Mutational landscape of aggressive cutaneous squamous cell carcinoma


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Title: Mutational landscape of aggressive cutaneous squamous cell carcinoma

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Abbreviations: Nonmelanoma skin cancer (NMSC), basal cell carcinoma (BCC), cutaneous squamous cell carcinoma (cSCC), perineural invasion (PNI), disease-specific survival (DSS), American Joint Committee on Cancer (AJCC), dinucleotide polymorphisms (DNP), head and
neck squamous cell carcinoma (HNSCC), lung squamous cell carcinoma (LUSC), melanoma (SKCM).

**Notes:** * These authors contributed equally.

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Abstract:

Purpose: Aggressive cutaneous squamous cell carcinoma (cSCC) is often a disfiguring and lethal disease. Very little is currently known about the mutations that drive aggressive cSCC.

Experimental Design: Whole exome sequencing was performed on 39 cases of aggressive cSCC to identify driver genes and novel therapeutic targets. Significantly mutated genes were identified with MutSig or complementary methods developed to specifically identify candidate tumor suppressors based upon their inactivating mutation bias.

Results: Despite the very high mutational background caused by UV exposure, 23 candidate drivers were identified including the well-known cancer-associated genes TP53, CDKN2A, NOTCH1, AJUBA, HRAS, CASP8, FAT1, and KMT2C (MLL3). Three novel candidate tumor suppressors with putative links to cancer or differentiation, NOTCH2, PARD3 and RASA1, were also identified as possible drivers in cSCC. KMT2C mutations were associated with poor outcome and increased bone invasion.

Conclusions: The mutational spectrum of cSCC is similar to that of head and neck squamous cell carcinoma and dominated by tumor suppressor genes. These results improve the foundation for understanding this disease and should aid in identifying and treating aggressive cSCC.

Translational Relevance:

The mutational spectrum of aggressive cutaneous squamous cell carcinoma contains a signature of exposure to UVB, which should aid in the definitive diagnosis for squamous tumors and metastases with ambiguous site of origin. Additionally, the mutational landscape is dominated by tumor suppressor genes, resembles that of head and neck squamous cell carcinoma, but includes novel candidate drivers. Many of the mutated genes are related to differentiation pathways.
KMT2C mutations are associated with poor outcome and could represent a new biomarker for aggressive disease. They also suggest an epigenetic component to this disease that could possibly be targeted. Mutations in HRAS and STK19 are candidate oncogenic events, but are not yet targetable. These findings expand our knowledge of this disease and should aid in the development of genomically driven treatments.

**Introduction:**

Cutaneous squamous cell carcinoma (cSCC) is the second most frequent cancer among Caucasians with an incidence of approximately one million cases per year (1). Cutaneous SCC (25%) and basal cell carcinoma (BCC) (75%) are the major subtypes of nonmelanoma skin cancer (NMSC) (2). Most cSCC arise in the head and neck region because it is frequently exposed to sunlight and its ensuing ultra-violet radiation-induced DNA damage, which is the major etiological factor (2). Immunosuppression, usually associated with organ transplantation, elevates the risk of developing cSCC by over 100-fold (3). Although cSCCs frequently respond well to conventional treatments, including electrodessication and curettage, cryosurgery, wide local excision, and radiotherapy, 3 to 5% of these tumors recur (4). According to a recent large study, patients with cSCC have a 3.7% risk of metastasis and 2.1% risk of disease-specific death (5). Clinically, aggressive cSCCs are characterized by frequent and multiple recurrences necessitating large surgical excisions, increased tendency for regional metastasis, and significant disease-related mortality (6). When aggressive or highly invasive cSCC occurs in the head and neck, surgical treatment can have profound functional, cosmetic, and psychosocial effects, sometimes leading to loss of an eye, ear, or a nose. This may require significant reconstruction and diminish quality of life.
In a large prospective study (6), a primary tumor size greater than or equal to 4 cm, the presence of perineural invasion (PNI), or invasion beyond the subcutaneous tissue were all associated with aggressive cSCC and significantly decreased three-year disease-specific survival (DSS). The American Joint Committee on Cancer (AJCC) has also recognized aggressive features of cSCC that lead to upstaging the disease and are associated with increased risk of recurrence or metastasis, including invasion of bone, tumor sizes greater than 2 cm, or presence of at least two high risk factors such as poor differentiation, PNI, depth of invasion greater than 2 mm, occurrence at a high risk site (i.e., ear or lip), or Clark level greater than or equal to 4 (7).

