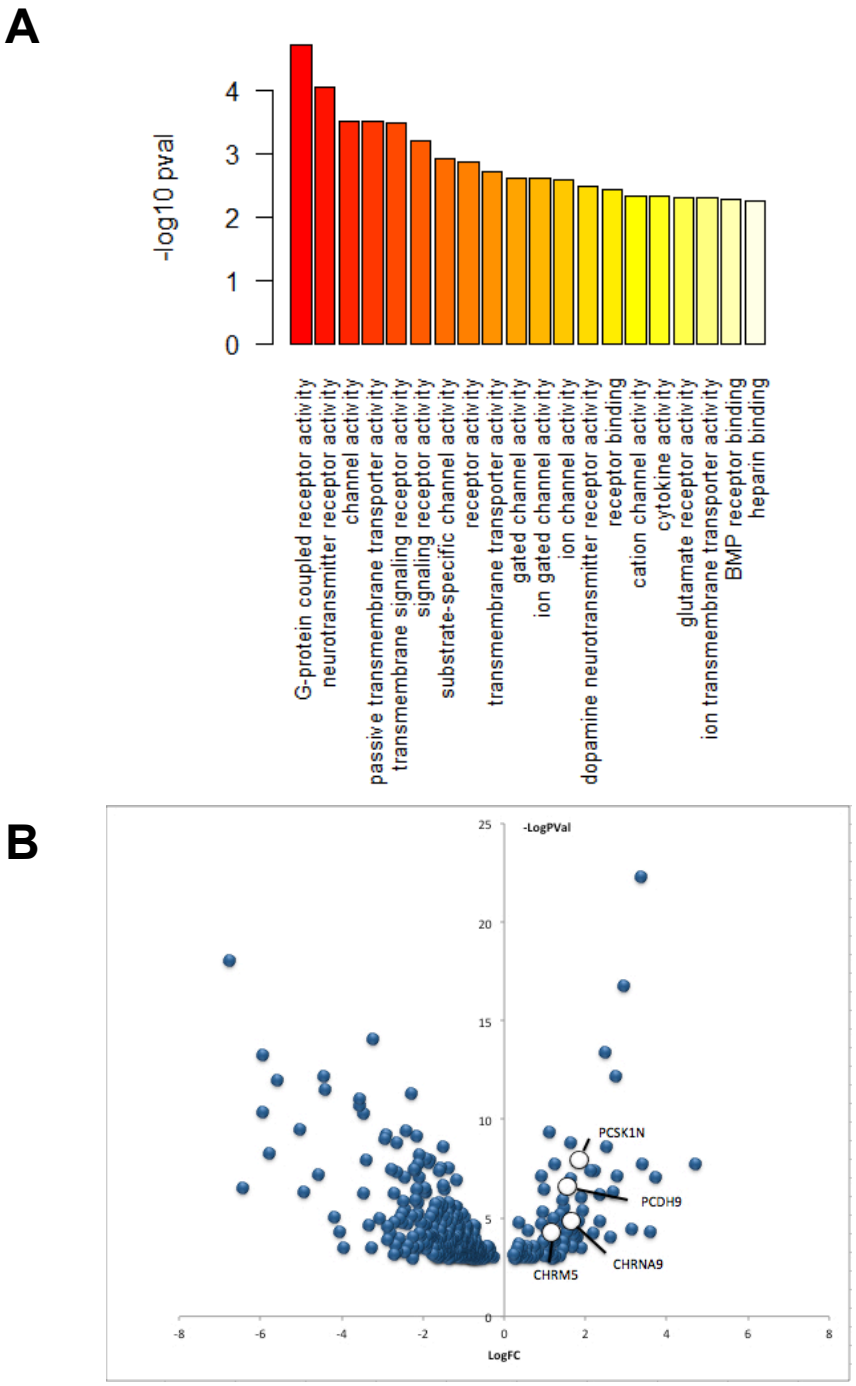

Figure 2.2. Gene expression data and pathway analysis from PNI+ tumors versus PNI- tumors. A) GO analysis reveals increased neurogenesis-related signaling as well as several neurologic related and sensory-related ontogenies; B) A waterfall plot of elevated transcripts reveals important neuropeptide (PCSK1N, PCDH9) and neurotransmitter signaling genes in PNI+ disease (CHRM5, CHRNA9).

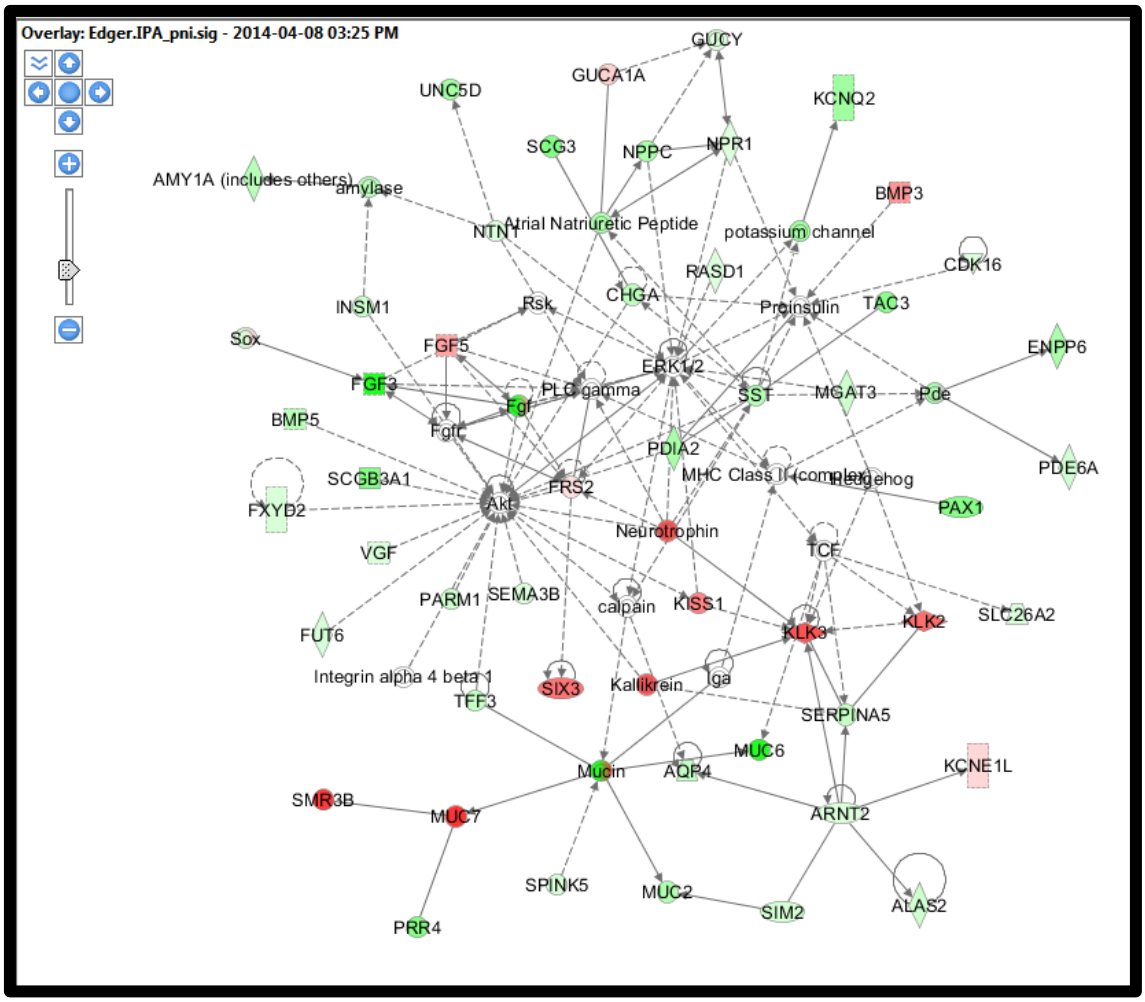

Figure 2.3. Pathway analysis shows increased neurotrophin and decreased netrin signaling in PNI+ HNC.

2.4 DISCUSSION

Perineural disease is clinically associated with poor outcome, difficult management, and brain metastasis in HNC; however, the mutations and gene expression patterns which differentiate perineural association versus local invasion are largely unknown. Our analysis confirms a poor prognosis in association with the perineural invasion in the largest fully sequenced cohort to date. While the mutational spectrum was similar between these sub-types and the number of non-silent mutations, PNI+ lesions were more aggressive clinically. We also observed a higher incidence of mutations in AJUBA and EP300 in non-perineural invasion-associated disease (PNI-). AJUBA encodes a LIM protein that interacts with critical regulators of epithelial to mesenchymal transition (EMT), a key regulator of invasiveness in HNC. LIM proteins are co-repressors of E-Cadherin via Snail family transcription factors (Langer et al., 2008). EP300 encodes a histone acetyltransferase protein that has recently been implicated in EMT and hypoxia (Chen et al., 2013). The associations between mutations in EMT-related genes in PNI suggest an alternative mechanism to traditional invasiveness in perineural involvement in HNC. These findings suggested to us a divergence in the expression profiles of the PNI+ tumors versus the tumors with no perineural involvement.

We next interrogated level III RNAseq data for difference in the transcriptome and revealed involvements of neurogenesis and nervous-system-related pathways in HNC. Recent genomic studies have greatly advanced the number of clinically actionable targets in HNC (Kandoth et al., 2013); Walter et

al., 2013; Stransky et al., 2011; Agrawal et al., 2011; Tamborero et al., 2013). Our unique study focused on Gene Set Enrichment Analysis (GSEA) and pathway analysis in exploring the importance of differential gene expression in perineural versus non-perineural disease. Various neurotrophic ontogenies which centered around cholinergic signaling, sensory perception in the periphery, and neurotrophin signals were significantly implicated. The translational importance of these findings is corroborated by recent studies that have demonstrated an emerging role of the peripheral nervous system in tumor control (Magnon et al., 2013). We also discovered a decrease in the inhibitory netrin receptor UNC-5 which serves as a repulsive molecule for neuronal axons in the periphery (Löw et al., 2008). Taken together with the increases observed in neurogenesis and neurogenic GO pathways, these decreases in signals that inhibit netrin action indicate that signals that stimulate innervation are enhanced in perineural disease. This hypothesis is further supported in lesions of the head and neck where perineural involvement, and the opportunity for tumors to nerves are high (Johnston, Yu, & Kim, 2012;Mendenhall et al., 1989; Lanzer et al., 2014). A better understanding of the molecular mechanisms pathways which drive aggressive PNI behavior and neurogenic spread are of great significance for our efforts to decrease the risk of locoregional failure and metastasis.

