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Insights into Cutaneous Squamous Cell Carcinoma Pathogenesis and Metastasis Using a Bedside-to-Bench Approach

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Insights into cutaneous squamous cell carcinoma pathogenesis and metastasis using a bedside-to-bench approach

By

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Dedications

To my Dad, who inspired me to become a physician-scientist

And whose memory continues to inspire my research in cancer

To my Mom, who has been with me every step of the way

with encouragement and support

To my sister, whose resilience and altruism inspires my research and career

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Abstract

Insights into cutaneous squamous cell carcinoma pathogenesis and metastasis using a bedside-to-bench approach

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Cutaneous squamous cell carcinoma (SCC) is the second most common skin cancer, affecting 1,000,000 people in the United States annually and causing approximately 9,000 deaths. SCC and basal cell carcinoma are the most common types of skin cancer arising in the superficial squamous cells or deeper basal cells of the epidermis, respectively. SCC is more likely to invade and metastasize, while basal cell cancer tends to grow locally. While the majority of cases of SCC are cured by surgery alone, approximately 2-5% of SCCs metastasize, at which point outcomes may be poor. Specific patient groups, particularly immunosuppressed patients, have a 60-250-times increased risk of SCC and an elevated risk of metastasis of up to 8%. There are also gene mutations known to affect SCC pathogenesis, however, less is known about the impact of mutations on metastasis.

Current staging systems for SCC include the Brigham and Women's Hospital (BWH) system and the American Joint Committee on Cancer (AJCC) 8th edition staging systems. While these staging systems help to risk stratify patients, the sensitivity (proportion of positives correctly identified) of AJCC8 and BWH for patients who will experience nodal metastasis or disease-specific death is only 0.78 and 0.73,

respectively, and the specificity (proportion of negatives correctly identified) of each is 0.85 and 0.93, respectively. With this knowledge, one aim of this project was to identify and quantify additional risk factors for SCC metastasis utilizing a large database of institutional data.

Treatment options for metastatic SCC are limited and often consist of excision followed by radiation and/or systemic therapy. Utilized systemic therapies include traditional cytotoxic chemotherapy, cetuximab (off-label), cemiplimab and pembrolizumab. However, overall response rates (ORRs) are currently low (34-78%). Therefore, additional therapeutic targets are needed to expand and improve treatment options. To identify additional therapeutic targets, next-generation sequencing was performed on a cohort of localized primary SCCs (n=10), metastatic primary SCCs (n=10), and matched nodal metastases from SCC (n=10). The localized primary SCCs were obtained from 10 unique patients and the metastatic primary SCCs and nodal metastases were obtained from an additional 10 unique patients, with each patient providing a primary tumor and metastatic sample, allowing for sample matching by patient. In addition to identifying actionable somatic mutations to further investigate, analyses of germline polymorphisms and mutational patterns in subsets of patient groups were performed.

To validate findings from the sequencing data, a comprehensive literature search was completed for all manuscripts that performed next-generation sequencing in SCC and had patient-level mutational data available. The data generated were used to confirm our previous findings and to identify additional mutational targets in SCC. For a two-tiered approach to identifying actionable targets, a literature review was performed to identify immunohistochemistry studies that found proteins that are differentially expressed in metastatic and localized SCC. Combining all approaches led to the investigation of *ALK* and *LRP1B* in the laboratory, where these findings were validated

and explored further. Notably, we identified a new therapeutic target, *ALK*, explored the mechanism by which it promotes SCC progression, and identified ceritinib, a commercially available molecular inhibitor, as a possible therapy for *ALK*-mutated SCC.

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List of Abbreviations

5AZA-DC	5-aza-2'-deoxycytidine
ABC	Avidin-Biotin Complex
AIC	Akaike Information Criterion
AJCC	American Joint Committee on Cancer
AK	Actinic Keratosis
ALK	Anaplastic Lymphoma Kinase
AML	Acute Myeloid Leukemia
ANOVA	Analysis Of Variance
AP	American Population
ARF	Alternative Reading Frame
BCC	Basal Cell Carcinoma
BD	Bowen's Disease
BIC	Bayesian Information Criterion
BWH	Brigham and Women's Hospital
CAPRI	R Package for Cancer Progression Inference
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CI	Confidence Interval
CLEC-2	C-Type Lectin-Like Receptor 2
CMax	Maximum Serum Concentration
CML	Chronic Myelogenous Leukemia
COSMIC	Catalogue Of Somatic Mutations in Cancer
CPT	Current Procedural Terminology

DAB	3,3’Diaminobenzidine
DAPI	4’,6-diamidino-2-phenylindole
dbSNP	Single Nucleotide Polymorphism Database
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNdScv	R Package using likelihood dN/dS models
DS	Double Stranded
E-Cadherin	Epithelial Cadherin
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
ESCC	Esophageal Squamous Cell Carcinoma
ExAC	Exome Aggregation Consortium
FACS	Fluorescent Activated Cell Sorting
FDA	United States Food and Drug Administration
FDR	False Discovery Rate
FFPE	Formalin-Fixed Paraffin-Embedded
GFP	Green Fluorescent Protein
GIST	Gastrointestinal Stromal Tumor
GRCh37	Genome Reference Consortium Human Build 37

GWAS	Genome-Wide Association Study
H&E	Hematoxylin and Eosin
HCC	Hepatocellular Carcinoma
Hg19	Human Genome version 19
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
HR	Hazard Ratio
HR	Homologous Recombination
IACUC	Institutional Animal Care and Use Committee
ICD-9,10	International Classification of Diseases Ninth/Tenth Revisions
ICP	Immunocompetent Patient
IHC	Immunohistochemistry
INK4A	Inhibitor of CDK4a
IRB	Institutional Review Board
ISP	Immunosuppressed Patient
KA	Keratoacanthoma
LDL	Low Density Lipoprotein
LRP1B	Low-Density Lipoprotein Receptor-Related Protein 1B
LUAD	Lung Adenocarcinoma
MAF	Mutant Allele Frequency
MMS	Mohs Micrographic Surgery
MPM	Malignant Pleural Mesothelioma
MRN	Medical Record Number
NA	Not Available
NCI	National Cancer Institute
NER	Nucleotide Excision Repair
NGS	Next-Generation Sequencing

NMSC	Non-Melanoma Skin Cancer
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
NOTCH1	Notch homolog 1
NSCLC	Non-Small Cell Lung Cancer
OR	Odds Ratio
ORR	Overall Response Rate
OSCC	Oral Squamous Cell Carcinoma
OTR	Organ Transplant Recipients
PBL	Peripheral Blood Lymphocytes
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PD-1/PD-L1	Programmed Cell Death-1 Receptor (Ligand)
PNI	Perineural Invasion
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta Analyses
RCC	Renal Cell Carcinoma
RDEB	Recessive Dystrophic Epidermolysis Bullosa
ROS	Reactive Oxygen Species
SCC	Cutaneous Squamous Cell Carcinoma
SD	Standard Deviation
SLNB	Sentinel Lymph Node Biopsy
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
TCGA	The Cancer Genome Atlas
TCL	T-Cell Lymphoma
TCR	T-Cell Receptor

TERT	Telomerase Reverse Transcriptase Gene
TERTp	TERT promoter
Ti	Transition
TMB	Tumor Mutational Burden
TME	Tumor Microenvironment
TNM	Tumor Node Metastasis Staging
TP53	Tumor Protein p53
TRONCO	R Package for Translational Oncology
TSA	Trichostatin A
TSH-R	Thyroid-Stimulating Hormone Receptor
Tv	Transversion
UNMC	University of Nebraska Medical Center
USC	University of Southern California
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UV-R	Ultraviolet Radiation
WLE	Wide Local Excision
XP	Xeroderma Pigmentosum

Chapter 1: Introduction to Cutaneous Squamous Cell Carcinoma (SCC)

1.1 Epidemiology of SCC

Cutaneous squamous cell carcinoma (SCC) is the second most common malignancy worldwide with an estimated 1,000,000 cases and 9,000 deaths annually in the United States.[1] Although the case-fatality rate is only approximately 1% for SCC, the overall mortality figures equal or exceed those for melanoma, which is considered more lethal, but less common.[2] In the last few decades, there has been a 50% to 200% increase in SCC incidence.[3]

Risk factors for SCC include older age, male gender, light skin, ultraviolet radiation exposure, and arsenic exposure.[4–7] Immunosuppression also poses an elevated risk, with an estimated 65-250-times increased risk, which is largely dependent on the degree of immunosuppression.[8–12] The evidence is mixed as to whether or not human papillomavirus (HPV) is implicated in SCC, however, studies that argue in favor of a positive association suggest that this may be through dysregulation of common pathways (e.g. notch signaling) and inflammation.[13–15]

In addition, several familial syndromes confer an increased risk of developing SCC. Xeroderma pigmentosum (XP) patients have a germline genetic defect in nucleotide excision repair (NER), placing them at a 1000-fold increased risk of developing skin cancer, with the first SCC developing at a median of 8-9 years.[16] NER is particularly relevant as it repairs DNA damage caused by exposure to UV light, the most prevalent injury to skin. Other familial cancer syndromes that confer an increased risk of SCC include Werner Syndrome, Bloom Syndrome, Epidermodysplasia Verruciformis, Ferguson-Smith Syndrome, Rothmund-Thomson Syndrome, Fanconi Anemia, and Oculocutaneous Albinism.[16]

1.2 Clinical and Histologic Presentation of SCC

SCC Precursors

Actinic keratosis (AK) is a precursor lesion to SCC that presents as a single lesion or multiple lesions on sun exposed areas, typically, the scalp, neck, and extremities. The most common clinical appearance is an erythematous, rough, and scaly lesion, however, variants such as hypertrophic and pigmented subtypes may be observed.[17–19] It is estimated that approximately 10% of AKs will eventually progress into SCC.[20] Squamous cell carcinoma in situ (also called Bowen's disease [BD]) presents as an enlarging scaly lesion that is confined to the epidermis.[19] BD has an approximate risk of transformation to invasive SCC of 3-8%.[21,22]

Clinical and histological appearance

SCC usually presents on sun exposed areas, such as the face, scalp, and extremities. The clinical appearance of SCC is a red, scaly plaque that may itch or bleed (**Figure 1**). Findings observed on dermoscopy include irregular vessels that may be linear, elongated, or dotted, and a lesion surrounded by a white rim (**Figure 2**).[23] Histologic findings of SCC include aggregates of eosinophilic keratinocytes with pleomorphic nuclei and mitoses, keratin pearls, and varying degrees of cellular differentiation (**Figure 3**). Inflammatory infiltrate consisting of plasma cells and lymphocytes may be present in varying amounts.[23]

Figure 1: Clinical Image of SCC

(Courtesy of NCI Visuals Online, Public Domain)

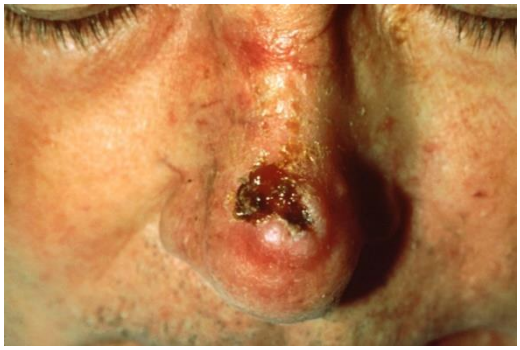

Figure 1 demonstrates the clinical appearance of SCC.

Figure 2: Dermoscopy of SCC

Images courtesy of DermNetNZ (https://creativecommons.org/licenses/by-nc-nd/3.0/nz/legalcode)

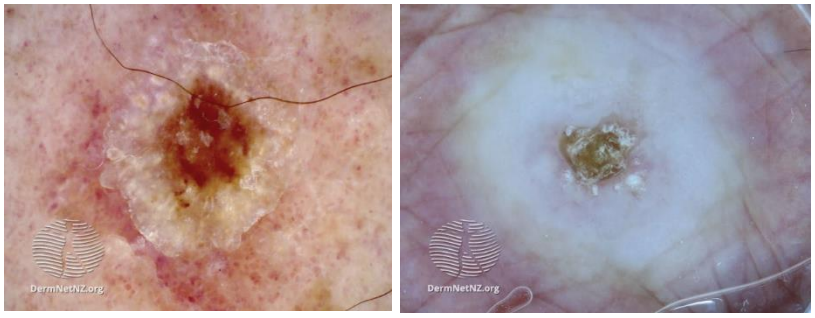

Figure 2 illustrates the dermoscopic appearance of SCC.

Figure 3: Histologic appearance of well-differentiated SCC with keratin pearls

Image Courtesy of the Creative Commons Attribution 4.0 International License and Yanovsky *et al*. (2011)[24]

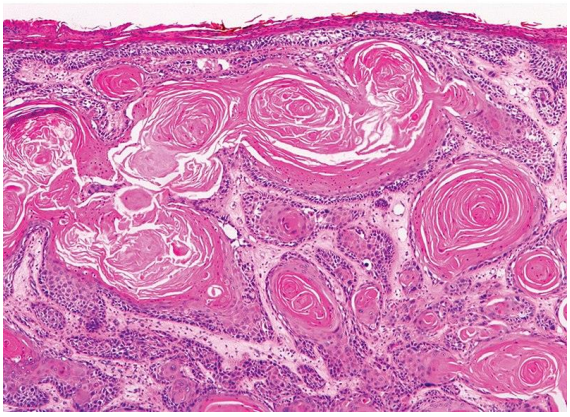

Figure 3 illustrates the histologic appearance of SCC.

Histologic subtypes of SCC

Acantholytic SCC

Acantholysis is defined as loss of intercellular adhesion that occurs secondary to detached intercellular bridges.[25,26] Acantholytic SCC appears grossly as a nodule with crusting, scaling, and/or ulceration, similar to typical SCC. Acantholytic SCC often has the typical histological features of SCC along with acantholysis, dyskeratosis, and glandular formation. It usually arises from acantholytic actinic keratoses in sun-exposed locations in older patients.[27]

Clear cell SCC

The clear cell variant of SCC is named for the hydropic changes exhibited from the accumulation of intracellular fluid in the cell. Cells may appear clear with nuclei present in the periphery.[27] There are three additional subtypes of clear cell SCC, which include keratinizing (type I), nonkeratinizing (type II), and pleomorphic (type III).[28] Like other variants, this is most commonly seen in older Caucasian men with a significant history of sun exposure.

Desmoplastic SCC

Desmoplastic SCC is an aggressive subtype of SCC characterized by a trabecular growth pattern and a desmoplastic stroma. Desmoplastic SCCs exhibit recurrence in 27.3% of cases, compared to 2.6% of cases for typical SCC.[27] Desmoplastic SCC has a metastatic rate of 22.7%, which is six times that of traditional SCC (3.8%).[27] Desmoplastic SCC frequently occurs on the ear.[29]

Keratoacanthomas

Keratoacanthomas (KAs) are rapid growing, crateriform nodules that can spontaneously regress. There are three clinical stages of KAs: proliferation, maturation, and involution.[27] There has been debate as to whether KAs should be considered their own entity or a variant of well-differentiated SCC. Due to reports of aggressive behavior, although rare, KAs are generally considered a variant of SCCs that have the potential to regress.[30,31]

Papillary SCC

Papillary SCCs exhibit rapid growth and may appear as fungating, exophytic, pedunculated masses.[32] On histology, papillary SCCs have been characterized by keratinocytes with eosinophilic cytoplasm, significant nuclear atypia, and fibrovascular projections over a thick epidermis.[33] Overall, this is a relatively rare variant that is only described in several case reports and series.

Pigmented SCC

Pigmented SCC is another rare variant that can be mistaken for melanocytic neoplasms or pigmented basal cell carcinomas.[34,35] Clinically, they may appear on sun damaged skin as crusted papules with fast growth.[36] The histological findings include both keratinized squamous cells with attendant dendritic (non-malignant) melanocytes that produce melanin.[27]

Signet Ring SCC

Signet ring SCC is a very rare variant of SCC. It is marked by the histological finding of signet rings, which are cells with intracellular mucin vacuoles that result in peripherally displaced nuclei.[37] However, despite the histologic appearance of mucin, several cases of signet ring SCC have reported negative mucicarmine staining.[38,39]

Utilizing a variety of immunohistochemistry stains is important for diagnosis and differentiation from other mucin-producing tumors.

Spindle Cell SCC

Spindle cell SCCs are marked by atypical spindle cells in a whorled pattern on histology. Immunohistochemistry may be used to differentiate spindle cell SCC from spindle cell melanoma, cutaneous leiomyosarcoma, and atypical fibroxanthoma. Spindle cell SCCs stain positive for at least one of markers 34βE12, AE1/3, and vimentin and negative for S-100, CD68, and SMA.[40]

Verrucous SCC

Verrucous SCC is an exophytic, low-grade variant of SCC with little metastatic potential. While growth is usually slow for this variant, recurrence and local destruction can happen.[41,42] Verrucous SCC is thought to be associated with human papillomavirus (HPV) positivity, however, this association has not held up in all studies.[43,44]

Staging systems in SCC

As of January 2021, the most commonly utilized staging systems in SCC are the Brigham and Women's Hospital (BWH) and American Joint Committee on Cancer (AJCC) 8th edition staging systems. The AJCC8 system has five levels. T1 tumors are those that are <2 cm in greatest diameter. T2 tumors are those that are greater than or equal to 2 cm but less than 4 cm in greatest diameter. T3 tumors are greater than or equal to 4 cm in greatest diameter or have any of the following: minor bone invasion, perineural invasion, or invasion beyond the subcutaneous fat (or 6 mm). Tumors with

gross cortical bone invasion or marrow invasion are upgraded to stage T4a. Tumors with skull invasion or skull base foramen invasion are upgraded to stage T4b.[45]

The BWH staging system is based on risk factors for progressive disease, which are: tumor diameter 2 cm or greater, poorly differentiated histology, perineural invasion of nerves measuring 0.1 mm or greater in diameter, and tumor invasion beyond the subcutaneous fat. T1 tumors have 0 high-risk factors, T2a tumors have 1 high-risk factor, T2b tumors have 2 or 3 high-risk factors, and T3 tumors have all 4 high-risk factors. Of note, bone invasion in the BWH system upgrades a tumor to T3 stage.[46] A comparative study by Ruiz *et al*. (2019) concluded that the BWH system was superior to AJCC8, as AJCC8 T2 and T3 stages had too similar of risks for nodal metastasis.[47] While BWH may be an improvement, SCC staging systems are still imperfect and improving upon these may lead to optimizing which patients undergo further workup for high-risk disease. Chapter 3 will further expand upon our contribution to identifying and quantifying risk factors.

1.3 Common Gene Mutations in SCC

SCC has the highest mutational burden of all solid tumors.[48,49] *TP53* mutations that are ultraviolet-induced are often the first or early mutation(s) in SCC.[50] The p53 protein was originally discovered by its association with simian virus 40 large T antigen in cancer cells.[51] The tumor suppressor function of *TP53* was uncovered by a series of mouse experiments in which it was observed that mice deficient in p53, the protein product of TP53, developed tumors at an abnormally high rate.[52,53] *p53* acts in part as a transcription factor and regulates cell division through dozens of pathways, including apoptosis and DNA repair.[54] In SCC, *TP53* mutations are found in 50-90% of all tumors.[50,55,56] These mutations are often UVB-induced and consist of C-to-T and CC-to-

TT transitions, which inactivate p53 and allow cancerous cells to avoid DNA-damage-induced apoptosis and promote clonal expansion of the *TP53* mutation.[57]

A whole exome sequencing study of 20 SCCs identified *NOTCH1* mutations as another early event in SCC pathogenesis.[58] Notch receptors are members of an evolutionarily conserved pathway that involves response to environmental stimuli in animals through cell-to-cell contacts.[59,60] It has been demonstrated that Notch and Wnt/β-Catenin signaling pathways are interconnected and cooperate in many cancers including colorectal and hepatocellular carcinomas.[61–63] In SCC, 85% of *NOTCH1* mutations were G-to-A transitions from UV radiation that occurred after *TP53* mutations, suggesting that *NOTCH1* acts as a tumor promoter rather than initiator in SCC.[64,65] The frequency of *NOTCH1* mutations in SCC ranges from 30-69%, making it one of the most common mutations.[48,66,67]

Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) encodes two proteins that regulate the cell cycle, p16 (INK4A) and p14 (in an alternative reading frame [ARF]).[68,69] Germline *CDKN2A* mutations have been identified as a predisposing factor to melanoma.[70] Somatic *CDKN2A* mutations have been found in 20-48% of SCCs, with metastatic SCCs generally on the higher end of that range.[56,66,71] In addition, *CDKN2A* promoter hypermethylation, which is associated with UVA radiation, has been reported in 35-78% of SCCs.[72–75] While there is significant data demonstrating high frequencies of mutations in *TP53*, *NOTCH1*, and *CDKN2A* in SCC, these genes have been difficult to target clinically.

Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that plays a role in cell proliferation, differentiation, and migration in many cancers.[76] *EGFR* mutations are reported in 0-5% of SCCs.[77–79] However, overexpression of EGFR has been found in 35-80% of SCCs and has been associated with poor prognosis.[80–82] This oncogene has been successfully targeted clinically. EGFR inhibitor cetuximab is used

off-label for advanced and/or metastatic SCC and has an overall response rate (ORR) of 33% as monotherapy and 58% when used in combination with surgery and radiation.[83] Additional EGFR inhibitors such as gefitinib have been tried with moderate success (e.g., complete response rate of 18%).

Telomerase reverse transcriptase gene (*TERT*) encodes the limiting factor for telomerase activity, which maintains telomeric DNA.[84] In an analytical study of publicly available sequencing data in 31 cancer types, Barthel *et al*. (2019) found *TERT* mutations in many cancers including ovarian cancer, lung squamous cell carcinoma and adenocarcinoma, and esophageal carcinoma.[85] Several recent studies found TERTp (TERT promoter) mutations in 31.6-50% of SCCs.[86–88] A study by Campos *et al*. (2019) found that TERTp mutations were independently associated with a higher risk of lymph node metastasis (OR=15.89; p=0.022).[87] While it is clear that there are several mutations well-known to be involved in SCC pathogenesis, such as *TERT* and the aforementioned genes, this dissertation focuses on novel mutations and potential therapeutic targets.

1.4 Current Treatments for SCC

Surgery

While some very low-risk SCCs may be treated with cryotherapy or local destruction, the majority of SCCs are treated with surgery, which is wide local excision (WLE) or Mohs Micrographic Surgery (MMS). MMS is a technique that removes a tumor in a series of steps, with examination of the complete peripheral and deep tumor margins (“comprehensive margin assessment”) under the microscope after each layer is taken, until the margins are completely clear. This process is both extremely effective in removing the cancer with high local cure rates and is also tissue-sparing and cosmetically favorable.[89] MMS is indicated for tumors with high-risk features or tumors in

high-risk locations.[90] The decision to treat with either surgical approach is typically dependent on tumor stage and location, with higher stage tumors and those in an anatomically high-risk location (e.g. head and neck) more likely to be treated with MMS. A study of three hundred and sixty-six T2a (BWH) tumors treated with MMS (n= 240) and WLE (n= 126) found that recurrence was significantly more likely in tumors treated with WLE (4% vs 1.2%, p=0.03), supporting the use of MMS in tumors with even a single high-risk feature.[91] In addition to excisional surgeries, radiologic or pathological nodal staging may be performed for high-risk tumors.

Radiation and Systemic Therapy

For unresectable and/or metastatic SCCs, therapies in addition to or instead of surgery may be considered. A review by Trodello *et al.* (2017) of cisplatin for SCC found a complete response rate of 22% and an overall response of 45%.[92] Radiation therapy has also been used in combination with surgical therapy, particularly for patients with perineural invasion.[93] More recently, targeted therapies have been replacing cytotoxic treatment regimens. One example of a small molecule targeted therapy used off-label for SCC is EGFR inhibitor cetuximab. Cetuximab is used for *EGFR*-mutated SCC and a systematic review by Trodello *et al.* (2017) found an overall response of 78%, which appears to be an improvement over cisplatin, however, other studies report much lower response rates.[92] Other small molecule EGFR inhibitors have been investigated in SCC, however, responses have been modest.[94]

The most recent advances in systemic therapy for SCC are immunotherapies, which primes the body to use its own immune system to attack cancer cells. As a result of successful clinical trials, in September 2018, the United States Federal Drug Administration (FDA) approved programmed cell death-1 receptor (PD-1) inhibitor cemiplimab for SCC.[95] An objective response rate was observed in 44% of patients, with

grade 3-4 adverse events occurring in 44% of patients.[95] Another PD-1 inhibitor, pembrolizumab, was FDA approved in June 2020 for recurrent and metastatic SCC after a successful clinical trial demonstrated a disease control rate of 52.4% and grade 3-5 adverse events in only 5.7% of patients.[96] While newer therapies represent an overall improvement, there is still additional research needed to treat the substantial number of patients who do not respond to current treatments and to discover alternatives with more tolerable toxicity profiles. Further, identification of patients with resectable disease at risk for nodal metastasis is necessary.

Chapter 2: Materials and Methods

Portions of this chapter are from the following manuscripts (with permission):

Lobl MB, Clarey D, Higgins S, Sutton A, Hansen L, Wysong A. Targeted next-generation sequencing of matched localized and metastatic primary high-risk SCCs identifies driver and co-occurring mutations and novel therapeutic targets. J Dermatol Sci. 2020 Jul;99(1):30-43.[56]

Lobl MB, Hass B, Clarey D, Higgins S, Wysong A. Next-generation sequencing identifies novel single nucleotide polymorphisms in high-risk cutaneous squamous cell carcinoma: A pilot study. Exp Dermatol. 2020 Jun 1.[97]

Lobl MB, Clarey D, Higgins S, Thieman T, Wysong A. The correlation of immune status with ultraviolet radiation-associated mutations in cutaneous squamous cell carcinoma: A case-control study. J Am Acad Dermatol. 2020 May;82(5):1230-1232.[98]

Lobl MB, Clarey D, Schmidt C, Wichman C, Wysong A. Analysis of mutations in cutaneous squamous cell carcinoma reveals novel genes and mutations associated with patient-specific characteristics and metastasis: a systematic review. Arch Dermatol Res. 2021 Mar 18.[99]

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2.1 A Case-control study to identify clinical and histologic predictors of SCC metastasis

This study is a multicenter matched case-control study at 1) the University of Southern California in Los Angeles, California (USC) (including patients seen at Keck Medical Center of USC), and 2) The University of Nebraska Medical Center (UNMC) in Omaha, Nebraska. All studies were approved by the Institutional Review Boards (IRBs) at these Centers. At USC, a database was compiled with patients seen by the Department of Pathology from 2013-2017. This database was searched for International Classification of Diseases, Ninth Revision (ICD-9) and Tenth Revision (ICD-10) diagnostic codes for squamous cell carcinomas, unspecified malignant neoplasms, or other specified malignant neoplasms of the following locations: skin of the lip,

ear/external auditory canal, eyelid, other/unspecified parts of face, scalp and neck, overlying sites of skin, and unspecified skin. Current Procedural Terminology (CPT) codes for excision procedures of salivary glands/ducts and malignant skin lesions were also searched. A total of 1,393 patient medical record numbers were identified. At UNMC, a database was created by using CoPath to retrieve all cases of specimens of skin with “squamous cell carcinoma” in the final diagnosis from 2010-2018. A total of 709 unique medical record numbers (MRNs) were identified. Between the two centers, there were a total of 2,102 cases returned. All identified cases of lymph node metastases arising from cutaneous SCC were included in this study. Non cutaneous SCCs were then excluded.

Patient charts were evaluated for evidence of cutaneous SCC and the presence or absence of lymph node metastasis at initial diagnosis or anytime thereafter. Lymph node metastasis was defined by medical record documentation with diagnostic imaging, fine needle aspiration, sentinel lymph node biopsy (SLNB), or lymph node dissection and confirmed by pathologic diagnosis. Histopathologic features were collected from either surgical pathology or dermatopathology reports of excisions or Mohs debulk specimens, respectively. If these reports were not available, biopsy reports were used.

Sixty-five cutaneous SCC tumors with lymph node metastasis were identified (cases). Each of these cases was matched with three cutaneous SCC tumors without nodal metastasis (controls). The cases and controls were matched by anatomic location, gender, and age within a 10-year range (or the age closest to a match, if a match was not available). Cases were matched by anatomic location, as there are particular locations (e.g., lip, ear) that portend a higher risk of lymph node metastasis. For the USC cohort, the average follow-up time was 17.8 months. For the UNMC cohort, the average follow-up time was 60.8 months.

A modified conditional logistic regression test for 3:1 matching was performed to identify factors that were significantly associated with metastasis. This was performed using univariate models followed by a multivariate model. After performing a matched conditional logistic regression, odds ratios (ORs), 95% confidence intervals (CIs), and p-values were calculated to quantify associations between each risk factor and lymph node metastasis. The backwards conditional regression technique was used for the multivariable model. SPSS (IBM), SAS version 9.4, and Excel (Microsoft) were used for statistical analysis. The modified conditional logistic regression methods are available: https://www.ibm.com/support/pages/conditional-logistic-regression-using-coxreg. The threshold of p=0.05 was used for statistical significance throughout the study.

2.2 Performing Next-Generation Sequencing

Sequencing for Chapter 4.1

This study was approved by an institutional review board (IRB). A cohort of 20 high-risk SCC patients was developed for this study, which utilized the Vela OncoKey Select Panel (**Table 1, Table 2**). Ten patients had localized disease only, at a minimum of two years follow up. Ten patients had confirmed lymph node metastasis. The localized SCC and metastatic SCC patients were matched by sex, age by decade, and BWH stage. Inclusion criteria were patients presenting to our academic medical center between 2014-2017 with histologically confirmed high-risk SCC. Exclusion criteria were patients with mucosal squamous cell carcinoma or patients with SCC without high-risk features. Primary formalin-fixed paraffin-embedded (FFPE) tissue was analyzed from our cohort. Hematoxylin and eosin stained sections were evaluated by board certified pathologists to determine percent tumor content in areas selected for extraction. Macro-dissections were made on unstained tissue sections and genomic DNA was isolated using the Maxwell FFPE DNA isolation kit (Promega Corp). Automated template

preparation, next-generation sequencing, analysis, and reporting were performed on the Sentosa SQ301 system (Vela Dx) using the Sentosa SQ Oncokey Select targeted cancer mutation 76 gene panel. The Veriti® Dx 96-Well Thermal Cycler was used for off-board PCR amplification (ThermoFisher Scientific). The PCR parameters were recommended by Vela Diagnostics in the user manual. The initiation step was carried out at 99°C (1 cycle for 2 minutes) followed by 18 cycles of amplification. Denaturation was carried out at 99°C (15 seconds each). Annealing and elongation was carried out at 60°C (4 minutes each). The final hold was carried out at 10°C overnight. Additional details regarding the sequencing, alignment, and coverage parameters are detailed in the user manual (veladx.com).

Table 1: Genes included in the next-generation sequencing panel (Sentosa SQ Oncology Panel)

Median coverage 500x, minimal coverage 300x

Table format:

Gene

Exon Coverage

AKT1 3	BRAF 11, 15	ERB B3 2, 3, 6, 7, 8	FOXL2 1	HRA S 2, 3, 4	KMT2 D 32, 33, 48, 53	NFE2L 2 2	RAC1 0, 2, 6	SMA D4 3, 9, 10, 11, 12	U2A F1 2, 6
AKT2 3	BRCA 1 3, 10	ESR1 4, 5, 7, 8	GATA3 4, 6	IDH1 4	KRAS 2, 3, 4	NOTC H1 6, 8, 26, 27, 34	RET 10, 11, 13, 15, 16	SMO 3, 6, 8, 9	
AKT3 2, 4, 5	BRCA 2 11, 27	EZHZ 16	GNA11 4, 5	IDH2 4	MAP2 K1 2, 3, 4, 6, 7, 11	NRAS 2, 3, 4	RHO A 2, 3	SRC 14	
ALK 20, 22,	CDKN 2A 0, 1, 2	FAT1 10, 15	GNAQ 4, 5	JAK2	MAP2 K2 3	PDGF RA	ROS 1 38	STK1 1	

23, 24, 35				12, 14, 16		12, 14, 18		Whole gene	
AR 5, 8	CTCF 3, 4, 5, 6, 7, 8, 10	FBXW7 4, 5, 7, 8, 9, 10, 11, 12	GNAS 6, 8, 9, 11	KDR 7, 8, 11, 15, 22, 23, 24	MAP3K1 4, 14, 17	PIK3CA 2, 5, 8, 10, 14, 21	SF3B1 14, 15, 16, 18	TERTp -	
ARAF 7	CTNNB1 3	FGFR1 4, 7, 12, 14, 15	H3F3A 2	KEAP1 4	MET 0, 14, 16, 19, 20, 21	PIK3R1 9, 10, 11, 12, 13, 14, 15	SMAD1 4	TP53 Whole gene	
ARID1A 5, 8, 14, 15, 16, 18, 20	EGFR 3, 7, 15, 18, 19, 20, 21	FGFR2 3, 7, 9, 12, 14	HIST1H3B 1	KIT 2, 8, 9, 10, 11, 12, 13, 14, 17, 18	MTOR 53, 56	POLE 9, 13	SMAD2 5, 8, 11	TSC1 15	

BAP1 0, 4, 7, 9, 10, 12, 13	ERBB2 8, 17, 18, 19, 20, 21, 22, 24	FGFR3 7, 9, 14, 16, 18	HNF1A 0, 1, 2, 3, 4	KMT2C 15, 34	NF1 9, 12, 34, 49	PTEN Whole gene	SMAD3 3, 6, 7, 9	TSC2 17, 30	

Table 2: Cohort of 20 patients for sequencing

	Localized	**Metastatic**
Average Age (years)	68.4	68.1
Males\Females	8\2	8\2
Location		
Cheek	2	0
Ear	2	5
Eye	1	0
Forearm	1	0
Maxillary	1	0
Neck	1	0
Nose	0	1
Lower Leg	1	0
Scalp	1	3
Supraorbital	0	1
AJCC8 Stage		
T1	1	1
T2	0	0
T3	7	7
T4a	1	1
T4b	1	1
BWH Stage		
T1	1	1
T2a	3	3
T2b	2	2
T3	4	4
Immunosuppressed?		
Yes	4	4
No	6	6

Positive and no template controls were provided by the The OncoKey KIT OncoKey System Control. Primary analysis (signal processing and base-calling) was performed by Sentosa SQ Suite software (Vela Diagnostics). Secondary analysis (variant calling, report generation) was performed by Sentosa SQ Reporter software (Vela Diagnostics) which filters to 5% allele frequency. Classification of variants was performed using published literature and public databases such as dbSNP (https://www.ncbi.nlm.nih.gov/snp/, accessed 11/1/19), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/, accessed 11/1/19), and COSMIC (https://cancer.sanger.ac.uk/cosmic, accessed 11/1/19).[100–102]

Patient Samples for Chapter 4.2

This study was approved by an IRB. A cohort of 10 metastatic SCC patients was developed for this study, which was composed of the same ten patients sequenced in chapter 4.1 (**Table 3**). Inclusion criteria were patients presenting to our academic medical center between 2014-2017 with histologically confirmed metastatic SCC. All patients had both primary tumor and metastatic tumor tissue available for analysis. Exclusion criteria were patients without histologically confirmed metastasis and patients with non-cutaneous squamous cell carcinomas. The sequencing was performed as stated in the methods for Chapter 4.1.

Table 3: Cohort developed for Chapter 3.2

Average Age	68.1
Males\Females	8\2
Location	**Primary Tumors:** Ear (5) Nose (1) Scalp (3) Supraorbital (1) **Metastases:** Lymph node (10)
BWH Stage	T1 (1) T2a (3) T2b (2) T3 (4)
Immune Status	Immunocompetent (6) Immunosuppressed (4)

Sequencing for Chapter 4.3 and 4.4

Sequencing was performed as stated in “Sequencing for Chapter 4.1”.

2.3 Analysis of Next-Generation Sequencing

Analyses performed for Chapter 4.1

Statistical analyses were performed with SPSS Statistics version 26 (IBM), R Studio version 3.6.1, and Excel version 16.35 (Microsoft). The R packages Maftools version 2.2.10 and GenVisR version 3.10 were used.[103,104] For the co-occurring mutations, the "somaticInteractions" function was used within the Maftools package. This performs pair-wise Fisher's Exact test to detect mutually exclusive or co-occurring events. The chi square variance of proportion test was used to determine if there were significant differences in the subtypes of mutations between localized and metastatic groups. A two-tailed student's t-test was used to evaluate if there was a significant difference between the mutant allele frequencies (proportion of a tumor with a mutation) and the frequencies of individual gene mutations (proportion of patients with a mutation) between localized and metastatic SCC. To determine the similarity of mutational signatures seen in our cohort compared to COSMIC signatures, the non-negative matrix factorization was run and the goodness of fit was measured using the signature analysis tools within Maftools. A p-value of 0.05 was used throughout as the threshold for significance.

Analyses performed for Chapter 4.2

Each subchapter employed various analytic techniques customized to obtain unique data specific to the two sampling techniques used in this study (matched by patient versus matched by age, gender, stage). Statistical analyses were performed with R Studio version 3.6.1 and Excel version 16.35 (Microsoft). Within the R Maftools package, various functions were used for data analysis: TiTv to calculate the distribution of base pair changes, trinucleotideMatrix, extractSignatures and plotSignatures to obtain

and visualize mutational signatures, and OncodriveCLUST to identify driver mutations.[103,105]

Alexandrov *et al*. (2013) described over 20 mutational signatures in various human cancers.[106] Using the signature analysis module in Maftools, mutational signatures were computed for our cohort and compared to previously described signatures using cophenetic correlation and non-negative matrix factorization. Cosine similarity is used to identify the signature(s) that are the best match(es) for the input data. Additional R packages used for analyses include TRONCO and dndSCv.[107,108] Within TRONCO, the Capri function was used. Capri takes results from mutation studies and constructs a proposed model of tumor evolution based on TCGA data and prior sequencing studies.[109] dNdScv works to detect driver mutations through quantitation of selection in cancer by maximum-likelihood dN/dS methods.[108] In a separate analysis, mutational concordance rates between primary and metastatic samples were calculated by counting the number of mutations that were seen in both matched primary and localized samples (concordant mutations), dividing this number by the total of all concordant and discordant mutations, and multiplying by 100.

Analyses performed for Chapter 4.3

Polymorphisms were detected in tumor tissue and differentiated from somatic mutations using previously validated methods (**Figure 4)**.[110] In total, 26 unique alleles were tested. We used a standard p-value of 0.05 and a Bonferroni-corrected significance threshold of p=0.0019. Statistical analyses were performed with SPSS Statistics (IBM) and Excel (Microsoft).

Figure 4: Methods for categorizing mutations

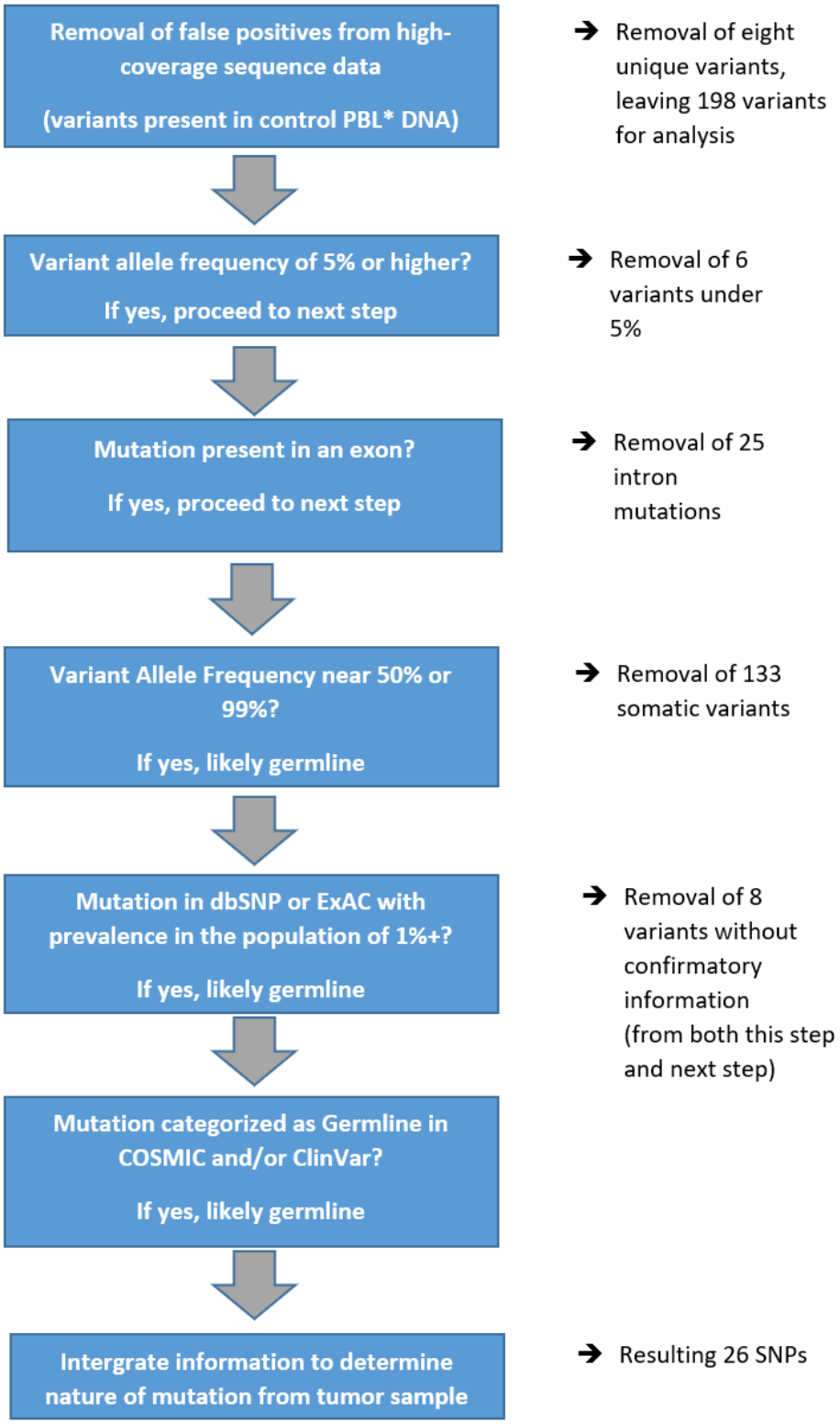


Figure 4 illustrates methods to categorize mutations as germline or somatic.

*PBL=peripheral blood lymphocytes

Analyses performed for Chapter 4.4

Mutations were categorized as being caused by UVA radiation, UVB radiation, reactive oxygen species (ROS) (thought to be secondary to UVA damage, likely due to deeper penetration in the skin), or 'other' based on methods by Agar *et al*.[111] Ensembl hg19/CRCh37 (https://uswest.ensembl.org/index.html) was used to locate specific base changes.

2.4 Methods for the Literature Review of All Sequencing Studies of SCC

Literature Search

A systematic literature review was performed in July 2019 according to PRISMA guidelines (**Figure 5**). Searches of The Cochrane Library (Wiley), EMBASE (embase.com) and MEDLINE ("Ovid MEDLINE and In-Process & Other Non-Indexed Citations 1946 to July 9, 2019") were performed on July 9, 2019. The complete search strategies are available at https://digitalcommons.unmc.edu/search/5. The total number of articles returned from the initial search was 3017, which was reduced to 1895 after duplicates were removed. After screening abstracts using inclusion and exclusion criteria, 127 articles remained (**Table 4**). These full text articles were screened for studies that had individual-level data available for inclusion in our study, which was typically found in a supplementary file. This left 26 full-text articles for inclusion. After screening these full-text articles by sequencing platform and for individual level data, nine articles remained for inclusion in our analysis.