While some attention has been given to targeting the epidermal growth factor receptor (EGFR) there are no standard effective treatments beyond surgery and radiation for cSCC (8, 9). There is an urgent need to identify new therapeutic targets for this group of patients. Knowledge regarding the genetic underpinning of this cancer remains largely rudimentary. Specific mutations in cSCC have been identified in TP53 (10), NOTCH receptors (11, 12), and RAS (13).

Efforts to characterize the genetic landscape of cSCC have been hampered by very high background mutation rates associated with UV damage (12, 14, 15), which can be 5 to 15 times greater than what is found for non-cutaneous tumors. The extraordinarily high background mutation rate makes it difficult to identify driver mutations from passengers. Although there have been some recent reports of exome data for cSCC (11, 12) and BCC (15), these cohorts were heterogeneous and small, making it difficult to identify potentially novel tumor drivers.
In the present study, we examined exomic mutations in a cohort of patients with aggressive cSCC. We hypothesized that the analysis of genomic data from a larger cohort of patients with clinically aggressive cSCC disease would permit more definitive characterization of the mutations that contribute to overall disease progression in this subset of disease with poorer prognosis.

**Methods:**

**Tissue processing.** Fresh-frozen surgically resected tumor and patient-matched normal lymphocytes were obtained from consented patients treated for cSCC of the head and neck region at The University of Texas M.D. Anderson Cancer Center, under an IRB approved protocol. Frozen tissue was embedded in OCT compound and completely sectioned. Tissue was washed once in PBS prior to isolating genomic DNA using an ArchivePure DNA purification kit (5Prime).

**Library Construction.** Genomic DNA samples were constructed into Illumina paired-end pre-capture libraries according to the manufacturer’s protocol (Illumina Multiplexing_SamplePrep_Guide_1005361_D) with modifications as described in the BCM-HGSC Illumina Barcoded Paired-End Capture Library Preparation protocol that is accessible from the HGSC website (https://hgsc.bcm.edu/sites/default/files/documents/Illumina_Barcoded_Paired-End_Capture_Library_Preparation.pdf).
Illumina Sequencing and Copy number. Four pre-capture libraries were pooled together and hybridized in solution to the HGSC VCRome 2.1 design1 (42Mb, NimbleGen) according to the manufacturer’s protocol NimbleGen SeqCap EZ Exome Library SR User’s Guide (Version 2.2) with minor revisions. Exomes were sequenced on an Illumina HiSeq 2000 platform to an average coverage of 115x. For both tumor and normal smaples, > 80% of bases achieved a q20 quality and 20x coverage. Details regarding library preparation and coverage for all samples are provided in the Supplemental Methods and Supplemental Table S4. Sequence analysis was performed using the HGSC Mercury analysis pipeline ([https://www.hgsc.bcm.edu/software/mercury](https://www.hgsc.bcm.edu/software/mercury)) to call mutations and generate BAM files (Supplemental Methods). Finalized BAM files generated from whole exome sequencing were then used to generate copy number data using an in house R package, BEDTools (16), and ABSOLUTE (17) to estimate the absolute copy number based on ploidy and purity (Supplemental Methods).

Significantly Mutated Genes: MutSigCV v1.4 which corrects for gene-specific background rates was run on maf files that included flanking regions. IntOGen v2.3.0 software (Universitat Pompeu Fabra), which examines whether genes are enriched for potential impactful mutations beyond what is expected by chance (18), was run as an online package. Two additional algorithms were developed to recognize a bias towards inactivating mutations (see Supplemental Methods).

Statistical analyses. Wilcoxon rank sum tests and Fisher’s exact tests were used to test associations between individual mutation and continuous/ordinal variables and nominal
variables, respectively. For analysis of primary site, tumors from the ear or lip were grouped into one high risk site category, and the remaining cases grouped as either preauricular, scalp, periorbital (including temple), cheek, or other. Pearson correlation coefficients and p-values were provided for assessing associations between total number of mutations and continuous/ordinal variables. Kruskal-Wallis tests were used to test associations between total number of mutations and nominal variables. Kappa coefficient correlations computed for each pair of genes and the results were summarized in a matrix plot. We used Cox model score test to assess if individual genes or total number of mutations are related with any of the time-to-event outcomes. All analyses were performed using SAS software (version 9.3; SAS Institute, Cary, NC).