In addition to the confirmation of clinical studies of PNI in HNC, these data hold translational importance for biomarker development and therapeutic targets for better management of perineural invasion-associated aggressive disease. Perhaps most actionable are the neurotrophin-related pathways implicated in this

unsupervised analysis. It has previously been demonstrated that neurotrophin activity is associated with increased invasiveness, poor outcomes, and resistance to chemotherapy (Søland et al., 2008; Yilmaz et al., 2010). Genomic patient-based studies have also implicated epigenetic silencing of neurotrophin-driven pathways in HNC (Panizza et al., 2013). These data complement other studies in demonstrating the genetic and gene expression differences between PNI+ and PNI- lesions and suggest targets for future investigation (Agrawal et al., 2011; Panizza et al., 2013; Kostareli et al., 2013; Kolkythas et al., 2010). It will be of great interest to investigate these targets in larger well-designed studies where PNI is specifically investigated by imaging, pathologic staging, and molecular analysis.

The aggressive nature of PNI-associated disease is further indicated and the need for actionable pre-clinical targets and surrogate markers – to complement conventional imaging studies and anatomic pathology – is paramount. Translating these markers into clinically actionable subtypes holds promise in the management of head and neck disease.

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CHAPTER 3

MASSIVELY PARALLEL SEQUENCING IDENTIFIES NOVEL MUTATATIONS IN NEUROENDOCRINE HNC

Merkel Cell Carcinoma (MCC) is a relatively rare neuroendocrine cancer with poor prognosis that is seen with increasing frequency in the USA (Schrama et al. 2012). MCC often presents in the sixth decade of life in patients of predominantly Caucasian ethnicity. Tumors arise in UV-exposed regions of the head and neck, upper thorax and extremities (Lemos et al. 2007). The disease often presents as nonpainful nodules with local invasion in immunocompromised patients. Recently, a great deal of attention has focused on MCC due to the discovery of a viral pathogenesis for the disease (Feng et al. 2008).

Merkel cell carcinoma is a neuroendocrine neoplasm involving somatosensory cells present within the epidermis. Merkel cells, also known as Merkel-Ranvier cells or APUD cells (apparently unrelated endocrine cells) contain neuroendocrine granules and detect coarse tactile stimuli. Their distribution occurs throughout the basal epidermis in both glabrous and haired skin including the nose, lips, and gingiva (Winkelmann & Breathnach 1973). Merkel cells often juxtapose hair follicle bulges and Langerhann's cells, and are increased in mechanosensory and tactile-sensory regions. As such, these cells are innervated by Aα nerve fibers of the peripheral nervous system (Boulais & Misery 2007) This compartment is innervated at approximately 50 cells per nerve

bouton and is principally served by mechano- (αβ), proprio(γ and C)-, and nociceptive (Aα and C) fibers (Lumpkin & Bautista 2005). Merkel cells are putative mechanosensory cells of the epidermis; however, the direct mechanistic role they play in neoplasia remains poorly defined at the molecular level.

MCC is considered a nonmelanotic skin cancer and has only within the past several decades gained better definition. MCC was first described by Cyril Toker as trabecular carcinoma of the skin and its diagnosis was greatly facilitated by the advent of reliable cytokeratin 20 immunohistochemical staining in the early 1990's (Toker 1972; Moll et al. 1992; Erovic & Erovic 2013). Additionally, MCC lesions are positive for various granular neuroendocrine markers including chromogranin A and synaptophysin. Interestingly, in 2008 a DNA polyomavirus was identified and classified as the Merkel cell polyomavirus (MCPyV) that has since been detected in the majority of MCC cases (Feng et al. 2008; Erovic et al. 2013). Infection with MCPyV is believed to be associated with nearly 100% of MCC. Reactivation of latent MCPyV in immunocompromised individuals has been posited as an essential underlying pathogenic mechanism (Wiedinger et al. 2014).

Merkel cell carcinomas are clinically challenging to manage and often recur locally within a short time following initial resection. Management often includes broad excision followed by concurrent chemoradiation including 5-Fluoroxyuridine (5-FU) and platinum-based regimen and there are currently no FDA-approved targeted therapies.

The management and understanding of MCC has remained limited due to the absence of deep sequencing studies to determine potential mutations within these tumors (Rodig et al. 2012; Nardi et al. 2012). To this end, we have implemented a massively parallel sequencing approach covering over 400 cancer-related genes in an attempt to further dissect some of the critical oncogene drivers in a cohort of MCC patients treated at our institution.

3.1 MATERIALS AND METHODS

Patient Selection

All aspects of the study were approved by the William Jennings Bryan Dorn VA Medical Center research department and institutional review board. Retrospective chart reviews conducted from 1993 to 2013 revealed a total of five patients diagnosed and treated for neuroendocrine Merkel cell carcinoma. Patient demographics, clinical metrics, history, progression free survival, and overall survival were analyzed.

Diagnostic Pathology

All cases were confirmed by a board certified pathologist for histopathological small cell differentiation as well as cytokeratin 20 (CK20), Synaptophysin (Syn), and Chromogranin A (CgrA). Additionally, Merkel cell polyoma virus (MCPyV) large T antigen was analyzed in slides from the retrospective cases and reviewed.

DNA Extraction and Massively Parallel Sequencing of 400 Cancer-related Genes.

Genomic DNA was extracted from archival formalin fixed paraffin embedded (FFPE) utilizing a FFPE DNA extraction kit as per manufacturer's recommendations (Qiagen). Briefly, tissue was sectioned at 4 μm with approximately 3 sections per block and deparaffinized in xylene. Tissue was then washed with ethanol and the sedimented samples were separated from residual solvent by evaporation. Samples were lysed in lysis buffer with 10% DNA Proteinase-K at 55 °C for 60 minutes with an additional reversal of DNA cross-links at 90 °C for 60 minutes. Lysed tissue was then added to DNA columns and submitted to a series of washes with elution in water.

AmpliSeq (Life Technologies) DNA libraries were then prepared following the manufacturer's instructions. Briefly, for each specimen, 40 ng of DNA was divided amongst four multiplex primer pools containing 10 ng of template DNA. Approximately 4000 target amplicons were amplified in each pool using the AmpliSeq Comprehensive Cancer Panel (CCP) primers and standard AmpliSeq manufacturers protocol. These amplicon pools were then combined and put through the remainder of the library preparation per the standard protocol. Libraries were diluted to 100 pM, combined in equal amounts, and used for template preparation of Ion Sphere Particles (ISPs) per the manufacturer's instructions. Prior to sequencing, the percent of templated ISPs was verified to lie between 10% and 25%, ensuring that the appropriate amount of library was added during the template preparation step. ISPs were then

enriched and deposited on to a Proton PI sequencing chip per the instructions given in the Ion PI Sequencing 200 Kit user guide. Six CCP libraries were sequenced simultaneously across two Proton runs, yielding between 15.5M and 28M aligned reads per library. Alignment and Variant identification was performed using Torrent Suite version 3.6.2 using high stringency somatic detection settings.

Statistical Analysis

All statistical analysis was carried out using the Torrent Suite version 3.6.2 as described above.

3.2 RESULTS

Patient Demographics and Clinical Course. Retrospective chart reviews conducted from 1993-2013 identified 5 patients treated at our institution and diagnosed with Merkel cell carcinoma. Patients fell within the typical diagnostic criteria with all being Caucasian males >60 years of age (Mean: 80, 61-89) (Table 3.1). Patients were referred to the clinic following identification of characteristic MCC nodules. Interestingly, 80% (4/5) patients presented with past history of colon adenocarcinoma and actinic keratosis (Table 3.1). One patient was incidentally diagnosed and treated with wide-band excision of the forehead. Three patients underwent direct biopsy with subsequent subtotal resection and 4/5 patients underwent salvage gross total resection. Post-operative scans demonstrated marked disease reduction. Two patients completed follow-up radiation therapy while one patient underwent chemotherapy

alone. Eighty percent of patients (4/5) presented with history of skin cancer and dermatological disorders at diagnosis (Squamous Cell Carcinoma: 2/5;40%; Melanoma: 1/5; 20%; Actinic Keratosis 4/5; 80%).

Patient Pathology. All diagnoses were confirmed by a board certified pathologist with chromogranin A (CgrA), synaptophysin (Syn), and cytokeratin 20 (Ck20) all common pathological markers of neuroendocrine Merkel cell carcinoma. All cases were positive for CgrA, Syn, and CK20 (Figure 3.1; Table 3.2). Additionally, where available, tissue was stained for the surrogate marker MCPyV large T antigen which was present at the reported frequency of 75% of the cases with the most commonly used antibody (3/4 specimens) (Figure 3.1f).

Massively Parallel Sequencing of Patient Samples. Adequate tissue for DNA extraction was available from four archival patient specimens and the blocks were selected for further analysis utilizing the Ion AmpliSeq Comprehensive Cancer Panel (CCP) on Ion Proton instrumentation to assess somatic mutational spectra. The CCP is a multiplex PCR-based 409 gene targeted panel with rapid high-throughput assessment of mutations based on the Wellcome Trust Sanger Institute's Cancer Gene Census. Within our data set, we called an average of 4606 variants per patient (*n*=4; 4472-4762). One sample was found to maintain elevated variants at 12226 called variants (Appendix B). This is not unexpected as this specimen was processed in higher concentration formalin had been archived for a prolonged period (Srinivisan et al. 2002; Williams et al. 1999). However, concomitant variants and deleterious mutations were detected within all genes that were interrogated.

Gene Mutational Spectrum Analysis. Next-generation sequencing analysis revealed a spectrum of mutations which were present at high mutant allele frequencies (MAF). The CCP contains exon-specific probes which specifically avoid homopolymers and we corrected for the possibility of G>C and T>A transversions by focusing only on high MAFs. Due to the retrospective nature of the cohort, matched blood samples to determine somatic mutational status were unavailable. We therefore focused our analysis only on the most significant and penetrant mutations within our population.

Nonsense mutations. Several nonsense mutations were detected in 75% of patients (*n*=3/4) within the fourth exon of PDE4DIP (Table 3.3). This gene encodes phosphodiesterase-4 interacting protein or myomegalin, a protein commonly associated with myelodisplastic disorders. Interestingly, phosphodiesterase-4D is a key interacting enzyme responsible for downstream inhibition of cyclic adenosine mono-phosphate (cAMP)-mediated activity within neural tissues (deVente et al. 2006). Other mutations in critical DNA damage response genes (PRKDC (50%; 2/4) were also detected but occurred at lower confidence MAFs (MAF=48.25 and 2.65, respectively) (Figure 3.3; Appendix B).

Insertion/Deletions and Missense Mutations. We next focused our analysis on single nucleotide polymorphisms (SNPs) resulting in missense mutations with a high MAF in our cohort (*n*= 45 Genes; MAF >86%). Importantly, many of these mutations are validated within functionally important coding domains based on the comprehensive cancer panel. A 1 bp indel (insT) was present in exon 14 of MLL3 at high allelic frequency across all 4 samples, resulting in a frame-shift

mutation at tyrosine 816 (Figure 3.2B). Because this mutation was present in all four samples, we wanted to ensure that this was not a systematic error in the sequencing data. For example, Ion Torrent sequencing is susceptible to miscalling indels within homopolymer stretches due to the difficulty in resolving signal amplitudes for multiple bases of the same type (Margulies et al. 2005). We analyzed the mutation and demonstrated that the indel identified in all four samples does not reside within a homopolymer, thus reducing the possibility that this mutation is a systematic error (S2). Furthermore, the coverage across this mutation was over 1000X in all samples, and there was no indication of strand or allelic bias. MLL3 encodes a nuclear-localized histone lysine-4 methyltransferase that is frequently deleted in myeloid leukemia and mutated in glioblastoma as well as pancreatic cancer (Balakrishnan et al. 2007).