Figure 5: PRISMA Diagram for Sequencing Study Review

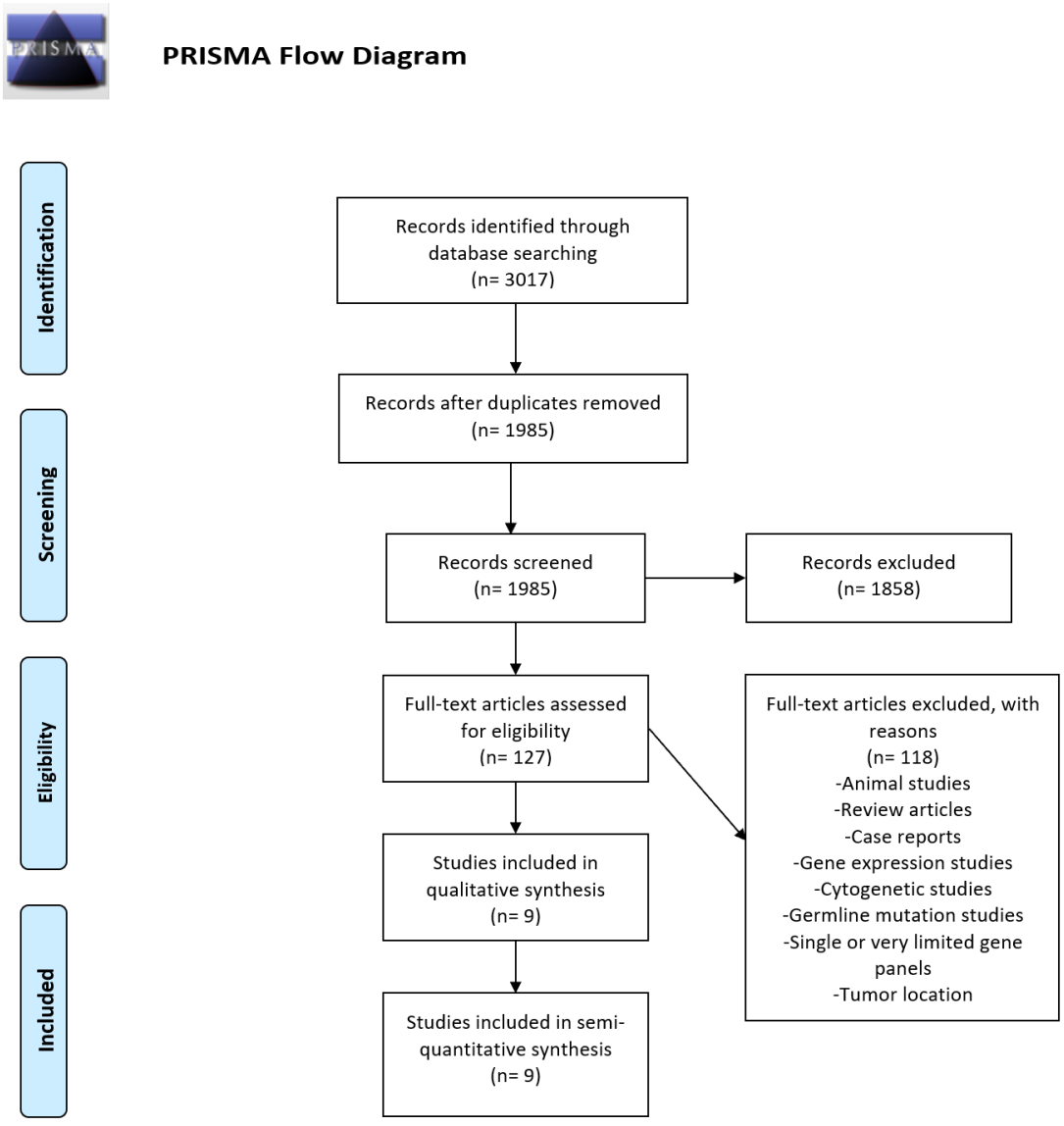


Figure 5 illustrates the search methods for the systematic sequencing study review.

Table 4: Inclusion and exclusion criteria for the SCC sequencing review

Inclusion Criteria	Exclusion Criteria
Studies that performed next-generation sequencing	Animal studies
Samples were primary localized or primary metastatic SCC tissue	Review articles
Studies with individual level data available	Case reports
	Gene expression studies
	Cytogenetic studies
	Inherited mutation studies

Sample Selection

From the nine articles compiled, there were several groups of primary tumor tissue with individual level sequencing data that were analyzed. Our study focused only on mutations that were present in primary SCC tumors that remained localized (follow-up periods ranged from 2-4 years) and primary SCC tumors that metastasized. Mutations found in the tumor at the metastatic site (ie., lymph node) were excluded from this analysis. Most studies returned from our search utilized the Illumina or other next-generation sequencing platforms. Studies that performed sequencing with PCR or microarrays were excluded as very few genes were included in these studies, precluding an unbiased dataset. The total number of samples from these studies was 279 (189 localized SCCs, 90 metastatic SCCs).

Data Synthesis

Data were checked for somatic versus germline mutations by M.L. in dbSNP or the original manuscript authors. Data synthesis and formatting were accomplished in two steps. Initial organization was done in Excel (Microsoft) and further formatting was done with R programs tidyr and dplyr.[112,113] The first step in this analysis focused on mutations in the entire gene; therefore, multiple base pair changes in a single gene within one sample were only counted as one mutation. That is, each sample either had a mutation in a particular gene (coded 1), did not (coded 0), or was not measured (coded NA). This method was used to determine which genes were the most highly mutated in SCC.

In order to evaluate the most common mutations in localized and metastatic SCC, we first selected genes that were mutated in greater than 5% of tumors. The 5% threshold was set by examining the literature to identify what percent of SCCs have a mutation in *EGFR*, which is the target of cetuximab, the only small molecule targeted therapy for SCC. A literature search was performed and found that *EGFR* mutations are

present in approximately 0-5% of SCCs.[77–79] We aimed for the mutations in our study to be present in more SCCs than the best current therapy, so we narrowed our focus to mutations present in more than 5% of tumors. The next step was to keep the data at a manageable size for computing and practical space limitations, therefore, we selected from this list the top 20 genes mutated in localized SCC and the top 20 genes mutated in metastatic SCC for additional analysis. Since there was overlap of some genes that were in the top 20 in both localized and metastatic SCC, 34 unique genes remained for further analysis. In the subsequent steps of our analysis, the individual protein changes were analyzed for a subset of the data when that information was available.

Semi-quantitative analysis of all sequencing studies of SCC

To calculate differences in mutational frequencies between localized and metastatic SCC, the chi-square for homogeneity of proportions test was used. The conservative Bonferroni-corrected p-value was also reported. The threshold used for significance throughout the study was p=0.05. R package Maftools and Excel (Microsoft) were used for data analysis and visualization.[103] The pattern counts table was generated with SAS 9.4.

2.5 Methods for analysis of SCC immunohistochemistry studies

Systematic Review

A search was performed in MEDLINE via PubMed for articles published in the last 20 years (January 1, 1999-June 30, 2019) using keywords “immunohistochemistry”, “metastasis”, “metastatic”, (“cutaneous squamous cell carcinoma” OR (“squamous cell carcinoma” AND “skin”)). Six hundred and fifty-three articles were returned from this search. The following were used as inclusion criteria for articles selected for this review: articles that were available in the English language, used human tissue, had available

data on outcomes, and included more than one sample (i.e., no single case reports) (**Figure 6**). After applying our inclusion criteria, 31 articles remained.

Figure 6: Search methods for IHC systematic review (adapted from PRISMA guidelines)

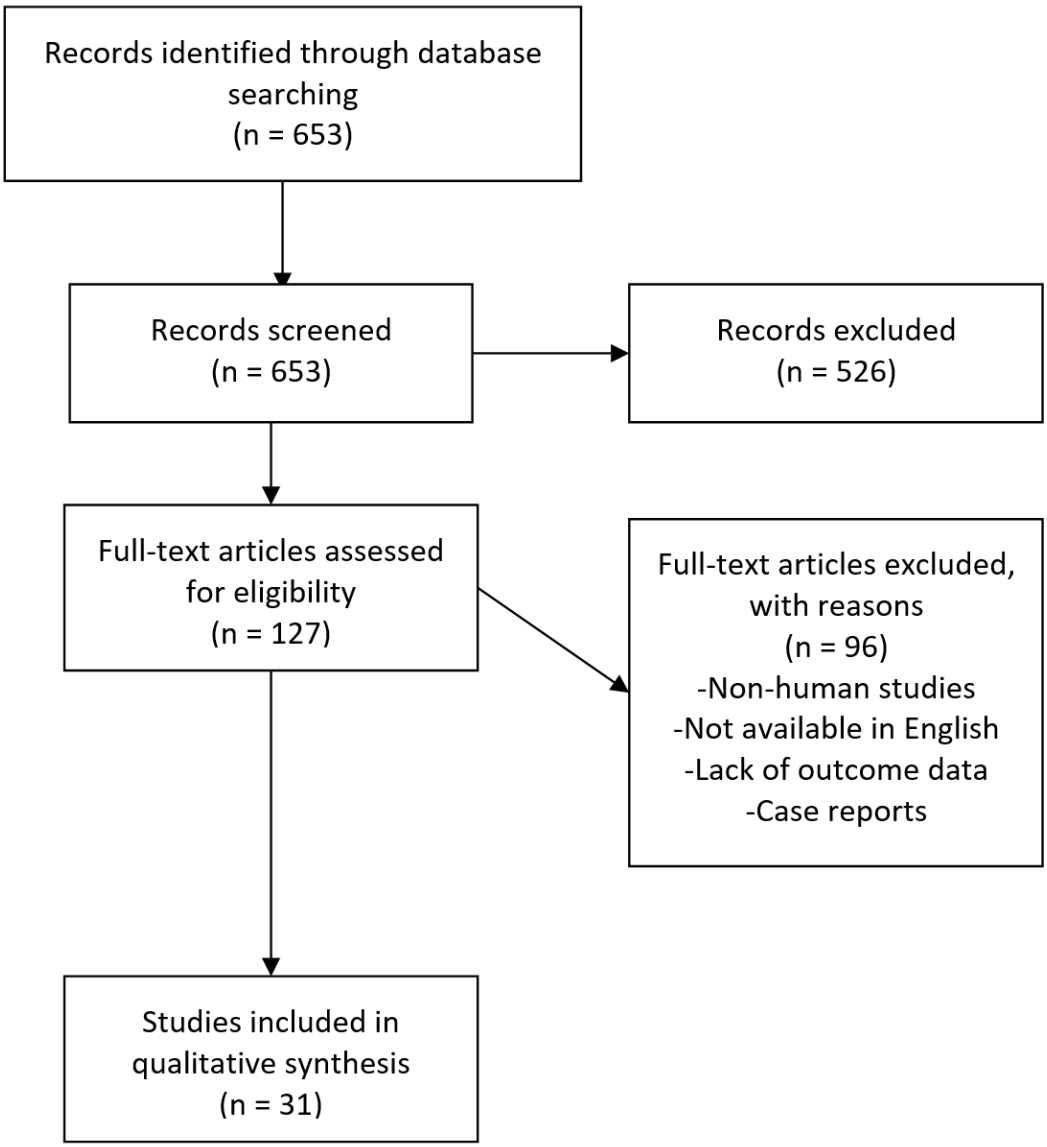


Figure 6 illustrates the search methods for the IHC systematic review.

Low-density lipoprotein receptor-related protein 1B (LRP1B) IHC Study

Institutional Review Board (IRB) approval was obtained prior to this study. Records of patients seen at the University of Nebraska Medical Center Department of Pathology were obtained by a CoPath search for specimens of skin with “squamous cell carcinoma” in the final diagnosis from 2010-2018. Inclusion criteria for our study included all cases of metastatic SCC (primary tumor and lymph node metastases) seen at our institution during the time period with tissue available. Exclusion criteria included any non-cutaneous SCCs or SCCs without confirmed metastasis. Fourteen cases of primary SCCs and fifteen patient-matched lymph node metastases were selected for inclusion. Normal epidermis tissue was included as a control.

Formalin-fixed paraffin-embedded (FFPE) sections were used to create tissue microarrays. Slides were deparaffinized and rehydrated to ddH_2O. Slides were then immersed in 3% H_2O_2 to block endogenous peroxidase activity. After washing in phosphate-buffered saline (PBS), citrate-based antigen retrieval was performed for 70 minutes under pressure at pH 6.0-6.2. After washing in PBS, blocking was performed with 0.03% casein prior to overnight incubation with primary antibody (LRP1B HPA069094 at 1:500, Atlas Antibodies) at 4 °C. After washing with PBS, slides were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:250), vector BA-1000 (Vector Laboratories). Additional washes with PBS were performed followed by incubation with avidin-biotin complex (ABC) detection reagent (Vector Laboratories). The chromogen reagent 3,3’Diaminobenzidine (DAB) was then applied for 3 minutes (Dako K3466). After counterstaining with hematoxylin and differentiation in 1% acid alcohol, slides were washed in tap water, dehydrated in a series of graded alcohols, and a coverslip was placed with Permount. The procedure was performed without primary antibody as an additional control for all experiments. Slides were evaluated and findings were confirmed by two board-certified Dermatopathologists. An H-Score was calculated

for each tumor. The staining was graded for intensity and percent of tumor covered. The intensity grading was from 0-3+ (0=no staining, 1+=weak staining, 2+=moderate staining, 3+=strong staining). The intensity score was multiplied by the percent of the tumor with that intensity of staining. Tumors with multiple intensities had all levels factored into the H-score. Statistical analyses were performed with Excel (Microsoft), SPSS (IBM), and SAS version 9.4.

2.6 Materials and methods for Chapter 7

Patient Samples

Institutional Review Board (IRB) approval was obtained prior to this study. Records of patients seen at the University of Nebraska Medical Center Department of Pathology were obtained by a CoPath search for specimens of skin with “squamous cell carcinoma” in the final diagnosis from 2010-2018. Nine cases of SCCs of various stages were selected to screen for *ALK* overexpression with immunohistochemistry (IHC). All metastatic cases (primary tumors and corresponding lymph node metastases) were screened for ALK expression (n=15). Nine cases of localized SCC of various stages were randomly selected to screen for *ALK* expression with immunohistochemistry (IHC).

Immunohistochemistry

IHC was done as described above with primary antibody ALK D5F3 at 1:250 (Cell Signaling) at 4 °C. Slides were evaluated under a light microscope with a board-certified Dermatopathologist.

Cell culture and transfection

SCC cell line Colo16[114] was a generous gift from Dr. Laura Hansen, PhD. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 7% penicillin-streptomycin (Gibco, 15140-122) (Complete growth medium). Cells were transfected with the plasmid *ALK*-F1174L (addgene) using lipofectamine 3000. Cells expressing ALK were identified and enriched using fluorescence activated cell sorting (FACS) for green fluorescent protein (GFP), which was also included in the transfected plasmid. Expression of *ALK* was confirmed by Western blot.

Protein isolation and western blot analysis

Cells were treated with 0.2 nM ceritinib (inhibitor of ALK kinase activity, Selleckchem LDK378) in dimethyl sulfoxide (DMSO) or the same amount of DMSO 6 hours prior to protein isolation. Protein was isolated from cells using lysis buffer (Tris + NaCl + DTT [dithiothreitol] + Na_3VO_4 + PMSF [phenylmethylsulfonyl fluoride] + NaF + Triton X-100) containing Roche complete protease inhibitor. Cells were scraped and pipetted into microfuge tubes and vortexed 3 times, every 5 minutes and placed on ice in between. Cells were centrifuged for 10 minutes at 13,000 RPM at 4 °C. The supernatant was collected and Laemmli + DTT was added before boiling samples. Samples were loaded onto 10% polyacrylamide gels for protein separation and transferred to a nitrocellulose membrane. Blocking was performed with 5% milk at room temperature for 30 minutes prior to incubation with the primary antibody (phospho-ERK: Cell Signaling T202/Y204; ERK: Cell Signaling 137F5; phospho-ALK: Cell Signaling, D6F1V; ALK: Cell Signaling, D5F3; Actin: Sigma Aldrich A2228) at 4°C overnight. After washing, the membrane was incubated with secondary antibody (anti-Rabbit or anti-Mouse) for 45 minutes. After three washes, SuperSignal ECL was applied for 1 minute (ThermoFisher) prior to visualization with x-ray film.

Colony formation assay

Cells were plated in 6-well plates at approximately 200 cells/well. After 6-8 days or when colonies were visible to the naked eye, wells were washed with PBS, fixed with 10% formalin, and stained with crystal violet solution. All cells were processed on the same day. Colonies were then quantified for size and number using ImageJ. When ceritinib inhibitor treatment was used, cells were treated every other day with ceritinib 0.2 nM in DMSO or the equivalent amount of dimethyl sulfoxide (DMSO). The 0.2 nM concentration was chosen based on the literature and on results of a series of proliferation assays that were performed with 0.0002 nM, 0.002 nM, 0.02 nM, 0.2 nM, 0.6 nM, 1 nM, and 2 nM concentrations. The concentration just below the one that appeared to cause decreased viability was selected. A two-tailed t-test was used to determine significant differences in proliferation between DMSO and ceritinib treated cells in a given cell line (i.e. DMSO versus certinib treated in 2C3 cells). Analysis of variance (ANOVA) with a post-hoc test was used to determine any differences between cell lines (i.e., between controls, 2C3, C7 lines).

Cell migration assays

Cells were resuspended in growth medium at 350,000 cells/mL. One cell insert (Ibidi #80466) (used to induce a reproducible gap in the cell monolayer) was adhered to the bottom of each well of a 6-well plate and 110uL of the cell suspension was pipetted into each of the 4 sections of the insert. The next day, the inserts were carefully removed and images were taken at each edge. Two mL of medium were added to each well and the imaging was repeated after 8 hours. Images were quantified in ImageJ by taking the area of the image without cells at 0 hours, subtracting the area of the image at 8 hours, dividing by the area at time 0, and multiplying by 100 to get the percent area closed by

migration at 8 hours. When ceritinib inhibitor treatment was used, cells were treated during initial plating and when fresh medium was added the next day it contained ceritinib 0.2 nM in DMSO or the equivalent amount of dimethyl sulfoxide (DMSO). When ERK inhibitor treatment was used, cells were treated during initial plating and when fresh medium was added the next day it contained 1 uM inhibitor in DMSO or the equivalent amount of dimethyl sulfoxide (DMSO) (Selleckchem, SCH772984). A two-tailed t-test was used to determine significant differences in migration between DMSO and inhibitor treated cells in a given cell line (i.e., DMSO versus inhibitor treated in 2C3 cells). Analysis of variance (ANOVA) was used to determine any differences between cell lines (i.e., between controls, 2C3, C7 lines).

Cell invasion assays

Cells were plated with an insert to cause a reproducible gap, as above, at 350,000 cells/mL (with 0.2 nM of ceritinib or an equal volume of DMSO). The next day, the inserts were carefully removed and Matrigel was combined with PBS at 1:1 and plated 0.8 mm high on top of the area previously enclosed by the insert. Images were taken at each edge. After 2 hours (to allow the Matrigel to set), 2 mL of medium (with 0.2 nM of ceritinib or an equal volume of DMSO) were added to each well and the imaging was repeated after 18 hours. Images were quantified in ImageJ by taking the area of the image without cells at 0 hours, subtracting the area without cells at 18 hours, dividing by the area at time 0, and multiplying by 100 to get the percent area closed by invasion at 18 hours. A two-sample t-test was used to determine significant differences in invasion between DMSO and ceritinib treated cells in a given cell line. Analysis of variance (ANOVA) with post-hoc analysis was used to determine any differences between cell lines.

Apoptosis assays

Cells were plated in complete growth medium in a 24-well plate at 70-80% confluency. Six replicates of six wells were used per cell line (control, 2C3, C7) with three wells treated with 0.2 nM ceritinib and the other three treated with an equal volume of DMSO. The cells were incubated overnight. The next day, 1uL of 4',6-diamidino-2-phenylindole (DAPI) was added to each well and the plates were incubated at 37 °C for 10 minutes. The cells were then imaged with fluorescence microscopy. Five representative images were taken per plate using DAPI and phase microscopy. Apoptotic cells in each image were identified (bright DAPI staining of condensed, fragmented nuclei) and divided by the total number of cells per field counted under phase contrast. The average percent apoptosis was calculated for each well. A two-sample t-test was used to determine significant differences in apoptosis in DMSO- and ceritinib- treated cells in a given cell line. Analysis of variance (ANOVA) with post-hoc analysis was used to determine any differences between cell lines.

In vivo experiments

Institutional Animals Care and Use Committee (IACUC) approval was obtained prior to this study. One million SCC-derived Colo16 ALK- cells or a clone with confirmed ALK expression (C7 cells) were suspended in 100 uL of Matrigel combined with the cells at 1:1 and injected subcutaneously into the bilateral flanks of NOD/SCID mice (Jackson Laboratories). There were two groups of mice with 6 mice per group. Group 1 was injected with parental ALK- cells and group 2 was injected with C7 ALK+ cells. Equal numbers of male and female mice were included and no sex-based differences noted. Mice were examined for tumor size with calipers three times per week until tumors reached 1 cm in diameter. When tumors reached the size threshold, mice were

sacrificed and tumor weight, size, and mouse weight were quantified. Tumors were examined grossly for signs of angiogenesis.

Chapter 3: Recurrence status, perineural invasion, and hypothyroidism are associated with lymph node metastasis in cutaneous squamous cell carcinoma: A case-control study

Introduction

Cutaneous squamous cell carcinoma (SCC) is the second most common malignancy worldwide with an estimated 1,000,000 cases annually in the United States.[115,116] While the majority of SCC cases have an excellent prognosis after surgical removal, there are high-risk features that are predictive of aggressive behavior.[117] Current staging criteria utilize a set of known high-risk features, including tumor diameter ≥ 2 cm, poorly or undifferentiated histology, presence of perineural invasion (nerve diameter >0.1 mm), invasion beyond subcutaneous fat, and depth of invasion > 6 mm.[118,119] While these high-risk tumor features are predictive of poor outcomes, the single most important prognostic indicator for mortality in patients with SCC is lymph node metastasis.[119] In a retrospective study of 136 patients, the presence of regional nodal disease at presentation was the only variable that was significant in multivariate analysis for recurrence or death (Hazard Ratio (HR)=7.64, $p<0.0001$).[119] Thus, features predictive of lymphatic involvement would have prognostic significance and potentially allow additional treatments to be initiated earlier.

The Brigham and Women's Hospital (BWH) tumor staging system has improved distinctiveness (outcome differences between stages), homogeneity (outcome similarity within stages), and monotonicity (outcome worsening with increasing stage) over American Joint Committee on Cancer (AJCC) 7th edition and is comparable to AJCC 8th edition with perhaps increased specificity.[120–122] However, it remains challenging to predict which individual tumors will metastasize, making it difficult to discern which tumors warrant additional work-up, nodal staging and surveillance. Also of note, the

initial and validation cohorts used in the development of current staging criteria had very few metastatic tumors.

Our group recently published the largest retrospective cohort of 53 cutaneous SCCs with lymph node metastasis, which verified the significance of currently utilized high-risk features and identified additional features that might be of prognostic significance.[123] In the current study, we identify the magnitude of the features associated with lymph node metastasis in SCC using a matched case-control design in an expanded multi-institutional study with a total of 260 SCCs. To the authors' knowledge, this is the largest and only study of this type and scope.

Results

Patient and tumor characteristics of the 65 cases and 195 controls included in the study are summarized, including the percentage of cases and controls with each risk factor and a univariate analysis (**Table 5**). Of the histopathologic high-risk features used in BWH staging, the most common feature in the present cases was invasion beyond subcutaneous fat or 6 mm depth (74.0%, 37/50), followed by size greater than 2 cm (70.2%, 40/57), perineural invasion (59.6%, 35/57), and poor histologic differentiation (29.5%, 18/61). Among the controls, the most common high-risk feature was invasion beyond subcutaneous fat or 6 mm (67.9%, 57/84), followed by size greater than 2 cm (48.6%, 71/146), perineural invasion (25.6%, 41/160), and poor differentiation (22.5%, 34/151). A modified univariate conditional logistic regression showed significant differences between cases and controls in the primary tumors characterized as recurrent (63.5% vs. 18.6% in controls, $p<0.001$), cases with perineural invasion (59.6% versus 25.6% in controls, $p<0.001$), cases with lymphovascular invasion (28.0% versus 1.5% in controls, $p=0.002$), cases with tumor size of 2 cm or greater (70.2% versus 48.6%, $p=0.008$), and in patients with hypothyroidism (33.3% versus 20.3% in controls, $p=0.03$).

Upon performing a multivariate analysis, recurrence, perineural invasion, and hypothyroidism all remained significant (**Table 6**). The odds ratios for metastasis were 6.3 for recurrent tumors (95% CI 2.6-15.3, $p<0.001$), 4.5 for tumors with perineural invasion (95% CI 1.7-11.8, $p<0.001$), and 2.7 for patients with hypothyroidism (95% CI 1.04-7.0, $p=0.04$).

Table 5: Patient and Tumor Characteristics for Case-Control Study

	Cases, n=65	Controls, n=195	P-value*
Age			
Average Age (SD)	74.0 (11.5)	73.3 (9.7)	matched
Gender			
Males	90.8% (59/65)	90.8% (177/195)	matched
Females	9.2% (6/65)	9.2% (18/195)	matched
Location			
Cheek	13.8% (9/65)	13.8% (27/195)	matched
Ear	21.5% (14/65)	21.5% (42/195)	matched
Extremity	1.5% (1/65)	1.5% (3/195)	matched
Eye	1.5% (1/65)	1.5% (3/195)	matched
Forehead	6.2% (4/65)	6.2% (12/195)	matched
Jawline	1.5% (1/65)	1.5% (3/195)	matched
Lip	4.6% (3/65)	4.6% (9/195)	matched
Neck	3.1% (2/65)	3.1% (6/195)	matched
Nose	7.7% (5/65)	7.7% (15/195)	matched
Temple	16.9% (11/65)	16.9% (33/195)	matched
Trunk	1.5% (1/65)	1.5% (3/195)	matched
Scalp	20% (13/65)	20% (39/195)	matched
Differentiation			
Well	10.8% (7/65)	22.1% (43/195)	P=0.05
Moderate	55.4% (36/65)	26.2% (51/195)	**P<0.001**
Poor	27.7% (18/65)	17.4% (34/195)	P=0.2
Unknown	6.2% (4/65)	34.4% (67/195)	NA

Size			
Average Size (SD)	4.0 (2.7)	2.4 (1.9)	**P<0.001**
Average Depth (SD)	11.4 (9.0)	9.1 (5.1)	P=0.8
Other Characteristics			
Recurrence	**63.5% (40/63)**	**18.6% (34/182)**	**P<0.001**
Perineural Invasion	**59.6% (35/57)**	**25.6% (41/160)**	**P<0.001**
Lymphovascular Invasion	**28.0% (14/50)**	**1.5% (2/137)**	**P=0.002**
Size of 2 cm or greater	**70.2% (40/57)**	**48.6% (71/146)**	**P=0.008**
Hypothyroidism	**33.3% (21/63)**	**20.3% (39/192)**	**P=0.03**
Poor Differentiation	29.5% (18/61)	22.5% (34/151)	P=0.2
Invasion Beyond Subcutaneous Fat (or 6mm)	74.0% (37/50)	67.9% (57/84)	P=0.5
Any Smoking History	58.7% (37/63)	56.4% (110/195)	P=0.8
Immunosuppression	31.7% (20/63)	33.9% (64/189)	P=0.9

* p-value calculated from univariate 1:3 conditional logistic regression modified per SPSS manufacturer instructions, see methods

SD= standard deviation

Table 6: Multivariate Analysis for Case-Control Study (Modified 1:3 Conditional Logistic Regression+)

Characteristic	**Odds ratio for metastasis**	**95% CI**	**P-value**
Recurrence	6.3	2.6-15.3	**P<0.001**
Perineural Invasion	4.5	1.7-11.8	**P<0.001**
Hypothyroidism	2.7	1.04-7.0	**P=0.04**

+modified per SPSS manufacturer instructions, see methods

Discussion and Conclusions

BWH staging utilizes four risk factors: tumor diameter ≥ 2 cm, poor differentiation, perineural invasion, and invasion beyond subcutaneous fat. The frequency of these features amongst the lymph node metastasis cohort confirms the risk they portend as invasion beyond subcutaneous fat, tumor diameter ≥ 2 cm, and perineural invasion were found in more than half of the tumors that metastasized. While size of 2 cm or greater is already recognized as an important risk factor, there are significant differences in value assigned to this factor for AJCC8 versus BWH staging systems, with greater weight given in the AJCC8 staging system.[122] This study confirms that tumors measuring 2 cm or greater have a significantly higher risk of nodal metastasis. Additional investigation into the magnitude of risk given by the tumor size may help to further refine staging systems. While the presence of perineural invasion in SCCs treated with Mohs micrographic surgery (MMS) has been reported in only 5.95% of cases overall, its presence has been reported to indicate a risk of occult disease of 15-20% of those without palpable lymph nodes.[124,125] The role of perineural invasion in nodal metastasis and death was further refined by Carter *et al*. (2013) who identified that involvement of large nerves >0.1 mm was associated with an increased risk of nodal metastasis (HR 5.6) and death (HR 4.5).[126] Thus, it is apparent that the literature and current staging systems recognize perineural invasion as a high-risk feature but the exclusion of perineural invasion in nerves measuring <0.1 mm in staging systems and the prognostic value assigned to perineural invasion in these systems is still up for debate given differences in value assigned to this risk factor. Our study confirms that tumors with perineural invasion are associated with increased odds of nodal metastasis (4.5 times), and further characterization of perineural invasion as a key risk factor is imperative in the development of reliable staging systems and identification of high-risk SCCs. Additionally, in our study, nerve sizes were available in a limited number of tumors.

Future studies investigating the association of nerve caliber with response to treatment and outcomes would be useful.

Several features not utilized in current staging systems, though reported to be high-risk, were significantly associated with lymph node metastasis in our study. Specifically, lymphovascular invasion, recurrent tumors, and tumors in patients with a diagnosis of hypothyroidism were significantly more common in the lymph node metastasis cohort. Lymphovascular invasion was reported by Moore *et al*. (2005) to be an independent predictor of nodal metastasis in SCC (OR 7.54).[127] In addition, Veness *et al*. (2007) found that 40% of patients with nodal metastasis from SCC had lymphovascular invasion in the primary tumor compared to 8% of node-negative patients.[128] The current study showed an impressive difference in prevalence of lymphovascular invasion between cases and controls with rates of 28.0% noted in the cases and only 1.5% in the controls, with an OR of 25.7 for metastasis (95% CI 3.3-198.6). While not significant in the multivariate analysis, likely due to the strong correlation of lymphovascular invasion with perineural invasion ($p<0.001$), our study supports lymphovascular invasion as associated with lymph node metastasis, the key determinant of survival in cutaneous SCC.[127] Physicians should consider lymphovascular invasion as a very high-risk feature that may warrant additional work-up. It is important to work closely with dermatopathology to evaluate for lymphovascular invasion in SCC not yet defined as high-risk, including potentially employing immunostains such as CD34 and D2-40, as these endothelial markers highlight vessels and have been shown to increase the detection of lymphovascular invasion relative to routine histology alone in various tumors.[129]

Recurrent lesions have been shown to have a greater propensity to metastasize relative to primary lesions of similar locations, with rates ranging from 15%-45%.[130,131] In

our study, recurrence showed a 6.3-fold increased chance of nodal metastasis in a multivariate analysis. The recurrent lesions with lymph node metastasis included in this study were reviewed for prior treatment with wide local excision (WLE) being the most common (6 with adjuvant radiation therapy), followed by MMS. Recurrent lesions with nodal metastasis were significantly more likely to have been treated with WLE than with MMS (p=0.03) initially. MMS is often the treatment of choice for SCC in critical locations with aggressive histology.[132] The cure rates for SCC treated with MMS are superior to standard excision, with local recurrence rates for high-risk lesions reported as low as 1.2%.[132] A recent study by Marazzo *et al*. (2019) examined outcomes for patients with high-risk SCC treated by MMS, and clinical and tumor characteristics that predict poor outcomes for this population.[133] Predictors of poor outcomes in patients with high-risk SCC treated with MMS were deep invasion beyond the subcutaneous fat and poor differentiation.[133] For T2b (BWH) patients treated with MMS, the local average recurrence rate was 7.8%. However, T2b patients treated with WLE with or without radiation had a local recurrence rate of 17.2%.[133] The overall rate of nodal metastasis reported in the Marazzo *et al*. study utilizing MMS is 4.8%, which is the lowest reported thus far for high-risk SCC using BWH staging.[133] The potential of MMS, as the primary surgical modality, to reduce the risk of nodal metastasis is promising and worth further investigation. Nonetheless, our data suggest that when recurrence is present, particularly in high-stage lesions, nodal evaluation may be warranted. Despite the significant association of lymphovascular invasion and recurrence in nodal metastasis of cutaneous SCC, neither of these features are utilized in current staging systems. When creating the BWH T staging system, recurrent lesions were excluded from the study and thus not considered as a risk factor for staging. This is traditional in oncologic staging which has been used almost exclusively for all primary tumors. However, it may be

useful to account for recurrence as a high-risk feature that was shown to have an increased odds (6.3) of nodal metastasis in our study.

In addition to tumor-specific factors, this study evaluated some patient-specific factors. Specifically, we found that a diagnosis of hypothyroidism conferred an increased risk of lymph node metastasis (OR 2.7, 95% CI 1.04-7.0). A retrospective study performed by Ahadiat *et al*. (2018) was the first to report that hypothyroidism is present in a higher percent of SCC patients than the general population ($p<0.05$).[134] However, this is the first study to report hypothyroidism as a potential risk factor for nodal metastasis in SCC. Slominski *et al*. (2005) studied thyroid-stimulating hormone receptor (TSH-R) expression in skin specimens and found significant expression in keratinocytes, epidermal melanocytes, and melanoma cells.[135] Ellerhorst *et al*. (2006) reported that TSH-Rs on melanoma cells are functional and hypothesized that TSH is a growth factor for melanoma cells.[136] In SCC, hypothyroidism may act in a similar way by increasing TSH-R expression or the effect may be a surrogate marker of immune dysfunction. Future studies to investigate potential mechanisms of hypothyroidism and metastasis in SCC are indicated. In addition to hypothyroidism, another patient-specific risk factor for nodal metastasis may be immunosuppression. While immunosuppression has previously been identified as a risk factor in SCC, the data are mixed on whether immunosuppression is associated with an increased risk of metastasis.[137] One possible explanation for the lack of significance in our study is that our cohort had a very high proportion of immunosuppressed patients, likely due to the institutions where the study was conducted.

The current study is limited by data that is derived from a retrospective analysis at two large academic institutions with variable follow up times. In addition, the evaluation of recurrence is limited by possible inconsistencies in how providers define recurrence. These inconsistencies, however, are not limited to retrospective reviews, but

rather, they span the skin cancer literature and have led to the publication of specific guidelines for defining skin cancer recurrence prospectively.[138] Future studies should continue efforts to refine current staging systems and to accurately identify risk factors for lymph node spread.

This study identifies novel risk factors, some of which are not currently included in staging systems, and suggests that perineural invasion, lymphovascular invasion, size of 2 cm or greater, characterization of primary tumor as recurrent, and a diagnosis of hypothyroidism are associated with lymph node metastasis in SCC. The results of this study may be used to refine clinical management and to guide future staging systems for cutaneous SCC. Ultimately, these findings may lead to optimized management and surveillance strategies for a high-risk subset of individuals with these aggressive features.

Chapter 4: Analysis of genomic landscape of cutaneous squamous cell carcinoma with next-generation sequencing data

This subchapter is published in the Journal of Dermatological Science and included here with permissions:

Lobl MB, Clarey D, Higgins S, Sutton A, Hansen L, Wysong A. Targeted next-generation sequencing of matched localized and metastatic primary high-risk SCCs identifies driver and co-occurring mutations and novel therapeutic targets. J Dermatol Sci. 2020 Jul;99(1):30-43.[56]

4.1 Analysis of somatic mutations in primary localized SCCs and primary metastatic SCCs

Introduction

Cutaneous squamous cell carcinoma (SCC) is the second most common type of skin cancer with an estimated one million cases per year in the United States, resulting in up to 9,000 deaths annually.[116] While only 3-5% of SCCs metastasize, those that do are associated with significant morbidity and mortality due to the lack of standardized and effective treatment options.[139] SCC is particularly challenging to manage due to the difficulty in determining which tumors will recur and metastasize and which tumors will be cured with surgery alone.

In order to help risk-stratify patients, the Brigham Women's Hospital (BWH) staging system is commonly used by clinicians.[120] This staging system uses risk factors of tumor diameter ≥2 cm, poorly differentiated histology, perineural invasion ≥0.1 mm, or tumor invasion beyond fat (excluding bone invasion, which automatically means T3 stage).[120] Clinically, high-risk tumors are those of T2b stage or T3 stage (2-3 risk factors and 4 risk factors or bone invasion, respectively). Low-risk tumors are typically those of T1 or T2a stage (0 risk factors or 1 risk factor, respectively).[120] Patients with high-risk tumors are more likely to have poor outcomes (local recurrence, metastasis, disease-

specific death).[120] Further, patients with other medical comorbidities, especially immunosuppressed organ transplant recipients, are more prone to develop metastatic disease, with 5-8% of immunosuppressed patients developing a metastasis.[140] Despite clinical staging and integration of patient characteristics, it is often difficult to determine the risk of any given SCC for metastasis. The role of gene mutations in the management of high-risk SCC patients has yet to be fully explored. While many studies have been published seeking to identify gene mutations in SCC, there is significantly less knowledge of the mutations seen in high-risk and metastatic SCC. This paper aims to better characterize the mutational landscape of SCC by presenting novel findings from our targeted mutation panel in a matched cohort of localized and metastatic high-risk SCCs.

Results

Study Design

The full experimental materials and methods are described (Chapters 2.2 and 2.3). A cohort of 20 patients with high-risk SCC was developed from an academic medical center. Ten patients had high-risk SCC with localized disease after a minimum of two years follow-up; these patients were case-matched with 10 patients with high-risk SCC with confirmed lymph node metastasis. We performed case-matching using age (by decade), gender, and Brigham and Women's Hospital (BWH) tumor stage to control for other patient- and tumor-specific characteristics aside from metastasis. Primary tumor tissue was obtained from both groups.

Summary of mutations seen in primary localized and primary metastatic SCC

Somatic mutations were differentiated from germline polymorphisms using previously validated methods (see Methods). The localized group (n=10) had a total of

51 somatic mutations, or an average of 5.1 mutations per tumor that were included in the panel. The metastatic cohort had a total of 41 somatic mutations, or an average of 4.1 mutations per tumor that were included in the panel. The mutations in the localized cohort were missense (51.0%, 26/51), nonsense (19.6%, 10/51), silent (19.6%, 10/51), and insertions/deletions (9.8%, 5/51) (**Figure 7A**). The mutations in the metastatic cohort were missense (68.3%, 28/41), nonsense (12.2%, 5/41), silent (14.6%, 6/41), and insertions/deletions (4.9%, 2/41) (**Figure 7B**). None of these differences were statistically significant (chi-square variance of proportion test). When silent mutations were excluded, the localized and metastatic cohort had a median of 4 and 3 mutations per sample, respectively (**Figure 7C, D**). Both cohorts had a predominance of C>T mutations when compared to other base pair changes. Transversion mutations accounted for 24.5% of mutations in the localized group and 28.5% of mutations in the metastatic group (**Figure 7E, F**). Of the total mutations, the localized cohort had 35.3% (18/51) pathogenic mutations and the metastatic cohort had 41.5% (17/41) pathogenic mutations as confirmed in COSMIC or ClinVar.[100,101] Of the confirmed pathogenic variants, no significant differences were seen in the prevalence of mutations between groups (chi-square variance of proportion test).

Figure 7: Summaries of Mutations in Localized and Metastatic SCC

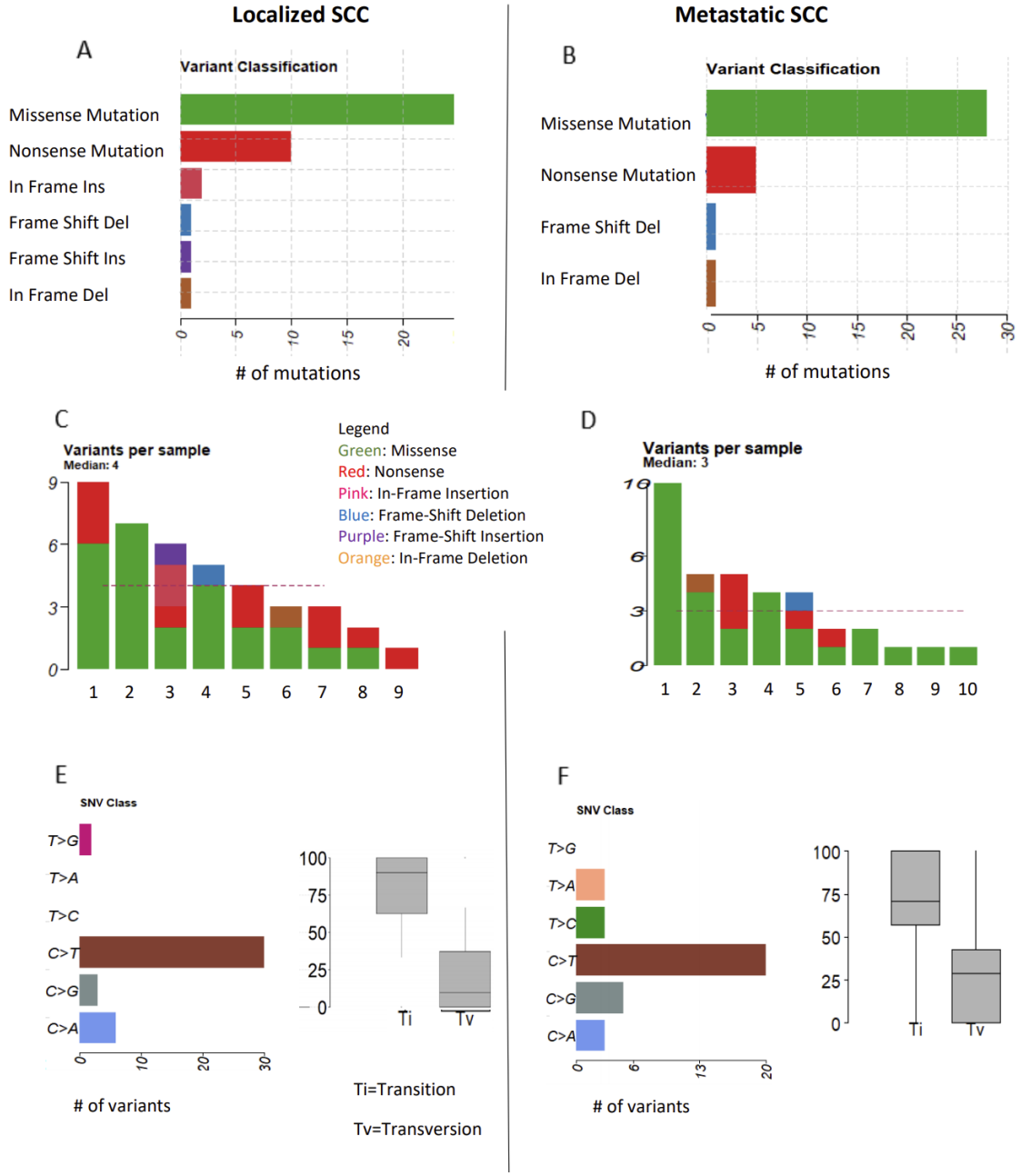


Figure 7: A and B-These plots illustrate variant classifications in localized and metastatic SCC. C and D-These plots illustrate the variants per sample as well as the mean number of variants per sample for localized and metastatic SCC. E and F-These graphs

illustrate the base changes that occurred, as well as the distribution of transition and transversion mutations in localized and metastatic SCC.

The summary of mutations along with the translational consequences can be appreciated alongside the clinical characteristics of each individual patient (**Figure 8**). While 19.6% and 14.6% of mutations were synonymous in the localized and metastatic cohorts, respectively, different synonymous and nonsynonymous mutations in *KDR* and *PTEN* were present in the same protein domains (**Figure 9**). These findings suggest that synonymous mutations may have greater functional consequences than previously thought and, in some cases, may result in similar effects as missense mutations.

Figure 8: A summary of gene mutations and clinical characteristics in our cohort

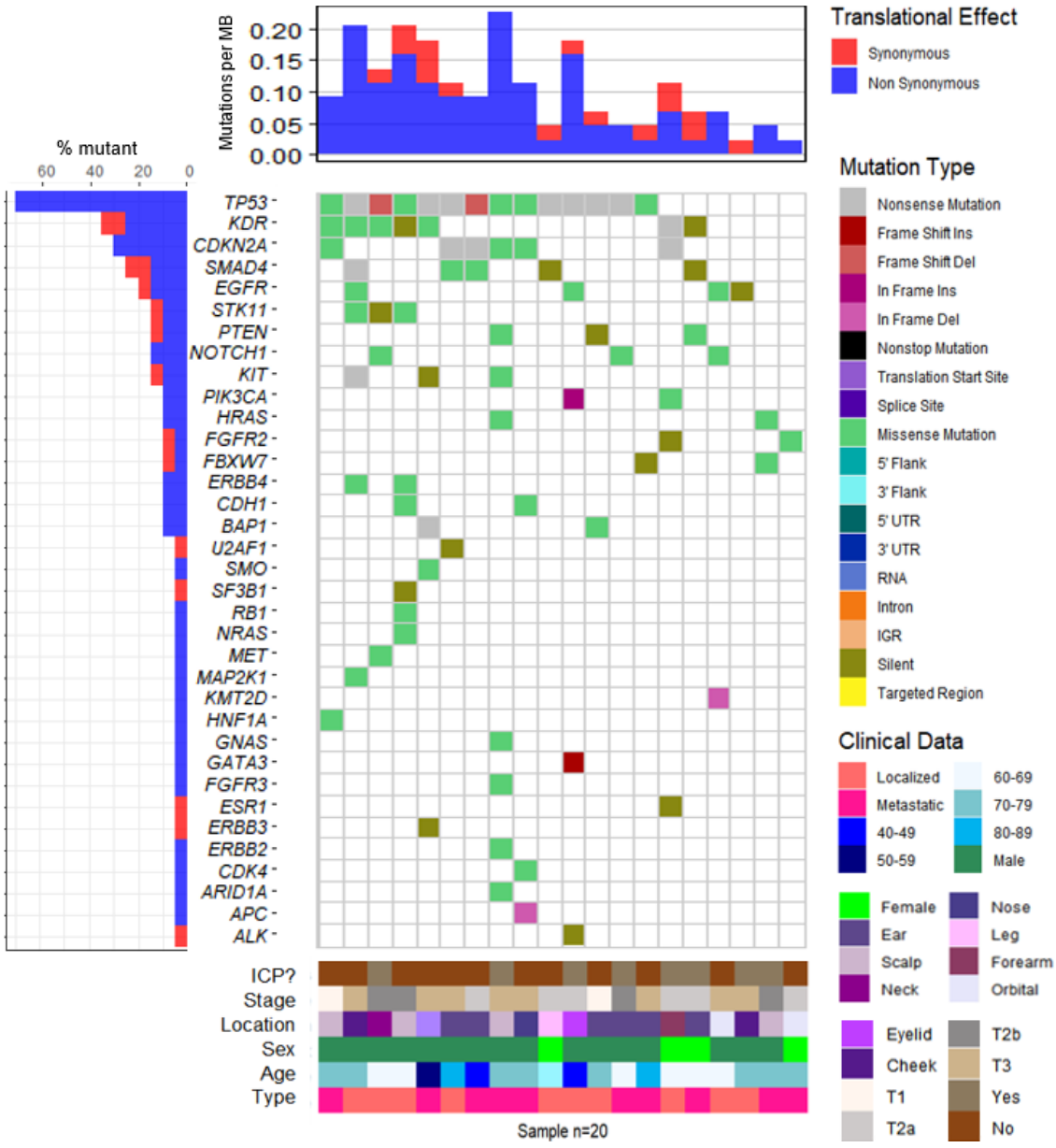
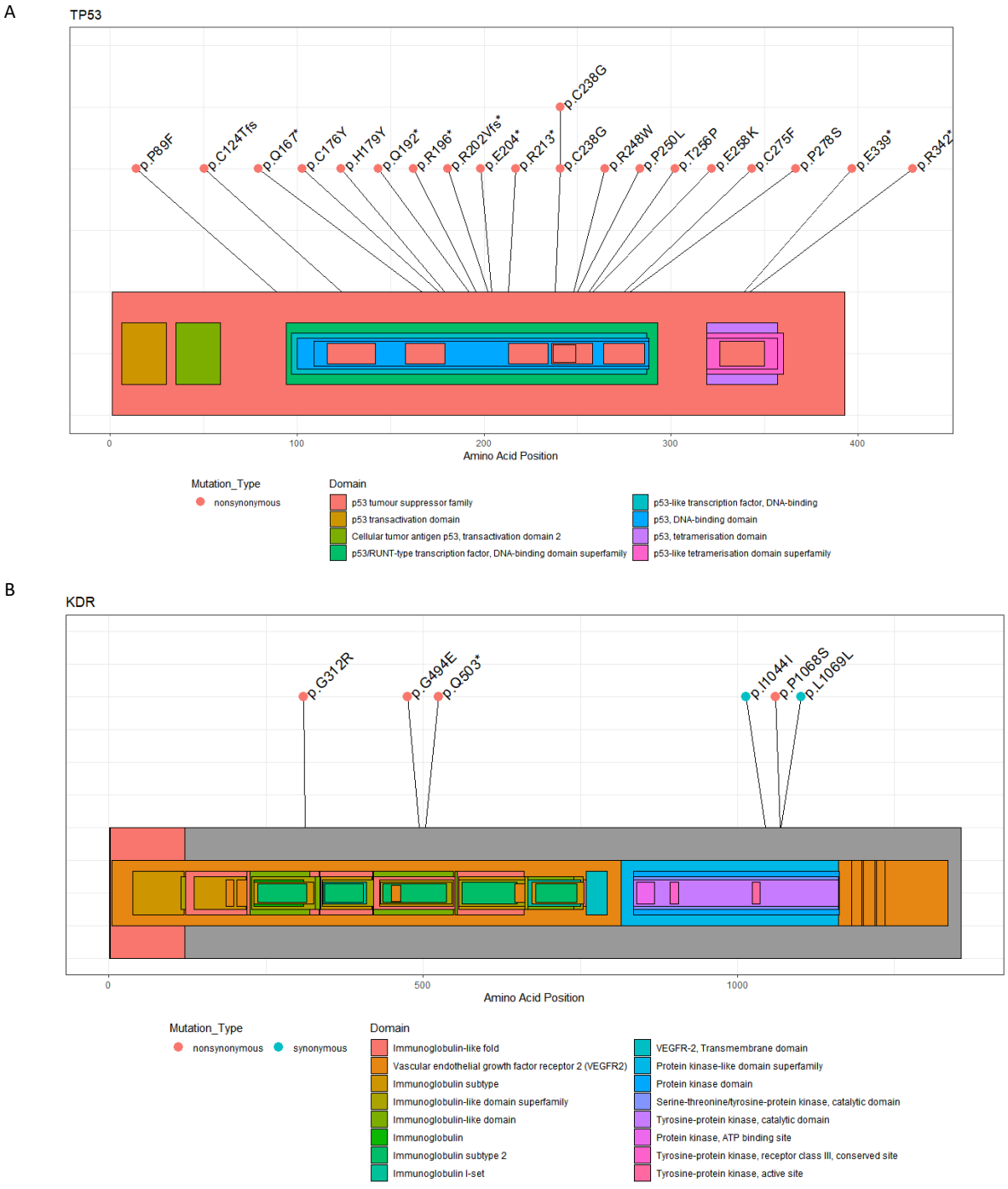


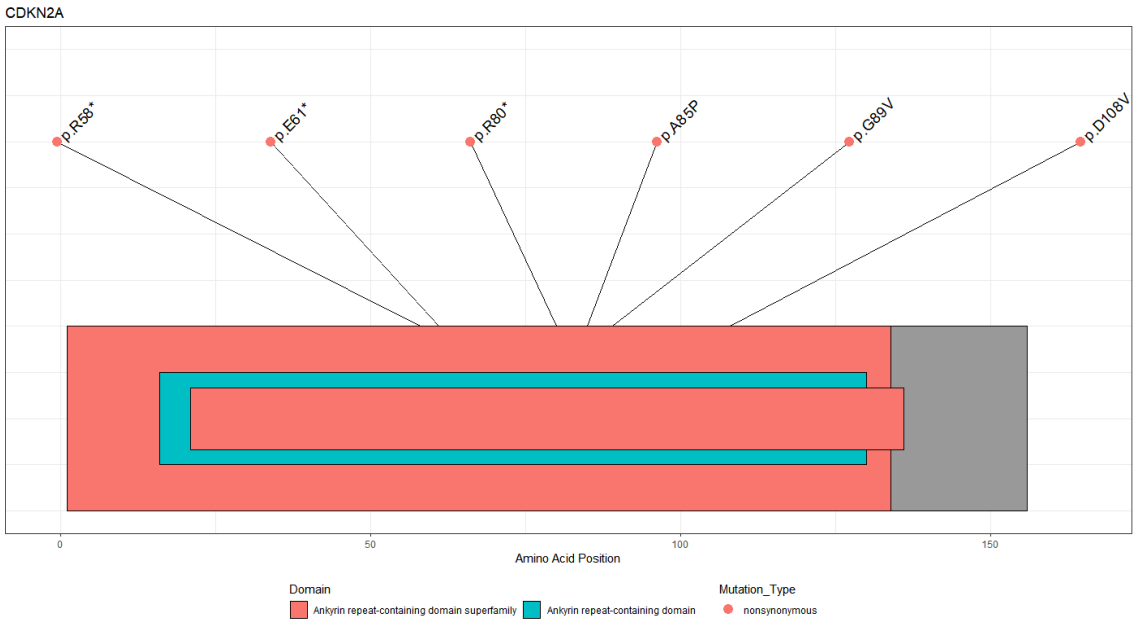
Figure 8 illustrates the clinical characteristics for each patient aligned with the mutational profile. ICP=immunocompromised patient

Stage=BWH Stage

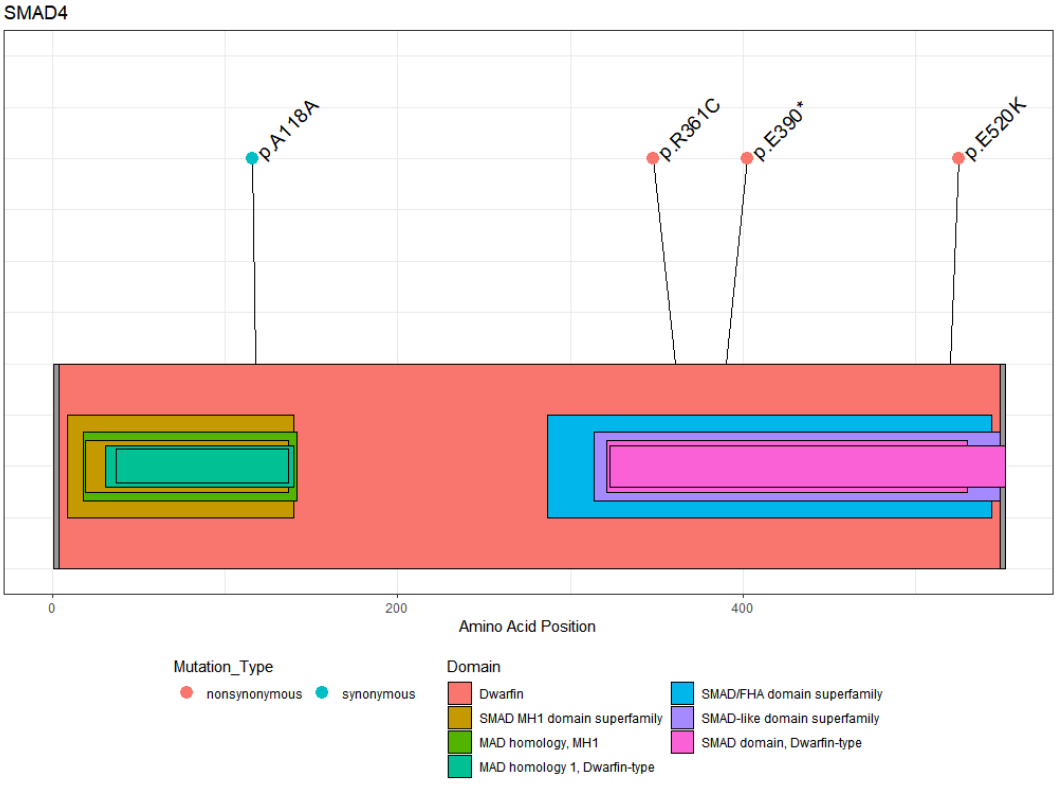
Figure 9: Affected domains of synonymous and nonsynonymous mutations.



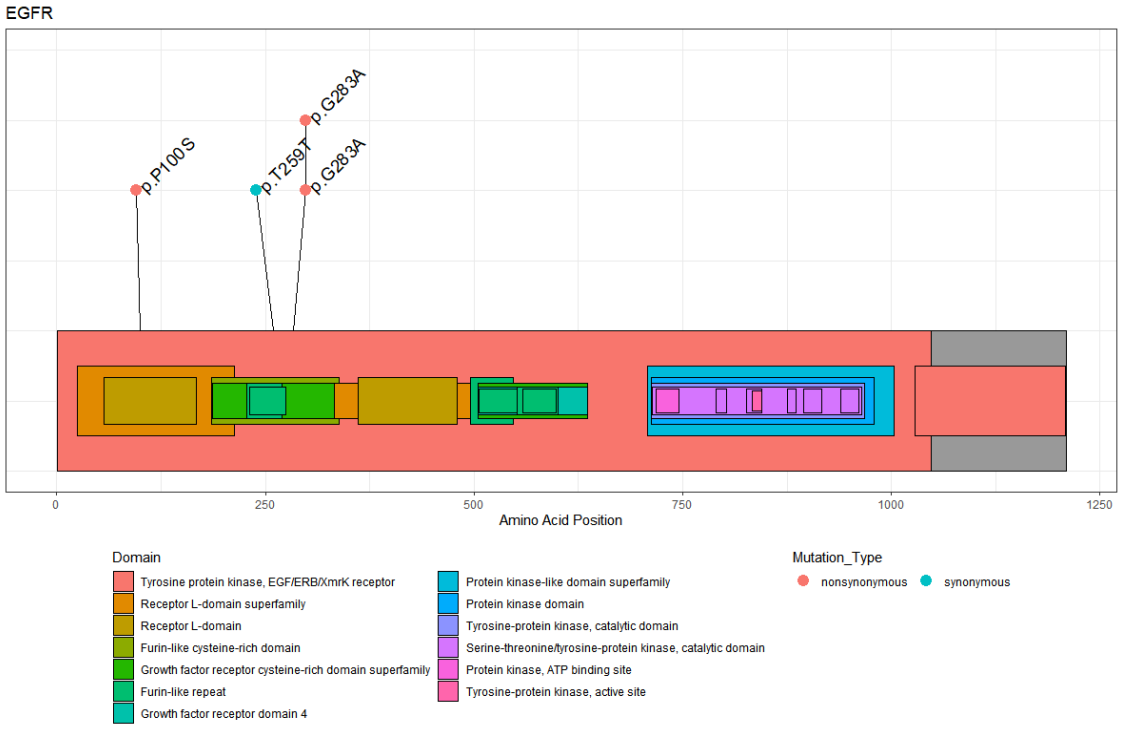
C


CDKN2A
p.R58*
p.E61*
p.R80*
p.A85P
p.G89V
p.D108V
0
50
100
150
Amino Acid Position
Domain
Ankyrin repeat-containing domain superfamily
Ankyrin repeat-containing domain
Mutation_Type
nonsynonymous

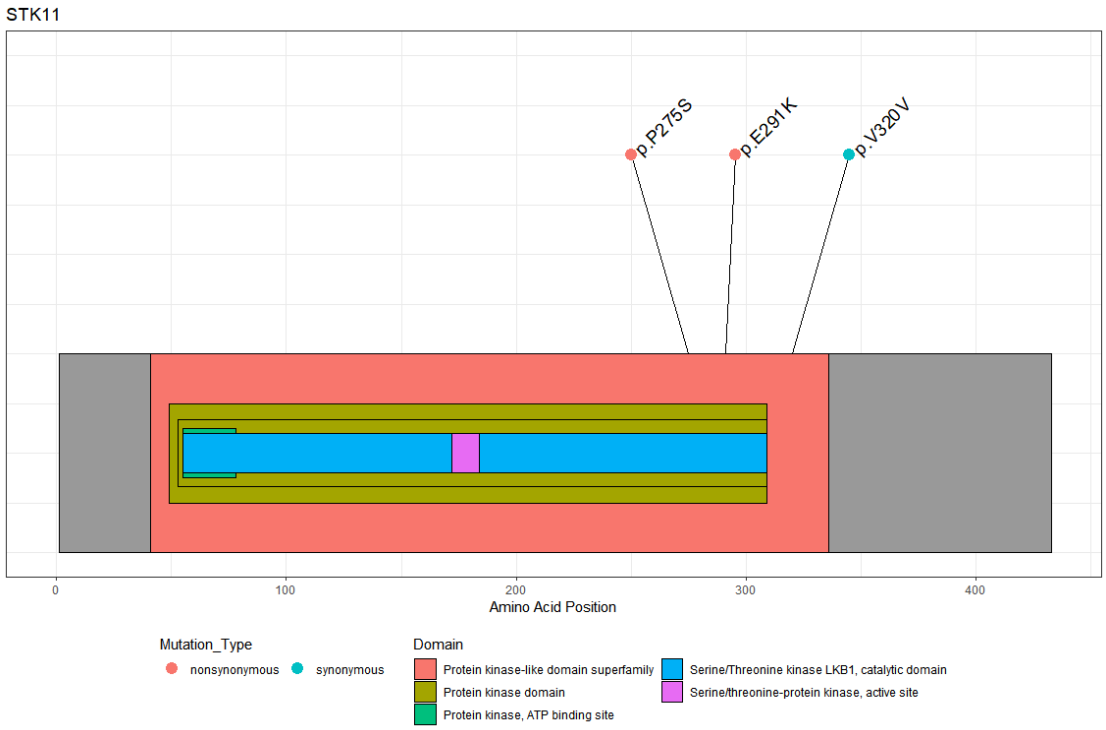
D


SMAD4
p.A118A
p.R361C
p.E390*
p.E520K
0
200
400
Amino Acid Position
Mutation_Type
nonsynonymous
synonymous
Domain
Dwarfin
SMAD MH1 domain superfamily
MAD homology, MH1
MAD homology 1, Dwarfin-type
SMAD/FHA domain superfamily
SMAD-like domain superfamily
SMAD domain, Dwarfin-type

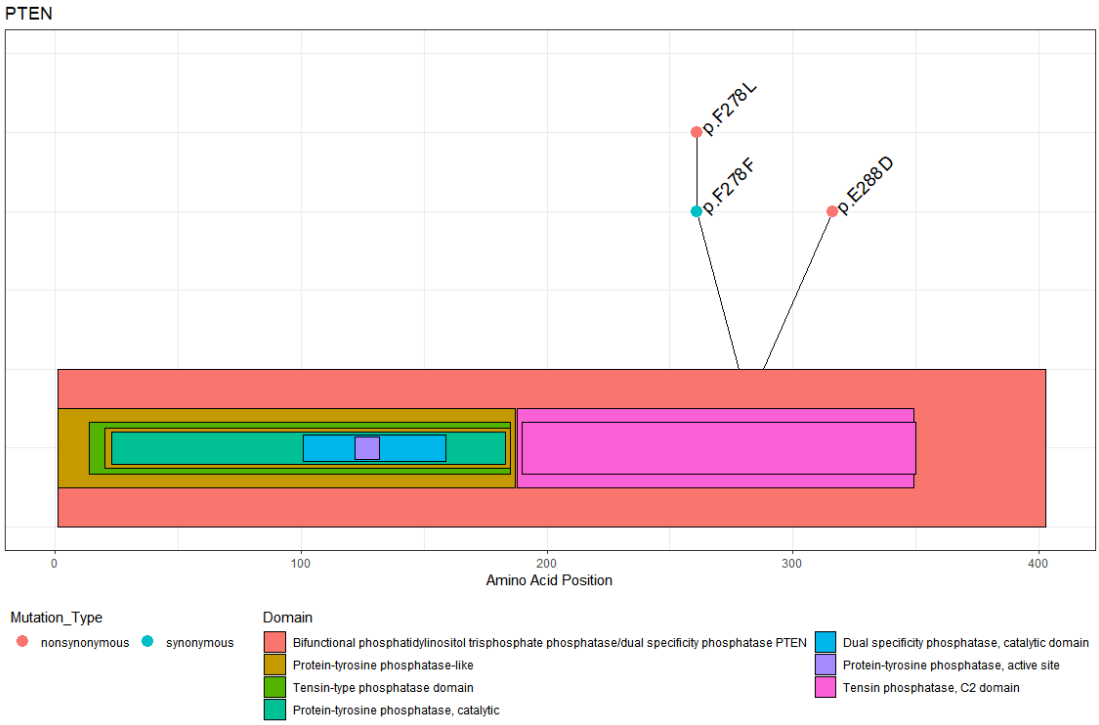
E


EGFR
p.P100S
p.T259T
p.G283A
p.G283A
0
250
500
750
1000
1250
Amino Acid Position
Domain
Tyrosine protein kinase, EGF/ERB/XmrK receptor
Receptor L-domain superfamily
Receptor L-domain
Furin-like cysteine-rich domain
Growth factor receptor cysteine-rich domain superfamily
Furin-like repeat
Growth factor receptor domain 4
Protein kinase-like domain superfamily
Protein kinase domain
Tyrosine-protein kinase, catalytic domain
Serine-threonine/tyrosine-protein kinase, catalytic domain
Protein kinase, ATP binding site
Tyrosine-protein kinase, active site
Mutation_Type
nonsynonymous
synonymous

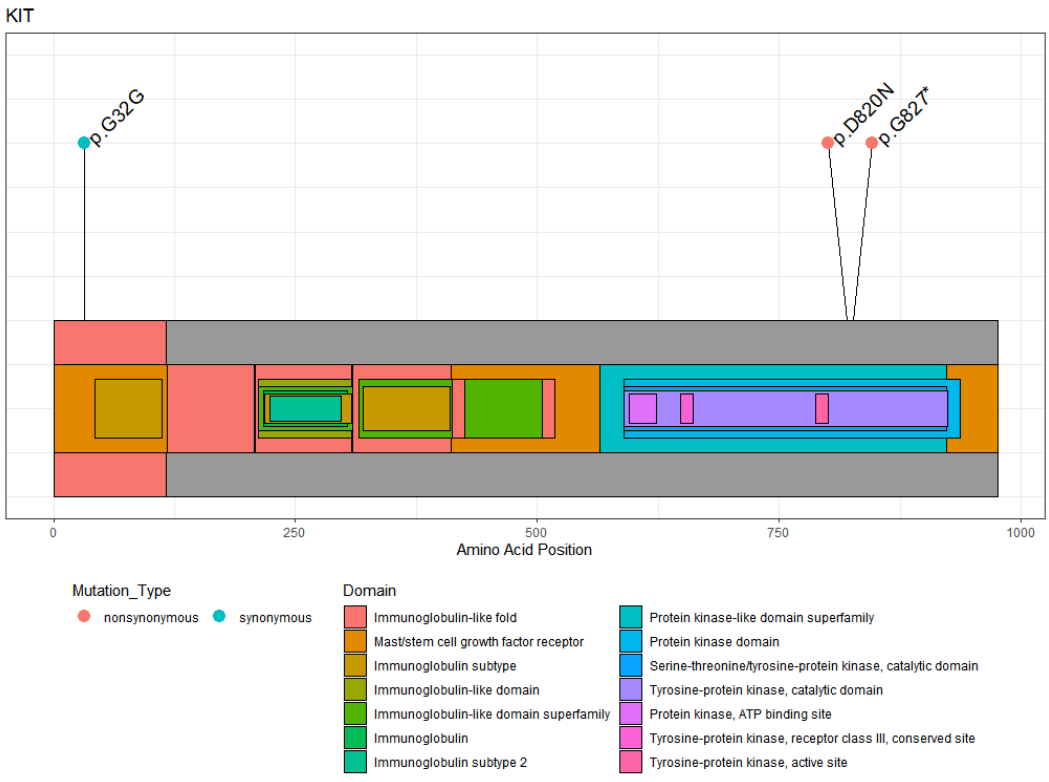
F


STK11
p.P275S
p.E291K
p.V320V
0
100
200
300
400
Amino Acid Position
Mutation_Type
nonsynonymous
synonymous
Domain
Protein kinase-like domain superfamily
Protein kinase domain
Protein kinase, ATP binding site
Serine/Threonine kinase LKB1, catalytic domain
Serine/threonine-protein kinase, active site

G


PTEN
p.F278L
p.F278F
p.E288D
0
100
200
300
400
Amino Acid Position
Mutation_Type
nonsynonymous
synonymous
Domain
Bifunctional phosphatidylinositol trisphosphate phosphatase/dual specificity phosphatase PTEN
Protein-tyrosine phosphatase-like
Tensin-type phosphatase domain
Protein-tyrosine phosphatase, catalytic
Dual specificity phosphatase, catalytic domain
Protein-tyrosine phosphatase, active site
Tensin phosphatase, C2 domain

H


KIT
p.G32G
p.D820N
p.G827*
0
250
500
750
1000
Amino Acid Position
Mutation_Type
nonsynonymous
synonymous
Domain
Immunoglobulin-like fold
Mast/stem cell growth factor receptor
Immunoglobulin subtype
Immunoglobulin-like domain
Immunoglobulin-like domain superfamily
Immunoglobulin
Immunoglobulin subtype 2
Protein kinase-like domain superfamily
Protein kinase domain
Serine-threonine/tyrosine-protein kinase, catalytic domain
Tyrosine-protein kinase, catalytic domain
Protein kinase, ATP binding site
Tyrosine-protein kinase, receptor class III, conserved site
Tyrosine-protein kinase, active site

I

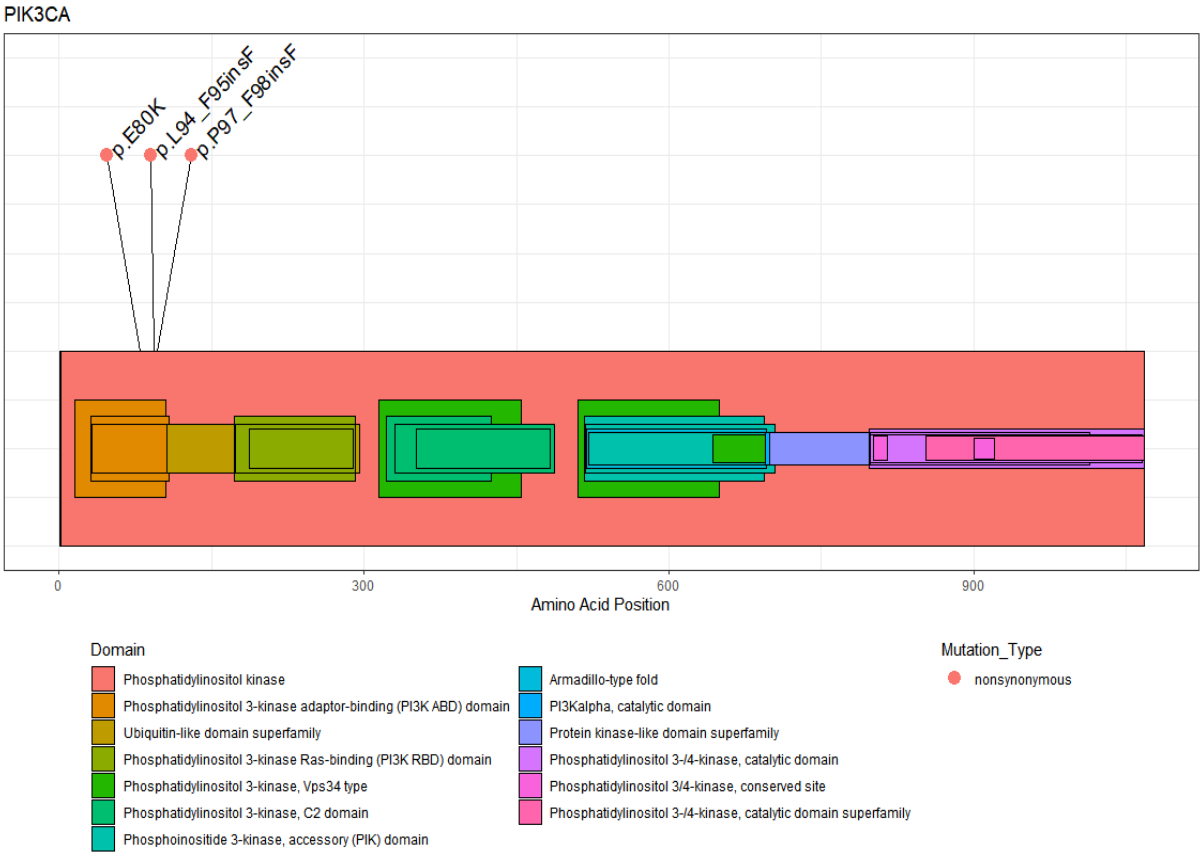


Figure 9 illustrates affected domains of synonymous and nonsynonymous mutations for genes with 3+ mutations and available transcripts in ENSEMBL. Two genes (*KDR*, *PTEN*) demonstrate the effects of synonymous and nonsynonymous mutations in the same protein domains.

The prevalence of specific somatic mutations was evaluated. The most frequently mutated genes in localized and metastatic SCC, respectively, were *TP53* (70% vs 70%), *CDKN2A* (20% vs 40%), *KDR* (40% vs 30%), *SMAD4* (30% vs 20%), *NOTCH1* (20% vs 10%), *PTEN* (10% vs 20%), and *KIT* (10% vs 20%) (**Figure 10**). In the localized group, *EGFR* mutations were seen in 40% of patients, *STK11* mutations were seen in 30% of patients, and *ERBB4* and *PIK3CA* mutations were seen in 20% of patients (**Figure 10**). In metastatic SCC, *HRAS* mutations were seen in 20% of patients (**Figure 10**). No statistically significant differences in individual gene mutation frequencies were found between groups (two-tailed student's t-test).

Figure 10: A direct comparison of the percentage of specific gene mutations in localized versus metastatic SCC

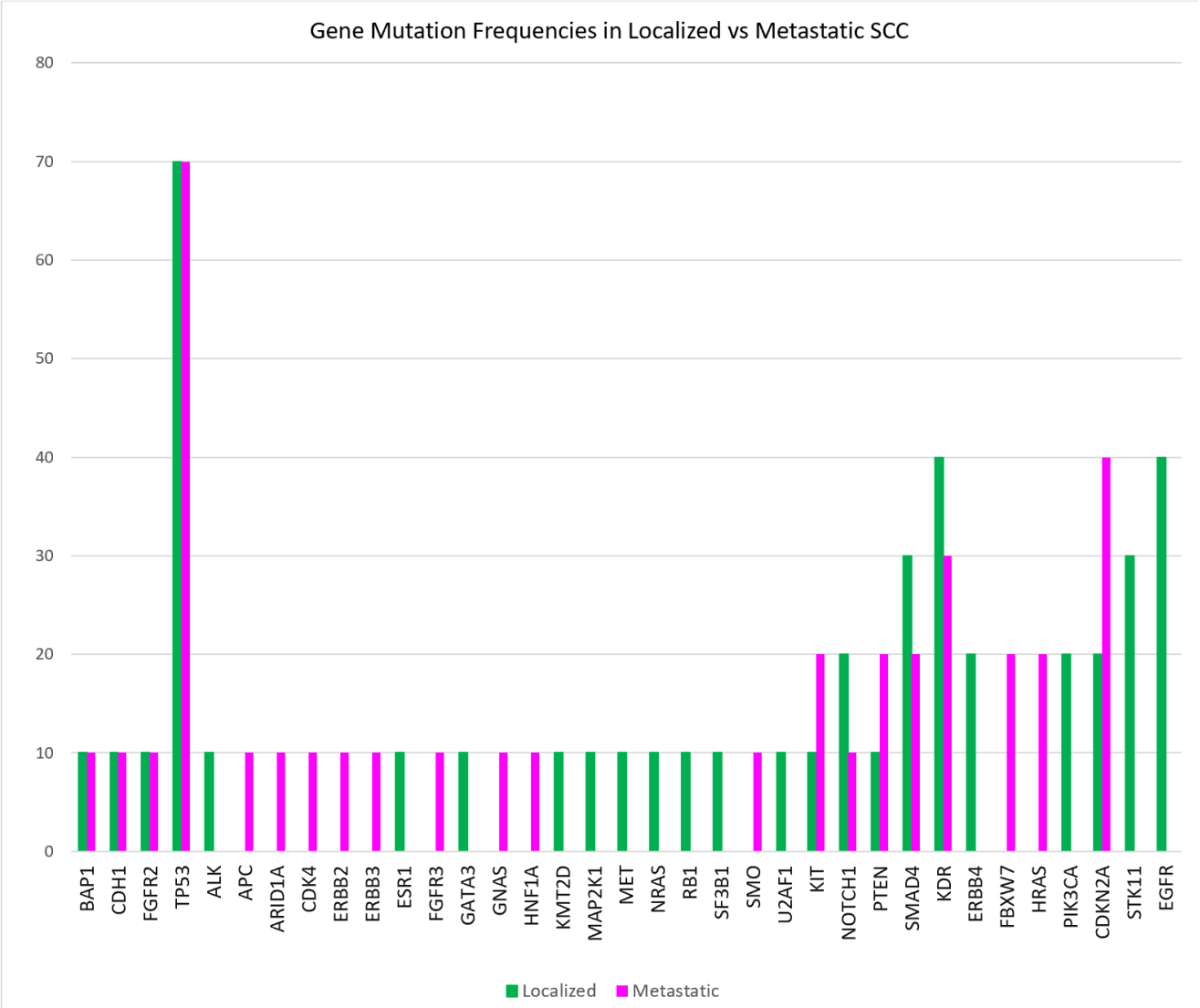


Figure 10 illustrates the frequency of each mutation in the localized (green) and metastatic (pink) samples.

The average mutant allele frequency (MAF) is higher in metastatic SCC than localized SCC

The MAFs between genes mutated in the localized and metastatic groups were explored after controlling for the percent tumor content in each sample. Overall, the average MAF was significantly higher in metastatic SCC than in localized SCC (45.6% versus 35%, respectively; p=0.04, two-tailed student's t-test). The study was not powered to evaluate differences in individual genes, although trends were observed (**Figure 11**).

Figure 11: The distribution of normalized (by percent tumor content) mutated allele frequencies in the most highly mutated genes in localized and metastatic SCC.

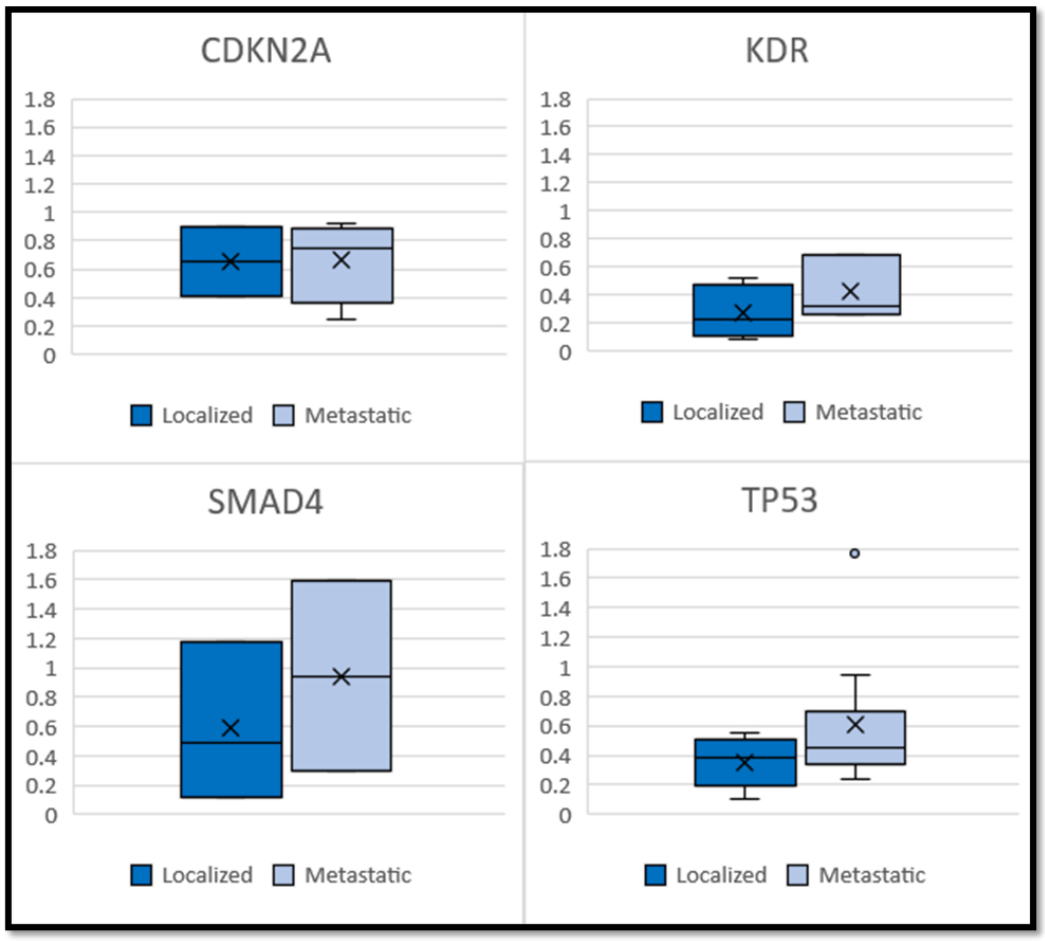


Figure 11 illustrates the MAFs for localized versus metastatic samples for the most frequently mutated genes in our cohort (CDKN2A, KDR, SMAD4, TP53). The MAFs were adjusted for percent tumor content.

Driver mutations are identified in metastatic and localized SCC

Driver genes in localized and metastatic SCC were identified using the algorithm OncodriveCLUST.[105] This algorithm works by analyzing the gene mutations in the context of spatial clustering. In localized high-risk SCC, the primary oncogenic cluster identified was *EGFR*. In metastatic SCC, the oncogenic cluster identified was *CDH1*, a gene responsible for making epithelial cadherin (E-cadherin) (**Figure 12**).

Figure 12: Altered oncogenic pathways identified using the Oncodrive algorithm in (1) localized SCC and (2) metastatic SCC.

EGFR was significant in localized SCC and *CDH1* was significant in metastatic SCC.

FDR=false discovery rate.

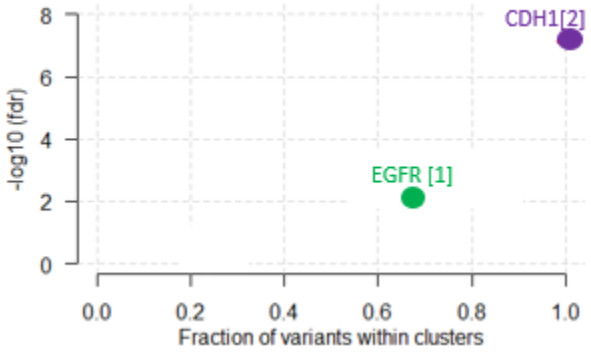


Figure 12 illustrates the driver mutations found using the OncodriveCLUST package

(Localized SCC: EGFR; Metastatic SCC, CDH1). FDR= false discovery rate.

Mutations in *ERBB4* and *STK11* co-occur in localized SCC

Mutually exclusive genes and co-occurring mutations were plotted (**Figure 13**). In three samples with localized SCC, *ERBB4* and *STK11* co-occurred, rendering this combination significant (pair-wise Fisher's exact test $p<0.05$).

Figure 13: Co-occurring mutations

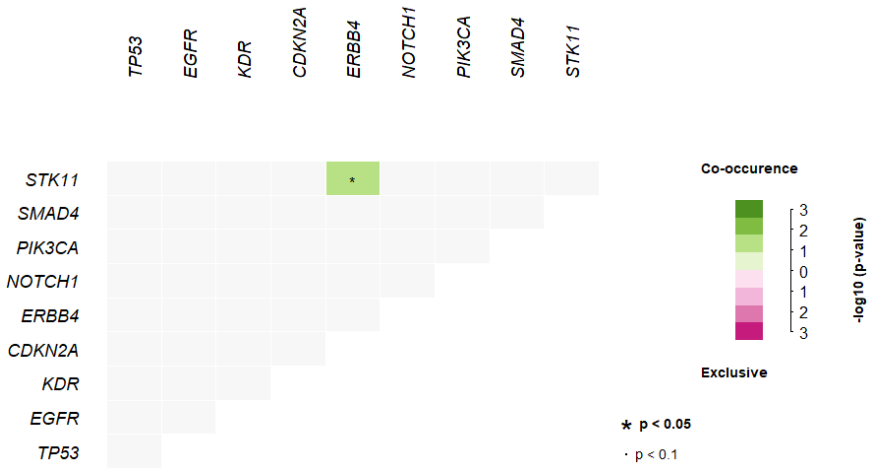


Figure 13 illustrates co-occurring mutations. It was found that ERBB4 and STK11 mutations had a significant pattern of co-mutation ($p<0.05$).

Altered pathways were identified in localized and metastatic SCC

Genes mutated in both groups were categorized into pathways, which are illustrated by the number of mutations and pathway size (**Figure 14A, Figure 14B**). In both groups, numerous mutations occurred in the *RTK*/*RAS* pathway (localized, 6 mutations; metastatic, 5 mutations). Both groups had mutations in the *TP53*, *TGF-β*, *NOTCH1*, *PI3K*, and cell cycle pathways. The metastatic groups had an additional enrichment in the Wnt pathway.

Figure 14: Altered pathways in both localized and metastatic SCC.

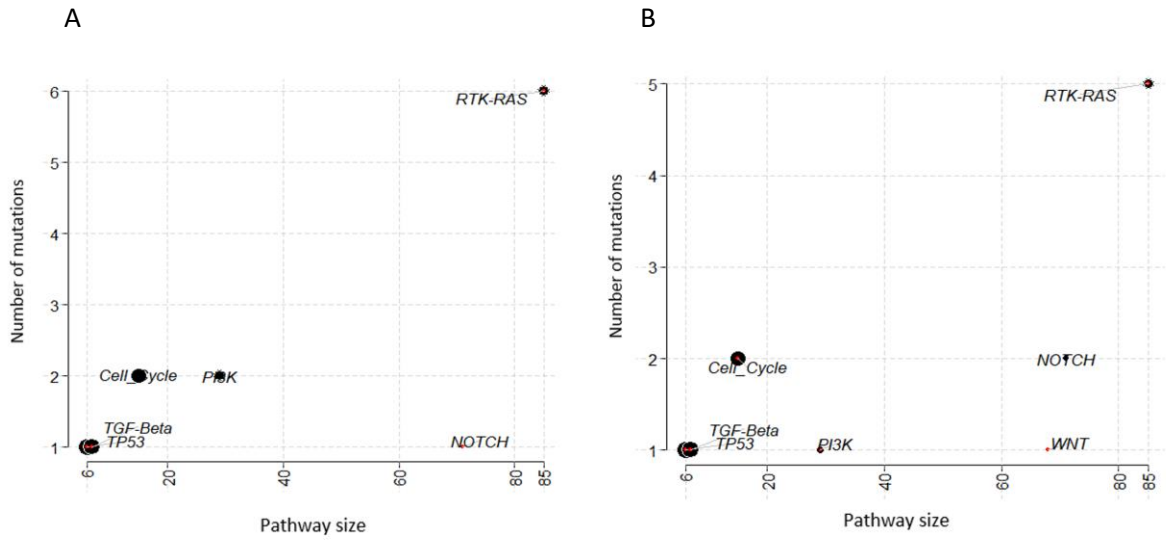


Figure 14 demonstrates that the TP53, TGF-β, NOTCH1, PI3K, and cell cycle pathways are altered in both localized and metastatic SCC. The Wnt pathway is also altered in metastatic SCC.

Mutational signatures differ between metastatic and localized SCC

There have been 30+ cancer-associated mutational signatures reported to date.[106] We sought to determine which of these signatures aligned most closely with high-risk SCC, and which signatures, if any, distinguished localized from metastatic SCC. The top five signatures with the highest cosine similarity to both cohorts are reported (**Figure 15A**-localized, **Figure 15B**-metastatic). Mutations seen in localized SCC corresponded best with Signature 23 (unknown aetiology), Signature 7 (UV exposure), Signature 3 (defects in DNA Double-Stranded (DS) repair by homologous recombination (HR), Signature 4 (exposure to tobacco mutagens), and Signature 1 (spontaneous deamination of 5-methylcytosine). Mutations seen in metastatic SCC best corresponded with Signature 5 (unknown aetiology), Signature 11 (exposure to alkylating agents), Signature 3 (defects in DNA DS repair by HR), and Signature 30 (unknown aetiology). Only 4 mutational signatures were returned for metastatic SCC.

Figure 15: Mutational Signatures in both localized (A) and metastatic (B) SCC.

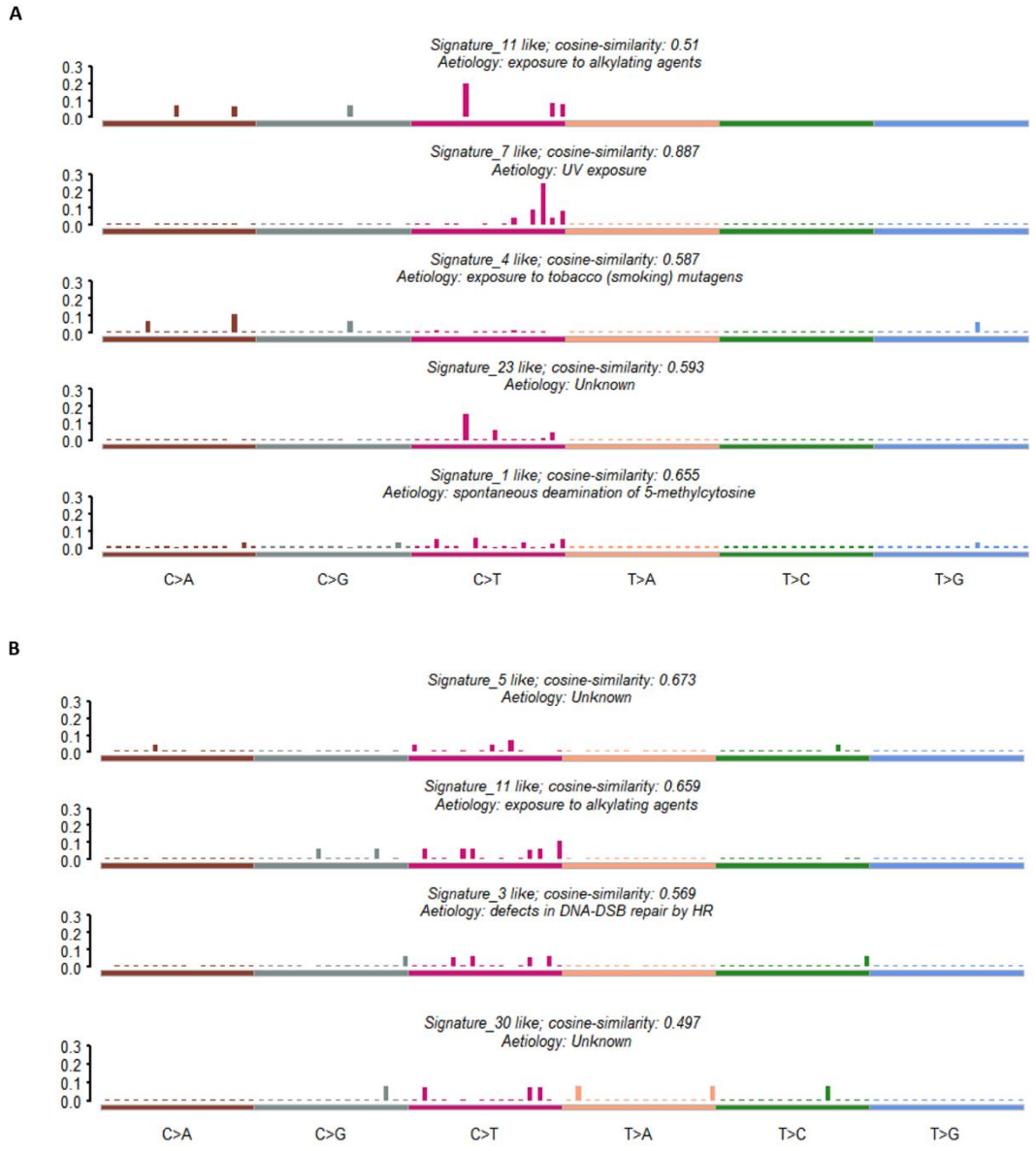


Figure 15 illustrates the mutational signatures that are the best fit for (A) localized SCC and (B) metastatic SCC. For localized SCC, signature 7 is the best fit; for metastatic SCC, signature 5 is the best fit.