Additional analysis revealed consensus frame-shift mutations within a variety of genes associated with DNA damage repair and apoptosis. A missense mutation in all of the samples at position 1053 (glycine to arginine) in the ERCC5 gene associated with xeroderma pigmentosum (MAF: >99%; 3/4) and an additional missense within the same region 1080G>R (MAF: 99.13 1/4) was observed. Intriguingly, this missense mutation is present upstream of the nuclear localization sequence and represents a highly conserved residue in the enzyme. ERCC5, also known as XR-5, is a critical regulator in the nucleotide excision repair pathway (NER) whereby it removes nucleotide adducts following chronic UV irradiation. It has been noted that MCC is most prevalent in pale-skinned UV-

exposed regions of the body wherein NER is critical to maintaining genomic integrity.

We also observed a missense mutation at $298^{M>T}$ (MAF:>99%; 4/4) in the aurora kinase β (AURKB) gene. Aurora kinase beta (AURKB) is a co-factor critical to maintaining mitotic-integrity that is activated in response to UV irradiation at the centromere and over-expression has been demonstrated to preclude chromosomal aneuploidy in colon cancer (Schjolberg et al. 2009; Arora et al. 2012). Furthermore, AURK β interacts with survivin at the centromere which has recently been described as clinical target in MCC (COSMIC 2013). An identical mutation in the large intestine has been documented in the AURK β gene and 3/4 of our patients intriguingly were treated previously for colon carcinoma (COSMIC) (Giampieri et al. 2013).

Missense mutations were observed in several other DNA repair pathways including ataxia telangiectasia rad6 related (ATR; 4/4) within the ATRIP-binding domain and the associated ATRX (3/4). This is an important regulator of cell cycle progression following DNA damage and ATRIP has been demonstrated to modulate the activity of ATR in initiating DNA damage repair (Figure 3.3).

Single-nucleotide polymorphism of the thyroid stimulatory hormone receptor (TSHR) were synonymously identified at position 727 $^{E>D}$ (MAF: >98%). Mutations in BCL2L2, an anti-apoptotic factor related to the BCL-2 family, demonstrated a glutamine to arginine mutation at position 133 (MAF: >97%; 4/4). Synonymous mutations in the COL1A1 ($1075^{T>A}$ MAF: >99%; 4/4) gene were

also detected with high frequency (Giampieri et al. 2013). Taken together, these germline mutations hold potential as novel targets that have not been previously described in MCC.

3.3 TABLES AND FIGURES

Table 3.1. Patient Demographics, Pathology, Treatment Parameters, and Relevant Clinical Comorbidities.

Demographics			**Pathology**		**Therapy**		**Comorbidities**			
Pat ***n=5***	Sex/ Race	Age	Site	Stage/ Grade	Resection Status 1: Bx 2: STR 3: ND 4: GTR	Treatment 1: Chemo 2: Radio	Alcohol/ Tobacco 1: Nonuser 2: Occasional 3: Chronic 4: Quit	Actinic Keratosis	Colon Cancer	Other Malignancy 1: SCC 2: BCC 3: Mel 4: CaP
I	M/C	61	Abd		1,2	-	2/3	X	X	1,4
II	M/C	82	Neck	T2N1M0/IIb	1,3(x2), 4	1	1/1	X	X	1,2
III	M/C	89	Ing		1	2	1/1			1,2,3
IV	M/C	85	Head	T1N0M0/Ib	1,4	2	1/4	X	X	1,2
V	M/C	82	Neck		2,3	-	1/1	X	X*	1,2
Average/N		79						4(80%)	4(80%)	

*Colon Polyps. Abbreviations: *Bx-Biopsy; Chemo-Chemotherapy; Radio-Radiotherapy; STR-Subtotal Resection; Ing-Inguinal; ND-Neck Dissection; GTR-Gross Total Resection; SCC- Squamous Cell Carcinoma; BCC- Basal Cell Carcinoma; Mel -Melanoma; CaP- Prostate Carcinoma*

Table 3.2. Immunohistochemical markers of Neuroendocrine MCC

Specimen	Syn	CgrA	CK20	MCPyV LT
I	+	+	+	+
II	+	+	+	+
IV	+	+	+	+
V	+	+	+	+

Abbreviations: Syn: Synaptophysin; CgrA: Chromogranin A; CK20: Cytokeratin 20; MCPyV LT: Merkel cell polyomavirus Large-T antigen.

Table 3.3. Nonsense mutations in PDE4DIP (3/4; 75%).

Specimen	Coverage		Mutation	AA^{Δ}	MAF	SNP db Ref
I	-	-	-	-	-	
II	1922	C>T	560W>*	22.1		
IV	1135	G>AG	622R>*	38.84	rs1778111	
V	1813	C>CT	506W>*	30.34	rs1698683	

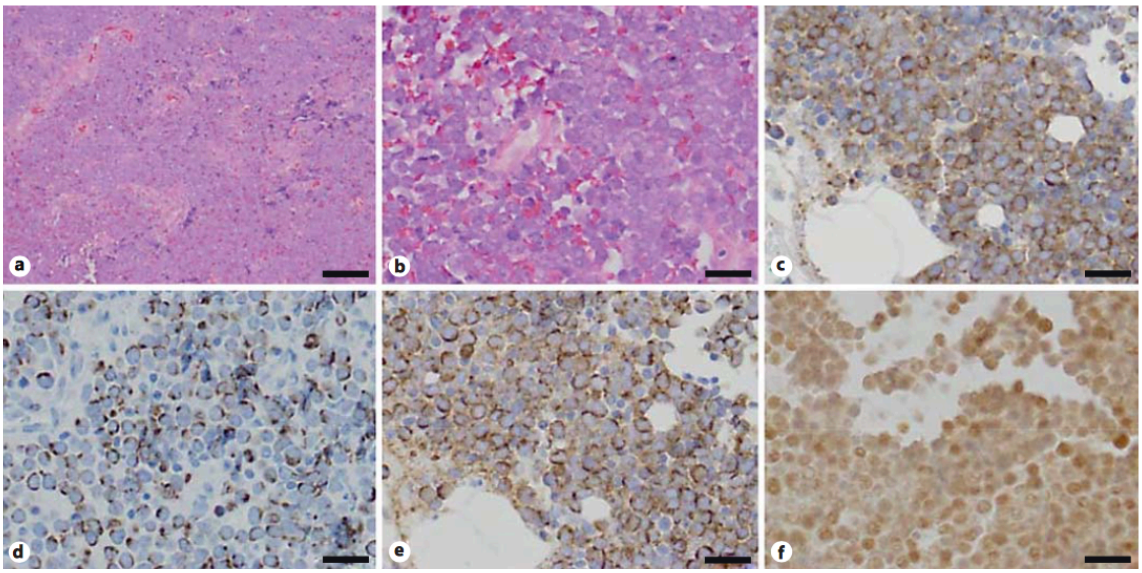

Figure 3.1. Representative diagnostic MCC anatomic pathology. a) H&E 10x, b) H&E 40x, c) Synaptophysin, d) CK20, e) Chromogranin A, and f) (MCPyV Large T Antigen Immunohistochemistry. 40x unless otherwise noted; bars represent 200 microns.

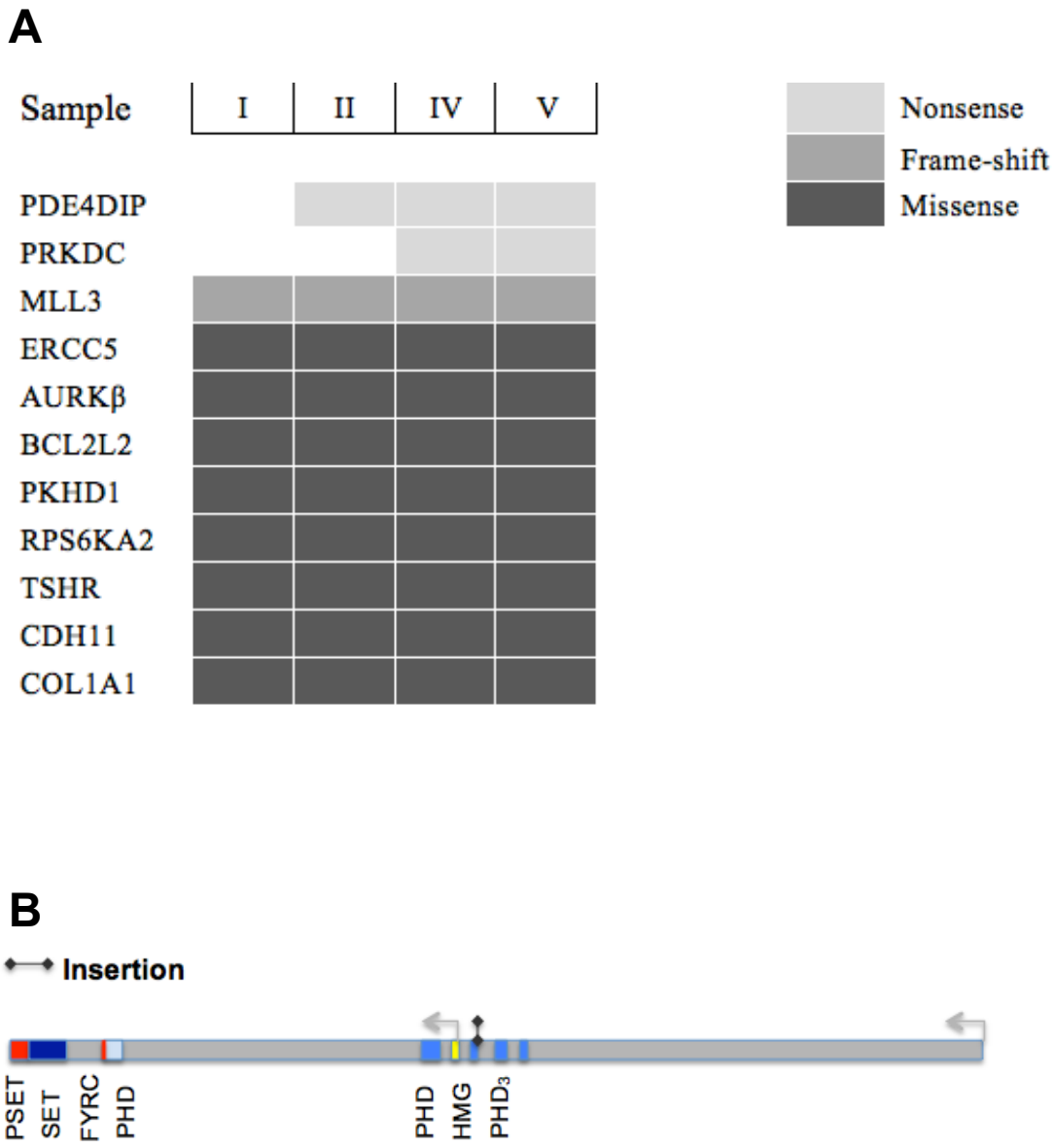

Figure 3.2. Mutations (MAF>86%) within the 4 sequenced samples. A) Distribution of mutations within various genes on the CCP. B) MLL3 gene with corresponding insertion in exon 14 within plant homeodomain (PHD) just up-stream of the high mobility group (HMG).