Discussion and Conclusions

SCC carries one of the highest tumor mutation burdens of all known cancers. Mutations in *TP53, and NOTCH,* many of which are UV-induced, are well-known driver mutations in SCC. [55,64] Yilmaz *et al*. (2017) performed whole-exome and targeted sequencing of metastatic and localized SCC and found higher *TP53* mutation frequencies in metastatic disease compared to localized disease (85% vs 54%, respectively; $p<0.0001$).[141] Another study by Li *et al*. (2015) performed targeted sequencing of lymph node metastases in SCC and found that, in addition to *TP53*, *CDKN2A*, and *NOTCH1* mutations, *MLL2, RIPK2, ARID2, ATM, ARID5B, CARD11, and SMARCA4* were mutated in 40% or more of metastatic nodal samples.[66] While some mutations detected in this study have been previously seen in SCC, other skin cancers, or the general oncology literature, many mutations observed have not yet been reported. This study aimed to contribute to the current literature, examine mutations specific to high-risk and metastatic SCC, and to gain insight into the mechanism of SCC development.

The most frequently mutated genes in our cohort of high-risk SCCs were *TP53*, *CDKN2A*, *KDR*, *SMAD4*, *NOTCH1*, and *KIT*. While our study was not powered to find statistically significant differences in individual mutations between our localized and metastatic cohorts, our summary data present the commonly mutated genes in high-risk SCC above, as well as mutations that were seen in only localized SCC (*EGFR*, *PIK3CA*, *STK11*, *ERBB4*) and only metastatic SCC (*HRAS*, *PTEN*). In addition, we found that metastatic SCCs had a significantly higher overall MAF than localized SCC. This may suggest that metastatic tumors have more clonal mutations whereas localized tumors have more polyclonal or subclonal mutations, implying greater tumor heterogeneity in localized samples compared to metastatic samples. This should be confirmed in studies with larger sample sizes. A study of MAFs in primary cutaneous melanomas and

corresponding visceral metastases detected an increase in MAFs in metastases compared to primary tumors; this was only true in *BRAF*-mutant tumors.[142] Further, a study of MAFs in primary and metastatic colorectal cancer also found increased MAFs in *BRAF*, *PIK3CA*, and *TP53*-mutant metastatic tumors compared to primary tumors.[143]

Utilizing pathway analyses, both the localized and metastatic groups had mutations in the *RAS*, *TP53*, *TGF-β*, *NOTCH1*, *PI3K*, and cell cycle pathways. The metastatic groups had an additional enrichment in the Wnt pathway. As such, the Wnt pathway may be a worthwhile target to investigate in treating metastatic SCC. A recent review of the literature suggests that Wnt signaling may support tumor metastasis.[144] Gao *et al*. performed a gene expression profiling study of SCC and found that Wnt pathway regulator *HOXB7* had increased expression.[145] Subsequent knockdown of *HOXB7* reduced protein levels of Wnt/*β* -catenin pathway genes and decreased SCC cell viability.[145] Further, *HOXB7* knockdown and Wnt pathway inhibitor IWR-1 suppressed cell invasion and migration, decreased cell viability, and decreased cell cycle progression.[145] The finding that *HOXB7* may promote SCC progression through the Wnt/*β*-catenin pathway may be clinically relevant, as there are several current and developing inhibitors of this pathway.[146]

Utilizing spatial plotting analyses, *ERBB4* and *STK11* were found to be significantly co-occurring in localized high-risk SCC. To our knowledge, this is the first time this association has been reported. The oncogene *ERBB4* is a member of the *ErbB* receptor tyrosine kinase family, which exerts effects including cellular proliferation, differentiation, and motility through homo- or hetero-dimerization.[147] The tumor suppressor gene *STK11* (also called *LKB1*) is known to impact carcinogenesis through its role in regulation of the tumor microenvironment, including T-reg functions.[148] Further, *STK11* suppresses angiogenesis and regulates oxidative stress.[149] *STK11* mutations are known to co-occur with *KRAS* in non-small cell lung cancer (NSCLC) in up to 29% of

cases.[150] Interestingly, in NSCLC, *STK11* mutations are inversely associated with *EGFR* mutations, another member of the *ErbB* receptor family.[151] Mutations in *TP53* and *STK11* have been reported to act synergistically and have also been reported in NSCLC.[152] While mutations of *STK11* appear to co-occur with other oncogenes and tumor suppressors, further work into potential mechanism of interaction with *ERBB4* would be useful.

The oncogene epidermal growth factor receptor (*EGFR*) induces cellular proliferation and differentiation upon ligand binding and is well-known to be implicated in many human cancers.[153] In our localized SCC cohort, 30% of cases harbored a non-synonymous *EGFR* mutation and *EGFR* was seen to be the primary driver mutation in spatial clustering analysis. As *EGFR* is frequently mutated in SCC, the *EGFR* inhibitor cetuximab is a current treatment option for patients with advanced or metastatic SCC. While some patients have a favorable response to treatment with cetuximab, studies have shown great variability with 0-67% of patients with inoperable tumors responding to this drug.[154,155] The *EGFR* inhibitor dacomitinib was approved by the FDA in 2018 for metastatic non-small cell lung cancer (NSCLC). A preclinical study in head and neck SCC by Ather *et al*. (2013) demonstrated that dacomitinib inhibits cell growth in the presence of an *EGFR* ligand; this was not observed with cetuximab.[156] As the presence of an *EGFR* ligand is associated with poor outcomes, dacomitinib may be an improved treatment option for some patients.[157] There is a current phase II clinical trial investigating dacomitinib for SCC.[158] If successful, dacomitinib may eventually present as a viable alternative to cetuximab for patients with *EGFR*-mutant high-risk SCC.

Utilizing the OncodriveCLUST algorithm[105], we identified *CDH1* as a driver mutation in our metastatic SCC cohort. *CDH1* is a gene responsible for production of E-cadherin, which is a key component of adherens junctions. E-cadherin functions to maintain cell-to-cell adhesion and epithelial cell phenotypes.[159] E-cadherin has been

investigated in many cancers, including SCC. In a study of colorectal cancer, *CDH1* expression was assessed by immunohistochemistry.[160] Loss of *CDH1* expression was positively associated with infiltrative growth (OR=2.02, p=0.01) and nodal metastasis (OR=1.73, p=0.001), however, it was not associated with distant metastasis or the patient prognosis.[160] Additional studies have investigated E-cadherin expression in primary metastatic versus primary localized SCC. Hesse *et al*. found that membranous E-cadherin expression was downregulated in primary metastatic SCC compared to primary localized SCC (p=0.031).[161] E-cadherin has been difficult to target therapeutically. A review was conducted by Song *et al*. that details compounds, some of which are natural compounds, and predominately preclinical studies that investigated the use of these compounds in various cancers.[162] While a promising avenue for further investigation, there are not any effective, currently available E-cadherin-targeted therapeutics on the market.

Over 30 cancer mutational signatures have been described to date, each with a unique aetiology and/or pattern.[106] UV-induced C>T mutations predominate Signature 7, which is seen in melanoma, head and neck squamous cell carcinoma (HNSCC), and oral gingivo-buccal squamous cell carcinoma [COSMIC], as well as our localized SCC group.[101] In a study that performed whole-exome sequencing on 40 SCC samples, this signature was found in 83% of samples (33/40).[163] Signature 1, also seen in our localized cohort, is also composed of many C>T substitutions at CpG dinucleotides (which may be caused by spontaneous deamination of 5-methylcytosine) and is especially prevalent in cancers derived from epithelia with a high turnover rate.[164] Signature 11 (exposure to alkylating agents) was found in both groups, and is typically associated with a history of treatment with chemotherapy. Signature 4 (exposure to tobacco) was enriched in our localized SCC cohort. This supports the somewhat contested association of smoking and SCC risk.[123,165] A better understanding of distinct

mutational signatures in SCC and therapeutic susceptibility may eventually inform treatment options.

Genes that were mutated in 10% or more of high-risk SCC samples and have a pharmacologic therapy available or in development are displayed (**Table 7)**. Mutations in genes in metastatic SCC are of particular interest with respect to the development of systemic therapies (*CDKN2A*, *HRAS*, ErBb family, *KIT*, *KDR*, *NOTCH1*, *PTEN*, and *TP53*). The oncogenes *EGFR*, *ERBB4*, *HRAS*, *KIT*, and *PIK3CA* all have available inhibitor therapies that are in clinical trials or already FDA approved for SCC and other cancers (**Table 7)**. Loss-of-function tumor suppressor genes are more difficult to target, but in recent years many therapeutic regimens have been developed for these mutations as well (**Table 7**). *CDKN2A* was mutated in a high percentage of SCCs in our cohort (20-40%), therefore, therapeutics directly or indirectly targeting this mutation would be of interest. Currently, CDK inhibitors are being investigated in pre-clinical and clinical trials.[166] *EGFR* inhibitor cetuximab has been used in high-risk SCC with efficacy over standard chemotherapeutics, and panitumumab is in phase II clinical trials for SCC with evidence that it may be an improvement over the current standard cetuximab.[167,168] The *HRAS* inhibitor tipifarnib is in phase II clinical trials and studies suggest efficacy in HNSCC.[169] *NOTCH1* was mutated in 15% of our cohort and potential downstream pathway inhibition (e.g., PI3K/mTOR) is a strategy to treat tumors harboring a *NOTCH1* mutation. One example, bimiralisib, is currently in phase II clinical trials for breast cancer, HNSCC, and other cancers.[158] This drug may also be considered in SCC. *PTEN* mutations were seen in metastatic SCC and disproportionally in immunosuppressed patients. *AKT*, Hsp, and *PI3K* inhibitors are suggested to treat cancers with *PTEN* loss.[170] Compounds MK2206, AZD6482, and 17-AAG have all shown pre-clinical efficacy in other cancers.[171]

Overall, the chapter presents novel gene mutation data from 10 primary localized and 10 matched primary metastatic SCCs using our targeted 76 gene oncology panel. Due to the scarcity of metastatic SCC data in the literature, as well as the high morbidity and mortality associated with metastasis, our study adds to this critically important area. Future directions should include larger validation cohorts and consideration of whole-exome or whole-genome sequencing studies to identify additional potential mutations of importance in high-risk localized and metastatic SCC. In addition, larger scale analyses of groups of mutations are necessary to better understand the pathogenesis of metastasis in SCC. Further investigation of new therapies in SCC is likely to lead to improved systemic treatment options for advanced and metastatic SCC.

Table 7: Mutated genes that can be targeted clinically with currently available or developing therapeutics

Gene Mutation & Percent Mutated in Metastatic SCC	**Pre-Clinical or Clinical Drug**	**Other Cancers Investigated**	**Mechanism of Action**	**Stage and Type of Trial**	**Clinical Efficacy (SCC or other cancers)**
*CDKN2A 40-42%**	Flavopiridol and Dinaciclib	SCLC, CDKN2A deficient lung cancers	CDK inhibitors	Pre-clinical studies	These compounds induced apoptosis and thus cyto-toxicity in cell lines[166]
	Ilorasertib ABT-348	Solid tumors	Kinase inhibitor targeting aurora kinases/VEGF/PDGFRA/SRC kinase	Phase II Clinical Trials	The compound acted as expected in Phase I clinical trials, no phase II trials results are available as of 3/21
	Abemaciclib	HNSCC, Breast Cancer, Renal Cell Carcinoma, Glioblastoma	CDK4/6 Inhibitor	Has reached some phase III clinical trials	Metastatic HR+, HER2- breast cancer patients had an overall response rate of 19.7% at 12 months[172]
*EGFR 5%***	Cetuximab	SCC	Monoclonal antibody against EGFR	Approved for use in SCC	Overall response around 50%[167]

	Panitumumab	SCC	Monoclonal antibody against EGFR	Phase II Clinical Trial	Of patients with SCC refractory to other treatments, the overall response rate was 31%[168]
ErbB Family Blockers/ *ERBB4 0-7%***	Dacomitinib	SCC	Orally available, small molecule pan-HER inhibitor	Phase II Clinical Trials	Of patients that failed to respond to prior treatments, the response rate was 28 (2% complete, 26% partial)[173]
	Afatinib	HNSCC, NSCLC, Esophageal SCC, Breast Cancer, Others	Potent and selective ErbB family blocker	Phase II Clinical Trials	In metastatic HNSCC, Afatinib had a 10% Improved progression-free survival (only) Over methotrexate[174]
*HRAS 13-20%**	Tipifarnib	HNSCC, cSCC and others	Farnesyltransferase inhibitor	Phase II clinical trials for HNSCC, showed efficacy in a proof-of-concept trial for cSCC	Seventy-one percent of HNSCC patients had a partial response with a mean duration of 14.1 months[175]
KIT 10%	Dasatinib	Hematologic Malignancies, cSCC, others	Small molecule inhibitor of SRC-family protein kinases,	Phase II Clinical Trials, has shown efficacy preclinically as a	Patients with chronic myeloid leukemia resistant to imatinib

			including c-KIT	topical therapy in cSCC	had 78% progression-free survival at 48 months[176]
*KDR 40%***	Apatinib	Sarcomas, Gastric Cancer, NSCLC, Ovarian, Others	Tyrosine kinase inhibitor that selectively inhibits VEGFR (KDR)	Phase II Clinical Trials	Refractory ovarian cancer patients had a median overall response rate of 41.4%[177]
*NOTCH1 10-50%**	Bimiralisib (PQR309)	HNSCC, Breast Cancer, Lymphoma, Others	Preclinical data suggests that patients harboring a NOTCH1 loss-of-function mutation by respond to Bimiralisib, a PI3K/mTOR pathway inhibitor	Phase II Clinical Trials	Bimiralisib showed antilymphoma effects in a preclinical study through inhibition of the PI3K/mTOR pathway[178]
*PIK3CA 0-14%**	Apelisib	Breast cancer, HNSCC, others	Orally bioavailable inhibitor of PI3k	Recently FDA-approved for HR+/Her2- breast cancer	In breast cancer patients, patients with PIK3CA mutations had a progression-free survival of 11 months versus 5.7 months (both arms combined with fulvestrant) at 20 months follow-up[179]
*PTEN 20%**	17-AAG (Hsp90 inhibitor)	SCC, Others	Inhibits production of	In a pre-clinical	In a pre-clinical study,

			UV-induced SCC	studies in SCC; Clinical trials for various other cancers	topical application of 17-AAG inhibited the production of UV-induced SCC[180]
*STK11/LKB1 30%***	Various Hsp90 inhibitors (i.e. AUY922)	Solid Tumors, Others	In preclinical studies, STK11 mutant cells showed an increased sensitivity to Hsp90 inhibitors	Several trials terminated, Some active Phase II trials	AUY922 demonstrated pre-clinical efficacy in reducing cellular proliferation and viability in hepatocellular carcinoma cells[181]
*TP53 70-85%**	APR-246	Gastrointestinal cancer, ovarian cancer, myeloid neoplasms, others	Re-activation of p53 and induction of apoptosis in cancer cells; PRIMA-1 Analogue	Predominately phase Ib/II trials currently	Pre-clinical studies on TP53-mutated acute myeloid leukemia cells demonstrated induction of apoptosis through re-activation of TP53[182]

*-Information from Yilmaz *et al* (2017)[141]

**-Information from Al Rohil *et al*. (2015)[183]

***-Information from Zilberg *et al*. (2017)[184]

Trial information from clinicaltrials.gov

4.2 Sequencing of cutaneous squamous cell carcinoma primary tumors and patient-matched metastases reveals *ALK* as a potential driver in metastases and low mutational concordance in immunocompromised patients

Introduction

Cutaneous squamous cell carcinoma (SCC) is the second most common skin cancer, with 1,000,000 cases and up to 9,000 deaths annually in the United States.[115,116,185,186] While a majority of SCCs remain localized, approximately 2-5% of tumors metastasize.[187,188] Organ transplant recipients (OTRs) are especially susceptible to developing SCC and have a risk of 65-100 times that of the general population.[189] In addition, OTRs generally have a higher risk of metastasis, estimated at 7.3-11.0%.[137] Metastasis and local invasion are responsible for significant patient morbidity and mortality in SCC.[190] As therapeutic options for advanced and metastatic SCC are currently limited, studying mutations specific to metastatic SCC may lead to improved and targeted treatments.

The literature describing genetic alterations in metastases arising from SCC is relatively sparse. However, several recent studies have begun to characterize these mutations. Li *et al*. (2015) performed targeted sequencing on 504 cancer-associated genes on 29 lymph node metastases arising from SCC.[66] Results demonstrated that C→T mutations were the dominant substitution, and *TP53*, *CDKN2A*, and *NOTCH1* were altered in over 50% of samples.[66] A study by Al-Rohil *et al*. (2015) also performed targeted sequencing on 11 lymph node metastases arising from SCCs and found many mutations in *TP53*, *TERT*, *NOTCH1*, *ASXL1*, *CREBBP*, *LRP1B*, and *MLL2*.[191] These studies provide useful information on gene mutations seen in metastases, however, the mutations that are conserved or altered from metastatic primary tumors to metastases have yet to be discovered. This is one of the first studies to sequence and compare genetic alterations between patient-matched SCC metastatic primary tumors and lymph node metastases.

Results

The full experimental methods are detailed (Chapter 2.2 and 2.3). Primary metastatic tumors harbored a total of 41 mutations (18 pathogenic), or an average of 4.1 mutations per tumor that were included in the panel. Nodal metastases harbored a total of 49 mutations (21 pathogenic), or an average of 4.9 mutations per tumor that were included in the panel. Several mutations had notable differences in mutational frequencies in primary tumors versus metastases (**Table 8**). For the primary tumors, 68.3% (28/41) of mutations were missense, 14.3% (6/41) of mutations were silent, 11.9% (5/41) of mutations were nonsense, 2.4% (1/41) of mutations were frameshift, and 2.4% (1/41) of mutations were deletions. For metastases, 57.1% (28/49) of mutations were missense, 24.5% (12/49) of mutations were silent, 16.3% (8/49) of mutations were nonsense, and 2.0% (1/49) of mutations were frameshift. There were no statistically significant differences between primaries and metastases with respect to mutation types. For primary tumors, tumor suppressor gene mutations composed 63.4% (26/41) of mutations, and oncogenes composed 36.6% (15/41) of mutations. Metastases had a very similar distribution, with 61.2% (30/49) of mutations arising in tumor suppressor genes and 38.8% (19/49) of mutations arising in oncogenes.

Table 8: Differences in Mutations Between Primary Tumors and Metastases

Gene	Primary (n)	Metastases (n)	P-value*
ALK	0	4	p=0.15
HRAS	2	0	p=0.15
NOTCH1	1	3	p>0.05
TP53	10	11	p>0.05
CDKN2A	4	3	p>0.05
KDR	3	2	p>0.05
FBXW7	2	1	p>0.05
KIT	2	1	p>0.05
PTEN	2	1	p>0.05
CDH1	1	2	p>0.05
APC	1	0	p>0.05
FGFR2	1	0	p>0.05
GNAS	1	0	p>0.05
AKT3	0	1	p>0.05
DDR2	0	1	p>0.05
EGFR	0	1	p>0.05
FAT1	0	1	p>0.05
GATA3	0	1	p>0.05
GNAQ	0	1	p>0.05
JAK2	0	1	p>0.05
MAP2K2	0	1	p>0.05
RB1	0	1	p>0.05
STK11	0	1	p>0.05
SMAD4	2	2	p>0.05
SMO	2	2	p>0.05
ARID1A	1	1	p>0.05
BAP1	1	1	p>0.05
CDK4	1	1	p>0.05
ERBB2	1	1	p>0.05
ERBB3	1	1	p>0.05
FGFR3	1	1	p>0.05
HNF1A	1	1	p>0.05

*paired samples t-test; multiple mutations in one sample are counted as one

The mutations with the greatest difference in frequency between primary tumors and metastases were *ALK* (four unique mutations in metastases and zero mutations in primary tumors), *HRAS* (zero mutations in metastases and two mutations in primary tumors), and *NOTCH1* (three mutations in metastases and one mutation in primary tumors). Further analysis using Maftools/Oncodrive revealed *ALK* as a driver mutation in metastases (**Figure 16**).[103,105] A second analysis was performed using R package dNdScv to evaluate the finding of *ALK* as a driver mutation. Using this package, *TP53*, *CDKN2A*, and *ALK* were all found to be significant driver mutations in metastatic SCC ($p<0.001$, $p<0.001$, and $p=0.003$, respectively; **Table 9**).[108]

Figure 16: The driver mutation found in nodal metastases with the oncoCLUST algorithm.

FDR=false discovery rate

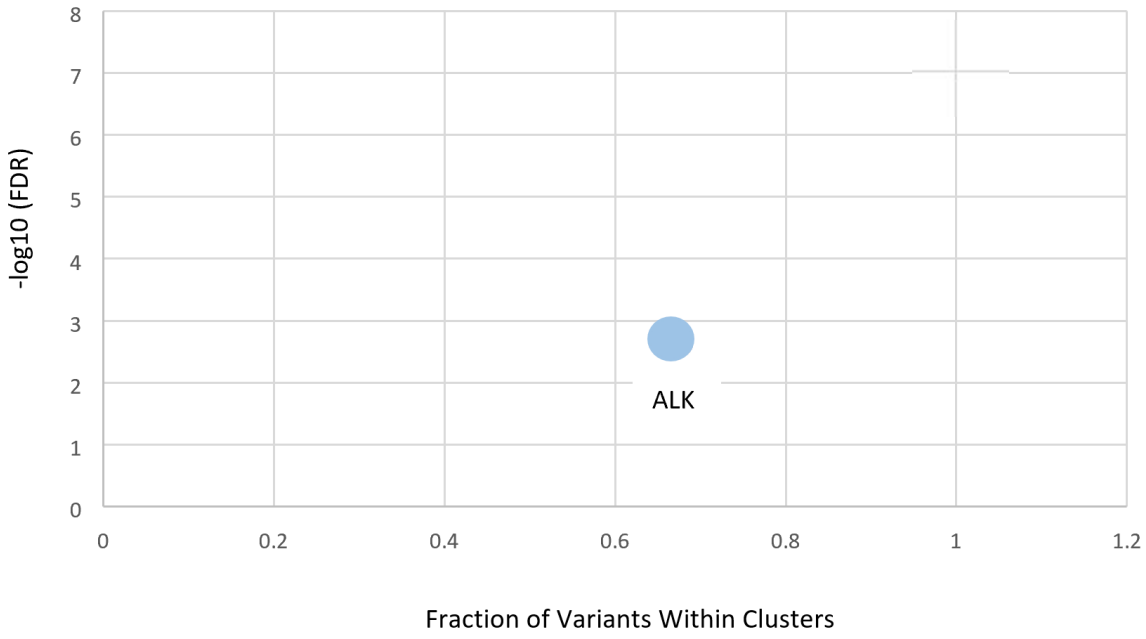


Figure 16 illustrates the results of the oncodriveCLUST algorithm, which revealed *ALK* as a potential driver mutation in metastases. FDR=false discovery rate.

Table 9: Significant Genes in our Cohort of SCC Lymph Node Metastases Identified in dNdScv

Gene	**Global q-value**
TP53	<0.001
CDKN2A	<0.001
ALK	0.003

Alexandrov *et al*. (2013) described over 20 mutational signatures in various human cancers that are described in COSMIC.[101,106] As these are largely dependent on base pair changes, base pair changes were analyzed in both groups and are illustrated (**Figure 17**). Using the signature analysis module in Maftools, it was determined that the best match signature for primary tumors was Signature 5 (unknown etiology, previously described[192]), and the best match signature for metastases was Signature 7 (UV exposure) (**Figure 18**).[103]

Figure 17: The distribution of mutations in primary tumors and metastases.

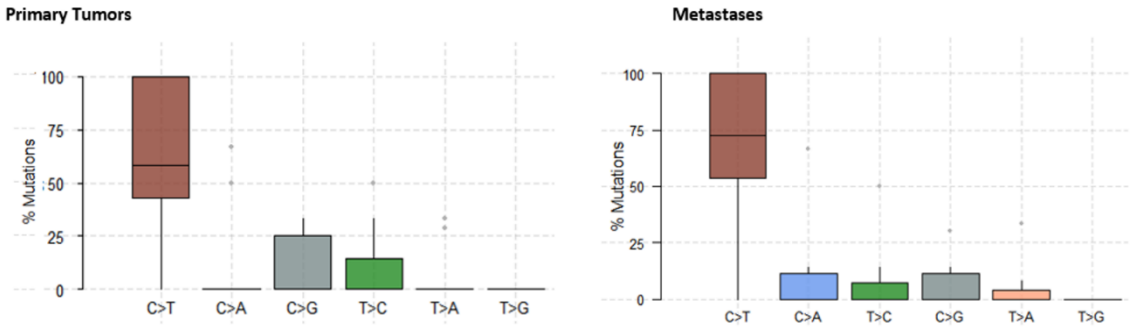


Figure 17 demonstrates the base pair changes observed in primary metastatic SCCs and metastases.

Figure 18: The COSMIC signatures most similar to the mutations seen in primary tumors and metastases.

The signatures for primary tumors are also presented in Chapter 4.1.

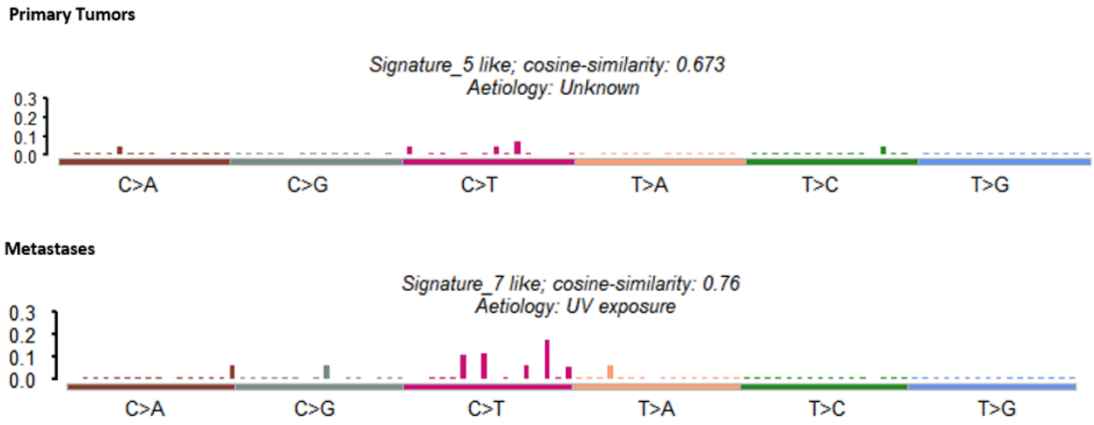


Figure 18 illustrates that the best match for primary metastatic tumors was signature 5, whereas the best match for metastases was signature 7.

When considering all mutations, the overall concordance rate between primary and matched metastases was 45.8%. However, the concordance rate for pathogenic mutations was considerably higher at 66.7%. Mutations in *SMAD4*, *SMO*, *BAP1*, *CDK4*, and *ERBB3* were concordant between primary tumors and matched metastases 100% of the time (**Table 10**). Mutations in *TP53*, *FBXW7*, *KIT*, and *PTEN* were concordant in 66.7% of cases (**Table 10**). *NOTCH1* mutations were concordant 50% of the time, *KDR* mutations were concordant 40% of the time; the remaining mutations were concordant in 0% of cases (**Table 10**).

Table 10: The rates of mutational concordance for all genes measured.

Gene	Concordant (n)	Discordant (n)	Concordant Mutations (%)	Total # Mutations	P=primaries M=metastases
SMAD4	4	0	100	4	Both P and M
SMO	4	0	100	4	Both P and M
BAP1	2	0	100	2	Both P and M
CDK4	2	0	100	2	Both P and M
ERBB3	2	0	100	2	Both P and M
TP53	14	7	66.7	21	Both P and M
FBXW7	2	1	66.7	3	Both P and M
KIT	2	1	66.7	3	Both P and M
PTEN	2	1	66.7	3	Both P and M
CDKN2A	4	3	57.1	7	Both P and M
NOTCH1	2	2	50	4	Both P and M
KDR	2	3	40	5	Both P and M
ALK	0	4	0	4	M only
CDH1	0	3	0	3	Both P and M
ARID1A	0	2	0	2	Both P and M
ERBB2	0	2	0	2	Both P and M
FGFR3	0	2	0	2	Both P and M
HNF1A	0	2	0	2	Both P and M
HRAS	0	2	0	2	P Only
AKT3	0	1	0	1	M only
APC	0	1	0	1	P Only
DDR2	0	1	0	1	M only
EGFR	0	1	0	1	M only
FAT1	0	1	0	1	M only
FGFR2	0	1	0	1	P Only
GATA3	0	1	0	1	M only
GNAQ	0	1	0	1	M only
GNAS	0	1	0	1	P Only
JAK2	0	1	0	1	M only
MAP2K2	0	1	0	1	M only
RB1	0	1	0	1	M only
STK11	0	1	0	1	M only

Mutation concordance was highly correlated to immune status. In immunosuppressed patients, 32.1% of mutations were concordant between primaries and metastases, whereas 54.9% of mutations in immunocompetent patients were concordant between primaries and metastases (p=0.04). When only considering pathogenic mutations, this was even more apparent as 41.7% of mutations were concordant in immunosuppressed versus 83.3% of mutations in immunocompetent patients (p=0.01).

Discussion and Conclusions

Several mutations detected in our cohort are well-established in SCC, including *TP53*, *NOTCH1*, and *CDKN2A*.[64,75,193] However, we present several unique findings that have not been previously reported. To our knowledge, this is the first report of *ALK* as a driver mutation in metastatic SCC. The oncogene *ALK* is a receptor protein-tyrosine kinase and member of the insulin receptor superfamily.[194,195] *ALK* mutations have been implicated in many humans cancers, including non-small cell lung cancer (NSCLC), breast cancer, ovarian cancer, colorectal cancer, and renal cell carcinoma (RCC), among others.[195]

The function of *ALK* has not been investigated in SCC with the exception of a recent study by Gualandi *et al*. (2020) that utilized a mouse model to demonstrate that *ALK* plays a role in the development of SCC.[196] It was demonstrated in mice that *ALK* exerts its tumorigenic role through cooperation with other well-known cancer-associated genes (*KRAS*, *TP53*, and *STAT3*).[196] The authors also investigated the effect of *ALK* mutations plus *TP53* loss. They concluded that this combination did not lead to metastasis, which is in contrast to our findings in which *ALK* mutations always co-occurred with *TP53* mutations in metastases.[196]

To understand the mechanism by which *ALK* drives metastasis, we analyzed the context of each mutation and performed an additional analysis with R package TRONCO using the CAPRI program.[107] In the model created for SCC lymph node metastases, the *ALK* missense mutation conferred an evolutionary advantage to the tumor that led to other downstream mutations in *FGFR3*, *JAK2*, *FAT1*, *ERBB2*, *TP53*, and *RB1* (**Figure 19**). As *ALK, FGFR3, JAK2,* and *ERBB2* are all part of the RTK/RAS/MAPK pathway, these data led us to further look at mutations in this pathway (**Figure 20**).

Figure 19: Evolutionary tumor model created with TRONCO CAPRI

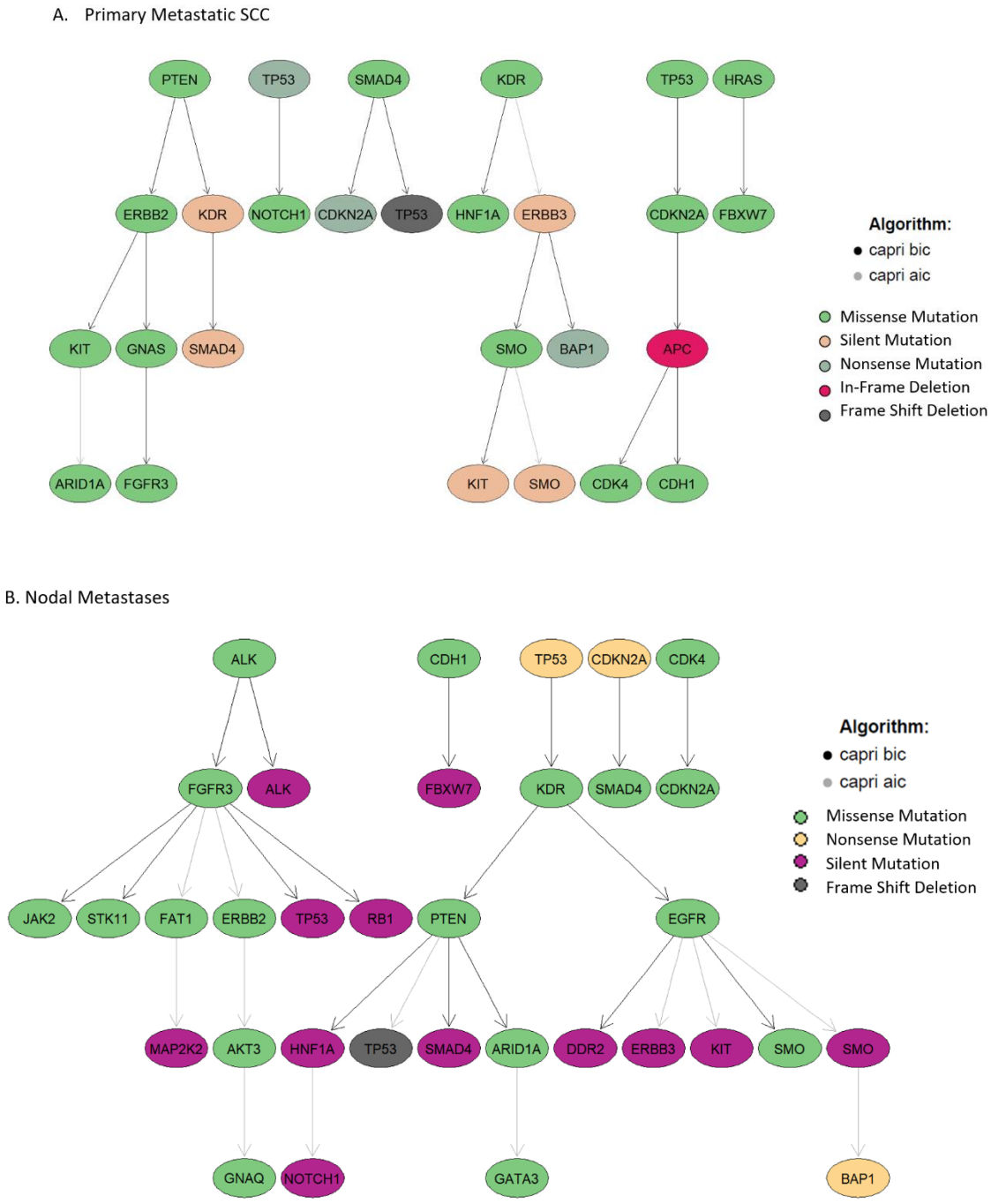


Figure 19 illustrates the proposed mutational evolution of (A) primary metastatic tumors and (B) metastases by the TRONCO package. AIC=Akaike information criterion; BIC= Bayesian Information Criterion.

Figure 20: Mutations in the RTK/RAS/MAPK pathway found in SCC metastases.

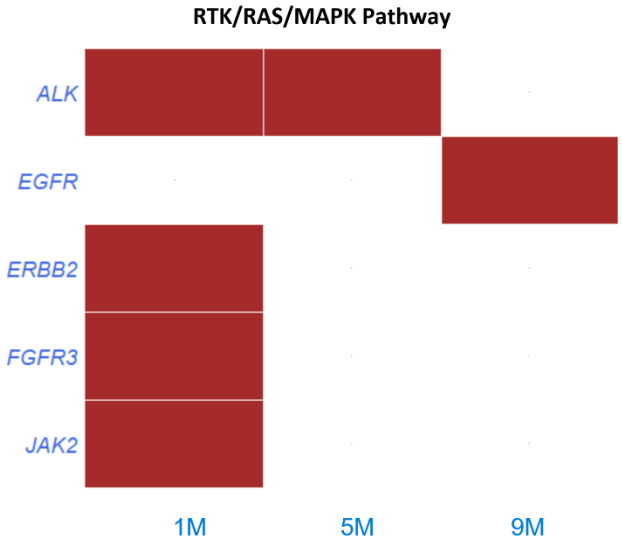


Figure 20 illustrates that three samples (1M, 5M, 9M) harbored mutations in the RTK/RAS/MAPK pathway and the specific mutations in each sample.

All *ALK* mutations occurred in patients with *ERBB2* mutations, and no *ERBB2* mutations occurred in patients without *ALK* mutations. In one patient *ALK* and *ERBB2* were co-mutated in a metastasis (this *ERBB2* mutation was characterized as pathogenic in COSMIC[101]); the other patient had an *ERBB2* mutation in the primary tumor and an *ALK* mutation in the metastasis. It is possible that *ALK* was also mutated in the primary tumor as a subclone below the 5% level threshold used to call variants in this study. *ALK* and *ERBB2* have been shown to act synergistically to promote tumor growth and survival in the studies using non-small cell lung cancer (NSCLC) cell lines.[197] A pathway analysis was performed to identify potential downstream targets of *ALK* and/or *ERBB2*. A common signaling pathway and possible mechanism for *ALK*-driven metastasis observed in this study is through the MAPK/ERK signaling pathway (**Figure 21**). Hrustanovic *et al*. (2016) studied models of lung adenocarcinoma (LUAD) and determined that *ALK*-positive LUADs were dependent on the MAPK/ERK pathway for tumor survival.[198] In addition, inhibition of this pathway along with *ALK* improved the magnitude and duration of response in preclinical models.[198] A study using T-cell lymphoma (TCL) cell lines determined that *ALK* fusion activates MEK1/2 and ERK1/2, corroborating our hypothesis that *ALK* may act through MAPK/ERK signaling.[199] Further strengthening this hypothesis, *ERBB2* is upstream activator of the MAPK/ERK pathway.[200] The MAPK/ERK pathway has been shown to play a role in metastasis for several cancers.[201,202] We hypothesize that *ALK* mutations, possibly in combination with *ERBB2*, activate the MAPK/ERK pathway, ultimately leading to growth, survival, and metastasis of SCCs.

Figure 21: Potential pathway activated in ALK-mutated metastatic SCC. Created with Biorender.

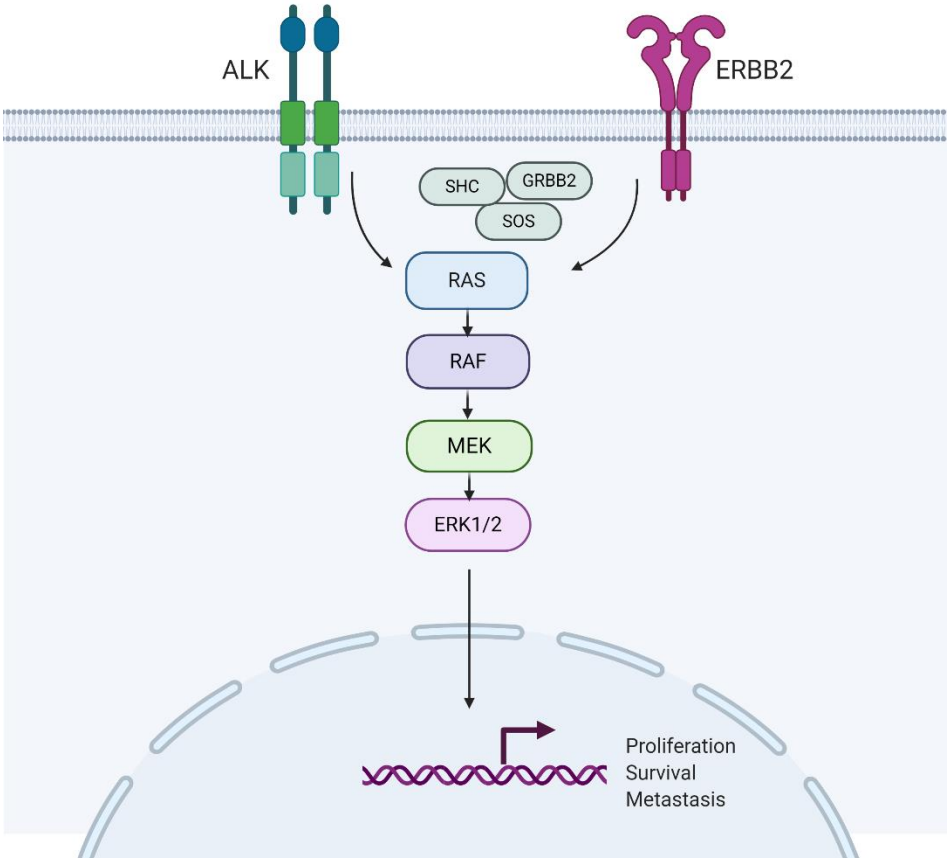


Figure 21 illustrates one hypothesized signaling pathway in ALK-mutated SCC (through the ERK pathway).

To interpret our findings within the context of the literature, we searched for sequencing studies of SCC metastases; however, these studies are limited. A study by Li *et al*. (2015) performed targeted sequencing on 29 SCC lymph node metastases and found that 27.6% (8/29) of samples had an *ALK* mutation, which is similar to our study in which 20% (2/10) of patients harbored an *ALK* mutation (4 unique mutations).[66] Li *et al*. also detected *ERBB2* mutations in 20.7% (6/29) of patients, 50% (3/6) of which co-occurred with *ALK* mutations.[66] Running Maftools program SomaticInteractions on this data revealed that *ERBB2* and *ALK* mutations have an odds ratio of co-occurrence of 5.3. However, this only leans towards statistical significance, which may be due to the relatively small sample size of 29 patients (p=0.16).[66,103]

A review of *ALK* mutations in localized SCCs demonstrates mutations in 10%-25% of localized tumors.[163,192,203] As *ALK* is mutated in many human cancers, there are FDA-approved and developing therapeutics (crizotinib, ceritinib, others) that targ*et ALK* mutations and have improved patient outcomes by blocking angiogenesis and metastasis.[195,204,205] Overall, these findings suggest that *ALK* is mutated in a significant number of SCCs, especially metastases. Our findings show the acquisition of *ALK* mutations as a driver mutation for metastases in our cohort of high-risk SCC. *ALK* may be a promising therapeutic target for adjuvant therapy of high-risk locally advanced SCCs and treatment metastatic disease. If further studies support a synergistic role of *ERBB2* and *ALK* in SCC growth and metastasis, inhibitors of *ERBB2* such as afatinib are used clinically in *ERBB2*-mutated cancers and may be effective in preventing *ALK*-inhibitor resistance.[206]

Identifying mutations with a 0% concordance rate between primaries and metastases, such as *ALK*, may be helpful to identify mutations that are important drivers in metastases and could serve as potential targets for adjuvant therapy in high-risk SCCs. Of note, *EGFR* was found to be mutated in a single sample of metastatic tissue

and not the matched primary. As *EGFR* inhibitor cetuximab is currently used off-label for high-risk and metastatic SCC, performing sequencing on both primaries and metastases and considering the optimal time for intervention may be important when selecting patients for targeted therapies.[154,167] Determining the mutational concordance between primary tumors and metastases is also important when selecting targeted therapies intended to target both lesions. One study by Yilmaz *et al*. (2017) performed whole exome sequencing on SCCs that included 6 pairs of matched primaries and metastases.[141] An overall concordance rate of 70.8% was found when looking at a subset of 26 genes previously determined to be mutated in SCC (34 concordant mutations, 14 discordant mutations).[141] This rate is very similar to the concordance rate for pathogenic mutations in our cohort (66.7% concordance). In our study, mutations in *SMAD4*, *SMO, BAP1*, *CDK4*, and *ERBB3* were concordant between primaries and metastases in 100% of cases, suggesting that these mutations likely occur early and may be consistent throughout the tumor progression. *CDK4* was previously demonstrated to have positive expression in 53.3% (16/30) of SCCs.[207] *CDK4* may be a promising therapeutic target in SCC as CDK4/6 inhibitor abemaciclib has shown benefit for metastatic breast cancer patients and is in many clinical trials for other metastatic cancers.[172]

To the best of our knowledge, this is the first study to examine the effect of immune status on mutational concordance between SCC primary tumors and metastases. A study of breast cancer matched primaries and metastases examined immune cells in the tumor microenvironment (TME) of primary and metastases and found notable differences, particularly with respect to PD-L1 expression.[208] We hypothesize that the TME has a greater impact on tumor progression in immunosuppressed patients. Thus, differences in the TME between primaries and metastases may have a greater impact on mutational concordance in

immunosuppressed patients, resulting in a lower proportion of concordant mutations in immunosuppressed patients. Clinically, this is especially relevant when selecting adjuvant therapies to treat both primary tumors and metastases. For example, sequencing is typically done on the primary tumor and the genes mutated in the primary are targeted by selected systemic therapies. However, the primary tumor is often excised surgically, leaving the metastases to be treated with systemic therapy. Given the substantial lack of mutational concordance between primary tumors and metastases, especially in immunosuppressed patients, sequencing of the metastases may be considered when identifying patient-specific adjuvant therapies.

Mutational processes in cancer generate unique combinations of mutations types, termed “mutational signatures”. Our data demonstrates that C>T mutations comprise a majority of total mutations in high-risk SCC and that COSMIC Signature 7 is the best fit for metastases. Signature 7 is associated with large numbers of CC>TT mutations at dipyrimidines that are typically repaired by nucleotide excision repair.[106] Mueller *et al*. (2019) performed whole genome sequencing on 15 SCC metastases (six parotid, nine neck lymph node) and found Signature 7 to correlate best with the somatic mutations.[209] The clinical utility of these mutational signatures was suggested by Mueller *et al*., as the UV signatures were able to differentiate metastases of mucosal origin from metastases of cutaneous origin.[209] In addition, Signature 7 may be helpful in prognosis and helping to identify a more high-risk subset of SCC.

The limitations of this sequencing study include that it was performed using one sample of tissue per primary tumor or metastasis at a single time point. Mutations detected in metastases but not in the matched primary may have been slowly acquired throughout tumorigenesis, developed from a subclone that was not reflected in limited tumor sampling, or passenger mutations that were not essential for clonal expansion and initial spread.[210] Targeted sequencing studies have several limitations and benefits.

While it is cost-effective and the depth of coverage and sensitivity is greater than would be possible than with whole-exome or whole-genome sequencing, the amount of DNA sequenced is much smaller and only reflects specific genes targeted by a specific panel. An additional limitation to this study was our limited sample size. Future studies with larger sample sizes would be helpful as a validation cohort.

In summary, we present results from targeted next-generation sequencing of 10 primary metastatic SCCs that were patient-matched with 10 lymph node metastases. We report *ALK* as a novel driver mutation for metastasis in SCC. *ALK* mutations were observed to co-occur with *ERBB2* mutations in our cohort suggesting a possible mechanism for *ALK*-driven metastasis is through the MAPK/ERK signaling pathway. In addition, we found that mutational concordance between primary and metastatic tumor was significantly lower in immunosuppressed patients. As these findings may have significant clinical implications, validation studies and evaluation of gene expression and pathways in metastatic SCC would be beneficial.

4.3 Germline mutations in SCC

This subchapter is published in Experimental Dermatology and included (with permission):

Lobl MB, Hass B, Clarey D, Higgins S, Wysong A. Next-generation sequencing identifies novel single nucleotide polymorphisms in high-risk cutaneous squamous cell carcinoma: A pilot study. Exp Dermatol. 2020 Jun 1.[97]

Introduction

Cutaneous squamous cell carcinoma (SCC) is estimated to affect 1 million people in the United States each year.[115] SCC can metastasize in 3-5% of cases, causing up to 9,000 deaths annually.[115,185] Known risk factors that predispose to SCC

development include fair skin, red hair, male gender, older age, chronic occupational sun exposure (particularly due to UVB), British or northern European ancestry, and immunosuppression.[211–213] In addition, several familial syndromes infer an increased risk of developing SCC, suggesting a genetic component (see Chapter 1).

Beyond familial cancer syndromes, there are otherwise "benign" single nucleotide polymorphism (SNPs) present in all cells in the body that have been implicated in elevating the risk of developing cancers, including lung, bladder, gastrointestinal, and hematologic. SNPs have also been found to impact patient survival of such cancers.[214–217] Several studies have investigated SNPs hypothesized to be linked to skin cancer. Chen *et al*. (2014) performed a meta-analysis of the *XRCC3* C18067T SNP for an association with non-melanoma skin cancer (NMSC) and concluded that it contributed to a decreased risk of both SCC and BCC (OR=0.81, p=0.01).[218] Nie *et al*. (2016) analyzed *VEGF* SNPs rs833061 and rs1570360 and concluded that the *VEGF* rs833061 SNP was correlated with a decreased risk of SCC (OR=0.36, p<0.001).[219] It was also determined that the *VEGF* rs1570360 SNP was correlated with reduced survival in SCC patients (23.88 months with the SNP versus 41.19 months wild-type, p=0.009).[219] A study by Asgari *et al*. (2016) reported a genome-wide association study (GWAS) of 6,891 patients with self-reported SCC and 54,666 controls; all patients were non-Hispanic whites residing in Northern California.[220] The pigmentation-related SNPs with the most significant difference between cases and controls on initial screen were *SLC45A2* (rs16891982), *IRF4* (rs12203592), *TYR* (rs1126809), *HERC2* (rs12916300), *DEF8* (rs4268748), and *RALY* (rs6059655) (p<0.05).[220] Other SNPs significant between groups on initial screen were *FOXP1* (rs62246017), *TPRG1/TP63* (rs6791479), *HLA-DQA1* (rs4455710), and *BNC2/CNTLN* (rs74664507) (p<0.05).[220]

While GWAS studies have been useful in uncovering SNPs that may be implicated in disease, there are clear limitations such as the inability to detect rare

variants and the lack of biological relevance of many SNPs detected. Our study design using targeted next-generation sequencing of cancer-related genes overcomes some of these limitations, by focusing on relevant pathologic SNPs with a higher sensitivity than would be possible with other sequencing technologies. Further, published work focuses on SNPs relevant in all SCC. Clinically, high-risk SCC must be differentiated from low-risk SCC. There is currently a lack of information regarding SNPs that may be implicated in particularly high-risk cases of SCC. Using high-coverage targeted sequencing of 20 high-risk SCCs, we hypothesize that our study may reveal SNPs relevant to high-risk SCC that would not be possible utilizing traditional GWAS studies.

Results

Targeted next-generation sequencing was performed on 20 high-risk SCCs using a 76 cancer-related gene panel (Vela Diagnostics, Fairfield, NJ). The full experimental methods are described (see Methods). Cohort characteristics are reported (**Table 2, Chapter 2.2**). Twenty-six coding SNPs were detected from sequencing. The SNPs with the greatest difference in frequencies between SCC and the American population (AP) are detailed (**Table 11**).

Table 11: SNPs with the greatest difference in frequency between the SCC cohort and the AP

Mutation	**Type**	**Pathogenic?**	**Frequency in the AP/high-risk SCC**	**P-value +**	**Bonferroni Adjusted p-value***	**Relevance of SNP in other cancers**
SF3B1 c.2631T>C rs788018 p.G877G	Silent	Unknown	47%/95%	**p<0.0001**	p=0.0019	-Acute myeloid leukemia[221]
KIT c.1621A>C rs3822214 p.M541L	Missense	Pathogenic (COSMIC), Benign/Likely Benign (ClinVar)	4.23%/20%	**p=0.0005**	p=0.0019	-Gastrointestinal stromal tumors[222]
KIT c.2586G>C rs3733542 p.L862L	Silent	Neutral (COSMIC), Benign (ClinVar)	5.45%/20%	p=0.0043	p=0.0019	-Acute myeloid leukemia[223]
SMO c.1164G>C rs2228617 p.G388G	Silent	Benign (ClinVar)	71.36%/100%	P=0.0046	p=0.0019	-None reported
EGFR c.2361G>A rs10501711 p.Q787Q	Silent	Pathogenic (COSMIC), Benign/Likely Benign (ClinVar)	54.92%/80%	p=0.0243	p=0.0019	-Colorectal cancer[224] -Hepatocellular carcinoma[225]
HRAS c.81T>C rs12628 p.H27H	Silent	Neutral (COSMIC), Benign (ClinVar)	36.8%/60%	p=0.0316	p=0.0019	-Bladder cancer[214] -Chronic myelogenous leukemia[216] -Gastric cancer[226]
FAT1 c.5004A>G rs35753072 p.T1668T	Silent	Neutral (COSMIC)	25.54%/5%	p=0.035	p=0.0019	-Oral squamous cell carcinoma[227]

+=P-value calculated using chi-squared difference in proportions test

*=Conservative Bonferroni adjusted p-value for multiple comparisons (n=26), required alpha of 0.0019 to reach significance

Discussion and Conclusions

SNPs predispose individuals to developing cancer and have been shown to impact patient outcomes. However, little research has focused on SNPs in high-risk SCC compared to other cancers. This work analyzes SNPs using a high-coverage targeted panel in cancer-associated genes and integrates our knowledge of the role of these SNPs in other cancers to hypothesize their impact on high-risk SCC.

SF3B1 rs788018 had a significantly higher frequency in high-risk SCC patients compared to the AP (95% versus 47%, respectively, p<0.0001; Bonferroni-adjusted p=0.0026).[102] *SF3B1* is known to be involved in splicing, however, the role of this gene and particular SNP remains to be fully understood.[228] One study utilizing a sample of 53 Asian AML patients found this SNP present in 86.8% of cases.[221] However, this was very similar to the frequency reported in the Asian population (78.6%-91.8%).[221] As more GWAS and sequencing studies are performed, we may gain a clearer understanding of the role of this SNP in cancer.

In our cohort, 20% of SCC patients had the SNP *KIT* rs3822214 compared to 4.23% in the AP (p=0.0005; Bonferroni-adjusted p=0.01).[221] *KIT* is responsible for production of receptor tyrosine kinases proteins. These proteins control many cellular processes, such as growth, division, and survival. SNP *KIT* rs3822214 has been implicated in predicting outcomes for gastrointestinal stroma tumors (GIST).[229] Specifically, a higher prevalence of this SNP was detected in patients who had metastatic disease at presentation (75% vs 17.2%, respectively, p=0.02).[229] Patients with the SNP also had a higher rate of relapse at 5 years (47% with SNP vs 14% wild-type, p=0.008).[229] SNP *KIT* rs3733542 displayed a similar trend to *KIT* rs3822214, as 20% of our cohort had this mutation compared to 5.45% of the AP (p=0.0043, Bonferroni corrected p-value >0.05).[102] A clinical trial of the *MEK* inhibitor selumetinib for advanced acute myelogenous leukemia (AML) found that this SNP was detected in significantly

more patients that responded to the therapy or had stable disease compared to the patients who did not respond to therapy (60% versus 23%, p=0.027).[223] MEK inhibitors can be co-administered with *BRAF* inhibitors to abrogate the risk of developing SCC from melanoma treatment.[230] In preclinical studies, Adelmann *et al*. demonstrated that *MEK* inhibition may also have therapeutic potential in SCC.[231] As the utilization of *MEK* inhibitors may increase in SCC, understanding of the impact of this SNP on therapeutic efficacy is highly clinically relevant.

Details and the potential clinical relevance of additional SNPs that were found to be significant initially, but subsequently did not meet the threshold with the conservative Bonferroni correction are as follows. In our cohort, SNP *SMO* rs2228617 was present in 100% of our cohort, while only present in 71.36% of the AP (p=0.0046).[102] *SMO* is a component of the hedgehog signaling pathway that is regulated by *PTCH1*.[232] When a mutation is present in the hedgehog pathway, *SMO* induces transcription factor GLI which promotes proliferation, differentiation, and survival of basal cells, which can lead to BCC.[233] A recent Polish case-control study analyzed SNPs in the hedgehog pathway and found that the presence of SNP *SMO* c. 349T>C statistically increased the risk of BCC (OR 87.9, p<0.001).[234] Our findings indicate that SNPs in *SMO* may affect not only BCC but SCC as well.

Another SNP that had a higher frequency in SCC compared to the AP was *EGFR* rs1050171. Epidermal Growth Factor Receptor (*EGFR*) mutations have been implicated in a variety of human diseases, including many cancers.[235] *EGFR* is known to induce cellular proliferation and differentiation as a result of ligand binding. SNP *EGFR* rs1050171 was common in the AP with an estimated frequency of 54.92%.[102] SNP *EGFR* rs1050171 was present in 80% (16/20) of SCC patients, which was higher than in the AP (p=0.024). Several studies have analyzed this SNP and aimed to determine a possible effect on cancer susceptibility and prognosis.[224,236] An increased frequency of

this SNP has been observed in colorectal cancer (82.7%, n=225) and hepatocellular carcinoma (81.5%, n=89).[224,236] Further, Bonin *et al*. reported that the GG variant of this SNP predicted response to *EGFR* systemic therapy (cetuximab and/or panitumumab) in metastatic colorectal cancer patients independent of RAS mutation status, with 6 month progression free survival declining from 81% (GG variant) to 34% (GA or AA) (p=0.01).[225] As such, this SNP may be important in both prognosis and risk assessment. Screening for SNPs may eventually improve patient selection for targeted therapy, such as use of the *EGFR* inhibitor cetuximab in treatment of advanced SCC.[154,167]

An additional SNP identified in our cohort was HRAS rs12628. *HRAS*, another gene commonly altered in cancer, is mutated in approximately 12% of SCCs.[141] *HRAS* functions primarily by regulating cellular division, acting as an activator in the Raf/ERK and PI3K pathways.[237] In our cohort, SNP *HRAS* rs12628 was present in 60% (12/20) of high-risk SCC patients compared to 36.8% of the AP (p=0.03).[102] SNP *HRAS* rs12628 has been implicated in several other malignancies, including chronic myelogenous leukemia (CML), gastric cancer, and bladder cancer.[214,216,226] A Chinese population based case-control study (n=744) analyzed the prevalence of this SNP with the risk of developing gastric cancer. It found that carriers of the C allele had an increased risk of gastric cancer (OR=3.65).[226] Another case-control study (n=200) examined the association of this SNP with chronic myeloid leukemia (CML).[216] Compared to the TT genotype, the CT genotype inferred over an 18-fold increased risk.[216] *HRAS* rs12628 has also been implicated in the risk and prognosis of bladder cancer. A study of 140 bladder cancer patients found a significant increase in the CT and CC genotypes compared to controls (CT: 30% to 15.6% and CC: 5.6% to 0%, respectively; OR=3.0).[214] Additionally, the patients with CT and CC genotypes tended to present with a higher grade (OR=5.4, p<0.0001) and advanced tumors (OR=3.3, p<0.05).[214] These studies

suggest that the *HRAS* rs12628 SNP may be more common in cancer patients and might play role in patient prognostication.

Tumor suppressor gene *FAT1* codes for a cadherin-like protein. When inactivated, it promotes Wnt signaling and tumorigenesis.[238] *FAT1* mutations have been implicated in a variety of human cancers in addition to SCC.[141,239,240] Our SCC cohort had a lower frequency of this SNP compared to the AP (SCC 5%, AP 25.54%, p=0.03).[102] To our knowledge, this is the first report of potential significance of this SNP in SCC. However, one study investigated the impact of *FAT1* rs28647489 as a risk factor for oral squamous cell carcinoma (OSCC) and found that patients with this SNP had a 1.32-2.09 OR of OSCC compared to the control group ($p<0.05$).[227] Further studies with larger cohorts may be helpful to determine the effects of this SNP in different cancers.

A limitation of our study design includes the small sample size due to the high expense of genetic sequencing. As such, only two SNPs reached statistical significance with the conservative Bonferroni correction for multiple comparisons. The SNPs with unadjusted significance and potential clinical utility were included in the manuscript. Future studies with larger sample sizes are needed to further validate the relevance of these SNPs in SCC. Our study utilizes publicly available databases to compare to our cohort. Although we used data from the AP, from which our cohort is also developed, this population is rather diverse and it is difficult to ensure that the population perfectly matched with our study. The next steps in validating the results of this pilot study are to expand our sample size and to collect matched control samples.

Overall, this subchapter presents novel data on 7 SNPs with a relationship to high-risk SCC that to our knowledge has not yet been reported. While there are limitations to our study, future studies should continue to investigate the prevalence and effects of influential SNPs in high-risk SCC and to utilize large sample sizes to validate findings in diverse patient populations.

4.4 Mutational differences between immunocompetent and immunocompromised patients with respect to UV-radiation

This subchapter is published in the Journal of the American Academy of Dermatology and included (with permission):

Lobl MB, Clarey D, Higgins S, Thieman T, Wysong A. The correlation of immune status with ultraviolet radiation-associated mutations in cutaneous squamous cell carcinoma: A case-control study. J Am Acad Dermatol. 2020 May;82(5):1230-1232.[98]

There are an estimated 1 million cases of cutaneous squamous cell carcinoma (SCC) per year in the United States.[115] Ultraviolet (UV) radiation contributes to the pathogenesis of SCC and causes characteristic pyrimidine-pyrimidine dimer mutations.[55] A study by Pickering *et al*. performed whole-exome sequencing of 39 aggressive SCCs and found 65% of mutations to be UVB-associated.[241] In addition to UV radiation, immunosuppression increases SCC risk. Organ transplant patients are 100x more likely to develop SCC secondary to underlying immunosuppression and toxicity from chemotherapy.[189] However, the pathogenesis of SCC in immunocompromised patients remains to be fully elucidated. We hypothesized that there are fewer UVB-associated mutations in immunocompromised patients compared to immunocompetent patients with SCC.

We performed next-generation sequencing (NGS) using a hotspot mutation panel covering 76 cancer-associated genes (Vela Diagnostics) in a cohort of 20 patients with high-risk SCC (**Table 1, Table 2**). We categorized mutations as being caused by UVA radiation, UVB radiation, reactive oxygen species (ROS) (thought to be secondary to UVA damage, likely due to deeper penetration in the skin), or other based on methods by Agar *et al*.[111] Exploring the pathogenesis of SCC development by stratifying for UVA,

UVB, and ROS mutations provides insight into the mechanism of SCC development in immunosuppressed patients.

Sixty-four percent (64.4%) of mutations in immunocompetent patients were UVB-associated, consistent with the literature (**Figure 22**). However, UVB mutations composed only 41.0% of mutations in immunocompromised patients; this was significantly different (p=0.04) (**Figure 22**). In contrast to literature suggesting fewer mutations in SCCs arising in immunocompromised patients, the number of mutations per patient was not significantly different between immunocompetent and immunocompromised patients (3.75 vs 4.88, respectively; p>0.05). This may be due to the use of a targeted panel with a limited number of genes. In a separate analysis, the percentage of UV mutations was also examined for tumors in different anatomical locations (**Table 12**). The proportion of both UVB and UVA/ROS mutations was significantly different based on tumor location, with high-risk area tumors (ear, lip, periorbital region, and nose, n=11) having more UVA/ROS mutations (30.77% vs. 11.62%, p=0.03) and fewer UVB mutations (41.03% versus 67.44%, p=0.02) than medium and low-risk area tumors (n=9).

Figure 22: Mutations by category for immunocompetent and immunocompromised patients

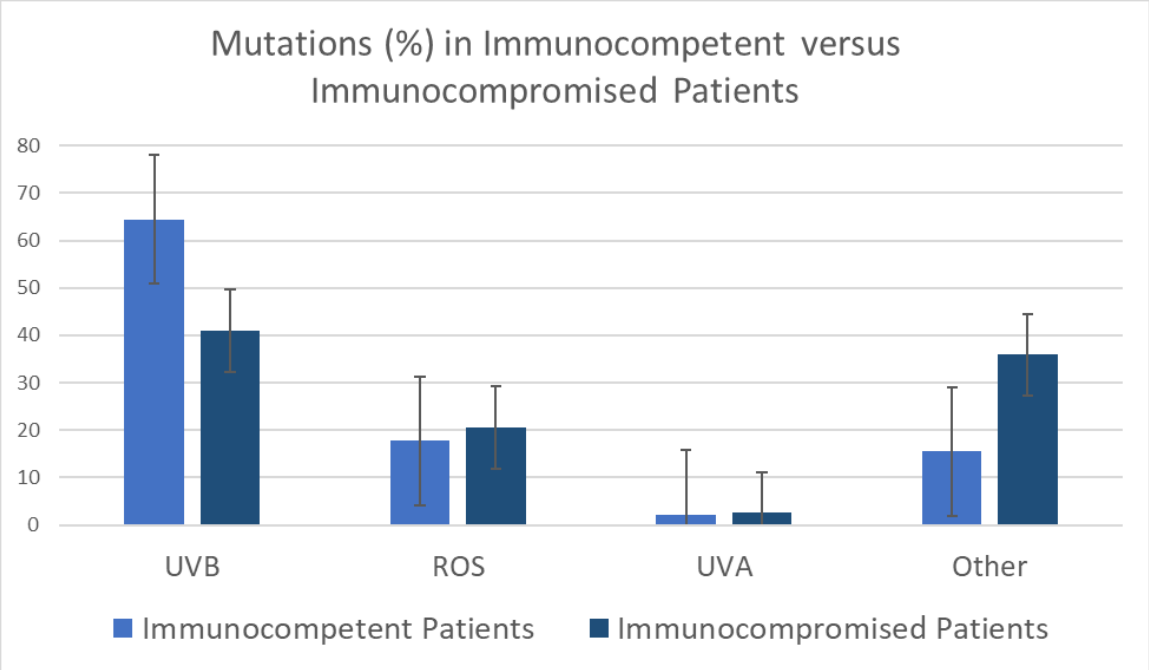


Figure 22 illustrates that 64.4% of mutations in immunocompetent patients were UVB-associated, while 41.0% of mutations in immunocompromised patients were UVB-associated (p=0.04).

Table 12: Patient and tumor characteristics separated by high versus medium and low-risk anatomic areas.

	Average Age	Gender	Location	AJCC8 Stage	BWH Stage	Immune Status*	Metastasis**
High-risk (n=11)	65.4	Male (9), Female (2)	Ear (7) Nose (1) Periorbital (3)	T1 (1) T3 (6) T2a (2) T2b (2)	T1(1) T2a (4) T2b (1) T3 (5)	27% Immunocompromised (3/11)	64% (7/11)
Medium/low-risk (n=9)	71.6	Male (7), Female (2)	Extremity (2) Cheek (2) Neck (1) Scalp (4)	T1 (1) T3 (8)	T1 (1) T2a (2) T2b (3) T3 (3)	56% Immunocompromised (5/9)	33% (3/9)

*A limitation of the study was that the medium/low- risk location group had more immunocompromised patients than the high-risk location group.

**While the high-risk group had a higher rate of metastasis, metastasis itself did not have a unique UV mutational signature compared to localized tumors.

These findings affirm our hypothesis that UVB radiation may contribute less to the pathogenesis of SCC in immunocompromised patients. Perhaps less UV radiation is required to be carcinogenic in immunocompromised patients secondary to their underlying immunosuppression. The literature surrounding SCC in immunocompromised patients also points to a permissive microenvironment to explain these findings. Further, the significantly higher contribution of UVA and ROS and lesser contribution of UVB to SCCs in high-risk areas of the face may be due to the thinner epidermal layer and shorter distance that UVA has to travel to become carcinogenic. As these anatomical areas are somewhat equally sun exposed, there also appears to be an inherent risk in certain locations due to patterns of vascularization and lymphatic drainage. Further studies are warranted to examine additional mutational differences between SCC in these groups of patients.

Chapter 5: Analysis of mutations in cutaneous squamous cell carcinoma reveals novel genes and mutations associated with patient-specific characteristics and metastasis: A systematic review

This chapter is published in Archives of Dermatological Research and included (with permission):

Lobl MB, Clarey D, Schmidt C, Wichman C, Wysong A. Analysis of mutations in cutaneous squamous cell carcinoma reveals novel genes and mutations associated with patient-specific characteristics and metastasis: a systematic review. Arch Dermatol Res. 2021 Mar 18.[99]

Introduction

Cutaneous squamous cell carcinoma (SCC) is the second most common type of skin cancer.[115,185,186] One million cases of SCC are diagnosed in the United States each year with up to 9,000 associated deaths.[115,116,185,186] While only 2-5% of SCCs metastasize, those that do metastasize tend to have poor outcomes.[139,187,188] SCC carries one of the highest mutational burdens of all cancers. Mutations in *TP53, NOTCH*, and *CDKN2A* are well-known driver mutations in SCC.[55,64,75] However, it is likely that there are additional driver mutations in SCC that have not yet been discovered. While there have been SCC sequencing studies recently published, no study has synthesized this information. This study aims to provide a comprehensive analysis of all published SCC sequencing studies to distill information regarding novel mutations, differences in mutations in localized versus metastatic SCC, and the relationship between mutations and patient- and tumor-specific characteristics. The methods of this study are detailed (Chapter 2.4).

Results

The search ultimately yielded nine articles for use in this study.[58,141,163,184,191,192,203,241,242] From these articles, there were 189 localized SCC cases and 90 metastatic SCC cases with individual-level patient information. The demographic and tumor characteristics from each study are detailed (**Appendix 4**). The most common mutations in localized and metastatic SCC, as well as the statistical result from performing the chi-square for homogeneity of proportions and conservative Bonferroni correction, are detailed (**Table 13**). The gene mutations that were statistically significant between the localized and metastatic groups are illustrated (**Figure 23**). Mutations that were present in a significantly different proportion of localized versus metastatic SCCs with the conservative Bonferroni correction were *TP53*, *TERT*, *SPEN*, *MLL3*, and *NOTCH2*.

Genes *CDKN2A*, *HRAS*, *NOTCH1*, and *TP53* were investigated in all studies and samples. Patterns of mutations more likely to occur in metastatic SCC are illustrated (**Table 14**). The mutational patterns seen the most often in metastatic SCC compared to localized SCC were pattern 1 (mutations in *CDKN2A* and *TP53* only, 60.7% of occurrences in metastatic SCC) and pattern 2 (mutation in *TP53*, *CDKN2A*, *HRAS*, and *NOTCH1*, 60% of occurrences in metastatic SCC). Of the total cases, 32.2% (90/279) were metastatic.

In the genes investigated in all studies (*TP53*, *CDKN2A*, *HRAS*, *NOTCH1*), we sought to determine the translational effects of each mutation. When available, we analyzed the protein changes and compared the distribution of mutation subtypes between localized and metastatic SCC (**Table 15, Figure 24**). Of note, silent mutations were found more in localized SCCs than metastatic SCCs (9.6% of all mutations in localized SCC versus 1.0% of all mutations in metastatic SCC, p=0.0003). Nonsense

mutations were found more in metastatic SCCs than localized SCCs (34.9% of all mutations in metastatic SCC versus 23.5% of all mutations in localized SCC, p=0.04).

From the studies that reported sex for the individual patient, there were 48 females and 190 males. Since not all studies investigated all mutations, we took the number of confirmed patients with the mutation and divided this by the number of patients who had the mutation investigated. *TP53* mutations were found in SCCs from men in a higher proportion than in women (*TP53*: 37.5% [18/48] female SCCs, 72.6% [138/190] male SCCs; p<0.0001, Bonferroni-corrected p<0.003). In males, 39.5% of tumors were metastatic, and in females, 29.8% of tumors were metastatic. Even so, the proportion of *TP53* mutations in males was higher than expected. As *TP53* mutations are UV-induced, we sought to evaluate these mutations by anatomic location, however, this information was not available for each individual tumor.

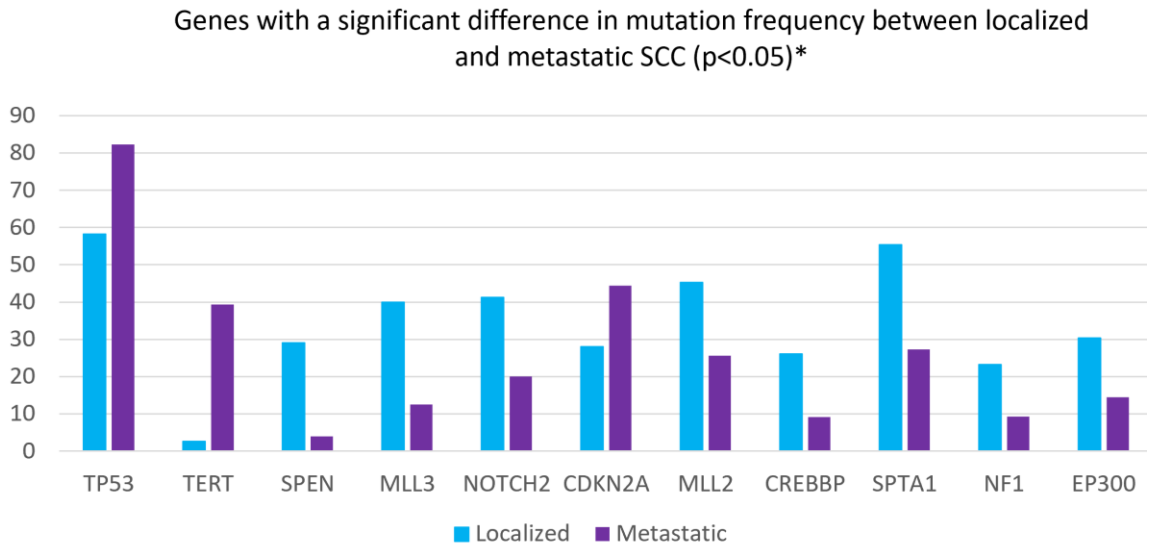
Patients in a study that reported patient age were stratified into an “older” category (above age 65, n=57) or a “younger” category (65 or younger, n=41). It was determined that *COL4A1* mutations were present in 22.7% (5/22) of older patients and 56.3% (9/16) of younger patients who had *COL4A1* measured (p=0.037, Bonferroni-corrected p>0.05). Patients were separated by immune status: (1) immunocompetent patients (n=64), and (2) patients with any type of immunosuppression (n=35). The number of mutations that occurred was divided by the total number of patients who had that mutation investigated for each group. *MLL4* mutations were present in 44.2% (19/43) of immunocompetent patients and 0% (0/27) of immunosuppressed patients (p=0.0001, Bonferroni-corrected p=0.003). *BRCA2* mutations were present in 0% (0/23) of immunocompetent patients and 17.1% (6/35) of immunosuppressed patients (p=0.038, Bonferroni-corrected p>0.05).

Table 13: Most common mutations in SCC and differences between localized and metastatic tumors in review of the literature.

Gene	Localized (%), n	Metastatic (%), n	P-value*	Bonferroni p-value	Odds ratio (OR) for metastasis	95% Confidence Interval for OR
TP53	(58.2), 189	(82.22), 90	0.0001	0.003	3.32	1.80 to 6.13
TERT	(2.6), 38	(39.4), 33	0.0001	0.003	24.05	2.93 to 197.47
SPEN	(29.1), 134	(4.0), 75	0.0001	0.003	0.10	0.03 to 0.34
MLL3	(40.0), 180	(12.5), 48	0.0004	0.01	0.21	0.09 to 0.53
NOTCH2	(41.2), 170	(20), 80	0.001	0.03	0.36	0.19 to 0.67
CDKN2A	(28.0), 189	(44.4), 90	0.007	NS	2.05	1.22 to 3.46
MLL2	(45.2), 84	(25.6), 9	0.007	NS	0.42	0.22 to 0.79
CREBBP	(26.1), 46	(9.2), 76	0.01	NS	0.29	0.10 to 0.80
SPTA1	(55.3), 38	(27.3), 33	0.02	NS	0.30	0.11 to 0.82
NF1	(23.2), 56	(9.3), 86	0.02	NS	0.34	0.13 to 0.88
EP300	(30.4), 46	(14.5), 76	0.04	NS	0.39	0.16 to 0.95
AHNAK2	(53.0), 134	(50), 4	NS	NS	NS	NS
NOTCH1	(49.2), 189	(42.2), 90	NS	NS	NS	NS
FAT1	(37.8), 172	(36.2), 47	NS	NS	NS	NS
LRP1B	(47.8), 46	(30.3), 76	NS	NS	NS	NS
MLL4	(35.8), 162	(37.5), 8	NS	NS	NS	NS
TRIO	(35.8), 134	(25), 4	NS	NS	NS	NS
MDN1	(32.1), 134	(75), 4	NS	NS	NS	NS
COL4A1	(33.6), 134	(25), 4	NS	NS	NS	NS
COL4A2	(31.3), 134	(25), 4	NS	NS	NS	NS
SVIL	(31.3), 134	(25), 4	NS	NS	NS	NS
HERC2	(30.6), 134	(25), 4	NS	NS	NS	NS
VPS13C	(31.3), 134	(0), 4	NS	NS	NS	NS
DST	(29.6), 142	(40), 5	NS	NS	NS	NS
DMD	(28.4), 134	(25), 4	NS	NS	NS	NS
DYSF	(26.9), 134	(50), 4	NS	NS	NS	NS
NOTCH3	(23.1), 134	(27.3), 33	NS	NS	NS	NS
ARID2	(23.9), 46	(13.2), 76	NS	NS	NS	NS
APC	(20), 65	(10.5), 86	NS	NS	NS	NS
TET2	(19.6), 46	(9.2) 76	NS	NS	NS	NS
RB1	(12.3), 65	(12.8), 86	NS	NS	NS	NS
ASXL1	(13.0), 46	(11.8), 76	NS	NS	NS	NS
BRCA2	(8.3), 48	(11.8), 85	NS	NS	NS	NS
HRAS	(8.5), 189	(8.9), 90	NS	NS	NS	NS

*p-value calculated using chi square variance of proportions

Figure 23: Genes with a significant difference in mutation frequency between localized and metastatic SCC



Blue= percent mutated in localized SCC

Purple= percent mutated in metastatic SCC

*p<0.05 before the Bonferroni correction

Figure 23 illustrates the difference in mutational frequencies for localized and metastatic tumors. The genes with the greatest difference are on the left and ordered left-to-right from greatest to least difference.

Table 14: Patterns of mutations observed in metastatic SCC

Pattern	CDKN2A*	HRAS*	NOTCH1*	TP53*	Number observed in metastatic SCC	Total number observed in all SCC	Percent that occurred in metastatic SCC
1	1	0	0	1	17	28	60.7
2	1	1	1	1	3	5	60
3	1	0	1	1	16	38	42.1
4	0	0	0	1	20	52	38.5
5	0	1	0	0	1	3	33.3
6	1	1	0	1	2	6	33.3
7	0	0	1	1	14	46	30.4
8	0	1	0	1	1	4	25
9	0	0	0	0	10	47	21.3
10	0	1	1	1	1	5	20
11	1	0	0	0	1	8	12.5
12	1	0	1	0	1	8	12.5
13	0	0	1	0	3	28	10.7
14	0	1	1	0	0	1	0
15	1	1	0	0	0	0	0
16	1	1	1	0	0	0	0

*0=not mutated, 1=mutated

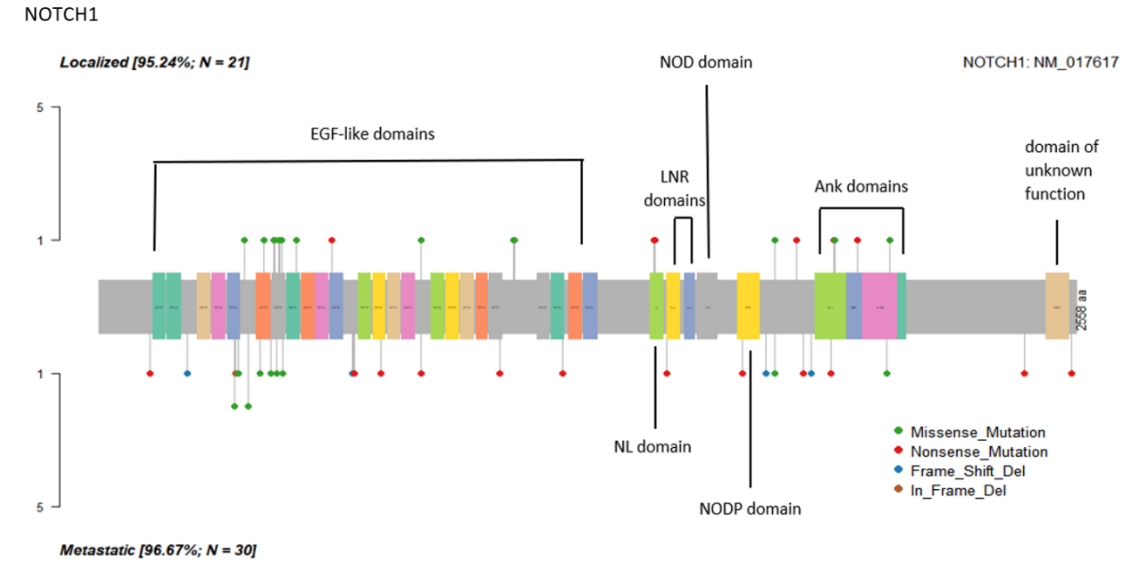
Table 15: Mutation subtypes by group

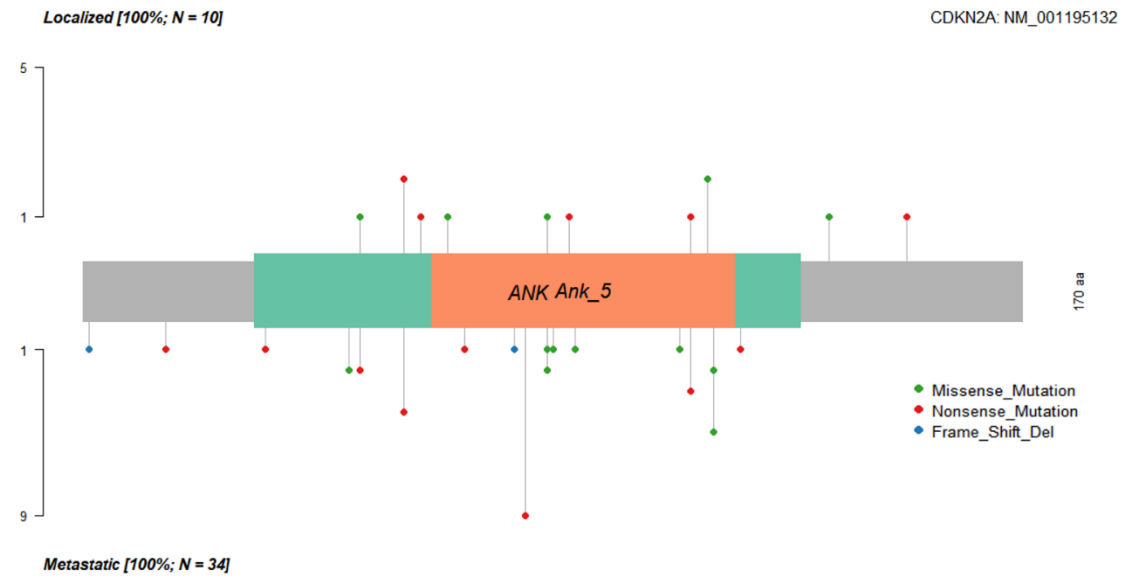
Mutation Type	**Localized (%), n**	**Metastatic (%), n**	**p-value***
Missense	64.3 (74/115)	56.9 (111/195)	NS
Nonsense	23.5 (27/115)	34.9 (68/195)	p=0.04
Silent	9.6 (11/115)	1.0 (2/195)	p=0.0003
Frameshift Deletion	2.6 (3/115)	6.7 (13/195)	NS
In-Frame Deletion	0.0 (0/115)	0.5 (1/195)	NS

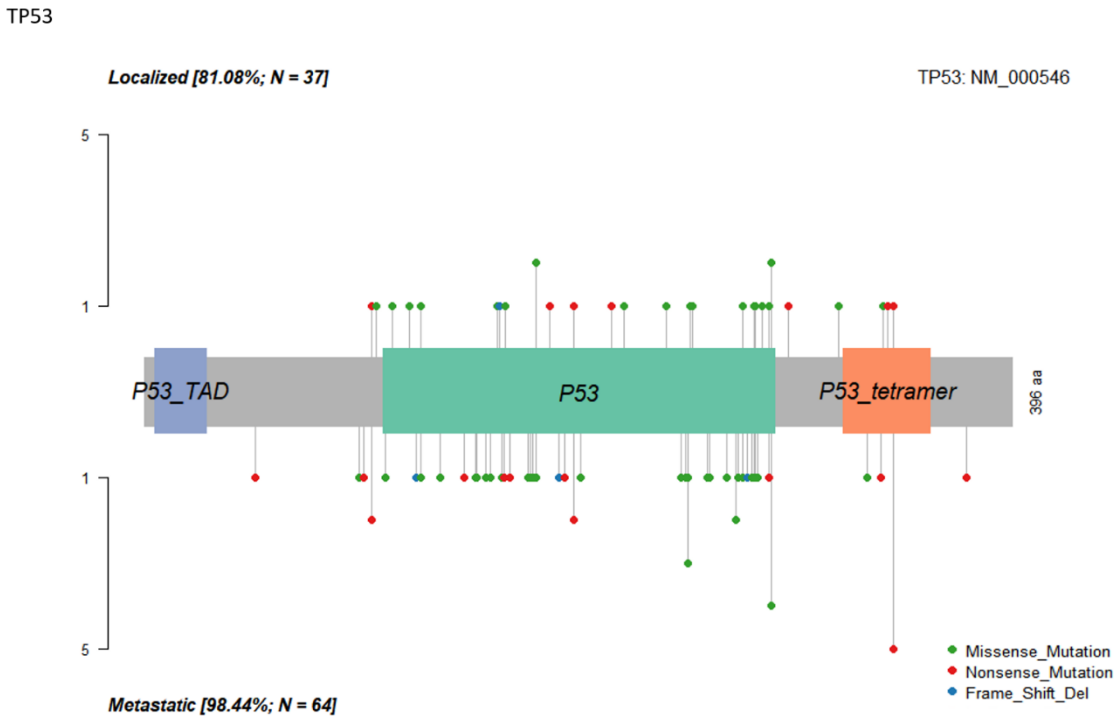
*p-value determined by chi-square variance of proportions test

NS= not significant

Figure 24: Distribution and subtypes of mutations by protein domain for mutations measured in all studies







HRAS was excluded due to the small number of mutations (<10)

Figure 24 demonstrates the subtypes and domains for the mutations measured in all studies (*TP53*, *NOTCH1*, *CDKN2A*). HRAS was excluded due to the small number of mutations (<10).

Discussion and Conclusions

While several well-known and previously described mutations occurred more in metastatic SCCs (*TP53*, *CDKN2A*, and *TERT*), we also observed mutations that occurred more in localized SCCs. As such, mutations in these genes (*SPEN*, *MLL3*, *NOTCH2*, *MLL2*, *CREBBP*, *SPTA1*, *NF1*, and *EP300*) in the absence of pathogenic, high-risk mutations may be predictive of a favorable patient outcome. Additional research is needed to investigate these associations. Further, we observed mutations in 25% or greater of SCCs in genes that have not been studied in-depth in SCC. Additional experiments studying these genes in SCC (*AHNAK2*, *LRP1B*, *TRIO*, *MDN1*, *COL4A2*, *SVIL*, *VPS13C*, *DST*, *DMD*, and *DYSF*) may eventually lead to additional targeted therapeutics. Currently, the only small molecule inhibitor therapy widely used for SCC is the *EGFR* inhibitor cetuximab. Epidermal growth factor receptor (*EGFR*) is a tyrosine kinase receptor that plays a role in cell proliferation, differentiation, and migration.[76] *EGFR* mutations are reported in 0-5% of SCCs.[77–79] Overexpression of EGFR has been found in 35-80% of SCCs and has been associated with a poor prognosis.[80–82] EGFR inhibitor cetuximab is used for advanced and/or metastatic SCC and has an overall response rate (ORR) of 33% as monotherapy, and 58% when used in combination with surgery and radiation.[167] Due to the substantial number of patients who do not respond, further investigation of significant genes in this review may be worthwhile in SCC.

Nonsense mutations occurred more often in metastatic SCC in our study ($p=0.04$). As nonsense mutations produce truncated proteins that rarely retain function, it is expected that these mutations may be more deleterious in SCC. A correlation of nonsense mutations and tumor metastasis has been reported in other cancers. A study of ovarian cancer reported that tumors with null *TP53* mutations (nonsense, frameshift, or splice site) had an 8-fold increase in distant metastasis compared to missense

mutations or the wild-type ($p<0.001$).[243] In contrast to nonsense mutations, silent mutations were present significantly more in localized SCCs relative to metastatic SCCs ($p=0.0003$). Oftentimes, silent mutations do not directly affect protein translation and are categorized as benign. However, more recent evidence suggests that they can act in a variety of ways, including modulation of splicing.[244] In addition, there were several patterns of mutations in *TP53*, *CDKN2A*, *NOTCH1*, and *HRAS* that occurred more often in metastatic SCC than localized SCC. Studies examining these gene mutations for mutual exclusivity or co-occurrence would provide important information on the pathogenesis of SCC.