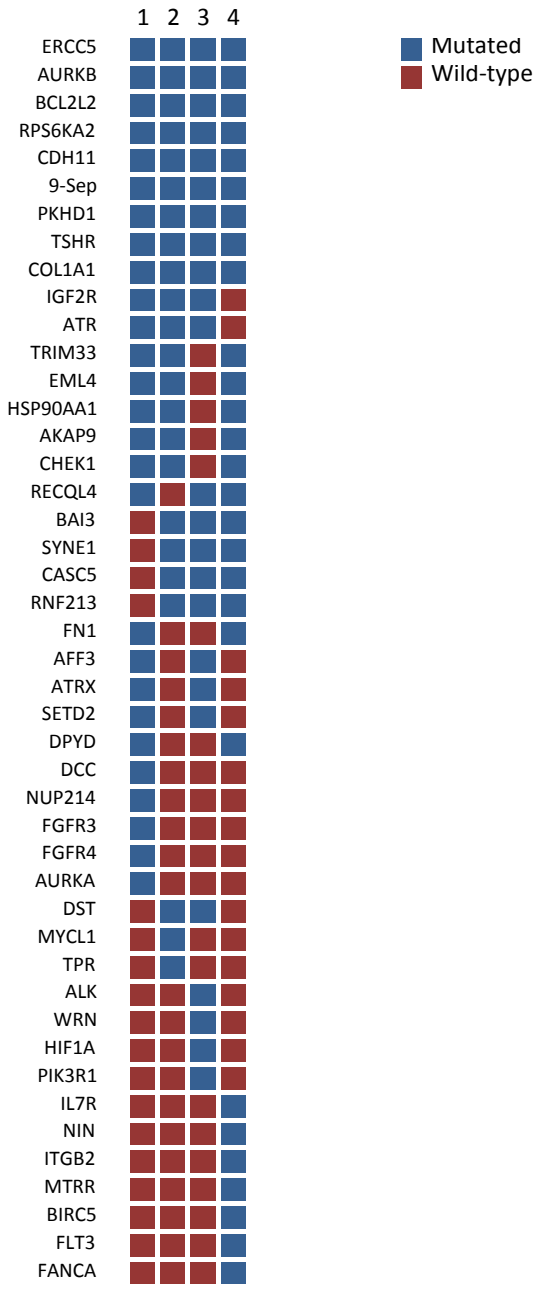

Figure 3.3. Overall missense mutations within the cohort of patients. Samples with missense mutations are noted in blue while non-mutated samples appear in red.

3.4 DISCUSSION

Several well-powered studies evaluating the mutational spectrum in Merkel cell carcinoma have recently been conducted; however, these studies

have not yet deeply sequenced tumors for a predicted mutational spectrum (Rodig et al. 2012; Nardi et al. 2012; Kartha & Sundram 2008). Within our focused cohort, we implemented a targeted 409-gene screen in four patients and demonstrated potential targets with relevance to Merkel cell disease. Importantly, we observed frequent nonsense mutations in PDE4DIP – better known as myomegalin – within 3/4 of the patients included in this study. Myomegalin is a predicted oncogene and gain of function mutations have been implicated in myeloproliferative disorders. All nonsense mutations occurred within the fourth coiled domain directly upstream of a breakpoint site within known PDE4DIP-PDGFRB fusion (Wilkinson et al. 2003).

Missense mutations within key DNA damage and apoptosis genes were also detected. H3K4 histone methyltransferase MLL3 demonstrated an insertion in our study in all the patients included in the study. This mutation adds a tyrosine residue upstream within the highly conserved plant homology domains (PHD) which flank the transactivation domain and are important in CREB-mediated gene expression (Ernst et al. 2001). Interestingly, MLL3 mutations have been previously demonstrated in other aggressive neuroendocrine tumors including pancreatic neoplasms as well as medulloblastomas (Parsons et al. 2011). Furthermore, MLL1 – a close analog of MLL3 and component of the methyl-tranferase complex – is essential for post-natal neurogenesis indicating a potential overlap of H3K4 methylation and over-activation in the neuroendocrine compartment (Lim et al. 2009). Our study demonstrated a potentially novel missense mutation in MCC within the ERCC5 gene proximal to the nuclear

localization sequence which was present in our cohort predominantly as a glycine to arginine substitution. In vitro work has recently linked ERCC5 signaling with ATR-ATRIP signaling in DNA damage responses to UV. In addition, UV induced damage repair pathway is known to activate aurora-kinase β which interacts with downstream BCL2L2 and anti-apoptotic pathways. Therefore, it will be of great interest to determine whether loss of function at these loci is incurred with these mutations (Lindsey-Boltz et al. 2014). In our cohort, a glutamic to aspartic acid mutation at position 727 of the TSHR gene was present in high allelic frequency. Interestingly, recent studies have indicated this SNP, denoted as rs1991517, to be associated with enhanced risk for thyroid cancer, DNA damage and RET-oncogene polymorphism in populations exposed to ionizing radiation (Sigurdson et al. 2009).

BCL2L2 is an antiapoptotic gene of great interest to oncologists. BCL2L2 is up-regulated downstream of NFκβ-mediated pro-survival signaling and is involved in nervous tissue responses to BDNF and NGF (Middleton et al. 2001). Interactions between UV-induced DNA damage response and apoptotic pathways (ERCC5, AURKβ, BCL2L2) and receptor responsive elements (PDE4DIP and TSHR) could provide potential preclinical targets for validation in MCC.

Randomized clinical trials incorporating targeted therapies such as pazopanib or imatinib are hindered by the rarity of MCC, but are necessary to improve outcomes (Desch & Kunstfeld 2013). Various immunotherapy trials are underway evaluating the utilization of IL-12 gene therapy and Large T Antigen

priming in MCC. Additionally, the potential PDGFRA-PDE4DIP fusion previously reported could also represent a potential clinical target. However, Swick and colleagues considered PDGFRA-KIT expression in a focused cohort of 23 tumors demonstrating a 95% and 65% expression of these markers, respectively, but found no activating mutations (2013). It will be of great interest to determine if the DNA damage and pro-survival pathways play a role in a larger cohort of this aggressive neuroendocrine cancer.

Within our small cohort of MCC cases, various genetic mutations were detected with potential relevance to MCC pathogenesis. UV exposure is a risk-factor for MCC and we describe a mutation in ERCC5, a gene which encodes a protein important in UV induced nucleotide excision repair (NER) and that is implicated in xeroderma pigmentosum (Senchenkov et al. 2007; Grotz et al. 2012). All of the patients sequenced in this cohort demonstrated other UV-associated dermatological conditions including actinic keratosis and skin cancers (Ferrara et al. 2010; Werner et al. 2013). Additionally, we found mutations in PDE4DIP, a gene which encodes a regulatory binding protein important in neural tissue associated calcium-mediated cAMP signaling. We also detected a high frequency of mutations within genes important in maintaining genomic stability including MLL3, AURK β, and BCL2L2. Further validation of these genes in larger associational cohorts and their significance as potential therapeutic targets and/or clinical prognostic indicators will better direct clinical decision and understanding of the underlying molecular drivers of Friedrich Sigmund Merkel's carcinoma (Merkel 1875).

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CHAPTER 4

DEEP LEARNING AND MOLECULAR PATHWAYS IDENTIFY TRANSLATIONAL TARGETS IN HNC

Tumors of the upper aerodigestive tract are closely associated with the peripheral nervous system. This likely contributes to the complex heterogeneity often appreciated in neoplasms that originate in the oropharynx, nasopharynx, and larynx. The etiological risk factors classically implicated in these diseases include genetics, tobacco and alcohol use, and infection with oncogenic viruses and co-morbid immunosuppression (Ang et al. 2010; Leemans et al. 2011; Kreimer et al. 2013). The oropharynx accounts for approximately 60% of all newly diagnosed aerodigestive tract tumors where exposure to oncogenic viruses such as the human papillomavirus (HPV) plays a significant role in disease progression (Hillbertz et al. 2012).

Recent evidence points to a significant role of neurotrophin signals from the peripheral nervous system in normal and neoplastic trans-differentiation (Fan et al. 2004, Bronzetti et al. 2006, Nakamura et al. 2007). The low-affinity nerve growth factor (p75NTR; LNGFR; CD271) is the principal neurotrophin receptor that signals via the nerve growth factor (NGF) (Lu, Pang, Woo 2005). These receptors classically heterodimerize with tyrosine kinases (Trk) A,B, and C promoting a diversity of receptor/ligand induced signals. p75NTR and various neurotrophins (Nerve growth factor (NGF); Brain-derived neurotrophic factor

(BDNF); Glial cell-derived Neurotrophic Factor (GDNF); Neurotrophin 3 (NT-3) and Neurotrophin 4 (NT-4)) are active in maintaining the somatosensory functions of the oropharynx (Bronzetti et al. 2006, others). Cancers of the head and neck are aggressive and often associate with the peripheral nervous system in perineural invasion and are associated with brain metastasis in as many at 6-18% of all cases (Tse 2013; Ruzevick et al. 2013). Recent studies have suggested an epigenetic role for TrkA and B and their cognate ligands in HNC that differentiates HPV-driven from HPV negative disease (Kostareli et al. 2013). Additionally, TrkB has been demonstrated to predict poor overall survival in oropharyngeal cancers and has been proposed as a target for clinical intervention (Kupferman et al. 2010; Lee J et al. 2012).

Taken together, the role that these neurogenesis-related drivers play in the pathogenesis of virally induced head and neck cancer might hold important insight into the molecular biology of the underlying tumors. We propose that these targets might provide a rationale for the involvement of the peripheral nervous system and offer potential targets for therapeutic intervention.

4.1 MATERIALS AND METHODS

Patient Specimen Collection

All patient specimens were collected under informed consent of the patient and the approval of the institutional review boards of the Medical University of South Carolina and the Dorn VA Medical Center.

DNA, RNA, and Protein Expression Analysis

DNA, RNA, and Protein were collected as previously described (Tomar et al. 2016). Briefly, flash frozen or RNA-later preserved surgical specimens were de-identified and cryostored at -80 C and -20 C, respectively. Nucleic acids and protein were collected using All Prep as per manufacturer's protocols (Qiagen). Nucleic acids and proteins were assessed for quality and quantity by spectrophotometer for all downstream analyses. cDNA was generated using iScript reverse-transcriptase for all patient samples (BioRad, USA).