To our knowledge, our study is the first to find a greater proportion of *TP53* mutations in SCCs from men than SCCs from women (72.6% versus 37.5%, $p<0.0001$, Bonferroni-corrected $p<0.003$). As *TP53* mutations are largely UV-induced, we hypothesize that this may be due to differences in sun exposure. Thus, SCCs in men may be present in sun-exposed locations (e.g. head and neck, dorsum of the hands) more often than in women. As many studies reported tumor locations in aggregate (i.e., individual locations were not available for each tumor), we cannot definitively make this conclusion. Future studies to further investigate this potential association would be interesting.

Several studies of other cancers have shown similar results with more *TP53* mutations in men than women. A cohort study of 152 patients who had developed early onset or multiple cancers found that the *TP53* polymorphisms conferred an increased risk of cancer in men but not women with DI or II genotypes ($p=0.0041$).[245] Another study analyzing colorectal cancer created several tumor classification groups largely based on mutations. The only group that was characterized by *TP53* mutations occurred more often in men (female versus male OR=0.62, $p=0.003$).[246] However, the opposite trend

has also been observed. A case-control study of malignant pleural mesothelioma (MPM) found that women were more likely to harbor *TP53* mutations compared to men (9% [18/200] of men versus of 23% [20/88] women; p=0.004).[247] If men with SCC are more likely to harbor *TP53* mutations, this may have clinical implications. For example, a randomized control trial of aggressive B-cell lymphomas found that patients with a *TP53* mutation were less likely to experience complete remission than those patients without a *TP53* mutation (61.9% versus 79.9%, p=0.007).[248] A study of head and neck squamous cell carcinoma (HNSCC) found that patients with *TP53* mutations that they classified as "high-risk" (using a previously validated computational approach) were 10-fold more likely not to respond to cisplatin-based therapy.[249] As cisplatin is used in SCC, investigating which patients are more likely to have *TP53* mutations may help individualize treatment for each patient.

BRCA2 mutations, while in a small proportion of overall tumors, were present in significantly more immunocompromised patients (17.1%) than immunocompetent patients (0%) (p=0.038, Bonferroni-corrected p>0.05). As only somatic variants were included in this study, the possibility of *BRCA2* mutations arising from particular immunosuppressants should be explored. A study of prostate cancer that used next-generation sequencing along with T-cell receptor (TCR) signaling found that in *BRCA2*-mutated tumors the CD8/FOXP3 ratio was lower (p=0.1), suggesting a possible association of *BRCA2* mutations with an immunosuppressed tumor microenvironment.[250]

In contrast to *BRCA2* mutations, *MLL4* mutations were present in 44.2% (19/43) of immunocompetent patients and 0% (0/27) of immunosuppressed patients (p<0.0001, Bonferroni-corrected p<0.003). *MLL4* (also called *KMT2D*) is known for its role as a histone H3 lysine 4 (H3K4) monomethyltransferase.[251] Few studies have examined the association between genes responsible for methylation and the tumor microenvironment;

however, a study analyzing tumor-draining lymph nodes demonstrated that a higher level of methylation was found in Th-2 skewed cells relative to Th-1 skewed cells, suggesting that methylation may be used to create an immunosuppressive environment to promote tumor growth ($p<0.0001$). In SCC, the lack of *MLL4* mutations in immunosuppressed patients may reflect the already immunosuppressive environment that is present to allow the tumor cells to grow, in contrast to immunocompetent patients, which would require a more immunosuppressive tumor microenvironment for SCC development. Additional studies would be useful to further investigate this association.

In our study, *COL4A1* mutations were found in 22.7% (5/22) of older patients and 56.3% (9/16) of younger patients who had *COL4A1* investigated ($p=0.037$). *COL4A1* encodes the alpha-1 subunit of type IV collagen found in the basement membrane of skin and has been shown to inhibit angiogenesis and tumor growth in vivo.[252] Using an in vitro model of melanoma, Kaur *et al*. (2019) demonstrated that changes in the aging skin extracellular matrix (ECM) can influence the tumor microenvironment and enhance the metastatic ability of tumors.[253] It is likely that in younger patients, collagen mutations disrupt a strong ECM, whereas older patients have an ECM structure that is already permissive for tumor growth. The importance of collagen mutations in SCC is illustrated by patients with recessive dystrophic epidermolysis bullosa (RDEB), a rare genetic skin disease characterized by systemic blistering due to *COL7A1* mutations. RDEB patients develop SCC early in life (67.8% by age 35 and 90.1% by age 55) which tend to be severe and oftentimes lethal.[254]

This study has several limitations. While there were thousands of gene mutations identified, this manuscript was only able to focus on a relatively small percent of the total number of mutations. However, we addressed this systematically to ensure that the mutations focused on were some of the most relevant. Another limitation is that this

manuscript focused on mutations in a binary way (e.g. mutated or not), however, each mutation is different and there are subtypes of mutations that were not appreciated in detail due to the broad scope study. Further, not all histological parameters were available for each individual tumor. For example, perineural invasion was often missing, which was found to be correlated with *FGFR2* mutations in a sequencing study by Zilberg *et al*. (2017).[184] The absence of some of this information is a clear limitation of this study.

Additionally, the sequencing studies in this review were all performed at different institutions and some with different panels and coverage. While this was a systematic review, we were unable to perform a true meta-analysis due to high between-study heterogeneity. While the heterogeneity of these studies made it impractical to perform multivariate analysis, we recognize the relative scarcity of metastatic SCC sequencing data in the literature and aimed to perform appropriate quantitative as well as qualitative analyses to contribute valuable information to the literature.

While this study has definite limitations, we identified genes mutated in a large percent of SCC samples, many of which have not been investigated in SCC. Further, patient characteristics such as age, immune status, and sex were hypothesized to be correlated with specific mutations. Many of the genes discussed have clinical relevance as therapeutic targets, predictors of therapeutic response, or for patient risk-stratification. Ultimately, we aim for these results to improve patient outcomes and inspire further research.

Chapter 6: Combining immunohistochemistry (IHC) and with mutational analysis in metastatic SCC

6.1 The correlation between immunohistochemistry findings and metastasis in SCC: A Review

This subchapter is published in Dermatologic Surgery and included (with permission): Lobl M, Grinnell M, Phillips A, Abels J, Wysong A. The Correlation Between Immunohistochemistry Findings and Metastasis in Squamous Cell Carcinoma: A Review. Dermatol Surg. 2020 Nov 3.[80]

Introduction

Cutaneous squamous cell carcinoma (SCC) is the second most common type of skin cancer after basal cell carcinoma (BCC), and the incidence has been steadily increasing.[3] Compared to BCC, SCC has increased potential for both regional and distant spread. Lymph node metastasis in SCC is estimated to occur in approximately 2-5% of cases.[127,139,187] Differentiating which SCCs will be cured with surgery alone from those that will metastasize is of great clinical importance, yet current methods are imperfect. There are several clinical and pathologic criteria that allow for differentiation of high-risk and low-risk SCCs. Features of high-risk SCCs that indicate an increased risk of metastasis include size >2 cm, depth of invasion > 4-5 mm or beyond subcutaneous fat, poor differentiation, perineural invasion, angiolymphatic invasion, anatomic location of the primary tumor on the ear, mucosal surface, site of previous inflammation or scar, and host immunosuppression.[118,121,123,255,256] However, the utility of immunohistochemistry (IHC) in risk stratifying patients with SCC has not been fully explored.

IHC is a commonly used technique by pathologists in the diagnosis of neoplastic skin lesions. While hematoxylin and eosin (H&E) is the most commonly used stain, there are a variety of IHC stains that may also have utility in the diagnosis of these lesions. Clinical outcomes, such as metastasis, have been reported to be associated with specific patterns of expression of individual IHC markers. This chapter aims to synthesize this data in order to explore the clinical utility of IHC in prognosticating patient outcomes and to suggest effective therapeutic strategies. The methods are described (Chapter 2.5).

Results and Discussion

Thirty-one studies described potential prognostic associations of IHC findings and metastasis (or high-risk features) in SCC. Many markers were only supported by a single study; however, several markers had multiple studies providing evidence for its use (see Appendix 1, Table S1, which provides details about these studies). Markers with only one study supporting their use are also detailed (see Appendix 2, Table S2, which provides details about these studies). Staining quantification and thresholds are described for all studies (see Appendix 3, Table S3, which describes quantification for each study).

Epithelial-Mesenchymal Transition (EMT) Markers

E-cadherin

E-cadherin is a member of the cadherin superfamily and is mainly found in epithelial tissues.[257] The interaction of the E-cadherin cytoplasmic domain with *β*-catenin maintains cell-cell contact and activates alpha-catenin, which links E-cadherin to the actin cytoskeleton.[258] Loss of membranous E-cadherin expression is a hallmark of the epithelial-mesenchymal transition (EMT). The EMT is characterized by the loss of

epithelial characteristics and the adoption of a mesenchymal phenotype that is known to impact tumor progression in many cancers.[259,260] Hypermethylation of *CDH1*, the gene encoding E-cadherin, has been suggested to impact patient outcomes of several cancers including esophageal cancer, invasive ductal breast cancer, and head and neck squamous cell carcinoma (HNSCC).[261–263] In SCC, there is evidence for a role of E-cadherin in tumor metastasis. Toll *et al*. (2013) found an increase in nuclear E-cadherin expression in primary metastatic SCC compared to primary localized SCC (65.3% (32/49) and 19.6% (10/51) of samples, respectively, $p<0.001$).[264] Hesse *et al*. (2016) found that membranous E-cadherin expression was significantly down-regulated in metastases compared to primary SCC ($p=0.031$).[161] This data suggests that nuclear E-cadherin is upregulated in metastatic SCC, while membranous E-cadherin is downregulated.

As there is strong evidence for the loss of membranous E-cadherin in tumor metastasis, therapeutic approaches are being explored. One such approach is to epigenetically target hypermethylated *CDH1*. A preclinical study using liver cancer cell lines found that epigenetic modifying compounds 5-aza-2'-deoxycytidine (5aza-DC) and pan-deacetylase inhibitor Trichostatin A (TSA) upregulated E-cadherin in vitro.[265] Additional research into epigenetic therapeutics involving this pathway may help identify novel adjuvant agents for use in high-risk SCC.

Podoplanin

Podoplanin is recognized for its ability to aggregate platelets and promote tumor metastasis, likely due to its pro-adhesion properties.[266] Podoplanin interacts with C-type lectin-like receptor 2 (CLEC-2) under pathological conditions.[267] In tumors cells, podoplanin binds to platelet CLEC-2, which activates platelets and has been shown to promote hematogenous metastasis.[267] A retrospective cohort study of metastatic SCC

performed by our group found that 53% of cases had angiolymphatic invasion.[123] Four studies have discussed the association of podoplanin expression with SCC metastasis. Canueto *et al*. (2017) determined that moderate/intense podoplanin staining correlated with a higher risk of nodal metastasis.[268] Primary tumors that didn't metastasize had moderate/intense podoplanin expression in 16.0% (13/81) of cases, while primary tumors that metastasized had moderate/intense expression in 46.2% (6/13) of cases (p=0.02).[268] In addition, patients with moderate/intense podoplanin staining had a shorter latency to developing lymph node metastases (4.5 months with moderate/intense podoplanin expression, 11 months with absent/weak podoplanin expression, p=0.008). Hesse *et al*. (2016) found that podoplanin was expressed in 76%-100% of the tumor in only 5.0% (4/80) of non-metastatic SCCs, while this degree of expression was found in 27.3% (6/22) of metastatic SCCs (p=0.04).[161] It was confirmed in a multivariate analysis that increased podoplanin expression was significantly associated with metastasis (p=0.04).[161] Toll *et al*. (2013) also determined that podoplanin expression was both positively correlated with metastasis, as expression was positive in 10.5% (standard deviation (SD)= 20.1) of non-metastatic SCCs and 37.1% (SD=37.0) of metastatic SCCs (p=0.001).[264] Kreppel *et al*. (2012) observed that increasing podoplanin expression was positively associated with lymph node metastasis (33% vs. 17%, p=0.005) compared to non-metastatic SCCs.[269]

All studies in this review investigating podoplanin found that increased expression was positively associated with metastasis, making it a potential prognostic biomarker in primary SCC tumors. In addition to serving as a potential biomarker, the podoplanin-CLEC-2 interaction may also serve as a possible target for cancer therapy, particularly for metastatic or high-risk cases. A pre-clinical study investigated the effect of a podoplanin inhibitor, SZ168, on the growth of pulmonary metastasis in melanoma, and it was shown to block the podoplanin-CLEC-2 interaction, preventing metastases.[270]

Given the strong role of EMT in tumor metastasis, therapeutics that can act on multiple targets in this process may be clinically useful in treating high-risk SCC. Metformin, a widely prescribed drug for type II diabetes, is being investigated in many cancers for its ability to inhibit multiple players in the EMT process. In recent preclinical studies of breast cancer, metformin downregulated EMT markers ZEB1, TWIST1, SNAIL, and Vimentin.[271,272] Further investigation of this agent, and others that target the EMT, as a preventive or therapeutic agent in high-risk SCC may be worthwhile.

Immune System Markers

CD8+ Cells

CD8+ cytotoxic T cells have the ability to induce apoptosis in cancer cells and are generally associated with a favorable patient prognosis. In SCC arising in transplant patients, which can be more aggressive, the Treg to CD8+ T cell ratio has been shown to be significantly higher and the average CD8+ T cell infiltrate significantly lower than in SCC arising in immunocompetent patients.[273] Azzimonti *et al*. (2015) analyzed tumors by grade and found that peritumoral CD8+ T cell infiltration was increased in low grade SCC tumors compared to high grade tumors (40.0% versus 30.5% positivity, respectively). However, this trend did not reach statistical significance.[274] CD8+ T cell counts have correlated with the prognosis of cancers of other organ systems, and this approach for cutaneous SCC requires further study.[275]

PD-L1

Programmed death-ligand 1 (PD-L1) interacts with inhibitory checkpoint molecule programmed cell death protein 1 (PD-1) to help tumors evade immune surveillance. Five studies discussed the relationship between PD-L1 expression and metastasis. Garcia-Diez *et al*. (2018) described positive PD-L1 expression in 26.0% (13/50) of primary non-

metastatic SCCs and 50.0% (23/46) of primary metastatic SCCs (p=0.02).[276] It was determined that PD-L1 expression was associated with an increased risk of metastasis, which was confirmed in a multivariate analysis (p<0.05).[276] Using a multivariate analysis controlling for clinicopathological factors, Kamiya *et al*. (2018) found that high intensity PD-L1 staining was an independent risk factor for lymph node metastasis (odds ratio (OR)= 22.6, p=0.009).[277] Garcia-Pedrero *et al*. (2017) observed that tumors with PD-L1 positivity ≥25% were at a significantly increased risk of metastasis compared to tumors with less than 25% positivity (6.54 Adjusted Hazard Ratio (HR), 95% confidence interval (CI) 2.28-18.78).[278] Amoils *et al*. (2019) found that primary tumors were more likely to have grade 1 PD-L1 staining (5-10% positivity), whereas metastases were more likely to have grade 2 PD-L1 staining (10-25% positivity) (grade 1 staining in 65% (20/31) of primary tumors, grade 2 staining in 46% (24/52) of metastases).[279] Slater *et al*. (2016) investigated PD-L1 expression in high-versus low-risk SCCs, as well as metastases.[280] It was determined that PD-L1 expression was present in 20% of low-risk tumors (4/20), 70% of high-risk tumors (14/20), and 100% of metastases (5/5).[280] All five studies support a higher percentage of cells positive for PD-L1 and higher intensity of PD-L1 staining correlating with an elevated risk of metastasis.[276–280]

PD-L1 is known to suppress the immune system, and therefore, it follows that tumors with higher and more intense expression would have a higher risk of metastasis. Inhibitors of PD-L1 have been developed in recent years and have improved survival in several cancers including non-small cell lung cancer and melanoma.[281,282] In 2018, the PD-1 inhibitor cemiplimab received FDA approval for advanced SCC. Cemiplimab proved to be efficacious, with 47% of patients with metastatic disease responding and 57% of responses exceeding 6 months.[283] High PD-L1 expression is typically associated with high response rates to PD-L1 inhibitor therapy, however, this association has not held true in all studies and is still a matter of debate.[284–286] Nevertheless, there is an

overall association of high PD-L1 expression with improved response to PD-L1-targeted therapy.

Cellular Proliferation and Replication

EGFR

Epidermal growth factor receptor (*EGFR*) induces cellular proliferation and differentiation when activated.[153] Four studies discussed the association of EGFR and metastasis. Ch'ng *et al*. (2008) determined that EGFR overexpression (3+ staining) was present in 36.0% (9/25) of primary non-metastatic SCCs and 79.0% (11/14) of primary metastatic SCCs.[287] EGFR overexpression was an independent prognostic factor for metastasis in multivariate analysis (p=0.05).[287] Canueto *et al*. (2017) also found that EGFR overexpression (3+ staining) was a prognostic factor for metastasis in logistic regression analysis (OR=7.1, p=0.004).[81] Shimizu *et al*. (2001) investigated the degree of staining positivity of EGFR in SCC and found that 80% (4/5) of primary SCCs demonstrated weak, focal EGFR positivity (+), while 80% (4/5) of metastases were strongly positive (+++).[82] Sweeny *et al*. (2011) found no correlation of EGFR expression with either metastasis or overall survival, with EGFR overexpression (2+ or 3+ staining) found in 56.0% (28/50) of primary tumors and 58.3% (7/12) of regional metastases.[288] When comparing primary non-metastatic SCCs to primary metastatic SCCs, high EGFR expression was observed in 53.8% (14/26) and 58.3% (14/24) of cases, respectively, which was not statistically significant (p>0.05).[288] One possible explanation for the differences in results seen in this study compared to other studies in the literature is that only advanced stage tumors (TNM stages 3 and 4) were included in this particular study. Overall, all studies except for Sweeny *et al*. (2011)[288] found that higher expression of EGFR was correlated with an elevated risk of lymph node metastasis.[81,82,287]

EGFR has been used as a marker of poor prognosis in several other cancers including esophageal squamous cell carcinoma, breast cancer, and gliomas.[289–291] Overexpression of EGFR in SCC has been established and the EGFR inhibitor cetuximab is currently used to treat advanced SCC. In addition, other EGFR therapies are being investigated in SCC. In a study by Foote *et al*. (2014), panitumumab (monoclonal antibody against EGFR) showed some efficacy (31% objective response rate) in a small study (n=16) of patients with incurable SCC.[168] The small molecule inhibitor of EGFR erlotinib has also been evaluated in advanced SCC, however, responses were not robust enough to warrant use or further investigation.[94] Another EGFR inhibitor, dacomitinib, is currently in phase II clinical trials for use in SCC. EGFR expression is another measure that may be useful in both predicting lymph node metastasis and response to future innovative therapies. A study by Pirker *et al*. (2012) found that lung cancer patients with high EGFR expression had an increased overall survival than patients with low EGFR expression when treated with cetuximab plus chemotherapy versus chemotherapy alone (p=0.011 high expression versus p=0.88 low expression).[292] It is thought that patients with high EGFR expression are more likely to develop a rash while on EGFR inhibitor therapy.[293] However, the development of a rash while on cetuximab or erlotinib therapy has been associated with a higher response to treatment and better survival.[294–297]

Cyclin D1

Cyclin D1 expression is frequently altered in human cancers and is hypothesized to act either independently under hormone regulation or by modifying cyclin-dependent kinase (CDK) activity to promote continuous proliferation.[298–300] Huang *et al*. (2012), described strongly positive (3+) expression of Cyclin D1 in 15.2% (5/33) of primary non-metastatic SCCs and 33.3% (3/9) of primary metastatic SCCs with cyclin D1 positively

associated with both depth of invasion and metastasis ($p<0.05$).[301] Utilizing a grading system based on the percentage of cells with positive staining, Mastoraki *et al*. (2009) found an inverse correlation between cyclin D1 expression and tumor differentiation, supporting the findings by Huang *et al*. (2012).[302] Overall, the literature surrounding Cyclin D1 and SCC suggests a correlation between increased Cyclin D1 expression and a more clinically aggressive phenotype.[302] Cyclin D1 expression may be of clinical interest, as cyclin kinase inhibitors are under investigation as therapeutic agents in several cancers. Li *et al*. (2015) performed targeted sequencing of 504 cancer-associated genes and found CDK4 mutations in 14% of lymph node metastases from SCCs, suggesting a potential role for CDK inhibitors in SCC.[66] A study by Cornell *et al*. (2019) investigated CDK4/6 inhibitor palbociclib in breast cancer cells and found that cells resistant to this drug had high levels of Cyclin D1 expression, highlighting the potential clinical importance of determining Cyclin D1 expression.[303]

Special Considerations: Immunocompromised patients

Immunocompromised patients are at an elevated risk of developing SCC. Organ transplant recipients (OTRs) are at an especially high risk, estimated at 65-100 times the risk of the general population.[304,305] Further, OTRs tend to develop particularly aggressive tumors and have an increased risk of developing lymph node metastasis.[140] As seen in this review, OTR SCCs had decreased CD8+ T cell infiltrate when compared to immunocompetent patient SCCs, highlighting the decreased immune-mediated killing of tumors cells that occurs in OTRs. With respect to treatment options for this population, additional precautions and studies are needed. While effective in many patients, immunotherapies cannot be safely used in all patients. In several case reports of immunocompromised patients, PD-L1/PD-1 inhibitors have caused acute graft rejection and graft failure.[306–308] However, a recent review (2019) demonstrated that graft rejection

was not the most common cause of death for OTRs treated with immunotherapies, thus it is not an absolute contraindication to receive immunotherapy.[309] IHC may play a role in investigating additional safe and efficacious therapeutic targets for this population.

Conclusions

Contemporary guidelines for staging and risk stratifying tumors are clinical and pathologic. Currently, pathologic guidelines are limited to H&E evaluation. While AJCC8 and BWH staging systems are an improvement over earlier versions of staging systems, a 2019 study evaluating both of these systems found that the sensitivity (true positive rate) of the AJCC8 and BWH systems to predict nodal metastasis and disease-specific death are 0.78 and 0.73, respectively.[122] Given the continued difficulty in determining which SCCs will metastasize, the use of IHC to help with prognosis and patient risk-stratification may be an appropriate, cost conscious option in select cases. Several markers in this review had multiple studies confirming an association with metastasis, including E-cadherin, podoplanin, CD8+ T cells, PD-L1, EGFR, and Cyclin D1. As systemic therapies are most often used in metastatic SCC cases, this review also highlights current and potential therapeutic targets for SCC. While targeted therapies have changed the therapeutic landscape of SCC, selecting patients who are most likely to benefit from therapy remains challenging. The findings in this review suggest that IHC may play a role in selecting the optimal therapy for each patient.

6.2 LRP1B expression is correlated with age and perineural invasion in metastatic cutaneous squamous cell carcinoma: A pilot study

Introduction

Cutaneous squamous cell carcinoma (SCC) is the second most common type of skin cancer after basal cell carcinoma.[115] Over 1,000,000 cases of SCC are diagnosed in the United States annually with up to 9,000 associated deaths.[1] While only 2-5% of SCCs metastasize, those that do may carry a poor prognosis.[187,188] SCC carries one of the highest mutational burdens of all known cancers. Mutations in *TP53, NOTCH*, and *CDKN2A,* many of which are ultraviolet radiation (UV)-induced, are well-known driver mutations in SCC.[55,64,75] *LRP1B* is a member of the low density lipoprotein (LDL) receptor family and is deleted in many malignancies.[310–313] A recent study published by our group indicates that *LRP1B* is mutated in 37% of all SCCs, making it one of the top 6 genes mutated in all SCCs.[314] Consistent with its proposed tumor suppressor function, *LRP1B* expression is reduced in several types of cancers through changes including *LRP1B* promoter methylation, histone de-acetylation, copy number loss, and homozygous deletions.[315–317] These findings and other evidence suggest *LRP1B* acts as a tumor suppressor gene. However, the biological significance of *LRP1B* mutations in SCC is not known. This study aims to evaluate LRP1B expression in a cohort of metastatic SCCs to understand its potential role in tumor progression.

Results

The characteristics of the SCC patients analyzed in this study are illustrated (**Table 16**). LRP1B expression was widespread in SCCs compared to normal epidermis. Expression in normal skin was restricted to a layer of basal keratinocytes and appeared

largely cytoplasmic (**Figure 25A**). Primary metastatic SCC and SCC invading a lymph node stained positively for LRP1B (**Figure 25B, C**). One tumor that exhibits both well and poor differentiation demonstrates weak (1+) LRP1B staining in the well-differentiated region, and moderate/strong (2+/3+) LRP1B staining in the poorly differentiated region (**Figure 25D**). An additional primary metastatic SCC tumor demonstrates staining of tumor cells, in contrast to keratin, which stains negative (**Figure 25E**). A localized, non-metastatic SCC was noted to stain negatively, furthering our hypothesis of stronger LRP1B staining correlating with increasing tumor aggressiveness (**Figure 25F**). The no primary antibody controls were negative with the exception of some non-specific staining in the basal layer of the normal epidermis (**Figure 25 G, H**).

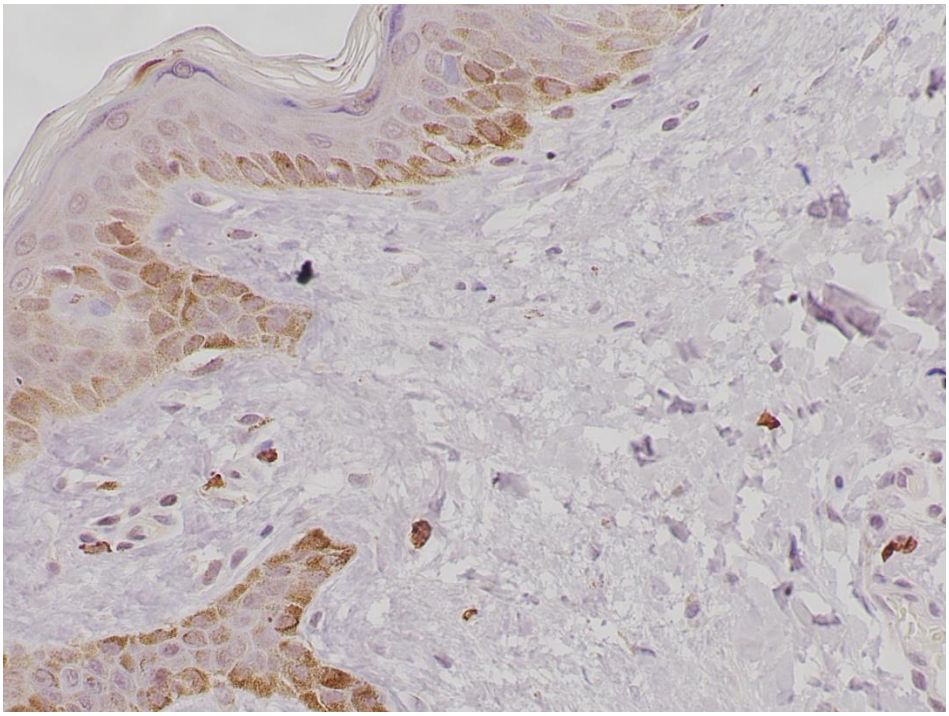
The H-Score is a measure of tumor positivity that takes into account the intensity of the staining and the percent of the tumor with each degree of intensity. There was a positive correlation between age and H-Score (**Figure 26**, R^2=0.44, linear regression p=0.01). There was also a correlation between perineural invasion (PNI) and higher LRP1B expression with a mean H-Score for tumors without PNI of 102 and 161 for tumors with PNI (p=0.03). There was no difference between primary metastatic SCCs and metastases with respect to LRP1B expression (paired samples t-test, p>0.05).

Table 16: Cohort characteristics for LRP1B IHC study

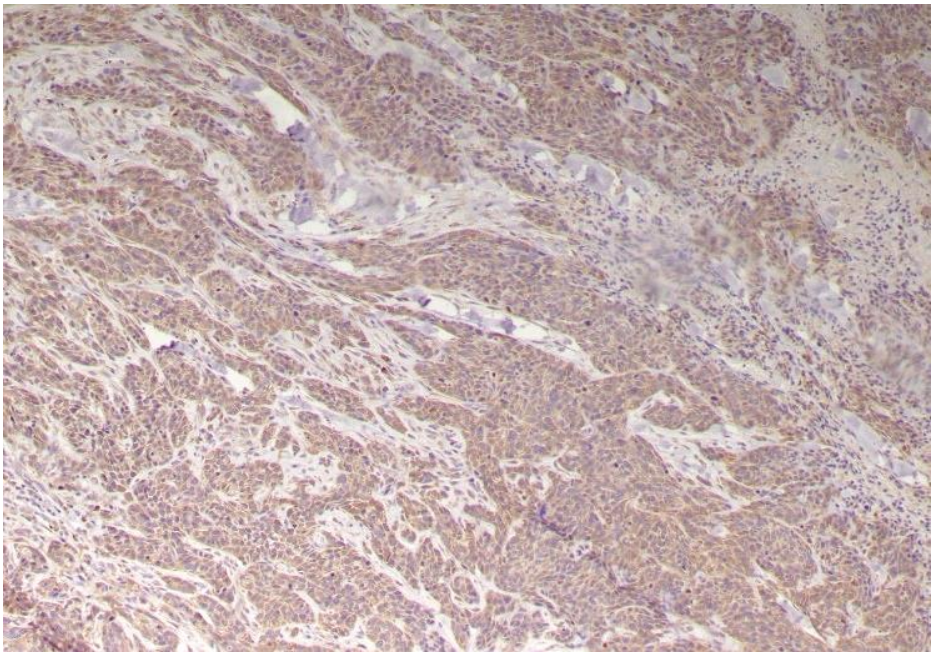
Specimen #	**Age**	**Sex**	**Location**	**H-Score**
1	76	Male	Preauricular area	40
2	62	Male	Lip	40
11	72	Male	Postauricular area	65
10	74	Male	Nose	72
12	57	Male	Temple	73
14	68	Male	Cheek	98
5	77	Male	Temple	98
3	94	Male	Cheek	120
9	86	Male	Cheek	130
7	76	Male	Scalp	135
8	85	Male	Ear	143
4	83	Male	Upper arm	146
6	93	Male	Cheek	180
13	80	Male	Temple	200

Figure 25: LRP1B IHC

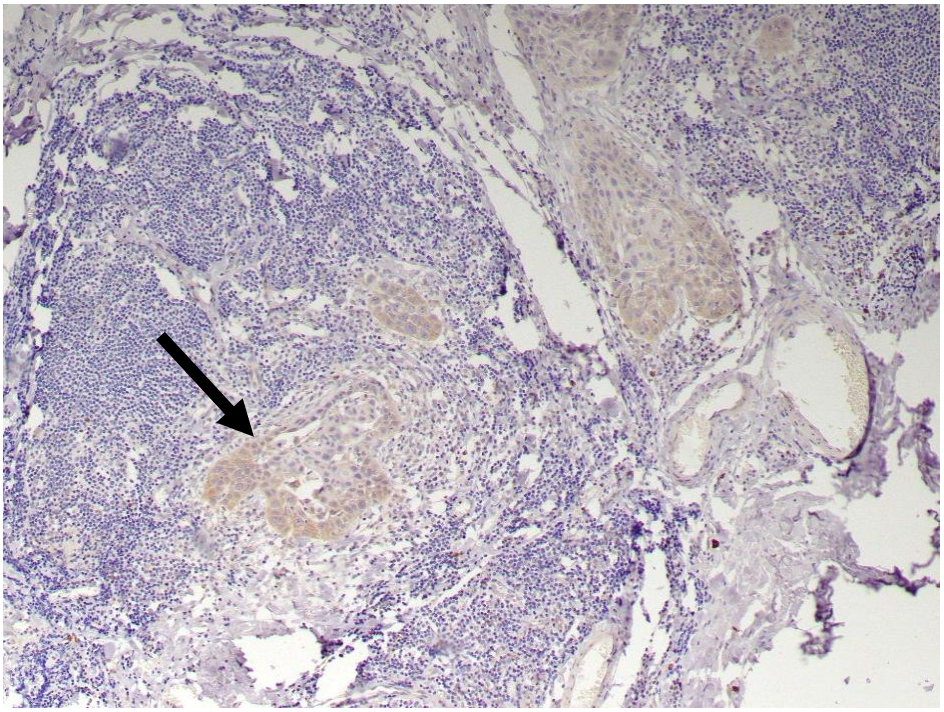
A



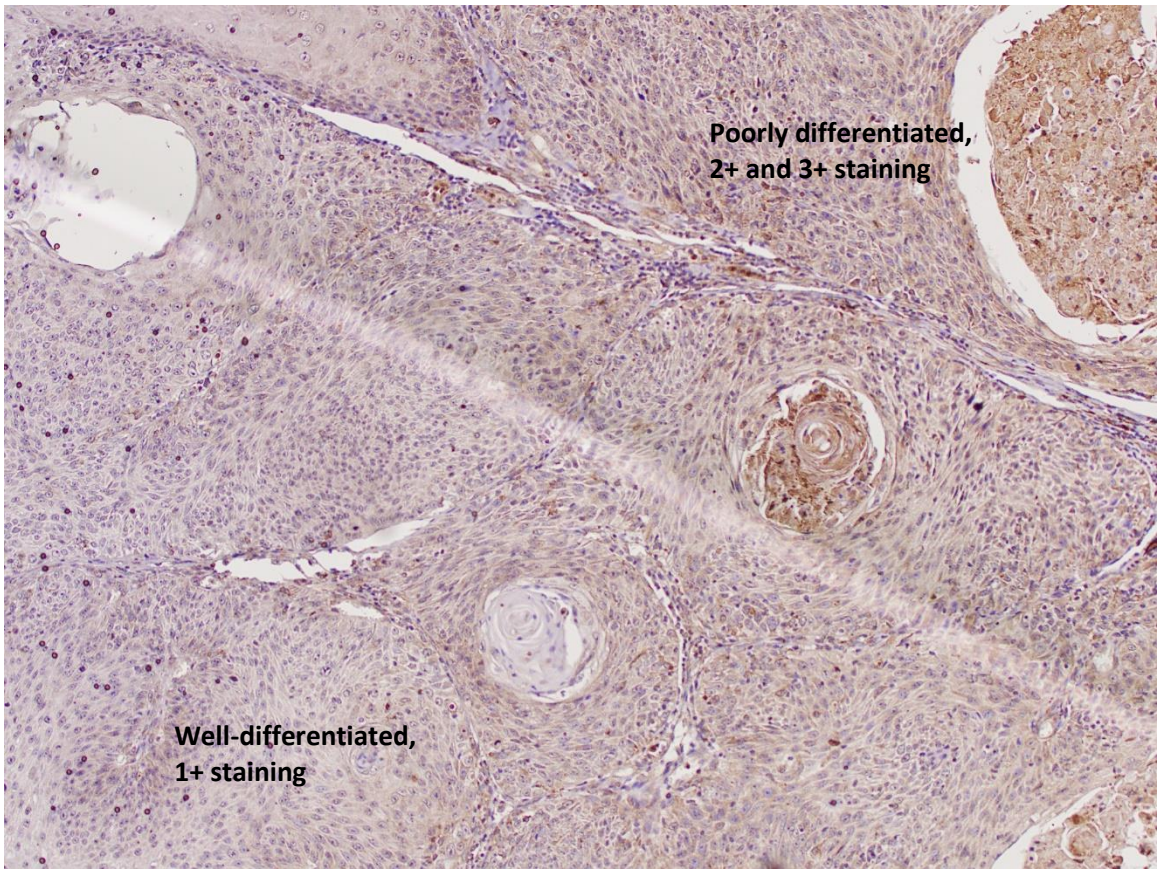
B



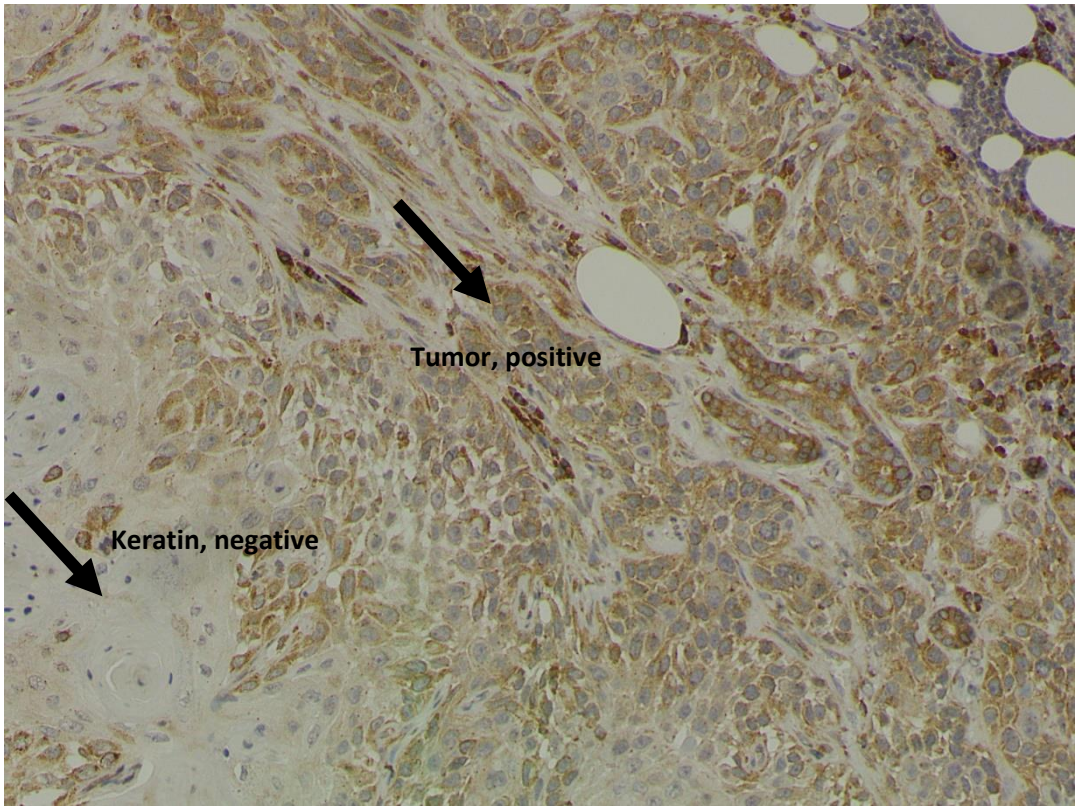
C



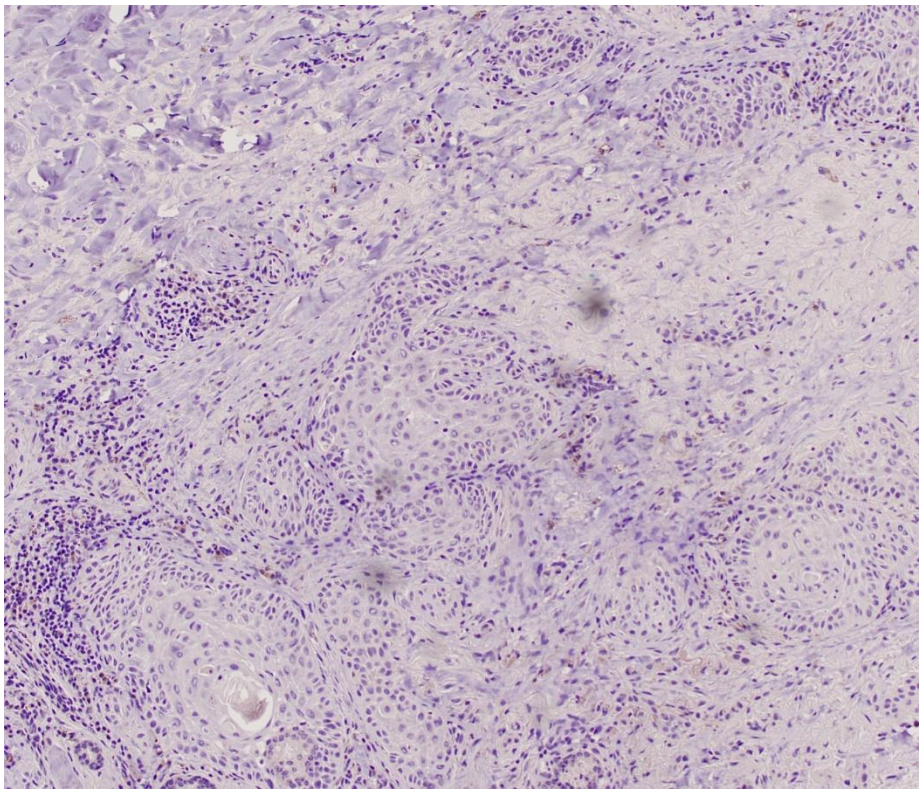
D


Poorly differentiated,
2+ and 3+ staining
Well-differentiated,
1+ staining

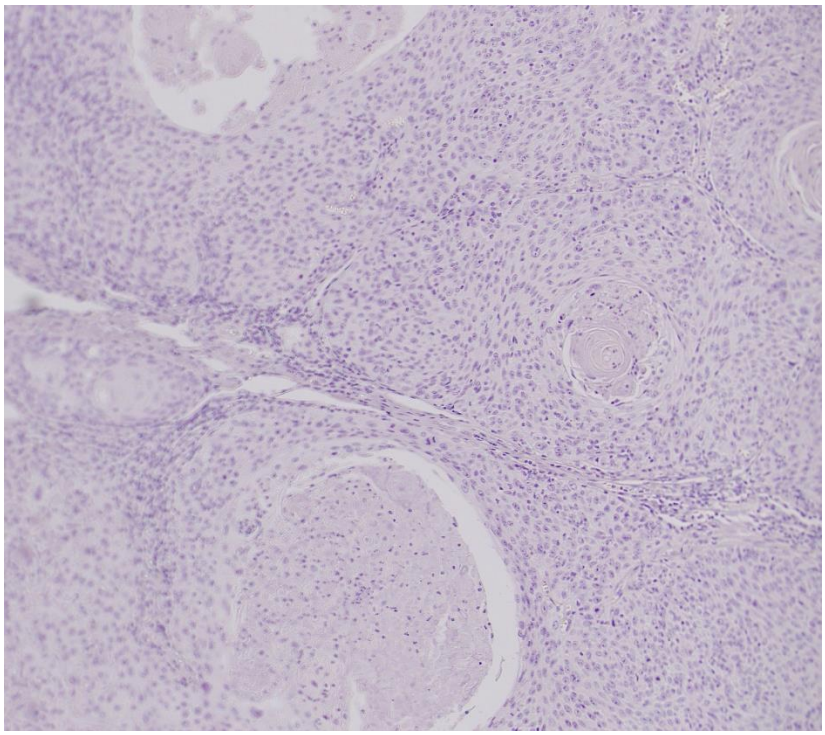
E



F



G



H

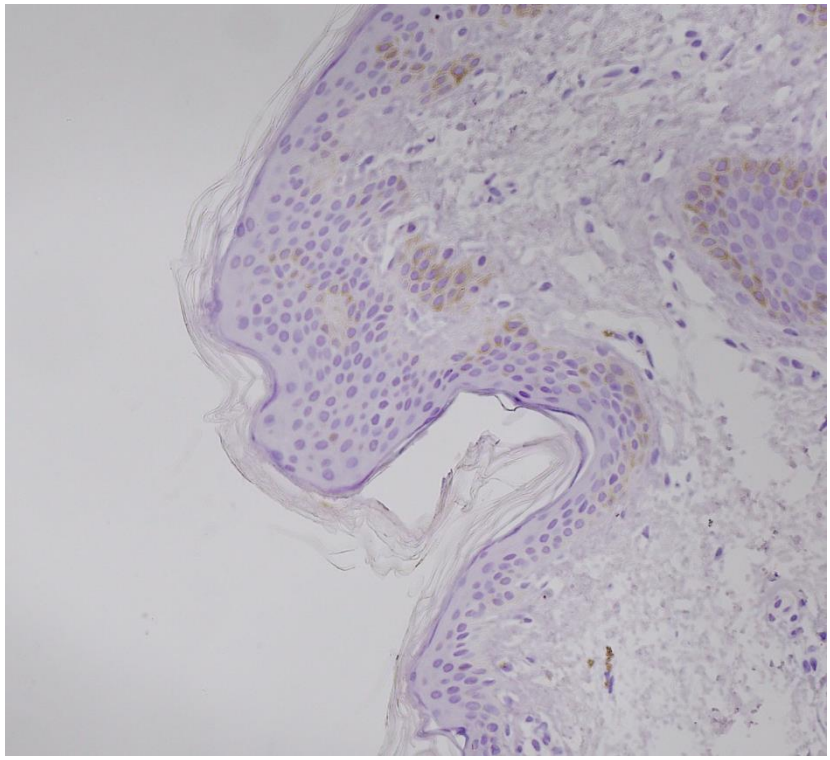


Figure 25A illustrates cytoplasmic LRP1B staining in the normal epidermis, 40x magnification. Figure 25B illustrates positive cytoplasmic LRP1B staining of a primary metastatic SCC, 10x magnification. Figure 25C illustrates positive cytoplasmic LRP1B staining in SCC that invaded into a lymph node, the arrows indicates tumor within node, 10x magnification. Figure 25D illustrates a SCC with well-differentiated characteristics (left) with 1+ LRP1B staining, the tumor becomes poorly differentiation (right side of image) and staining becomes 2+ and 3+, 10x magnification. Figure 25E illustrates a primary metastatic SCC with positive tumor cells and negatively staining keratin, 20x magnification. Figure 25F illustrates a localized SCC tumor negative for LRP1B, 10x magnification. Figure 25G illustrates the negativity observed in the no primary control, 10x magnification. Figure 25H illustrates normal epidermis tissue stained as a no primary control, 20x magnification.

Figure 26: LRP1B H-Score and Age

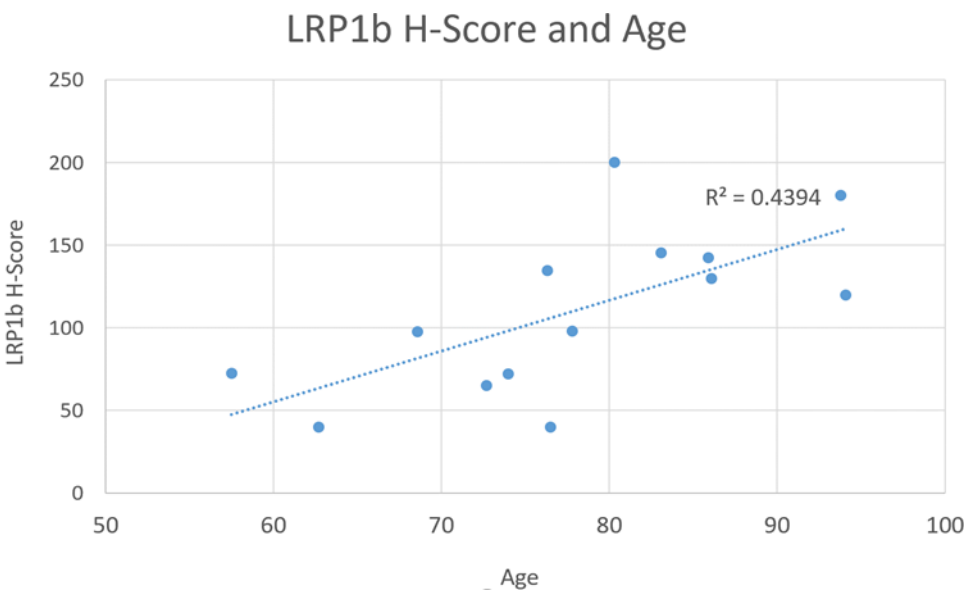


Figure 26 illustrates the positive linear correlation of LRP1B H-Score and Age.

Discussion and Conclusions

While mutated in 37% of SCCs, this is the first study to look at protein expression of LRP1B in SCC. To better understand the functional relationship between *LRP1B* mutations and protein expression, we searched the literature for data on LRP1B gene expression in SCC. An RNA sequencing study by Chitsazzadeh *et al*. published gene expression for patient samples of normal skin, actinic keratosis, and SCC. There was no difference in LRP1B expression with respect to age in SCC (R^2 <0.10). Also, there was no difference in LRP1B mRNA expression when compared between normal skin (n=7), AKs (n=10), and SCCs (n=9) (p=0.2, one way ANOVA).[318] However, this data should be interpreted with caution as it represents a small sample size and because our cohort consists of metastatic SCCs, which behave very differently from the more common, low-risk SCCs.

The association of age and LRP1B status has not been thoroughly investigated in the literature with the exception of several recent studies. One study of ovarian cancer by Zhang *et al*. (2021) found that *LRP1B* mutations were statistically associated with age of the patient, with older patients (60+) having a higher prevalence of *LRP1B* mutations (p=0.04), supporting the findings from our study.[319] On the other hand, a study of hepatocellular carcinoma found an inverse association between age and LRP1B expression (p=0.04). However, the age cut-off used in this study was 50 and the underlying causes of HCC may vary significantly between age groups.[320]

It has been demonstrated that ultraviolet radiation (UV-R) causes cancers with the highest tumor mutational burdens (TMBs).[321,322] *LRP1B* mutations have been associated with higher TMBs in several cancers.[319,323] A study based off of The Cancer Genome Atlas (TCGA) investigated mutational signatures in cutaneous melanomas and

found that *LRP1B* was one of ten genes that served as a surrogate for UV-R damage.[324] As cumulative UV-R exposure increase with age, the association of age and LRP1B expression that we observed may be due to the subsequent increase of TMB seen with longer lifetime UV-R damage. It is uncertain as to whether *LRP1B* mutations are associated with high TMBs due to the very large size of the gene increasing the likelihood that it is mutated in a tumor with many mutations or if there is biological function of *LRP1B* that leads to the development of an increased number of mutations in a particular tumor. One such hypothesis supporting the biological function theory points to the highly inflammatory microenvironment of *LRP1B*-mutated tumors, suggesting that this modulation is permissive for additional mutations.[317]

A majority of studies in the literature suggest a tumor-suppressive role of *LRP1B* in cancer, with low levels of expression correlated with tumorigenesis and in some cases, a worse prognosis.[325,326] This study observed increased expression with perineural invasion, a feature indicative of aggressive SCCs, as well as increased expression from well to poor differentiated regions of SCC. This is the first study to report an association of LRP1B overexpression with perineural invasion. However, this has been observed with LRP-1, which shares 60% of the same amino acid residues with LRP1B. An in vitro study in renal interstitial fibroblasts demonstrated that matrix metalloproteinase-9 (MMP-9) is overexpressed downstream of LRP1 activation through an ERK-dependent pathway.[327] The increased activity of MMP-9 may lead to the degradation of collagens, which are components of Schwann cell basement membrane. This may be the mechanism by which tumors with LRP1B overexpression are able to invade into the nerve sheath.[328]

Supporting the possibility of LRP1B activation rather than silencing as pathogenic, a recent study (2020) of hepatocellular carcinoma (HCC) performed IHC on samples and found that LRP1B had increased expression in HCC, similar to findings in

this study.[320] Another study of rhabdomyosarcoma found that gains and overexpression of LRP1B were associated with MycN amplification, which is typically associated with tumor progression and poor outcomes.[329,330] Overexpression of very closely related member LRP1 has been associated with a poor prognosis in renal cell carcinoma.[331] Overall, these data suggest that expression of LRP1B may be cancer-specific, and additional research is needed to further investigate these findings.

This study observed predominately cytoplasmic LRP1B staining in primary metastatic SCCs. Due to the transmembrane nature of the LRP1B protein, membranous staining would be expected. However, LRP1B has a canonical nuclear localization sequence (KRKRRTK) and can also undergo regulated intramembrane proteolysis (RIP), a process in which intramembrane proteases perform a second protein cleavage that releases an active cytoplasmic fragment to allow for nuclear localization.[332,333] While this current study was not powered to detect statistical differences between cellular staining patterns, future studies with larger sample sizes to investigate the relationship between LRP1B cellular localization and clinical characteristics in SCC would be interesting.

This pilot study has several limitations. While the mutation rate of *LRP1B* in SCC is high, the functional consequences of these mutations have yet to be determined. The high expression observed in this study may have the same outcome as absence of expression, which may depend on the domain affected in each individual mutation. Further, the relatively small sample size in this study is too small to draw definite conclusions, and future studies with larger sample sizes are needed to validate these findings.

In summary, this study demonstrates that LRP1B has increased expression in SCC with perineural invasion and that LRP1B expression increases linearly with age in SCC. We explore hypotheses behind these findings, including the possible connection

between UV-R damage, age, TMB, and LRP1B. Future studies with larger cohort sizes should continue to investigate the relationship between LRP1B expression, patient and tumor characteristics, and response to therapy in SCC. Given that LRP1B status predicts response to immunotherapy in other cancers, and the recent approval of several immunotherapies for SCC, these studies may have significant clinical value.

Chapter 7: Anaplastic lymphoma kinase (*ALK*) promotes tumor cell features, migration, and invasion in cutaneous squamous cell carcinoma (SCC) cells

Introduction

Cutaneous squamous cell carcinoma (SCC) is the second most common skin cancer after basal cell carcinoma, and it affects approximately 1 million people in the United States annually.[1] While SCCs identified in early stages typically have good outcomes, approximately 2-5% of SCCs metastasize.[188,334] Systemic therapies for metastatic SCC may include cytotoxic regimens, radiation therapy, targeted systemic therapy, and more recently, immunotherapy.[335] Currently, the only widely used systemic targeted small molecule inhibitor therapy for SCC is cetuximab, which targets mutant *EGFR*. This treatment yields responses of up to 58% when used in combination with surgery and radiation.[83] Immunotherapies pembrolizumab and cemiplimab may be used to treat metastatic SCC and have objective response rates of 34.3% and 44% of patients, respectively.[95,96] As it is challenging to predict which patients will have a favorable response, additional targeted systemic therapies would be of significant value.

We performed targeted next-generation sequencing on patient-matched metastatic SCC primary tumors and metastases and identified *ALK* as a potential driver mutation of metastasis. A subsequent review of the SCC literature confirms *ALK* mutations reported in approximately 10-25% of all SCCs.[163,192,203] Given the relatively high number of SCC patients that may harbor an *ALK* mutation and availability of United States Food and Drug Administration (FDA) approved therapy ceritinib for *ALK*-mutated tumors, this study employed human SCC tumor cell lines to study the possible mechanistic role of *ALK* in SCC.[336]

Results

Immunohistochemistry

Primary human SCC samples archived as formalin-fixed paraffin-embedded tissue were assessed for ALK protein expression. The characteristics of the nine patients with localized SCC are described (**Table 17**). Immunohistochemistry of nine SCC samples revealed one ALK-positive sample (11.1%). This patient was a 77 year-old male with a Brigham and Women's Hospital (BWH) stage T2b tumor (high-risk). The tumor was characterized by poor differentiation and invasion into the subcutaneous fat. All patients with metastatic SCC seen at UNMC in the last 10 years were screened for ALK expression (n= 15, **Table 18**). Of the 15 lymph node metastases evaluated, 1/15 (6.7%) was positive for ALK expression. For both positive tumors, the staining pattern for ALK consists of mostly 1+ and 2+ intensity staining that was granular and cytoplasmic in nature (**Figure 27A, B**). Staining of the normal epidermis and a known ALK+ anaplastic lymphoma were performed as controls (**Figures 27C, D**). Staining of the normal epidermis was negative with the exception of some patchy, 1+ positivity along the stratum basale, which is likely non-specific and expected based on results from Veija et al. (2017) who reported negative ALK staining of the epidermis also with clone D5F3.[337] No primary control slides were negative.

Table 17: Characteristics of the nine SCC samples stained for ALK

Patient ID	**Sex**	**Age**	**Location**	**Differentiation**
1	Male	84	Scalp	Moderate
2	Male	75	Cheek	Moderate
3	Male	67	Forehead	Moderate
4	Male	62	Temple	Poor
5*	Male	77	Temple	Poor
6	Male	79	Temple	Poor
7	Male	78	Forehead	Poor
8	Male	79	Neck	Poor
9	Male	75	Scalp	Poor

*denotes patient with + ALK expression

Table 18: Characteristics of the fifteen metastatic SCC samples stained for ALK

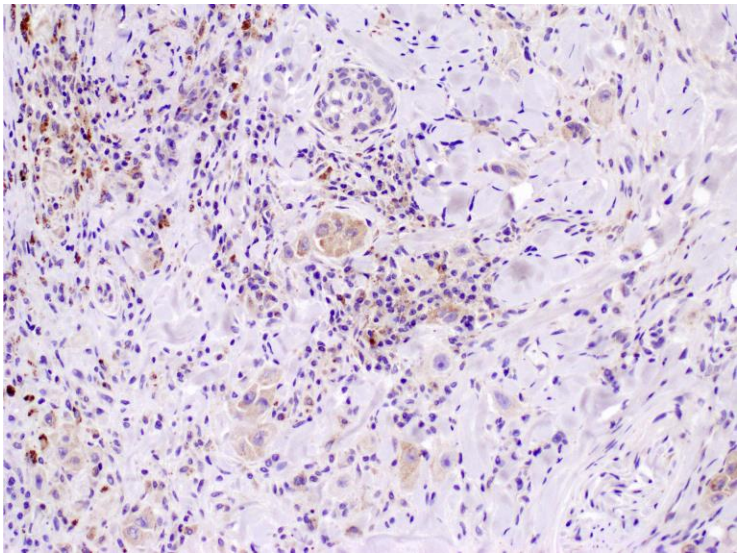
Patient ID	**Sex**	**Age**	**Primary Tumor Location**
1M	Male	83	Upper extremity
2M*	Male	42	Temple
3M	Male	77	Temple
4M	Male	68	Cheek
5M	Male	57	Temple
6M	Male	80	Temple
7M	Male	86	Cheek
8M	Male	72	Post-Auricular
9M	Male	85	Ear

10M	Male	62	Lip
11M	Male	86	Scalp
12M	Male	76	Preauricular
13M	Male	93	Cheek
14M	Male	93	Cheek
15M	Male	64	Nose

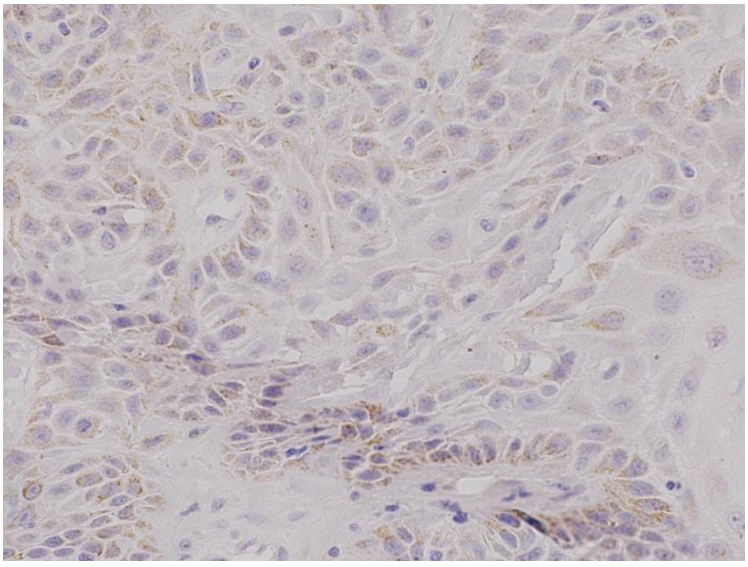
*denotes patient with + ALK expression

Figure 27: ALK IHC

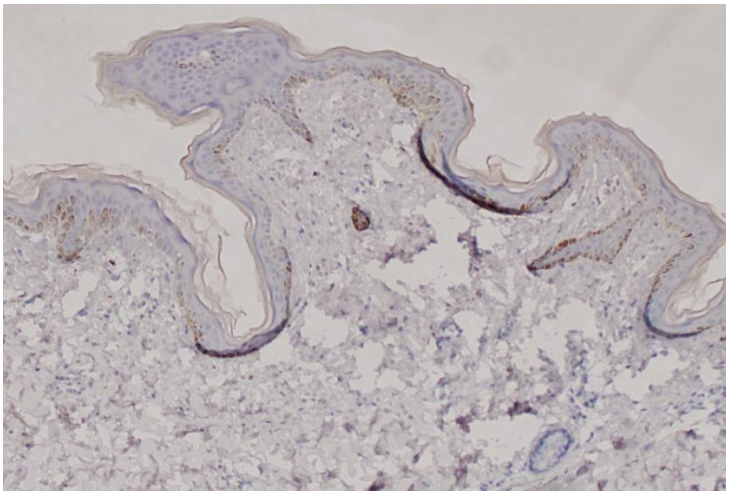
A



B



C



D

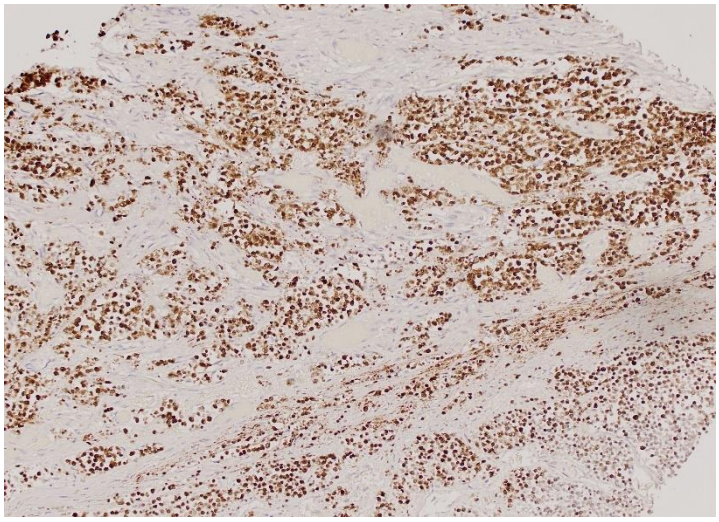


Figure 27A and B illustrates the granular cytoplasmic staining observed in ALK stained SCC. The ALK staining is brown with hematoxylin and eosin counterstaining in blue/purple. (A) localized SCC, 20x magnification and (B) metastatic SCC, 40x magnification. Figure 27C illustrates ALK staining of the normal epidermis, which is negative with the exception of some patchy staining of the stratum basale that is likely

non-specific, 10x magnification. Figure 27D represents staining of ALK+ anaplastic lymphoma as a positive control, 10x magnification.

Generation of ALK+ Stable Cell Lines

Colo16 cells are a human-derived SCC cancer cell line. We demonstrated that Colo16 cells lacked ALK protein expression by immunoblot. Parental cells were transfected to express ALK with a Phe-to-Leu activating mutation at position 1174 (ALK-F1174L). pHAGE-ALK-F1174L was a gift from Gordon Mills & Kenneth Scott (Addgene plasmid # 116108 ; http://n2t.net/addgene:116108 ; RRID:Addgene_116108).[338] Stable clones of the Colo16 cells (2C3, C7) showed strong ALK protein expression (**Figure 28**). Knowing the sequence we transfected and size of ALK, we expect the molecular weight to be 180 kDa. However, reports show that the protein migrates as a doublet and that both forms can be activated by phosphorylation.[339] We find that the faster migrating form is phosphorylated and anticipate that this is the active kinase, although we can't rule out that both have kinase activity. Evidence that these bands are truly ALK comes from several observations: (1) the expression is only present when the sequence is introduced via transfection (2) bands at both 140 and 200 are reactive to an anti-ALK antibody, and (3) the faster migrating form is reactive to an independent antibody specific to p-ALK. A similar pattern with 140 and 200 kDa forms was seen in HEK293 cells transfected with the same vector (data not shown). Thus, given that our migration pattern matches that in the literature and two independent antibodies, we provide evidence that ALK expressed in 2C3 and C7 clones is authentic.

Colony Formation Assays

We assessed proliferation in Colo16 cells lacking or expressing ALK. Control cells, 2C3 cells, and C7 cells were counted for the number of colonies formed in each well. Control cells formed an average of 126 colonies, 2C3 cells formed an average of 247 colonies, and C7 cells formed an average of 160 colonies. The number of colonies formed by 2C3 was significantly greater than the number formed by C7 or control cells ($p<0.001$, **Figure 29A**). There was no significant difference between the number of colonies formed by the cell lines treated with the 0.2 nM of ALK inhibitor ceritinib or an equal volume of DMSO, suggesting the inhibitor did not cause single-agent cell death. However, there was a statistically significant difference in the colony size formed by 2C3 cells treated with 0.2 nM ceritinib versus an equal volume of DMSO, consistent with decreased proliferation (47.7 versus 70.9 pixels squared, $p=0.02$, **Figure 29B**). The C7 cells treated with 0.2 nM ceritinib formed colonies at an average size of 98.1 pixels squared compared to 148.0 pixels squared for the C7 cells treated with an equal volume of DMSO. However, this difference in colony size was not statistically significant ($p=0.1$). For parental ALK-negative cells, there was no meaningful difference in the area of colonies formed between cells treated with DMSO or ceritinib (59.8 versus 71.6 pixels squared, respectively, $p=0.3$).

Figure 28: Western blot

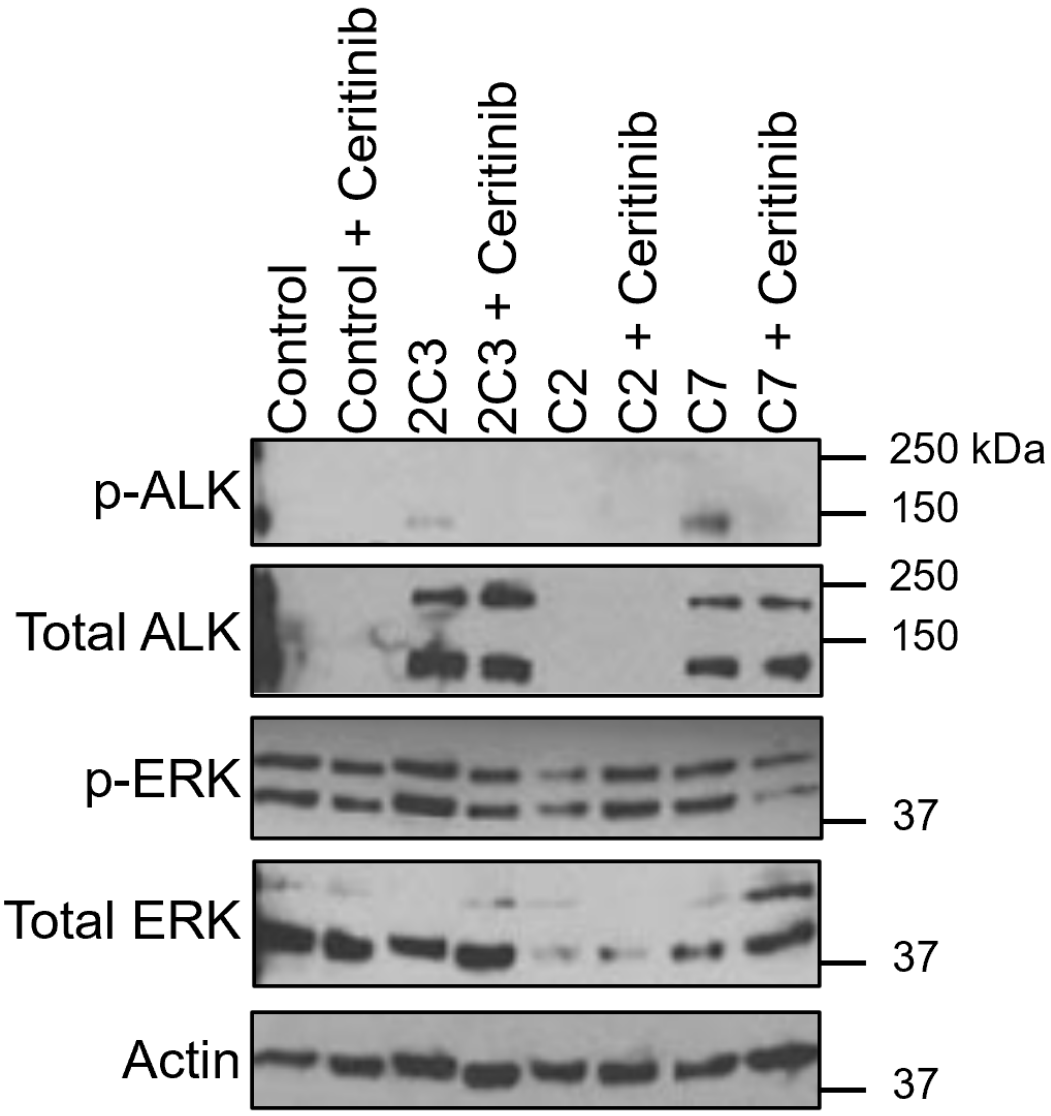
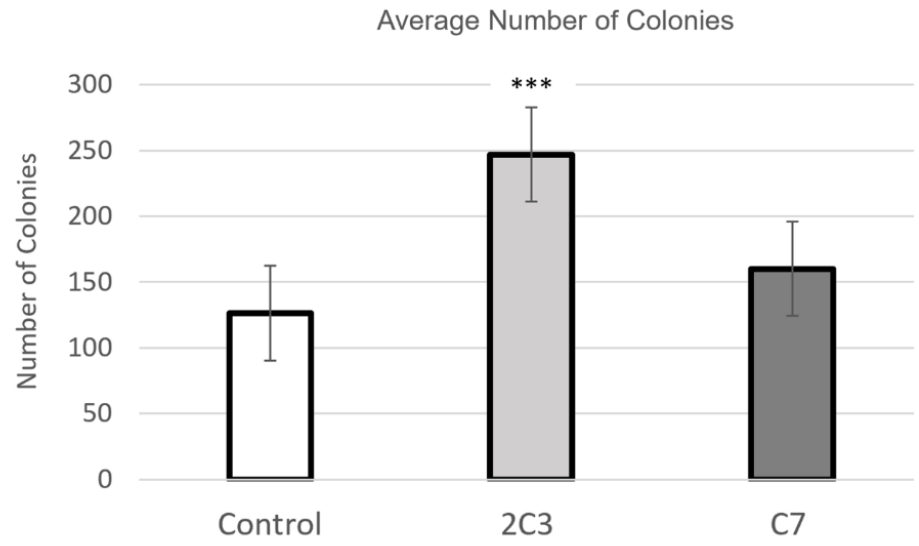


Figure 28 illustrates the positive ALK expression in 2C3 and C7 clones as well as the ALK activity (phospho-ALK) for these lines. Phospho-ERK is decreased upon treating these cells lines with ceritinib. This experiment was repeated three times.

Figure 29: Colony Formation Assays

A



B

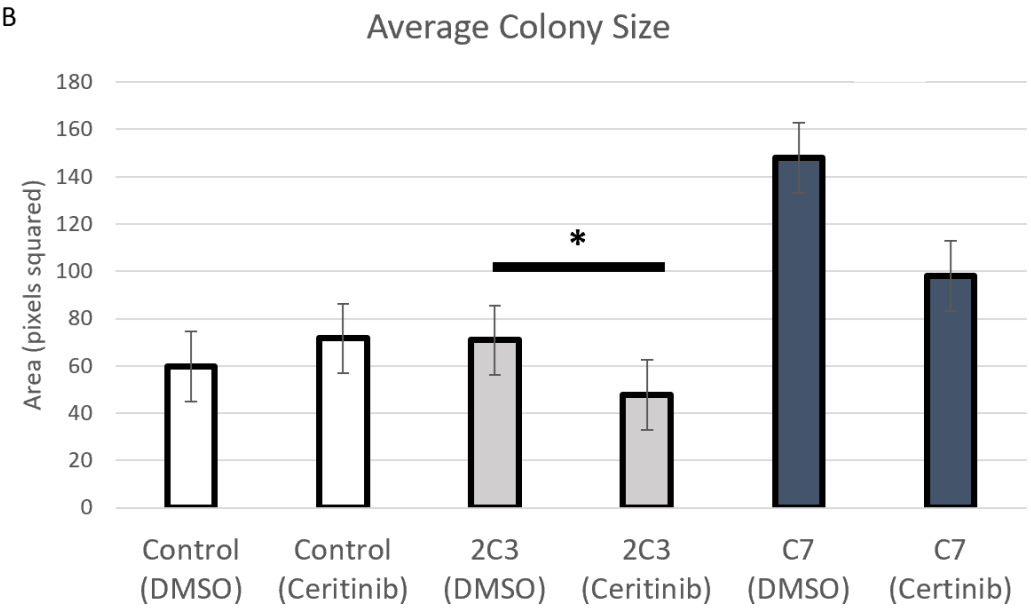


Figure 29A illustrates that the average number of colonies formed by 2C3 was significantly higher than control or C7 cells. Figure 29B illustrates that the average colony size for ALK+ 2C3 cells was decreased upon treatment with ceritinib. * indicates

p<0.05; *** indicates p<0.001. Six replicates were performed per cell line per treatment. These experiments were repeated twice.

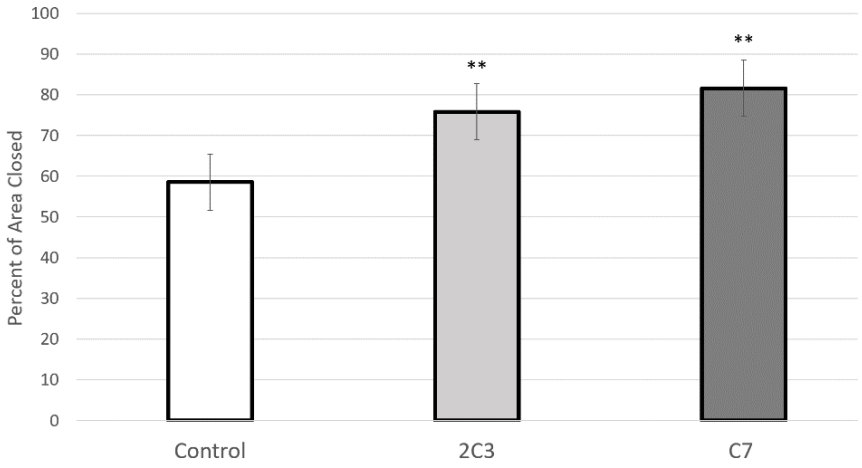
Migration Assays

Colo16 cell migration was tested by measuring closure of a gap in a confluent cell monolayer. Closure was measured by the percent of the original cleared area that was closed by migrating cells in 8 hours after removal of the silicone insert (Ibidi #80466). The first set of experiments looked at the percent of area closed by control cells, 2C3 cells, and C7 cells. The average percent area closed was 58.6%, 75.8%, and 81.6% for control cells, 2C3 cells, and C7 cells, respectively. The percent closed by 2C3 cells and C7 cells was significantly different from the control cells (**Figure 30A**, p=0.002).

To determine if the enhanced migration was due to the expression of activated ALK, we next tested gap closure in the presence or absence of the ALK inhibitior ceritinib (0.2 nM) or an equal volume of DMSO. The percent closed by the control cells, 2C3 cells, and C7 wells with DMSO was 42.4%, 38.8%, and 64.1%, respectively. The percent closed by C7 cells was significantly higher than both areas closed by control and 2C3 cells (**Figure 30B**, p=0.007). It is not known why the 2C3 cells showed faster closure in some experiments but not all. The percent area closed by the control cells treated with DMSO versus ceritinib was very similar at 42.4 and 37.4%, respectively (p=0.2). The same trend was observed for the 2C3 cells, although the percent closed by cells treated with DMSO and ceritinib were 38.8% and 20.9%, respectively (p=0.2). The C7 cells treated with DMSO had an average percent closed of 64.1% versus 37.4% for the ceritinib-treated cells (**Figure 30B**, p=0.009). Thus, ceritinib treatment reversed the advantage in cell migration due to ALK expression in C7 cells.

Figure 30: Migration Assays

A



B

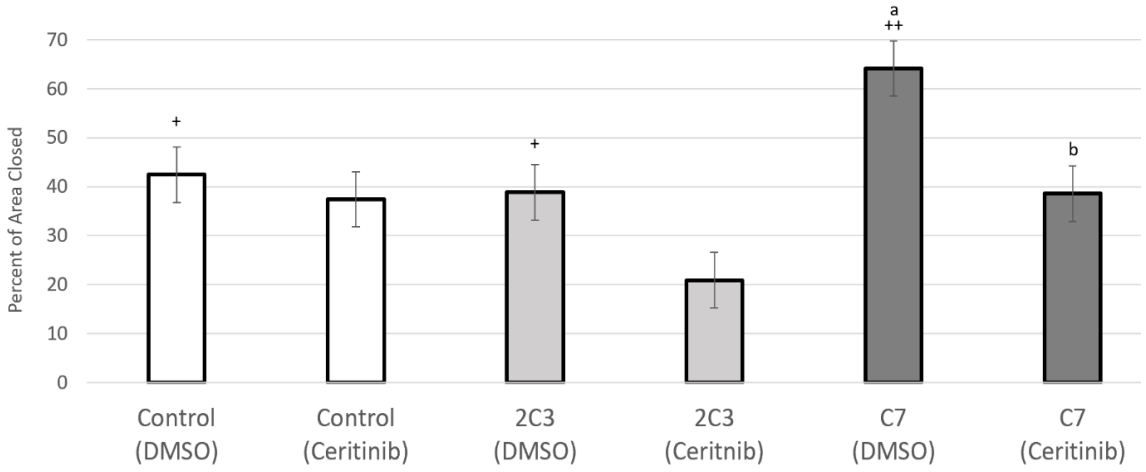


Figure 30A illustrates that migration was higher in 2C3 and C7 cells relative to the controls. ** indicates $p<0.01$. Figure 30B illustrates that migration in C7 cells was higher than in control or 2C3 cells and that treatment with ceritinib led to decreased migration in C7 cells. + versus ++ indicates $p<0.01$. A versus B indicates $p<0.01$. Imaging was performed at 0 and 8 hours. Three replicates were performed for each treatment of each cell line. These experiments were repeated twice.

Invasion Assays

The invasion assays examined the ability of ALK-positive clones and ALK-negative control cells to invade through Matrigel and close the defect left by the insert. Cells were treated with 0.2 nM ceritinib or an equal volume of DMSO. The percent closure by the control cells, 2C3 cells, and C7 wells treated with DMSO was 50.5%, 71.8%, and 69.3%, respectively, again indicating an advantage for the cells expressing activated ALK. The percent area closed by the control cells treated with ceritinib was very similar at 56.7% (p=0.7). The same trend was observed for the C7 cells, with the percent closed by cells treated with DMSO and ceritinib being 69.3% and 68.7%, respectively (p=0.9). The 2C3 cells treated with DMSO had an average percent closed of 71.8% versus 46.8% for the ceritinib-treated cells (**Figure 31**, p=0.04). Migration through the matrigel matrix involves both cell movement and remodeling of the extracellular matrix. We interpret these data to indicate that ALK activation supports invasion and migration of SCC tumor cells, and this can be reversed by ALK kinase inhibition.

Figure 31: Invasion Assays

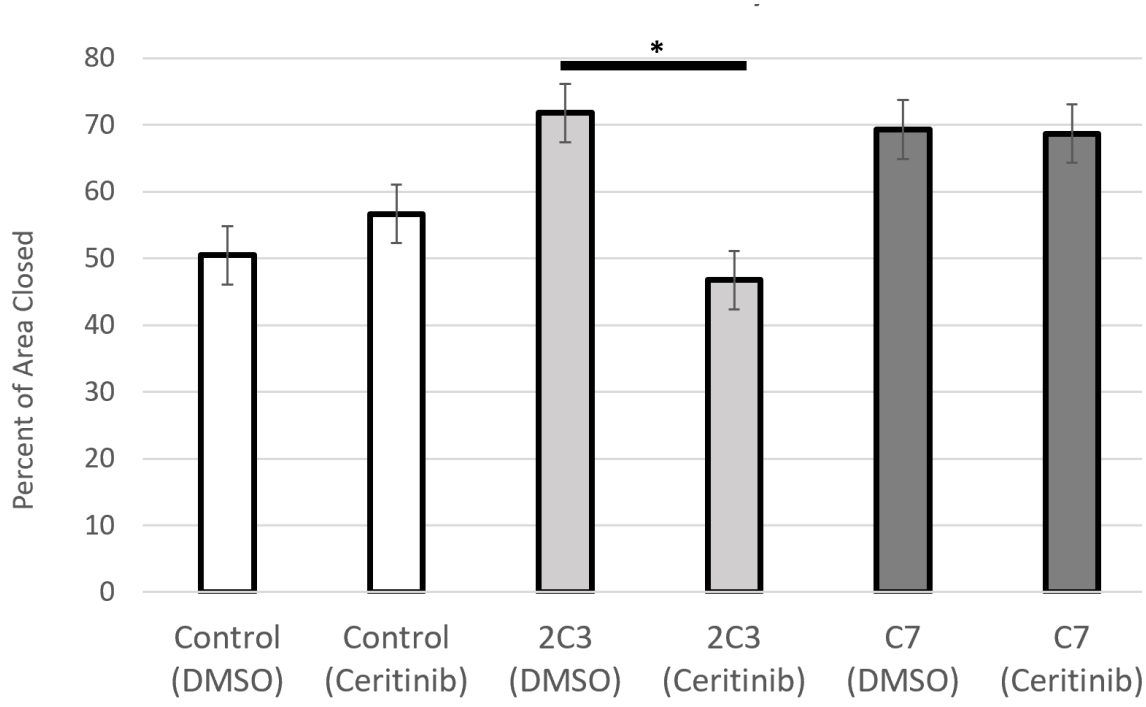


Figure 31 illustrates that treatment of 2C3 cells with ceritinib decreased invasion of cells through a matrix. * indicates $p<0.05$. Imaging was performed at 0 and 18 hours. Three replicates were performed for each treatment of each cell line. This experiment has not yet been repeated.

Apoptosis

Based on colony formation and observation of treated cells, we did not anticipate increased cell death in ALK-positive cells upon ceritinib treatment. To test apoptosis, we visualized nuclei in unfixed cells in culture by addition of the fluorescent nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI). DAPI is commonly excluded from healthy cells and only accumulates in dying cells, providing a bright signal. Additionally, apoptotic cells stained with DAPI demonstrate nuclear condensation (pyknosis) and fragmentation (karyorrhexis). In all cell lines undergoing 0.2 nM ceritinib treatment or treatment with an equal volume of DMSO, the percent of cells undergoing apoptosis (strong condensed or fragmented DAPI signal) was under 10%. The average percent of apoptotic cells for the controls were 1.7% and 3.8% for DMSO- and ceritinib-treated cells, respectively ($p>0.05$). For 2C3 cells the average percent of apoptotic cells for the controls were 2.0% and 2.4% for DMSO- and ceritinib-treated cells, respectively ($p>0.05$). For C7 cells the average percent of apoptotic cells for the controls were 3.5% and 7.2% for DMSO- and ceritinib-treated cells, respectively ($p=0.02$). The percent of apoptotic cells for C7 cells was significantly higher than for control or 2C3 cells ($p=0.02$, Tukey's test for post-hoc comparisons, **Figure 32**). Given the rate of apoptosis in cultured cells is commonly in the range of 2-10%, we did not interpret the observed changes to be biologically meaningful.

Figure 32: Apoptosis Assay with DAPI

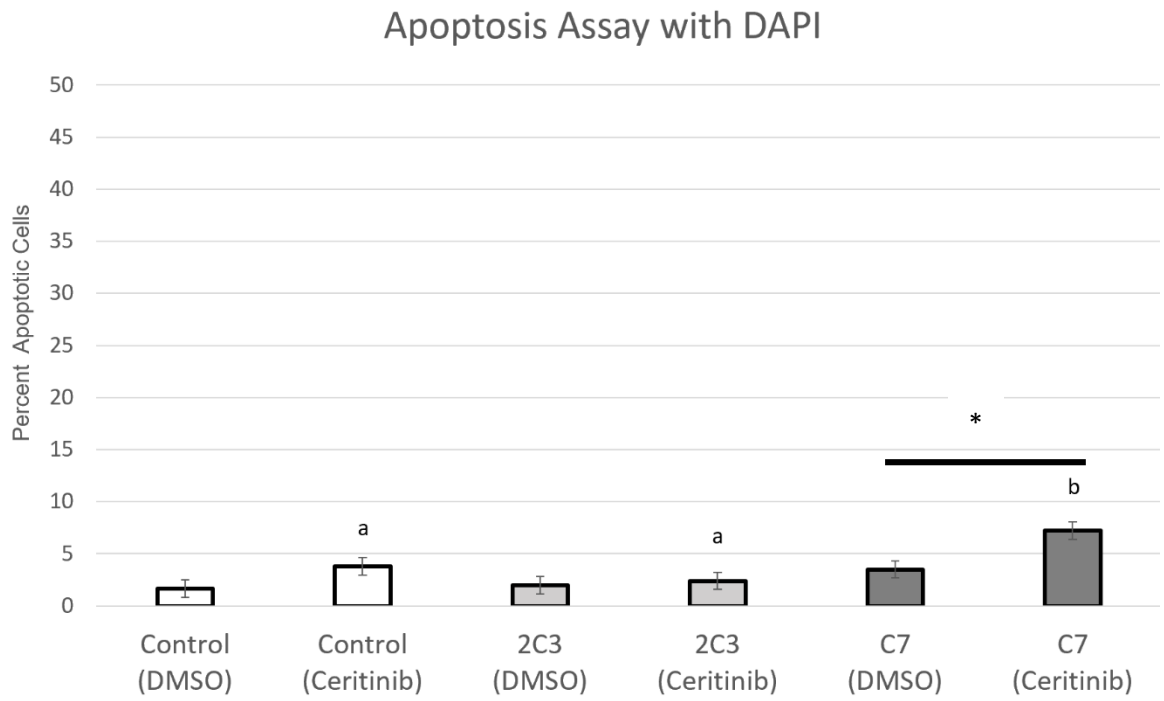


Figure 32 illustrates that the percent of cells undergoing apoptosis that were treated with DMSO or ceritinib was under 10%. The C7 cells treated with ceritinib had a higher rate of apoptosis than the control or 2C3 cells treated with ceritinib ($p=0.02$). The ceritinib treatment of C7 cells caused an increase in apoptosis relative to the C7 cells treated with DMSO ($p=0.02$). * indicates $p<0.05$. A versus B indicates $p<0.05$. Fifteen replicates were performed for each treatment of each cell line.

Kinase signaling

Colo16 cells with and without ALK expression were tested for kinase pathway activation. Here, a clone selected for with antibiotic resistance that did not express ALK, C2, is included as an additional ALK-negative cell along with parental control cells. Western blot analysis was performed for ALK-negative (parental and C2 cells) and ALK-positive (2C3 and C7 cells) treated with 0.2 nM ceritinib or an equivalent volume of DMSO for 6 hours prior to protein isolation (**Figure 28**). Blotting for ALK confirmed

positive expression in clones 2C3 and C7 and negative expression for control cells and clone C2. Blotting for phospho-ALK demonstrated bands only in the 2C3 and the C7 lysates, confirming that ceritinib treatment inhibited ALK activation in our cells. In order to investigate which pathways may be altered by ALK inhibition, we blotted for phosphorylated and total ERK. In the 2C3 and C7 clones, p-ERK expression decreased with ceritnib treatment. This was not observed with the control or C2 (ALK-) cells. In summary, expression of mutated ALK resulted in phosphorylation of ALK and ERK in SCC cells, and activation was sensitive to ceritinib. To further evaluate the role of ERK in *ALK*-mutated SCC, we performed migration assays with ERK inhibitor treatment SCH772984 (Selleck Chem). Migration of 2C3 cells with ERK inhibition was decreased significantly relative to the migration with an equal volume of DMSO (p=0.01, **Figure 33**), supporting the hypothesis that *ALK*-mutated SCC may be driven through ERK signaling.

Figure 33: Migration with ERK Inhibition

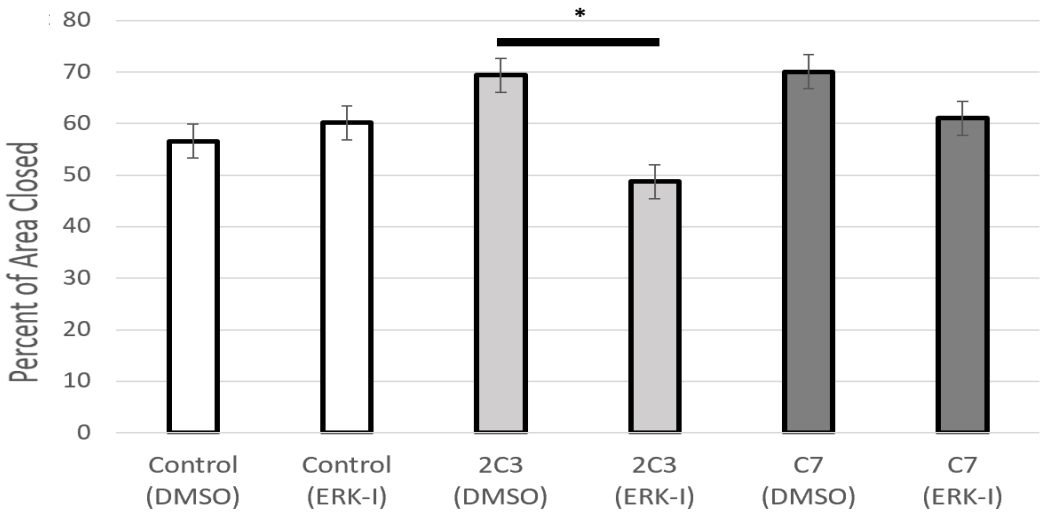


Figure 33 illustrates migration in control cells, 2C3 cells, and C7 cells treated with 1 uM of an ERK inhibitor or an equal volume of DMSO. * indicates a significant difference (p=0.01) observed between 2C3 cells treated with an ERK inhibitor versus an equal

volume of DMSO. Imaging was performed at 0 and 8 hour time points. Three replicates were performed per cell line per treatment. This experiment has not yet been repeated.

In vivo experiments

One million SCC cells with confirmed ALK expression (or parental cells) were injected subcutaneously into the bilateral flanks of NOD/SCID mice. There were two groups of mice with 6 mice per group and each received bilateral injections (group 1= injected with SCC ALK- cells, group 2= Injected with SCC ALK+ C7 cells). Tumor growth was so fast that the experiment had to be stopped at 10 days post-injection, which was shorter than our expectation of 28 days. At 10 days, the mice were sacrificed and tumor size, weight, and mouse health were quantified. There were no significant differences in tumor size, tumor weight, or mouse weight between the mice injected with parental versus ALK+ cells. Tumors were examined grossly for signs of angiogenesis. Of the 12 tumors injected with parental (ALK-) cells, 6/12 (50%) appeared vascularized. Of the 12 tumors injected with ALK+ C7 cells, 9/12 (75%) appeared vascularized.