HPV-Serotyping and Activity Analysis

All samples were typed for HPV-DNA using a linearized reverse hybridization assay as previously described (INNO-LiPa, Fujirebio) as previously described. Briefly, gDNA was globally amplified using primers directed to the L1 region od 27 HPV types. The resulting amplimers were then hybridized to linear indicators and results were quantified. HPV-Activity was assessed by quantitative real-time PCR (qPCR) as described in Tomar et al. 2014. Briefly, primer sequences for HPV16 E7 (Forward: 5' — CCG GAC AGA GCC CAT TAC AAT – 3' 5' — CCG GAC AGA GCC CAT TAC AAT – 3'; Reverse: 5' — ACG TGT GTG CTT TGT ACG CAC – 3') were utilized to amplify cDNA synthesized on the template of tumor-derived RNA (Lamarcq et al. 2002). qPCR conditions were as follows: initial denaturation (95°C x 180 s.) followed by 40 iterative cycles of denaturation (95°C x 10 s) and primer annealing and elongation, respectively (60s).

Agilent cDNA Microarray Analysis

Microarray experiments were performed using the Agilent Technologies (Santa Clara, CA) platform. Total RNA samples were amplified and labeled using Agilent's Low Input Quick Amp Labeling Kit according to the manufacturer's recommendations. Briefly, mRNA contained in 200 ng of total RNA was converted into cDNA using a poly-dT primer that also contained the T7 RNA polymerase promoter sequence. Subsequently, T7 RNA polymerase was added to cDNA samples to amplify the original mRNA molecules and to simultaneously incorporate cyanine-3 labeled CTP into the amplification product (cRNA). In the next step, labeled cRNA molecules were purified using Qiagen's RNeasy Mini Kit (Valencia, CA). After spectrophotometric assessment of dye incorporation and cRNA yield, samples were stored at −80°C until hybridization. Labeled cRNA samples (600 ng) were hybridized to SurePrint G3 Human Gene Expression 8×60K v2 Microarrays at 65°C for 17 h, using Agilent's Gene Expression Hybridization Kit according to the manufacturer's recommendations. After washes, arrays were scanned using a High Resolution Agilent DNA Microarray Scanner and images saved in TIFF format.

Deep Learning Validation of Microarray Gene Expression

Data were extracted from images with Feature Extractor Software version 10.7.3.1 (Agilent) where background correction was also performed. Background-corrected data were uploaded into GeneSpring GX version 11.5.1 for analysis. In this process, data were log2 transformed, quantile normalized and

base line transformed using the median of all samples. Then, data were filtered by flags in a way that 75% of the samples in at least one of the treatment groups have a “detected” flag. Differentially expressed genes were determined by analysis of the data using the non-parametric Mann-Whitney unpaired test. Cutoff values of 0.02 and 2 were used for p-value and fold-change, respectively. Gene ontology (GO) pathway analysis and hierarchical cluster analysis were also performed with GeneSpring. For the hierarchical cluster analysis, Euclidean similarity metrics and Wards linkage rule were used along with Kruskal-Wallis non-parametric ANOVA. Kaplan-Meier survival curves were generated using GraphPad PRISM version 6.0 (GraphPad, Software, La Jolla, CA). A p-value of 0.05 or less was considered significant.

Immunohistochemistry Validation of HPV+ vs. HPV- Cancers

Formalin-fixed paraffin embedded (FFPE) samples were cut at 5 μm thickness on a microtome and antigen-recovered at 75 ^{o}C. Specimens were then probed using antibodies to NGFR (Clinical Pre-dilute; Biocare), POU4F1 (1:200; Millipore) NEFH (1:1000; Biocare), Ki67 (1:2000, Cell Signaling Technologies). DAB chromogen was utilized for detection of antigen (Abcam). Freshly resected HNC tumor lesions were flash frozen and embedded in OCT mounting medium. Specimens were fixed in cold methanol, serially sectioned on a cryostat at 7 μm and incubated with anti-mouse NEFH-HRP (1:500; Biocare) to visualize innervation of the tissue specimens.

4.2 RESULTS

HPV Positive and HPV Negative Tumors Differentially Express Neurogenesis-Related Genes. Craniofacial development occurs early in embryonic development and the connective tissue of the head and neck originates from the neural crest. A stem cell niche of de-differentiated basal oral keratinocytes (bOK) are maintained to promote wound healing and tissue regeneration throughout life (Jones & Klein 2013). This niche has been suggested as the target of oncogenic HPV viruses within the oral mucosae and transformation and neoplasia are associated with poor differentiation of the epithelium (Syrjänen 2010). These slowly proliferating ($Ki67^{lo}$) cells express the NGFR ($p75NTR^{hi}$) and cytokeratins (CK 5/14/19) as well as other important developmentally regulated genes and are the targets of oncogenic papillomaviruses (Leemans et al. 2011). We discovered a preponderance of known and novel neural-related genes in our data set when analyzing differences between HPV driven and HPV- tumors (Figure 4.1; Table 4.1). We further assessed these neural-associated genes in the Walter et al. TCGA data-set and discovered a significant concordance in expression (Figure 4.2) (Walter et al., 2013). These data led us to further consider the role HPV16 infection might play in de-differentiated bOKs and activation of neural pathways in head and neck cancer.

Infection with HPV16 suggests a de-differentiated and neurogenic program in cancers of the head and neck. The abundance of significantly up-regulated neural genes concomitant with viral infection suggested to us that this might be

an important mechanism in the pathogenesis in HNC. We chose to focus our analysis on the peripheral nervous system (PNS) and gene components of the sympathetic and somatosensory compartments. We found significant differences when comparing HPV16 positive versus HPV16 negative expression which included GABA receptor Pi (GABRP (40 Fold)), Glutamic acid decarboxylase 1 (GAD1(10 Fold)) Single-minded (SIM2 (9.8 Fold)), NGFR (p75NTR (6.7 Fold)), POU4F1 (POU4F1 (6.2 Fold)), neurofilament heavy chain (NEFH; 200 kDa) in HPV positive and HPV patients amongst others (Figure 4.3).

Anatomic Markers of Peripheral Nerves are Up-regulated in HPV+ HNC pathology. To validate these gene expression changes in HPV+ cancers, we next examined the expression of NEFH. Because NEFH was up-regulated in our original dataset in HPV+ versus HPV- cancers and it is considered a gold-standard immunohistochemical marker for peripheral nerves, we performed IHC staining on HPV+ versus HPV- tumor samples. Utilizing clinical-grade antibodies, we stained for NEFH and observed a statistically significant increase in the number of cases that were positive for NEFH in HPV+ versus HPV- cancers (42% versus 22%, respectively; $p<0.05\%$, t test, Figure 4.4A). Furthermore, our IHC study indicated an increase in peritumoral invasion microanatomy in HPV+ HNC (Figure 4.4B) versus HPV- cancers.

Nerve growth factor receptor (NGFR) is Up-regulated in HPV+ HNC. To begin to determine a functional role and to validate machine learning data, we next evaluated the expression of the low-affinity neurotrophic receptor, NGFR. Because NGFR is a cytoplasmic tyrosine kinase receptor, we utilized clinically

validated antibodies to assess whether gene expression changes observed were present in HPV+ versus HPV- cancers. Our data demonstrated a marked difference in the prevalence of NGFR expression in HPV+ HNC versus HPV- cancers (75% versus 8%, respectively; Figure 4.5A. p=0.0078).

These factors have previously been demonstrated to play a role in HNC (p75NTR), HPV-associated malignancies (POU4F1), and esophageal cancers (NEFH), respectively. We observed an abundance of targets downstream of critical neural crest transcription factors (see schematic representation in Figure 4.6). Together, these data suggested that these neural-related genes might be targets in the pathogenesis of virally induced HNC.

4.3 TABLES AND FIGURES

Table 4.1. Selected neurogenesis-related genes up-regulated > 2.0 fold in HPV+ versus HPV- HNC.

Gene	**FC vs. HPV$^-$**
Neural Crest Markers	
POU4F1	6.02
ISL1	10.24
NGFR	6.52

Perineural Invasion Markers	
PTN	7.58
MDK	2.85
SRGAP3	5.34
Pain-Receptors	
TRPV6	5.27

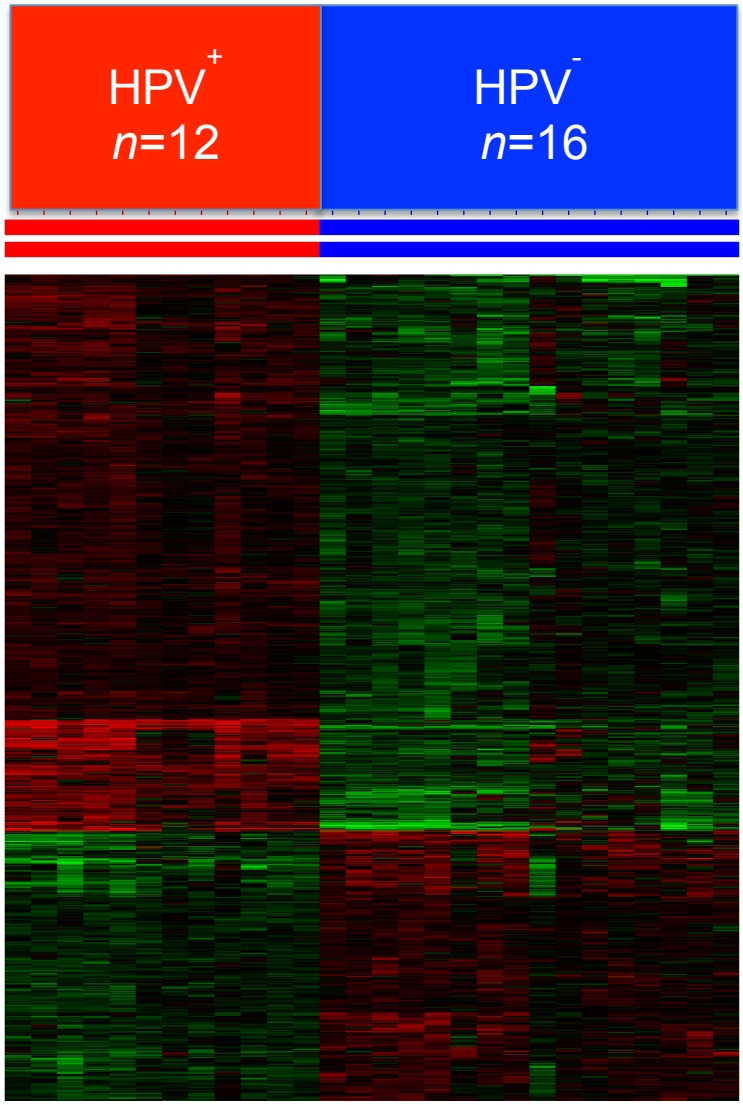
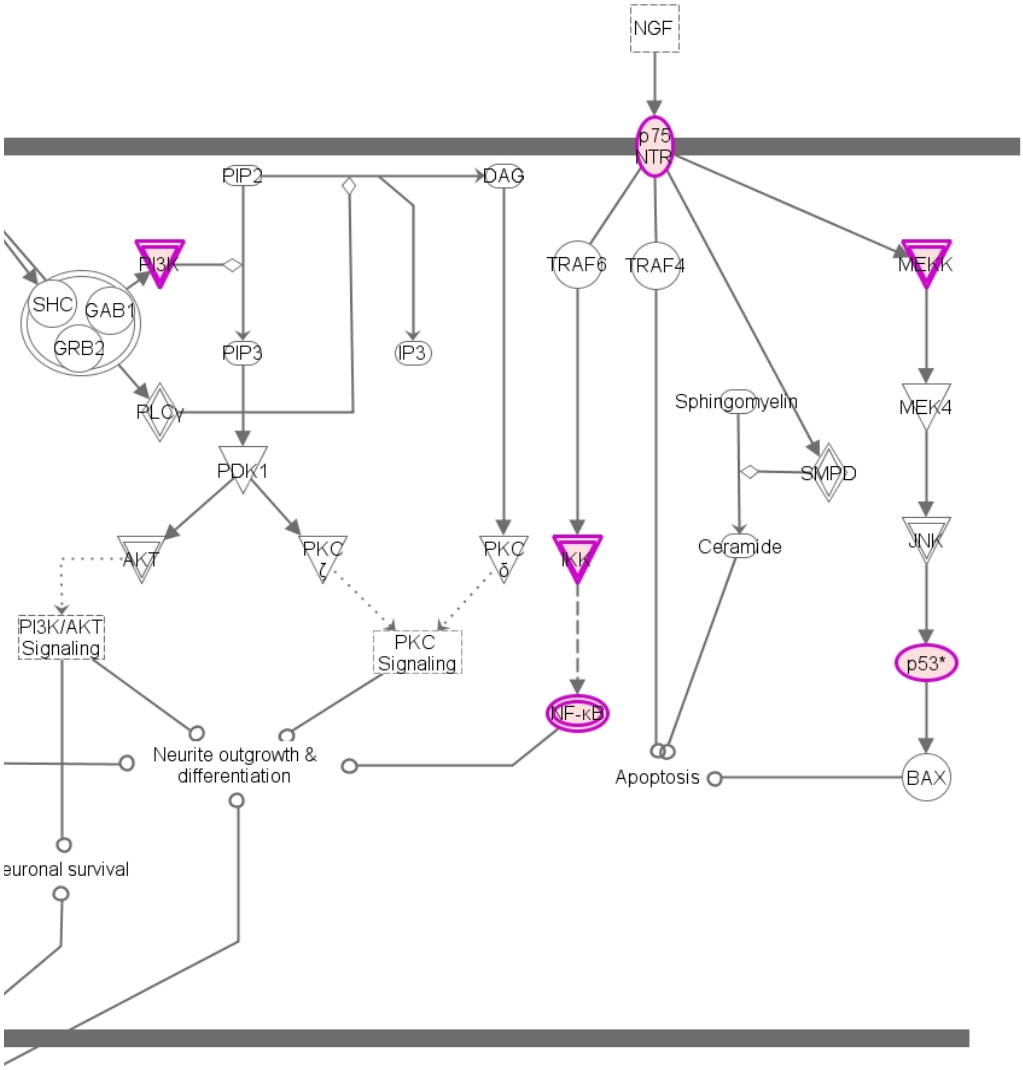


Figure 4.1. HPV+ and HPV- Tumors have distinct gene expression profiles. Unsupervised hierarchical clustering demonstrates divergent gene expression profiles in HPV+ vs. HPV- tumors $p<0.02$; FC ≥ 2 Mann-Whitney.

A


NGF
p75 NTR
PIP2
DAG
PI3K
SHC
GAB1
GRB2
PIP3
IP3
PLCγ
TRAF6
TRAF4
MEKK
Sphingomyelin
MEK4
SMPD
PDK1
AKT
PKC ζ
PKC δ
IKK
Ceramide
JNK
PI3K/AKT Signaling
PKC Signaling
p53*
NF-κB
Neurite outgrowth & differentiation
Apoptosis
BAX
euronal survival

B

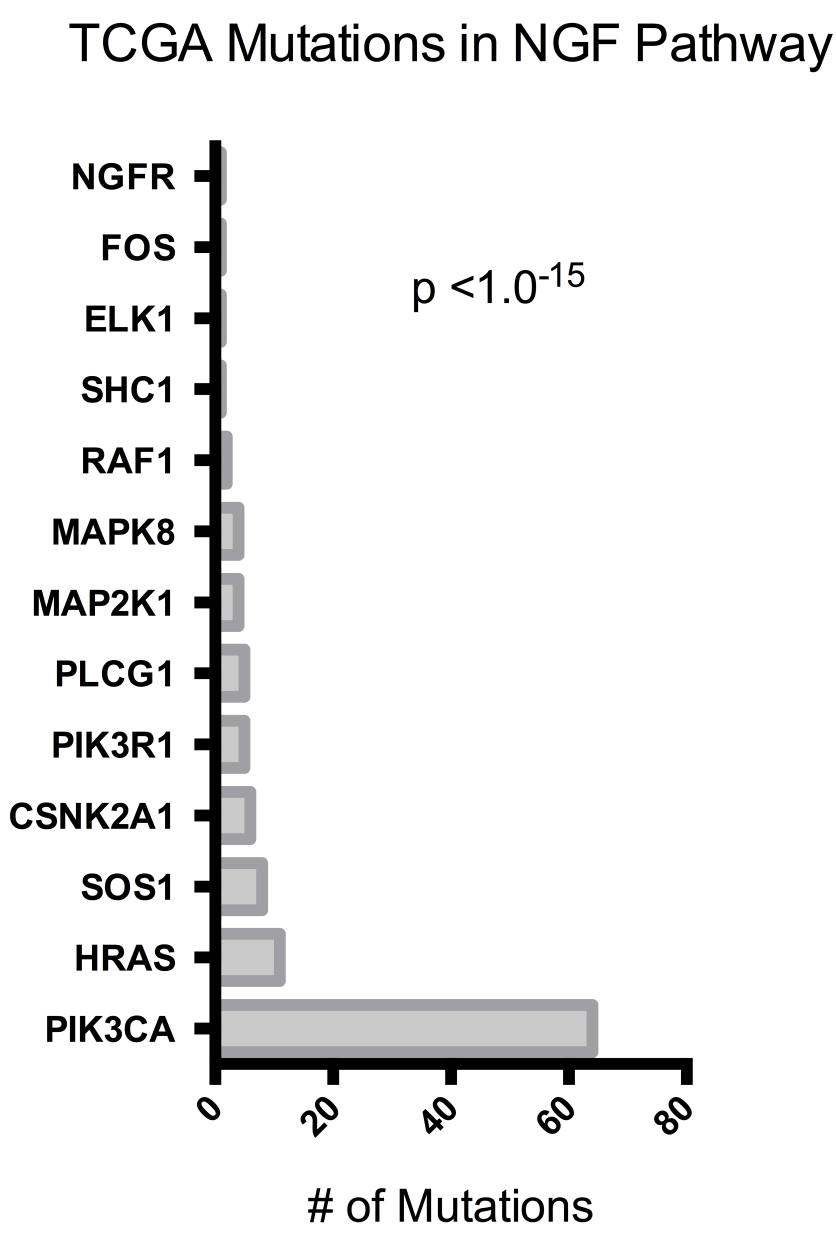


Figure 4.2. Key neural crest genes are mutated in an independent TCGA HNC dataset. (A) Screenshot from TCGA HNC dataset implicates NGF activation in P75NTR. (B) Mutations in HPV+ HNC that are significant for NGFR and TrkA signaling and are associated with the neurotrophin signaling cascade. $p<0.05$; FC ≥ 2 Mann-Whitney, Student's *t*-Test.

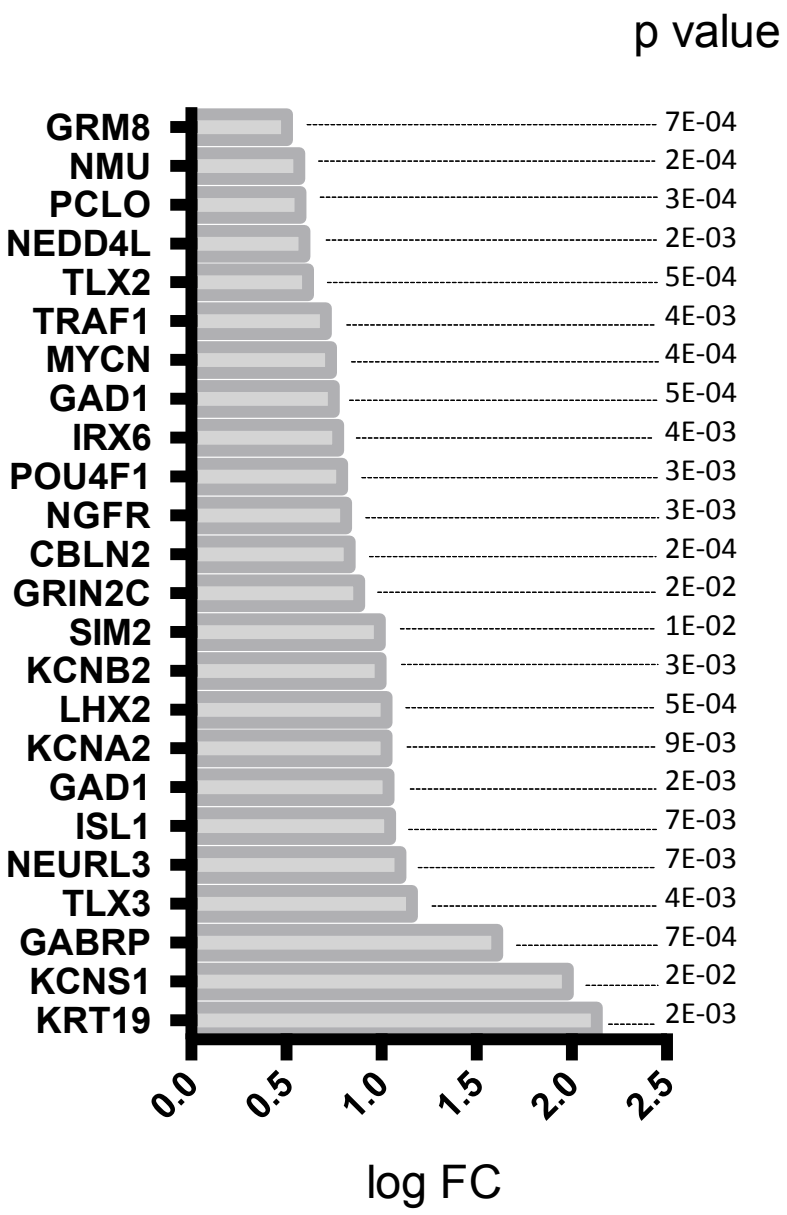
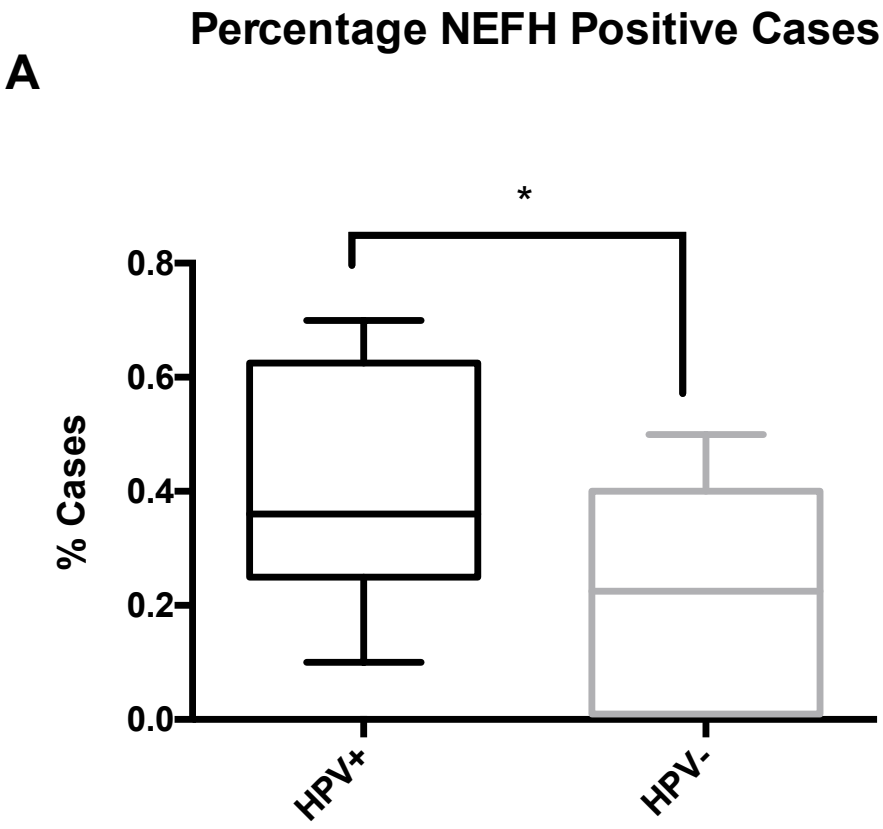