Discussion and Conclusions

This study investigated tumor cell features in SCC cells that were driven by an activating ALK mutation, Phe-to-Leu mutation at 1174. The principal findings presented here relate to tumor cell colony number and size, migration, invasion, and signaling through ERK. We demonstrated ALK expression in one of nine human primary SCC tumors. Enforced expression of ALK increased cell proliferation based on colony size, increased migration and invasion, and activated ERK phosphorylation.

With the exception of one study by Gualandi *et al*. (2020) that utilized very different techniques from those in this study, the role of *ALK* in SCC has not been

investigated.[196] The Gualandi study was based on data from inducing conditional expression of *ALK*^{F1174L} (the same one used for our mechanistic cell studies) in the skin of mice using 4-hydroxytamoxifen and performing RNA sequencing to identify possible pathways associated with *ALK*-mutated SCC. Overall, it was found that *ALK* cooperates with oncogenic *KRAS* and loss of *TP53* to promote SCC progression.[196] This current study further investigates the role of *ALK* using immunohistochemistry and cell culture studies utilizing the transfection of SCC lines with *ALK*^{F1174L}.

This is the first study to examine the effect of an ALK inhibitor on *ALK*-mutated SCC. The literature on ALK inhibitor therapy is heavily focused on non-small cell lung cancer (NSCLC), for which several ALK inhibitor therapies are used including crizotinib, ceritinib, alectinib, and brigatinib.[340] A text mining study that used publicly available data and computational tools explored potential therapies for SCC given mutated genes and altered pathways. Crizotinib and ceritinib were both returned as candidate drugs suggested to treat SCC.[341] Crizotinib resistance has been documented in neuroblastoma cell lines with the mutation *ALK*^{F1174L}. As this mutation is relatively common in cancer and is the mutation transfected into our SCC lines, we elected to treat with ceritinib, a more specific ALK inhibitor that has demonstrated success in treating NSCLC patients who have failed treatment with crizotinib.[342]

As SCCs are often amenable to treatment with surgery until they metastasize, understanding and treating metastasis would address the leading cause of death in SCC.[190] Therefore, this manuscript focused on the effect of *ALK* mutations on the hallmarks of metastasis, which include migration and invasion.[343] Proliferation was also investigated, although this is one of the more general hallmarks of cancer and not metastasis-specific.[344] *ALK* has been demonstrated to promote these hallmark events in other cancers, for example in neuroblastoma and anaplastic lymphoma.[345,346] Treatment with ALK inhibitors has been shown to reverse these effects. For example, a cell culture

study of pancreatic cancer cells demonstrated that treatment with ceritinib plus gemcitabine inhibited proliferation and migration.[347] Another study of hepatocellular carcinoma cells (HCC) evaluated the effect of crizotinib and ceritinib on cells and found that proliferation was inhibited.[348] Our data were similar to previous studies in other cancers in that we found that *ALK*^{F1174}-positive clones had increased cell proliferation, migration, and invasion compared to ALK-negative controls. Further, treatment with the ALK inhibitor ceritinib blocked these effects.

We did not find evidence that effects on proliferation by ceritinib were due to increased apoptosis. In all cell lines and treatments, apoptosis was below 10%, suggesting that cell death is not the primary mechanism of action of ceritinib on SCC cells. A study by Salminen *et al*. (2016) demonstrated that treating primary rat hepatocytes with ceritinib caused the widespread induction of apoptosis, and the difference in our observation may be due to concentrations employed (Salminen employed higher amounts than here) or due to cell- or tumor-type differences.[349] A study of rhabdomyosarcoma cells treated with ceritinib found that only cell lines with high levels of ALK expression demonstrated apoptosis upon inhibitor treatment.[350] We observed that C7 cells had the highest level of ALK activity (measured by phospho-ALK), which may explain the (small but detectable) increase in apoptosis for C7 cells treated with ceritinib.

ALK activated ERK in our SCC cell lines. The role of the ERK1/2 pathway has been previously demonstrated in SCC as well as the role of the ERK pathway activator Ras.[351,352] The inhibition of ERK phosphorylation by ceritinib corroborated the hypothesis by Gualandi *et al*. that KRAS cooperates with *ALK* in promoting SCC progression.[196] A study of neuroblastomas found that *ALK* activating mutations were dependent on ERK1/2 signaling to drive tumorigenesis.[353] A recent study by Bhagwat *et al*. (2020) investigated ERK inhibitor LY3214966 and found that it demonstrated impressive anti-

tumor activity in cell lines with ERK alterations.[354] As dual inhibitor therapy is often more efficacious in treating patients; treatment with an ALK inhibitor plus an ERK inhibitor could be considered to enhance the therapeutic approach.[355]

Immunohistochemistry of twenty-four human samples of SCC demonstrated that two case were positive for ALK (one metastatic SCC, one localized high-risk). Without a larger sample size, we cannot conclude that ALK expression correlates with higher-risk SCC tumors. We do find suggestions that ALK may promote aggressive behavior in the literature (10-25% of SCCs with *ALK* mutations, with approximately half being pathogenic).[163,192,196,203] The relatively high percent of tumors with an *ALK* mutation and the availability of FDA-approved ALK inhibitors makes *ALK* an appealing target for adjuvant therapy or for recurrent SCC. The only small molecule inhibitor widely used for advanced and metastatic SCC is cetuximab, an inhibitory antibody which targets EGFR mutations (only present in approximately 2.5% of SCCs).[356] Currently, SCCs are sequenced to determine eligibility for possible targeted therapeutics, however, this process is expensive and lengthy. In addition to *ALK* mutations being present in a high percent of SCCs, if these activating mutations are detectable downstream by IHC, this may be a cheaper and faster alternative to screen for eligibility for ALK inhibitor treatment compared to sending a tumor for gene sequencing.

Our *in vivo* experiments were preliminary but do provide some possible insight into the pathogenesis of *ALK*-mutated SCC. One possibility for our findings is that too many tumor cells were injected, not allowing for any meaningful differences to be observed in growth due to the short length of the experiment. Another possibility is that in an *in vivo* model, the role of *ALK* is more geared toward tumor metastasis rather than proliferation. This hypothesis supports findings in our patient data. To further draw conclusions regarding *ALK* and angiogenesis, IHC of the tumors utilizing stains such as CD31 and D2-40 should be performed. Additional *in vivo* experiments that may further

elucidate the role of *ALK* in SCC include utilizing models of metastasis such as lymph node injections, tail vein injections, intrasplenic injections, and experiments with ALK inhibitor therapy.

The current study has several limitations. Migration and invasion experiments were done using a fixed number of cells per cell line, but we did not inhibit proliferation during this experiment, possibly allowing for an effect from proliferation on migration assessment. Further, there are some inconsistencies with regards to the statistical significance of each cell line in proliferation, migration, and invasion experiments. These experiments will be repeated with higher numbers of replicates to investigate these discrepancies. This study is also limited by the relatively small number of clinical samples available and the single cell line used to generate ALK-positive clones for experiments. Future studies to continue to investigate *ALK*-mutated SCC are important to validate the findings in this study.

Chapter 8: Overall Discussion, Conclusions, and Future Directions

Cancer is the second most common cause of death in the United States after heart disease.[357] While the incidence of several cancers has decreased in the past 50 years, this has not been the case for skin cancer. An epidemiological study in Norway found that cutaneous squamous cell carcinoma (SCC) rates have increased 6-fold for males and 9-fold for females over the past 50 years.[358] Another epidemiological study in Australia, Germany, and the United States found that mortality is stable or increasing.[359] However, non-melanoma skin cancers are not reportable and are therefore difficult to track. In other cancers (reportable subtypes), increased prevention and new therapeutics has resulted in declining mortality rates since the early 1990s by 33.6% among men and 23.6% among women.[360] With the increasing incidence of SCC and stable or increasing mortality, research is needed to help discover new therapies to bring down mortality rates.

In this dissertation, we have taken a unique bedside-to-bench approach to identify and investigate new targets in SCC. We began with a clinical approach where new risk factors for metastasis in SCC were discovered. We identified individual high-risk tumors and matched them by patient and clinicopathologic staging features to identify novel mutations associated with metastasis. A translational component of the work, next generation sequencing, allowed for the analysis of mutational differences between localized and metastatic primary tumors as well as metastatic primary tumors and nodal metastases. Potentially relevant new polymorphisms in high-risk SCC were found. We also stratified our cohort by immune status and found that immunosuppressed patients have fewer UVB-associated mutations. A review of the literature and laboratory experiments demonstrated additional utility that LRP1B (and other markers) immunohistochemistry may have in SCC. In the laboratory, we confirmed

ALK as a novel target that is likely to be clinically actionable. The ultimate goal of such a project is to translate research findings to the clinic so that patients may benefit. There are several additional studies that may be important prior to initiation of clinical trials of *ALK* inhibitors for SCC.

Due to practical limitations of time and resources, *ALK* validation experiments were performed in one cell line. A next step in this project would be to replicate the cell culture experiments with additional SCC lines. In addition, another preclinical animal study would help to confirm the significance of *ALK* in an *in vivo* model system. Since *ALK* was found to promote characteristics of tumor metastasis, an *in vivo* model of metastasis would be particularly useful. One such way to model this would be to perform mouse experiments with lymph node injections of *ALK*-mutated SCC cells and observe if these mice develop metastatic lesions more than SCC cells without an *ALK* mutation. Another approach to study the effect of *ALK* on metastasis would be to utilize intrasplenic or tail vein injection approaches designed to model metastasis. Anti-ALK treatment should also be utilized to determine if its efficacy translates to an *in vivo* model system. If these proposed experiments further reinforce *ALK* as a dominant signaling pathway in SCC, a clinical trial may be an appropriate future step.

The decision to pursue *ALK* as a target over the many other potential targets identified was largely due to the availability of FDA-approved inhibitor therapies that have shown success in other cancers. The overall percent of clinical trials that ultimately succeed is quite low. Several studies have attempted to quantify the percentage of clinical trials that succeed, with estimates ranging from 9.6%-13.8%.[361–363] There are many reasons that clinical trials fail, including lack of safety, efficacy, financial barriers, or patient recruitment. A study by Hwang *et al*. found that lack of safety in advanced trials caused 17% of them to fail.[364] Investigating a drug that already is FDA-approved

increases the likelihood of success, by decreasing the chance it will fail due to safety issues, as it has already been tested and a maximum tolerated dose (MTD) has likely been determined. However, the safety and other causes of failures such as lack of efficacy are still possibilities.

One growing area of research that is related to the topic of this dissertation is how gene mutations modulate tumor response to therapeutics, both newer immunotherapies and traditional targeted or cytotoxic therapies. Programmed death 1 (PD-1) receptor and ligand (PD-L1) targeted therapies have shown impressive efficacy in treating many cancers in the past decade. Two immunotherapies that target PD-1, pembrolizumab and cemiplimab, have recently received approval for the treatment of advanced/metastatic SCC.[96,365] For patients who respond, some achieve a complete response. However it is challenging to predict which patients will respond versus those that will fail therapy.[96,365] A clinical study by Gainor *et al*. (2016) analyzed NSCLC patients treated with PD-1/PD-L1 therapy and found that tumors harboring an *ALK* rearrangement or *EGFR* mutation had low rates of response to immunotherapy.[366] One possible explanation proposed is that the authors observed lower PD-L1 and CD8+ expression in these tumors. Non-smokers have ALK rearrangements and EGFR mutations more often than smokers, and also have less inflammatory tumor microenvironments.[366,367] As smoking has been associated with SCC (RR 2.3, $p<0.0001$), this association would be interesting to further investigate in SCC.[368] Another mutation investigated in this dissertation, *LRP1B*, has been demonstrated to impact response to therapy, especially immunotherapies.[369,370] In addition to *ALK* and *LRP1B*, there are many other possible targets identified in this dissertation that may be interesting to investigate as predictors of response to therapy.

Final Thoughts

This dissertation demonstrates an effective approach for identification and investigation of new targets in skin cancer. This approach can be applied across many types of sequencing technologies and in numerous other cancers. We began this project with patient samples, performed next-generation sequencing, employed bioinformatics and statistical methods to identify key drivers, and employed basic science techniques to validate these findings in the experimental laboratory setting; bedside to bench. While our results reveal two potential genes (*ALK* and *LRP1B*) and one therapeutic that may be utilized in SCC, there were many genes identified that have yet to be explored. Additional studies are needed to further investigate these findings and to ultimately expand efficacious treatment options for patients with SCC.

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Appendices

Appendix 1: Table S1, Markers Included in Chapter 6.1 of this study

Key: Smith *et al*., 2011_1= study by Smith *et al*. in 2011, marker 1 studied by that author in that study

Author, Year	**Marker**	**Study Type**	**Expression in SCC**	**Type of Tissue Used**	**Multivariate Analysis Performed?**
Toll *et al*., 2013_1[264]	E-cadherin	Retrospective	Nuclear e-cadherin expression was positive in 19.6% (10/51) of non-metastatic SCCs and 65.3% (32/49) of metastatic SCCs ($p<0.001$).	Primary SCCs that did not metastasize (n=51) were compared to primary SCCs that did metastasize (n=49).	Authors suggest multivariate analysis was not necessary for several markers since recurrence, tumor stage, perineural invasion, and other markers were so closely matched.
Hesse *et al*., 2016_1[161]	E-Cadherin	Retrospective	Metastatic tissue demonstrated downregulated membranous E-cadherin expression compared to the corresponding primary SCC ($p=0.031$).	Patient-matched primary tumor tissue and metastatic tissue were analyzed (n=14 both groups, 10 skin metastases, 4 lymph node metastases).	A multivariate analysis was not reported.

Cañueto *et al*., 2017_A[268]	Podoplanin	Retrospective and Prospective	Primary tumors that did not metastasize had moderate/intense podoplanin expression in 16.0% (13/81) of cases, while primary tumors that metastasized had moderate/intense podoplanin expression in 46.2% (6/13) of cases. Moderate/intense podoplanin expression in the primary tumor was correlated with higher risk of nodal metastasis compared to	Primary SCCs that did not metastasize (n=81) were compared to primary SCCs that did metastasize (n=13).	Results were confirmed in a multivariate analysis.

			absent/weak expression (p=0.02).		
Hesse *et al*., 2016_2[161]	Podoplanin	Retrospective	Podoplanin staining in non-metastatic SCC was present in 0% of the tumor in 6.3% (5/80) of cases, 1-25% of the tumor in 50.0% (40/80) of cases, 26-50% of the tumor in 21.3% (17/80) of cases, 51-75% of the tumor in 17.5% (14/80) of cases, and 76-100% of the tumor in 5.0% (4/80) of cases.	Primary SCCs that did not metastasize (n=80) were compared to primary SCCs that did metastasize (n=22).	Results were confirmed in a multivariate analysis.

			Podoplanin staining in metastatic SCC was present in 0% of the tumor in 13.6% (3/22) of cases, 1-25% of the tumor in 18.2% (4/22) of cases, 26-50% of the tumor in 36.4% (8/22) of cases, 51-75% of the tumor in 4.5% (1/22) of cases, and 76-100% of the tumor in 27.3% (6/22) of cases. Podoplanin expression was significantly correlated with metastasis (p=0.04).		

Toll *et al*., 2013_2[264]	Podoplanin	Retrospective	Podoplanin expression was positive in 10.5% of non-metastatic SCCs and 37.1% of metastatic SCCs (p=0.001)	Primary SCCs that did not metastasize (n=51) were compared to primary SCCs that did metastasize (n=56).	Authors suggest multivariate analysis was not necessary for several markers since recurrence, tumor stage, perineural invasion, and other markers were so closely matched.
Kreppel *et al*., 2012[269]	Podoplanin	Retrospective	Primary tumors that did not metastasize had no podoplanin expression in 43.8% (21/48) of cases, weak expression in 27.1% (13/48) of cases, moderate expression in 12.5% (6/48) of cases, and strong expression in	Primary SCCs that did not metastasize (n=48) were compared to primary SCCs that did metastasize (n=15).	Results were confirmed in a multivariate analysis.

			16.7% (8/48) of cases. Primary tumors that did metastasize had no podoplanin expression in 13.3% (2/15) of cases, weak expression in 6.7% (1/15) of cases, moderate expression in 46.7% (7/15) of cases, and strong expression in 33.3% (5/15) of cases. Podoplanin expression was significantly higher in cases that metastasized (p=0.005).		

Zhang *et al*., 2013[273]	CD8+ T cells	Prospective	The presence of fewer CD8+ T cells in transplant SCC (TSCC) was associated with a more aggressive tumor phenotype of lymph node metastasis (TSCC had 48.22±8.38 cells/$\mu m^2 \times 10^5$ versus SCC, which had 95.70±9.92 cells/$\mu m^2 \times 10^5$; normal skin had 6.88±2.56 cells/$\mu m^2 \times 10^5$ ($p<0.05$).	TSCC (n=10) were compared with non-transplant SCCs (n=11).	A multivariate analysis was not reported.

Azzimonti *et al*., (2015)[274]	CD8+ T cells	Retrospective	Peritumoral CD8+ T cell infiltration was higher in low grade* SCCs (40.0%) compared to high grade** SCCs (30.5%), however, this did not reach statistical significance (p=0.08).	Primary SCCs that were well-differentiated (n=20) were compared to primary SCCs that were moderately or poorly differentiated (n=20).	A multivariate analysis was not reported.
Garcia-Diez *et al*., 2018[276]	Programmed death ligand 1 (PD-L1)	Retrospective	PD-L1 was associated with an increased risk of metastasis; positive PD-L1 expression was found in 26% (13/50) of non-metastatic cases and 50% (23/46) of metastatic	Primary SCCs that did not metastasize (n=50) were compared to primary SCCs that did metastasize (n=46).	Results were confirmed in a multivariate analysis.

			cases ($p<0.05$).		
Kamiya *et al*., 2020[277]	PD-L1	Retrospective	High intensity PD-L1 expression (staining scores of 2 or 3) was present in 27.8% (10/36) of non-metastatic SCCs and 80% (8/10) of metastatic SCCs. High intensity PD-L1 expression was an independent risk factor for lymph node metastasis in a multivariate analysis (OR= 22.6, p=0.009).	Primary SCCs that did not metastasize (n=36) were compared to primary SCCs that did metastasize (n=10).	Results were confirmed in a multivariate analysis.
Garcia-Pedrero *et*	PD-L1	Retrospective	Tumors cells with PD-L1 positivity of	Primary SCCs that did not	Results were confirmed in a multivariate analysis.

al., 2017[278]			≥25% were at significantly increased risk of nodal metastasis (Adjusted HR 6.54, 95% CI 2.28-18.78).	metastasize (n=50) were compared to primary SCCs that did metastasize (n=50).	
Amoils *et al*., 2019[279]	PD-L1	Retrospective	Sixty-five percent (20/31) of primary tumors had grade 1 staining, while grade 2 was observed in 46% (24/52) of metastases.	Primary tumor tissue was collected from locally aggressive or regionally metastatic SCCs (n=31). Regional metastatic tissue was also collected (n=52).	A multivariate analysis was not reported.

Slater *et al*., 2016[280]	PD-L1	Retrospective	PD-L1 expression was present in 20% (4/20) of low risk tumors. PD-L1 expression was present in 70% (14/20) of high-risk tumors.*** PD-L1 expression was present in 100% (5/5) of metastases.	There were 3 groups of tissue collected: 1) primary low-risk SCCs (n=20) 2) primary high-risk SCCs*** (n=20) 3) locoregional metastatic tissue (n=5)	A multivariate analysis was not reported.
Ch'ng *et al*., 2008[287]	Epidermal growth factor receptor (EGFR)	Retrospective	Thirty six percent (9/25) of primary non-metastatic SCCs demonstrated 3+ staining, while 79% (11/14) of primary metastatic SCCs	Primary SCCs that did not metastasize (n=25) were compared to primary SCCs that did metastasize (n=14).	Results were confirmed in a multivariate analysis.

			demonstrated 3+ staining. EGFR overexpression (3+ staining) was an independent prognostic factor for metastasis (p=0.05).		
Cañueto *et al*., 2017_B[371]	EGFR	Retrospective and Prospective	Overexpression of EGFR (+++ staining) was associated with the development of lymph node metastasis (OR=7.1, p=0.004).	Primary SCCs that did not metastasize (n=81) were compared to primary SCCs that did metastasize (n=13).	Results were confirmed in a multivariate analysis.
Shimizu *et al*., 2001[82]	EGFR	Retrospective	Expression of EGFR was strongly positive (+++) in metastases, (4/5) while weak (+)	Primary tissue that metastasized (n=5) and nodal metastatic tissue was	A multivariate analysis was not reported.

			expression was observed in primary tumors (4/5). One primary tumor and one metastasis was negative for EGFR expression.	analyzed (n=5).	
Sweeny *et al*., 2011[288]	EGFR	Retrospective	EGFR overexpression (2+ or 3+ staining) was found in 56.0% (28/50) of primary tumors and 58.3% (7/12) of regional metastases, p>0.05. EGFR overexpression was observed in 53.8% (14/26) and 58.3% (14/24) of	Primary tumors (n=50) were compared to metastatic tissue (n=12). Primary non-metastatic tissue (n=26) was also compared to primary metastatic tissue (n=24).	A multivariate analysis was not reported.

			cases, respectfully (p>0.05).		
Huang *et al*., 2012_1[301]	Cyclin D1	Retrospective	Positive expression of Cyclin D1 was positively associated with metastasis. Cyclin D1 was strongly diffuse (3+) in 15.2% (5/33) of non-metastatic SCCs and 33.3% (3/9) of metastatic SCCs (p=0.024).	Primary SCCs that did not metastasize (n=33) were compared to primary SCCs that did metastasize (n=9).	Bivariate analysis of clinicopathological factors was performed.
Mastoraki *et al*., (2009)[302]	Cyclin D1	Retrospective	Cyclin D1 expression was inversely correlated with the degree of tumor differentiation (Pearson correlation=	Grade I SCCs (well-differentiated, n=13), grade II SCCs (moderately differentiated, n=12), and grade III	A multivariate analysis was not reported.

			0.67, p<0.001)	SCCs (poorly differentiated, n=7) were compared.	

Abbreviations:
CI=confidence interval
OR=odds ratio
HR=hazard ratio
*low grade tumors were well-differentiated
**high grade tumors were moderately or poorly differentiated
***high-risk features included tumor diameter of 2 cm or greater, histologic grade 2 or greater or tumor thickness of 4 mm or greater

Appendix 2, Table S2, Markers not included in Chapter 6.1
Key: Smith *et al*., 2011_1= study by Smith *et al*. in 2011, marker 1 studied by that author in that study

Author, Year	**Marker**	**Study Type**	**Expression in SCC**	**Type of Tissue Used**	**Multivariate Analysis Performed?**
Toll *et al*., 2013_3[264]	Vimentin	Retrospective	Vimentin expression was positive in 31.4% (16/51) of non-metastatic SCCs and 67.9% (38/56) of metastatic SCCs (p<0.001).	Primary SCCs that did not metastasize (n=51) were compared to primary SCCs that did metastasize (n=56).	Authors suggest multivariate analysis was not necessary for several markers since recurrence, tumor stage, perineural invasion, and other markers were so closely matched.
Toll *et al*., 2013_4[264]	Twist	Retrospective	Twist expression was positive in 0% (0/51) of non-metastatic SCCs and 40% (22/55) of metastatic SCCs (p<0.001).	Primary SCCs that did not metastasize (n=51) were compared to primary SCCs that did metastasize (n=55).	Authors suggest multivariate analysis was not necessary for several markers since recurrence, tumor stage, perineural invasion, and other markers were so closely matched.
Toll *et al*., 2013_5[264]	Zeb1	Retrospective	Zeb1 expression was positive in 19.6% (10/51) of non-metastatic SCCs and 48.1% (26/54) of metastatic SCCs (p=0.004).	Primary SCCs that did not metastasize (n=51) were compared to primary SCCs that did metastasize (n=54),	Authors suggest multivariate analysis was not necessary for several markers since recurrence, tumor stage, perineural invasion, and other markers

					were so closely matched.
Toll *et al*., 2013_6[264]	Beta-catenin	Retrospective	Nuclear β-catenin was positive in 0% (0/51) of non-metastatic SCCs and 33.3% (16/48) of metastatic SCCs ($p<0.001$).	Primary SCCs that did not metastasize (n=51) were compared to primary SCCs that did metastasize (n=48).	Authors suggest multivariate analysis was not necessary for several markers since recurrence, tumor stage, perineural invasion, and other markers were so closely matched.
Huang *et al*., 2012_2[301]	PC cell-derived growth factor (PCDGF)	Retrospective	Positive expression of PCDGF was positively associated with metastasis. PCDGF was strongly diffuse (3+) in 24.2% (8/33) of non-metastatic SCC and 77.8% (7/9) of metastatic SCC ($p=0.003$).	Primary SCCs that did not metastasize (n=33) were compared to primary SCCs that did metastasize (n=9).	Bivariate analysis of clinico-Pathological factors was performed.
Suiqing *et al*., 2005[372]	Phospho-Stat3 (p-STAT3)	Retrospective	The positivity rate of p-STAT3 correlated with metastasis.	Primary SCCs that did not metastasize (n=21) were	A multivariate analysis was not reported.

			Forty-eight percent (10/21) of non-metastatic SCCs were positive for p-STAT3, while 77.8% (7/9) of metastatic SCCs were positive for p-STAT3 ($p<0.05$).	compared to primary SCCs that metastasized (n=9).	
Liu *et al*., 2013[373]	Tumor Suppressor in Lung Cancer 1 (TSLC1)	Retrospective	TSLC1 expression was inversely correlated with metastasis. Thirty-one percent (23/74) of non-metastatic SCCs had TSLC1 expression compared to 0% (0/13) of metastatic SCCs ($p=0.02$).	Primary SCCs that did not metastasize (n=74) were compared to primary SCCs that metastasized (n=13).	A multivariate analysis was not reported.

Wang *et al*., 2012[374]	P68	Retrospective	Strong p68 expression (3+) was seen in all metastases; primary SCC cases all had weak (1+) or moderate (2+) p68 expression. The H-score was 2.61±0.37 for the metastatic cases and 0.83±0.46 for the primary cases ($p<0.05$).	Primary tumors samples (n=13) and metastatic samples (4 soft tissue, 7 lymph node) were compared.	A multivariate analysis was not reported.
Keehn *et al*., 2004[375]	Protein C-ets-1 (Ets-1)	Retrospective	There is an increase in Ets-1 nuclear expression as SCC transforms from precancerous and well-differentiated lesions to moderately differentiated lesions to poorly differentiated/metastatic disease (mean labeling intensity of 0.4 ±	Primary tumor tissues were analyzed (n=15). Lesions were well-differentiated (n=5), moderately differentiated (n=2), poorly differentiated (n=5), and poorly differentiated and metastatic (n=3).	A multivariate analysis was not reported.

			0.3 versus 1.8 ± 0.6 versus 2.8 ± 0.2, respectively).		
Munguia-Calzada *et al*., 2019[376]	Focal adhesion kinase (FAK)	Retrospective	FAK overexpression (2+) was a significant risk factor for nodal metastasis with crude and adjusted HRs of 2.04 (p = 0.029) and 2.23 (p = 0.047), respectively.	Primary SCCs that did not metastasize (n=50) were compared to primary SCCs that metastasized (n=50) (4 year follow-up).	Results were confirmed in a multivariate analysis.
Kang *et al*., 2009[377]	N-methyl-D-aspartate-receptor subunit-NR1 (NMDAR-1)	Retrospective	NMDAR-1 expression was inversely correlated with metastasis; high NMDAR-1 expression (equal or stronger than normal epidermis) was present in 61.5% (16/26) of non-metastatic SCC and 16.7% (1/6)	Primary SCCs that did not metastasize (n=26) were compared to primary SCCs that metastasized (n=6).	A multivariate analysis was not reported.

			of metastatic SCC (p=0.049).		
Khandelwal *et al*., 2016[378]	PS6	Retrospective	Higher pS6 positivity and higher staining intensity was seen in patients with parotid metastasis (H-score 9.158 ± 0.4137) compared to SCC without parotid metastasis (H-score 7.895 ± 0.3966) (p=0.034).	Primary SCCs that did not metastasize (n=17) were compared to primary SCCs that metastasized (n=20).	Results were confirmed in a multivariate analysis.
Sekulic *et al*., 2010[379]	Inositol poly-phosphate 5-phosphatase (INPP5A)	Retrospective	Decreased INPP5A expression (using a 0-3 standard scoring system) was observed from primary metastatic SCC to metastatic tissue in 35% (6/17) of samples, while there was no	Patient-matched primary tumor tissue and regional metastatic tissue were analyzed (n=17).	A multivariate analysis was not reported.

			change in staining intensity in the remaining 65% (11/17) samples.		
Hernandez-Ruiz *et al*., 2018_1[380]	Enhancer of zeste homolog 2 (EZHZ)	Retrospective	In non-metastatic SCC, no expression of EZHZ was seen in 34.7% (17/49) of tumors, weak expression was seen in 36.7% (18/49) of tumors, moderate expression was seen in 28.6% (14/49) of tumors, and strong expression was seen in 0% (0/49) of tumors. In metastatic SCC, no expression of EZHZ was seen	Primary SCCs that did not metastasize (n=49) were compared to primary SCCs that metastasized (n=48).	A multivariate analysis was not reported.

			in 10.4% (5/48) of tumors, weak expression was seen in 39.6% (19/48) of tumors, moderate expression was seen in 35.4% (17/48) of tumors, and strong expression was seen in 14.6% (7/48) of tumors. Overall, higher EZHZ was seen in tumors that metastasized ($p<0.01$).		
Hernandez-Ruiz *et al*., 2018_2[380]	Ring1B	Retrospective	In non-metastatic SCC, no expression of Ring1B was seen in 25.5% (13/51) of tumors, weak expression was seen in 47.1% (24/51) of tumors, moderate	Primary SCCs that did not metastasize (n=51) were compared to primary SCCs that metastasized (n=54).	A multivariate analysis was not reported.

			expression was seen in 25.5% (13/51) of tumors, and strong expression was seen in 2.0% (1/51) of tumors. In metastatic SCC, no expression of Ring1B was seen in 20.4% (11/54) of tumors, weak expression was seen in 18.5% (10/54) of tumors, moderate expression was seen in 29.6% of (16/54) umors, and strong expression was seen in 31.5% (17/54) of tumors. Overall, higher Ring1B was seen in tumors that		

			metastasized (p<0.01).		
Tanemura *et al*., 2005[381]	Leucine rich repeats and immunoglobulin-like domains protein 1 (LRIG-1)	Retrospective	LRIG-1 staining intensity of class 1 (stronger staining, defined Supplementary Table 1) was present in 70% (21/30) of non-metastatic SCC and 25% (2/8) of metastatic SCCs, and thus was negatively correlated with metastasis (p=0.02).	Primary SCCs that did not metastasize (n=30) were compared to primary SCCs that metastasized (n=8).	Authors suggest multivariate analysis as the next step.
Santos-Juanes *et al*., 2019[382]	Lectin-like transcript 1 (LLT1)	Retrospective	LLT1 strong expression was a significant risk factor for nodal metastasis with crude and adjusted HRs of 3.40 (95% CI 1.39-9.28) and 3.25 (95% CI 1.15-9.16).	Primary SCCs that did not metastasize (n=50) were compared to primary SCCs that metastasized (n=50) (4-year follow-up period).	Results were confirmed in a multivariate analysis.

Chen *et al*., 2014[383]	p300	Retrospective	High expression of p300 (55%+ positive tumor cells) was detected in 47.0% (63/134) of non-metastatic SCCs and 74.2% (23/31) of metastatic SCCs (p=0.006).	Primary SCCs that did not metastasize (n=134) were compared to primary SCCs that metastasized (n=31).	Results were confirmed in a multivariate analysis.
Muchemwa *et al*., 2006[384]	Heat shock protein 105 (HSP105)	Retrospective	HSP105 was highly expressed in 60% (12/20) of primary SCCs that did not metastasize and 100% (3/3) of metastatic SCCs. The mean H-score for primary SCC was 132 and for metastatic SCC was 270.	Primary SCCs that did not metastasize (n=20) were compared to primary SCCs that metastasized (n=3).	A multivariate analysis was not reported.

Lai *et al*., 2015_1[385]	FOXP3+	Prospective and Retrospective	FOXP3+ cells were more abundant in primary SCCs that metastasized than primary SCCs that did not metastasize, present in 49.3% ± 13.8% versus 23.5% ± 11.0% of immune infiltrate, respectively (p<0.0001).	Primary SCCs that did not metastasize (n=26) were compared to primary SCCs that metastasized (n=29) (5 year follow-up period).	A multivariate analysis was not reported.
Lai *et al*., 2015_2[385]	OX40+	Prospective and Retrospective	OX40+ cells were more abundant in primary SCCs that metastasized than primary SCCs that did not metastasize, present in 17.0% ± 10.7% versus 11.7% ± 6.9% of immune infiltrate, respectively (p=0.0041).	Primary SCCs that did not metastasize (n=49) were compared to primary SCCs that metastasized (n=48) (5 year follow-up period).	A multivariate analysis was not reported.

Abbreviations:

CI=confidence interval

HR=hazard ratio

Appendix 3, Table S3, Thresholds and quantification of expression for Chapter 6.1
Key: Smith *et al*., 2011_2-4= study by Smith *et al*. in 2011, markers 2 through 4 studied by that author in that study; studies denoted with “A” or “B” indicate a different manuscript by the same author in the same year

Author/Year	**Thresholds for Expression**
Toll *et al*., 2013_1-6[264]	Scoring was based on the % of positive cells: The threshold for podoplanin and nuclear E-cadherin was 1%+ positively staining cells. The threshold for vimentin expression was 10%+ positively staining cells. The threshold for beta-catenin, twist, and zeb1 positivity was 5%+ cells with nuclear expression.

Hesse *et al*., 2016_1-2[161]	Staining was evaluated by intensity (relative to the normal epidermis): 0= no staining 1= lower 2= equal 3= stronger 4=very strong And by quantity: 0= 0% 1= 1-25% 2= 26-50% 3= 51-75% 4= 76-100%
Canueto *et al*., 2017_A[268]	Podoplanin intensity was defined: 0= none 1= weak 2= moderate 3= intense The percentage of positive cells was quantified as a percentage of stained cells: 0= <25% 1= 26–50% 2= 51–75% 3= >76%

Kreppel *et al*., 2012[269]	Staining intensity was classified from 0-3: 0 = no podoplanin expression 1 = weak expression 2 = moderate expression 3 = high expression The percent of positive cells was determined using the same scoring scheme as intensity. Scores were added up, divided by two, and rounded down if necessary.
Zhang *et al*., 2013[273]	Positive cells were counted using Image J software.
Azzimonti *et al*., (2015)[274]	The inflammatory cells outside tumor islands were analyzed as the peritumoral infiltrate. Ten random high-power fields (HPFs) were selected for quantification using the Image-Pro Plus 6.0 software technology.
Garcia-Diez *et al*., 2018[276]	The follow scoring system was used for staining intensity: 0= negative 1= weak 2= moderate 3= intense Tumors with weak (+) staining intensity and at least 1%+ cells staining positively were considered to be positive for PD-L1. The percentage of tumor cells with partial or complete membranous staining were also quantified.

Kamiya *et al*., 2018[277]	The follow scoring system was used for staining intensity: 0= negative 1= weak 2= moderate 3= intense High intensity scores were considering staining scores of 2 or 3. The percentage of positive cells was also evaluated and categorized into the following groups: < 1%, 1-49%, ≥50%
Garcia-Pedrero *et al*., 2017[278]	PD-L1 expression was scored: Negative= <1% stained cells Very low= ≥1% to <10% stained cells Low= ≥10% to <25% stained cells Intermediate= ≥25% to <50% stained cells High= (≥50%) stained cells A threshold of 25%+ of positive cells was met for tumors at an increased risk of metastasis
Amoils *et al*., 2019[279]	Staining was scored as follows: Grade 1= 5-10% positivity Grade 2= 11-25% positivity Grade 3= >25% positivity Tumors were considered PD-L1+ if at least 5% of tumor cells demonstrated membranous staining.

Slater *et al*., 2016[280]	The follow scoring system was used for staining intensity: 0= negative 1+= weak 2+= moderate 3+= intense Tumor proportion scores (TPS) were calculated as follows: No expression= TPS < 1% Low PD-L1 expression= 1-49% High PD-L1 expression= 50%+ Staining was evaluated using TPS and the percentage of cells with at least 1+ staining.
Ch'ng *et al*., 2008[287]	The follow scoring system was used for staining intensity: 0= negative 1+= weak 2+= moderate 3+= intense Protein overexpression was defined by cases with 3+ staining.
Cañueto *et al*., 2017_B[371]	The follow scoring system was used for staining intensity: += weak ++= moderate +++= strong

	Protein overexpression was defined by cases with 3+ staining.
Shimizu *et al*., 2001[82]	Scoring was as follows: Negative= no reactivity Weakly positive= up to 25% tumor stained Moderately positive= 25-75% of tumor stained Strongly positive= 76%+ of tumor stained
Sweeny *et al*., 2011[288]	Scoring was as follows: 0 = none to <10% of the tumor cells staining positively 1+ = light (intensity) and incomplete (quality) staining in ≥10% of the tumor cells 2+ = moderate and complete staining of ≥10% of the tumor cells 3+ = intense and complete staining ≥10%
Huang *et al*., 2012[301]	The degree of positive staining was graded as follows: 1+= weak/focal 2+= moderate/focal or diffuse 3+= strong diffuse The threshold for positivity was 5% of cells staining positive.
Mastoraki *et al*., (2009)[302]	Tumors classified as positive by two pathologists were quantified in five high-power fields as follows: 0 = <0.5% of cells expressing Cyclin D

	+ = 1-10% of cells expressing Cyclin D ++ = 10-20% of cells expressing Cyclin D +++ = >20% of cells expressing Cyclin D
Suiqing *et al*., 2005[372]	Pink brown or yellow staining= 1 Dark brown staining= 2 Positive cell rate <20%= 1 Positive cell rate 20–50%= 2 Positive cell rate >50%= 3 Scores were added and graded into one of three categories: Score 2–3= (+) Score 4–5= (++) Score >5= (+++) Completely negative= (–)
Liu *et al*., 2013[373]	The percent of positive cells were assigned a score: 0= <5% 1= 5–25% 2= 26–50% 3= 51–75% 4= >75% Intensities were scored: 1+= weak 2+= moderate 3+= intense

	The two scores were multiplied to produce a weighted score.
Wang *et al*., 2012[374]	Staining intensity was graded: 0= none 1= weak 2= moderate 3= strong The H-score was calculated by the sum of all the intensities multiplied by the proportion of cells with that intensity.
Keehn *et al*., 2004[375]	Staining was scored: 0= no staining 1= weak nuclear staining 2= moderate nuclear staining 3= intense nuclear staining Averages were calculated for each slide.

Munguia-Calzada *et al*., 2019[376]	Staining was scored based on intensity: 0= negative 1= weak 2= moderate 3= strong Scores of 2+ were considered “overexpression”.
Kang *et al*., 2009[377]	Staining was graded relative to the normal epidermis: - = completely negative staining intensity ± = lower staining intensity + = more or less overlapped staining intensity in the differentiated areas ++ = slightly higher staining intensity +++ = considerably higher staining intensity
Khandelwal *et al*., 2016[378]	Staining was graded: 0= no staining 1+= weak or focal staining 2+= moderate staining 3+= strong staining A modified H-score method was then used, which multiplies the staining intensity and the percentage of cells staining positively in the tumor: 1= 0%-25% 2= 26%-50% 3= 51%-75% 4= 76%-100%

Sekulic *et al*., 2010[379]	Intensity was scored from 0-3 using the standard scoring system: 0= no staining 1= weak staining 2= moderate staining 3= intense staining
Hernandez-Ruiz *et al*., 2018_1-2[380]	Staining intensity was scored: 0= negative or trace 1= low 2= medium 3= strong
Tanemura *et al*., 2005[381]	Staining was graded in comparison to the normal epidermis: -= negative +/- = less staining intensity + = equal or slightly higher staining intensity ++ = considerably higher staining intensity Samples were categorized into two groups:

	class 1= equal to stronger staining class 2= negative to weaker staining
Santos-Juanes *et al*., 2019[382]	Staining was categorized: None/weak Moderate Strong
Chen *et al*., 2014[383]	The threshold for high p300 expression was 55%+ tumor cells staining positively.
Muchemwa *et al*., 2006[384]	The H-score was calculated by Pi(i + 1) i= staining intensity (0-2) Pi= estimate percent of stained tumor cells Overexpression was defined as an H-score of 100+.
Lai *et al*., 2015_1-2[385]	The percent of cells in immune infiltrate were counted using Image J software.

Appendix 4: Cohort Characteristics for Chapter 5
ICP=Immunocompetent Patient

ISP=Immunosuppressed Patient

Study	N (this study)	Localized n\ Metastatic n	Males\ Female	Age (average)	ICP\ISP	Location
Al-Rohil et al., 2015	71	0\71	60\11	64.9	NA	NA
Durinck et al., 2011	8	8\0	7\1	74.6	7\1	Cheek: 2 Ear: 1 Lip: 1 Scalp: 2 Temple: 1 UE: 1
Inman et al., 2018	9	9\0	6\3	70	8\1	Cheek: 1 Forehead: 1 LE: 2 Nose: 1 Trunk: 1 UE: 3
Lee et al., 2014	100	100\0	44\22 (NA=32)	NA	NA	Ear: 8 Face (other): 1 Forehead: 6 LE: 11 Mandible: 1 Nose: 1 Orbital: 1 Periauricular: 1 Scalp: 11 Temple: 3 Trunk: 6 UE: 14
Lobl et al., 2020	20	10\10	16\4	68.2	12\8	Cheek: 2 Ear: 7 Eyelid: 1 LE: 1 Maxillary: 1 Neck: 1 Nose: 1 Scalp: 4 Supraorbital: 1

						UE: 1
Pickering et al., 2014	32	28\4	29\3	66.5	32\0	Cheek: 2 Ear: 7 Lip: 1 Neck: 1 Nose: 4 Periorbital: 6 Preauricular: 7 Scalp: 4
South et al., 20114	20	16\4	20\0	66.2	4\16	Cheek: 1 Ear: 3 Forehead: 2 Jawline: 1 LE: 2 Neck: 2 Scalp: 2 Trunk: 3 UE: 4
Yilmaz et al., 2017	9	8\1	7\2	61.9	9\0	Ear: 1 Face (other): 1 LE: 2 Lip: 1 Neck: 1 Trunk: 1 LE: 2
Zilberg et al., 2018	10	10\0	8\2	55 or under (2), 56-65 (2), 66 and above (6)	NA	Ear: 2 Forehead/scalp: 4 Lip: 2 Nose: 1 Pre-auricular: 1

Appendix 5: A comment on SCC in skin of color

While this thesis focused predominately on SCC in Caucasian patients, it is important to recognize differences in SCC between races. It is predicted that by 2045, African Americans, Asians, and Hispanics will compose 50% of United States population, highlighting the need for additional research in skin of color. While overall less common in skin of color, SCC is the most common skin cancer in African Americans.[386] African Americans have a tendency to develop SCCs at sites of prior wounds (Marjolin ulcer), which tend to be more aggressive.[386,387] In Black patients, a study of 58 SCCs reported that 61% developed in areas not exposed to the sun and 41% developed in areas of burn scars of chronic inflammation.[388] Additional data suggest that outcomes are worse in African Americans than Caucasians, which may result from delayed diagnosis due to varied clinical presentations, as well as the social determinants of health. In Hispanics, SCC is the second most common skin cancer.[386] SCCs in Asians have a higher likelihood of metastasis, which may be due to delays in diagnosis as they can occur more often in anatomic locations not exposed to the sun.[389]

It has been demonstrated that cancers behave differently in patients of different races. A review by Özdemir *et al*. (2017) examined several driver mutations in cancer, including *ALK*, and the difference in prevalence of these by race.[387] In lung cancer, *ALK* mutations were found in 4% of African Americans, 5.6% of Caucasians, and 4.9-67% of Asians.[387,390–394] The only skin cancer included in this review was melanoma, and the prevalence of *BRAF* mutations was variable between races, with 8% of African Americans, 21% of Caucasians, and 24-25.5% of Asians harboring this mutation.[387,395,396] A pilot study by Lobl *et al*. (2021) examined genetic intratumoral differences in basal cell carcinoma between Caucasians, Hispanics, and Asians and found that *GATA3* mutations and frameshift mutations were most common in the Hispanic cohort (Lobl *et al*., 2021).

When considering race and ethnicity, it is important to recognize that racism and systemic racism affect patient outcomes. In some cancers, differences in prognosis may relate to access, trust, diagnostic diligence, and treatment inequities rooted in racism. Race is a social construct and thus genetic differences are unlikely to account for cancer disparities. In skin cancer, skin color itself is a biologically significant variable and differences may be even more pronounced as the variations in pigmentation and UV protection are directly related to skin color. The particularly large differences in mutations noted between races highlights the need for sequencing studies with inclusive cohorts to identify possible differences in driver mutations between races that may impact therapeutic options and responses. Clinical trials under sample minority patients, with only an estimated 10% of clinical trial participants being minorities.[397] Improving recruitment and patient trust are necessary and an important part of research.