Figure 4.3. Neurogenesis-related genes associated with neurotrophin signaling, neurotransmitter signaling, and neuritogenesis are significantly overexpressed in HPV+ cancers versus HPV- cancers.



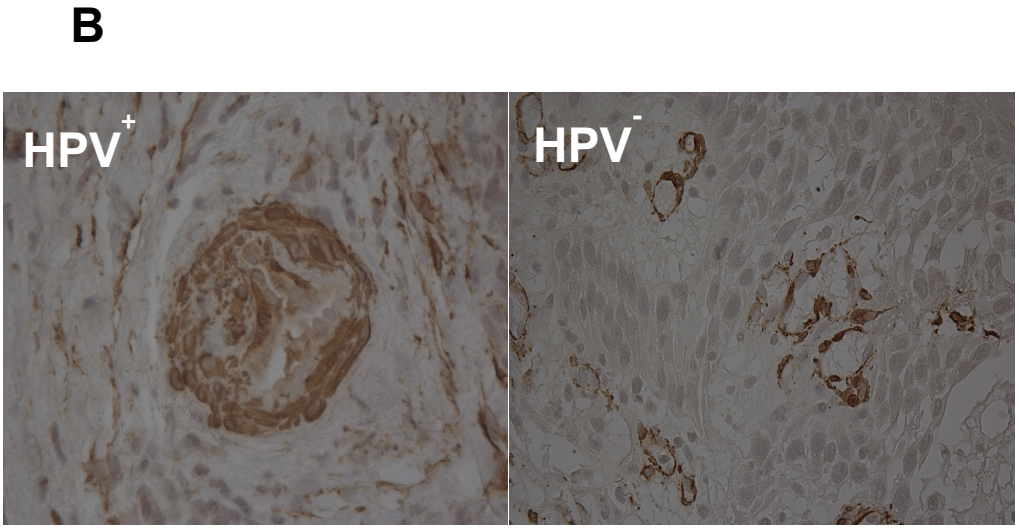
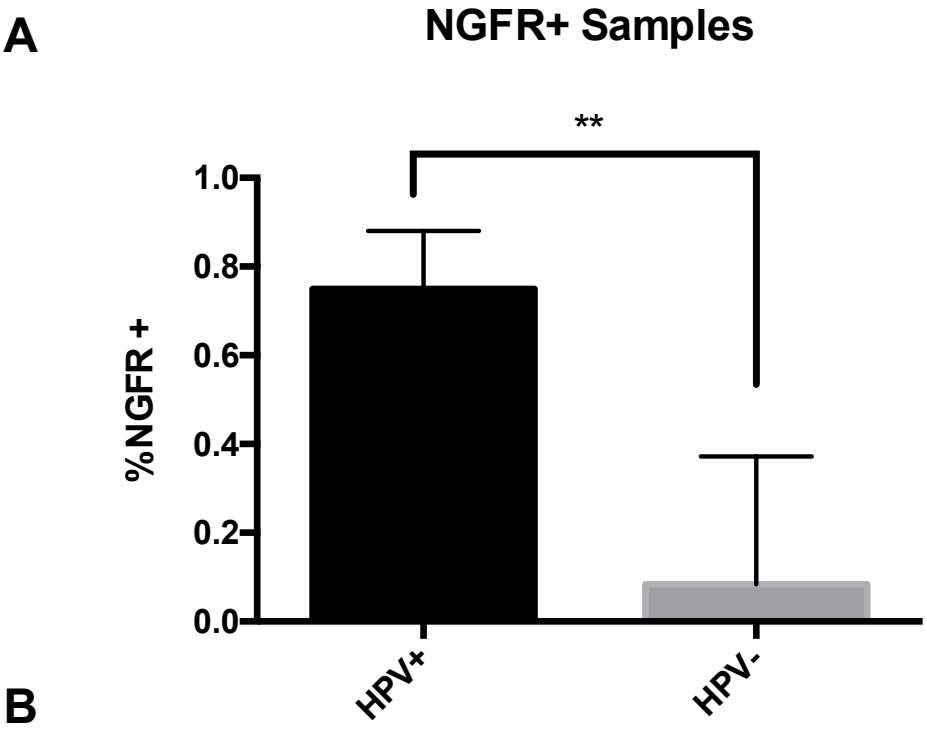


Figure 4.4. HPV+ tumors demonstrate increased interactions with the peripheral nervous system as labeled by NEFH. (A) Percentage of HPV+ and HPV- cancers that stain positive for NEFH (Mann-Whitney *t-test*, *p= 0.0404). (B) 40x representative images of NEFH staining in HPV+ and HPV- cancers.



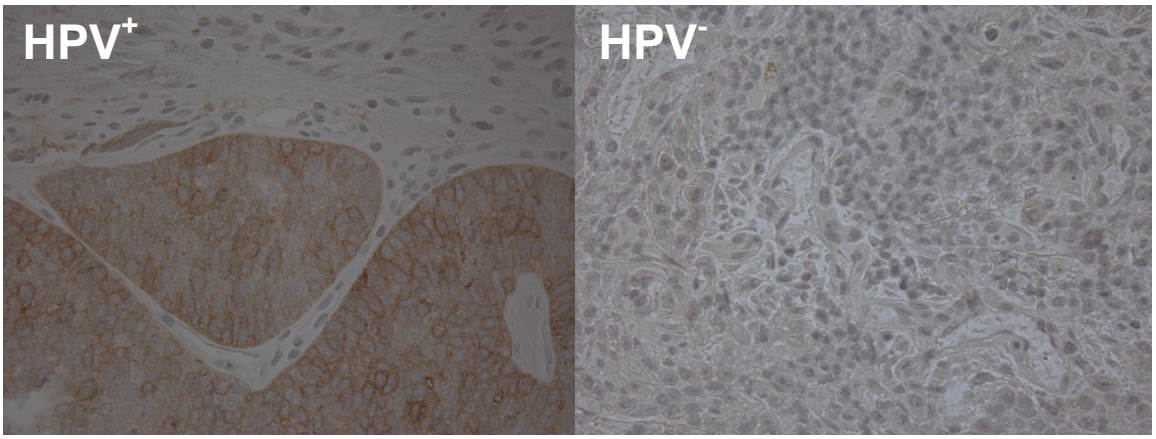


Figure 4.5. HPV+ tumors have high NGFR expression more often than HPV- tumors (A) % of HPV+ and HPV- cancers with positive NGFR staining (Wilcoxon t-test p=0.0078) (B) 40x representative images of NGFR staining in HPV+ and HPV- cancers.

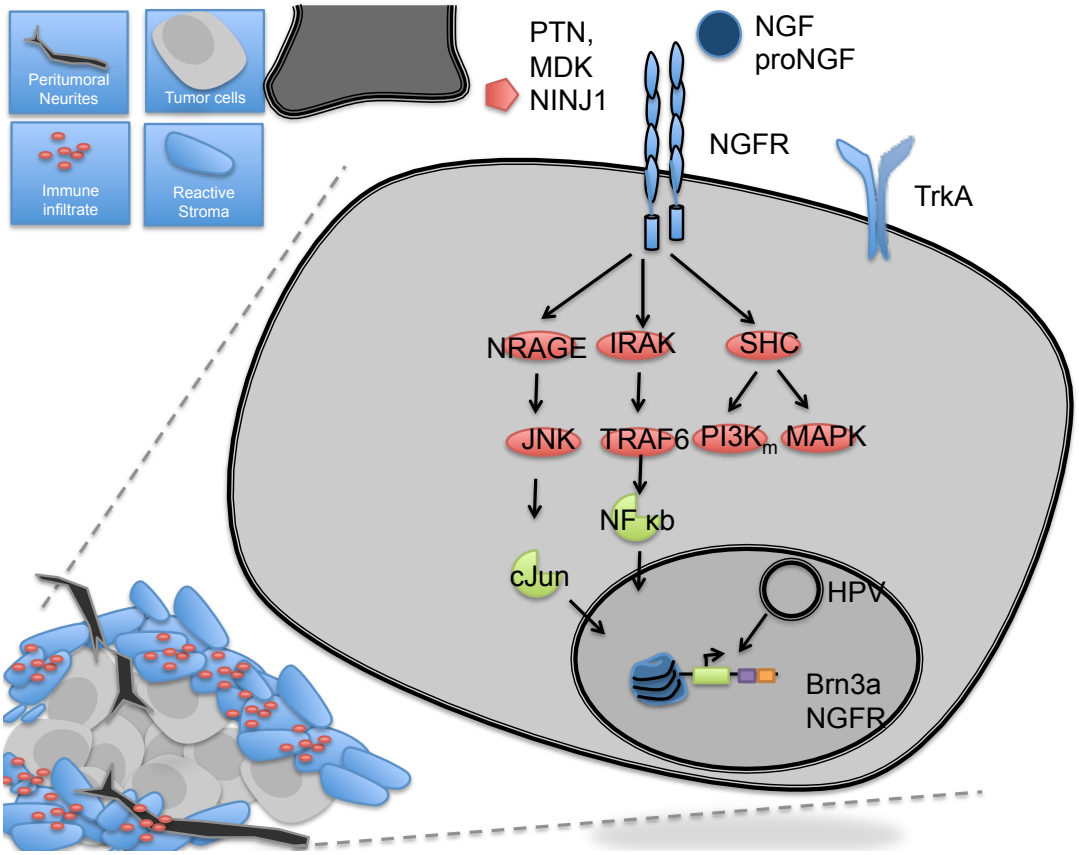


Figure 4.6. Proposed mechanism for peritumoral neurogenesis and signaling via the neurotrophin receptor pathway.

4.4 DISCUSSION

The proximity of tumorigenic cells to nerves is universally associated with poor prognosis in head and neck cancers (Tse 2012). Neurotrophism-associated genes (such as NGF, BDNF, etc.) have been demonstrated to play a key role in neurogenic drive in the peripheral nervous system (PNS) (Kolokythas et al. 2010). In addition, it has been previously posited that HPV transformed HNC carry a proclivity toward neuroendocrine differentiation in a subset of patients (Kraft et al. 2012). However, the involvement of the PNS in tumorigenesis and metastasis remains a novel area of investigation. Ruzevick et al. recently demonstrated increased brain metastasis in HPV-positive head and neck cancers where the major anatomic site was the base of tongue (2013). The tongue is highly innervated by the branches of the trigeminal, facial, glossopharyngeal, and vagus and has recently been demonstrated to be the most prevalent anatomic site in perineural invasion (this Thesis; Tai et al. 2012). Our data suggest that the virally-induced homeobox transcription factor POU4F1 plays a role downstream of HPV-infection in inducing p75NTR expression as well as other critical neurotrophism-related genes. Additionally, our data present the first mechanistic insight into the importance of p75NTR signaling in HPV-active versus HPV-negative. Interestingly, our data not only clearly support a role for NGF but also implicate downstream GABA and Glutamate signaling within the periphery. GAD1, the key synthetic enzyme that converts glutamate to GABA, has recently been shown to promote invasion and metastasis in oral cancers (Kimura et al. 2013). Furthermore, activity of glutamate receptor ionotrophic 2C

(GRIN2C), glutamate receptor metabotrophic 8 (GRM8), and various aspects of the glutamate signaling cascade provide intriguing targets for further investigation. The glutamatergic system has been implicated in GWAS studies by Vink et al. including these aforementioned targets which are increased in smokers versus non-smokers (Vink et al. 2009). It is well accepted that cancer patients on β-adrenergic blockers have lower rates of recurrence and diminished mortality (Barron et al. 2011; Melhem-Bertrandt et al. 2011; Lemeshow et al. 2011; Grytli et al. 2013; Grytli et al. 2013). Recent mechanistic evidence in prostate cancer suggests a dualism between the sympathetic and parasympathetic disease (Magnon et al. 2013). Neurotrophins are expressed in abundance in saliva and play pleiotrophic roles in maintaining the sympathetic/parasympathetic tempo within the upper aerodigestive tract (Laurent et al. 2013). Interestingly, acetylation of the H3 in proximity to POU4F1 has been suggested as a mechanism of control in regulating sensory ganglia development (Eng et al. 2007).

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CHAPTER 5

FUTURE DIRECTIONS AND TRANSLATIONAL PERSPECTIVES

Taken together and in light of our big data observations in patients with HNC we suggest a critical role for the involvement of the nervous system in neoplasia of the head and neck. Despite inclusion of HPV screening in the pathological work-up of all patients, HNC remains a disease intricately associated with neurostress-related behaviors and outcomes.

Our data collectively suggest an increased role of the peripheral nervous system in HNC including how it might relate to relevant clinical subtypes (e.g., HPV+, HPV-). Centering around the nerve growth factor receptor (NGFR) and associated downstream targets, we suggest potential molecular targets for future therapeutic development including the NGFR/ NFκβ /ERK pathway. Based on the finding that p75NTR levels were significantly up-regulated in HPV+ vs. HPV- HNC a more complete analysis of the functionality of the p75NTR/TrkB receptors in virally driven versus non-virally driven HNC cells is warranted. NGFR has previously been shown to be increased in HNC but not in the context of HPV infection (Søland et al. 2008). Because p75NTR has previously been suggested to play a growth suppressive role in maintaining the bOK niche, we hypothesize that HPV-mediated trans-differentiation might result in aberrant NGFR signaling.

ProNGF induces a pro-apoptotic and growth suppressive signal via NFκβ (Rabizadeh S et al. 1993). ProNGF treatment substantially reduces cell viability and induces increased TUNEL and cleaved caspase-3 activity, indicative of apoptosis. BDNF treatment results in a similar effect. Because the BDNF promoter was recently demonstrated to be hypermethylated in HPV-driven HNC (Kostareli et al. 2013) and because we demonstrated changes in NGFR, a common cognate receptor to the TrkB BDNF promoter, GDNF regulation of the basal compartment in HPV infected cells might promote aberrant neurogenesis.

The preponderance of neural crest-related genes suggest another intriguing target. To determine critical neural gene expression targets, we conducted a full deep learning analysis of our dataset and discovered several important neural crest transcription factors. POU4F1 is an essential transcription factor best known for the role it takes in regulating neurogenesis and neural development in concert with various factors (Islet 1,ISL1; LIM-Homeobox 1, LHX1; Paired-box Homeobox 1, PAX1) within the sensory ganglia and throughout the craniofacial axis (Eng et al. 2007). POU4F1 is also implicated as an HPV-induced transcription factor in cervical cancer and has been shown to be increased in response to HPV16 infection (Ndisang et al. 2001; Ndisang D et al. 2006). The presence of POU4F1 and other neurogenic factors might lead us to consider the involvement of POU4F1 and it's principal binding partner ISL1 in HPV16-positive and HPV16-negative cell lines and downstream druggable targets in HNC. POU4F1 and ISL1 were up-regulated in HPV+ versus HPV- HNC suggesting involvement of this highly conserved transcriptional program.

Finally, with pain as a sentinel symptom of many HPV-driven HNC cases (McIlwain et al. 2014), we discovered several pain-associated receptors including TRPV6, GRIN2C as well as involvement of GABRP and GAD suggesting an important role of the nervous system. Expanding upon these machine-learning findings with pathologist-confirmed PNI cases might present additional translational targets or validate known interventions, such as surgical or chemical denervation strategies, in HNC.

APPENDIX A

TCGA MACHINE LEARNING QUALITY CONTROL

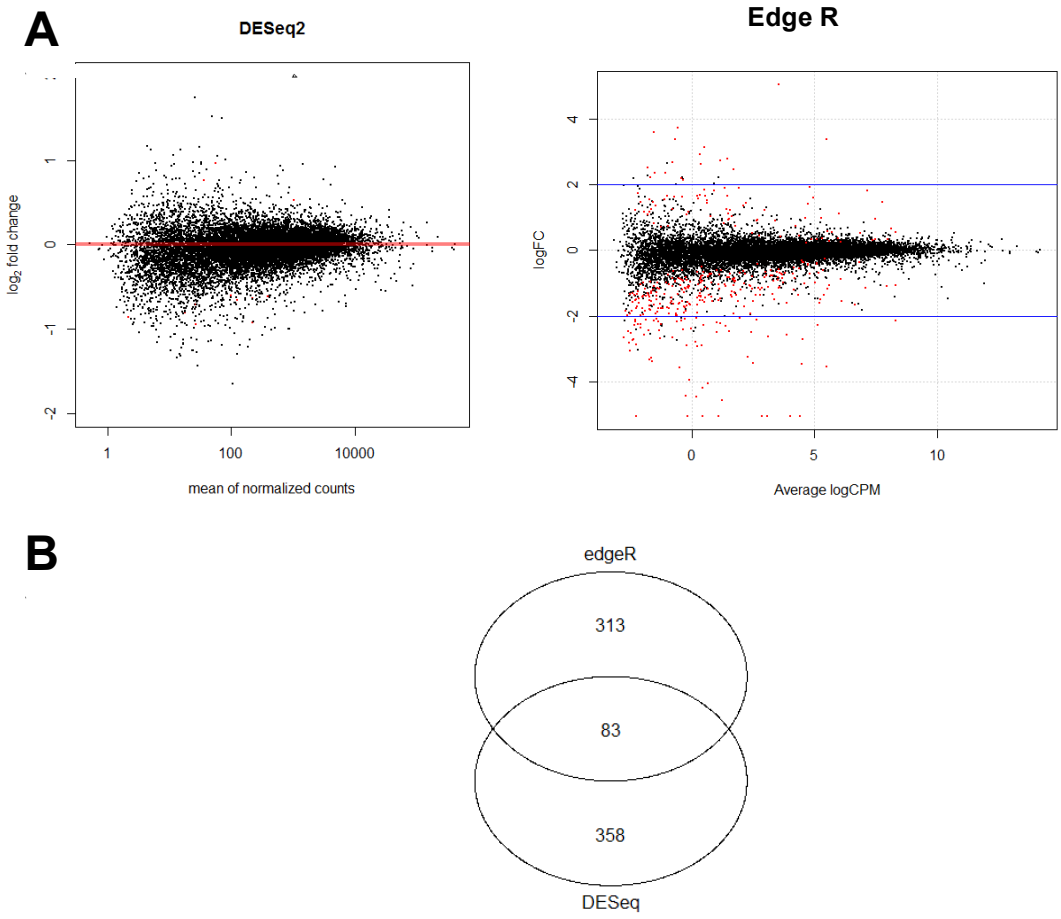

Figure A.1. Comparison of DeSeq2 and Edge R log FC versus mean normalized counts, demonstrating high concordance between methods and 83 overlapping neurogenesis genes in the PNI+ dataset (B).

APPENDIX B

MASSIVELY PARALLEL SEQUENCING PARAMETERS

USC1	
Number of mapped reads	14,433,987
Percent reads on target	87.30%
Average base coverage depth	645.9
Uniformity of base coverage	84.90%
Target base coverage at 1x	99.60%
Target base coverage at 20x	97.51%
Target base coverage at 100x	88.77%
Target base coverage at 500x	40.40%

USC2	
Number of mapped reads	20,440,292
Percent reads on target	92.69%
Average base coverage depth	1,010
Uniformity of base coverage	85.39%
Target base coverage at 1x	99.50%
Target base coverage at 20x	97.65%
Target base coverage at 100x	92.81%
Target base coverage at 500x	63.21%

USC4	
Number of mapped reads	16,819,389
Percent reads on target	88.85%
Average base coverage depth	773.2
Uniformity of base coverage	71.93%
Target base coverage at 1x	98.73%
Target base coverage at 20x	90.55%
Target base coverage at 100x	77.37%
Target base coverage at 500x	44.84%

USC5	
Number of mapped reads	27,196,570
Percent reads on target	91.79%
Average base coverage depth	1,315
Uniformity of base coverage	83.07%
Target base coverage at 1x	99.63%
Target base coverage at 20x	97.87%
Target base coverage at 100x	93.15%
Target base coverage at 500x	67.10%