Results:

Patient Characteristics

To comprehensively characterize the somatic mutations in aggressive cSCC, we performed whole exome sequencing on DNA from snap frozen tumors and matched normal blood from 39 patients. These cases were considered aggressive because they presented with regional or distant metastasis or had at least one of the features associated with increased mortality previously described by Clayman et. al. (6), (Figure 1, Supplemental Tables S1 and S2). All cSCCs studied arose on the head or neck, with the majority from the ear, preauricular, scalp, or periorbital regions: 38.5% were recurrent, 25.6% were persistent, and 35.9% were previously untreated. Seven of the samples were from metastatic sites. The majority of cSCCs (71.8%) had invaded beyond the subcutaneous space, 48.7% had PNI present, 43.6% were poorly differentiated, and 100% of evaluable cases had Clark levels ≥ 4.
Exome Sequencing

Prior to sequencing, samples were evaluated by a trained pathologist and found to have an average tumor cellularity of 49.4% ±22.4% as assessed visually (Supplemental Table S3). All specimens had greater than 10% tumor nuclei by pathology and sequencing analysis. Samples were sequenced to 115x average coverage (Supplemental Table S4). 108,034 somatic alterations were detected in 16,588 genes (Supplemental Table S5). The total number of mutations/patient ranged from 86 to 12112. A median of 61.2 mutations/Mb were detected in this cohort (Figure 2A). This mutation frequency is one of the highest mutation rates ever detected, is more than 4 times as high as the rate in melanoma (14), and is higher than the rate in other squamous tumor types (Figure 2A). These tumors did not contain functionally relevant *POLE* mutations (19). Additionally, the tumors were largely clonal. Less than 15% of mutated genes were found to have a low allele fraction (Supplemental Table S6), and only 4 samples had a subclonal genome fraction >15% (Supplemental Table S3). Although many of our tumors were previously treated there was no difference in the number (Supplemental Figure S1A) or types (Supplemental Figure S1B) of mutations between treated and untreated tumors. Instead, the vast majority of mutations appear to be caused by exposure to UV light, as is expected for skin tumors. UVB exposure is known to cause C>T transitions often following a pyrimidine base. When averaged across the cohort 75% of events were C>T transitions (Figure 2B & C) and 87% of those were at a C following a pyrimidine base. Additionally, 5.6% of events were dinucleotide polymorphisms (DNPs), which are another signature of UV exposure (Figure 2B & C). A few select tumor types are shown for comparison (Figure 2B).
Interestingly, 4 of the tumors did not appear to have a signature of UVB exposure (Figure 2C). They had a much lower rate of C>T mutations (average 39% C>T) and a lower overall number of mutations (average 294). This mutation signature is more similar to that of HPV negative head and neck squamous cell carcinoma (HNSCC), with a C>T frequency around 40% (Figure 2B)(20). These tumors represent all of the nose tumors in the cohort. Subsequent review of these clinical histories was ambiguous as to the source epithelium for the lesion, cutaneous or mucosal. It is possible that these tumors arose from the mucosal surface of the nose but grew out to the skin surface.

Given the high background rate of mutations in cSCC it is difficult to identify candidate driver genes. Previous sequencing studies of cSCC did not include enough cases for statistical determination of drivers and relied on frequency of mutations alone. We performed MutSig analysis (21) and identified 11 genes with a q-value < 0.1 (Table 1, and Supplemental Table S7); however, two of these (RBM46 and DCLK1) had low allelic fractions suggesting they may not be true drivers (Supplemental Table S6). Because of the high background rate we undertook additional methods to identify candidate driver genes. Analysis using the Integrative Onco Genomics (IntOGen) package (18), which scores genes according to the cumulative predicted functional impact of missense and other non-synonymous mutations, identified 292 candidate genes below the FDR cut-off of 0.1. Only seven of the genes detected as candidate drivers by IntOGen were also significant by MutSig. Because of the large number of significantly mutated genes detected by IntOGen (Supplemental Table S8) it is likely that many were false positives or passengers. We therefore sought out additional methods to identify drivers that would have increased specificity and not be affected greatly by a high mutation background. Tumor
suppressor genes frequently have a high proportion of inactivating mutations, and this signature has been used by others to identify putative tumor suppressors that may be drivers from cancer sequencing data (22). We developed two algorithms to detect a bias towards inactivating mutations. The first method treats all inactivating mutations (i.e., splice, frameshift, and nonsense) equally and uses the global frequency of inactivating and non-inactivating mutations actually observed in the cohort along with a chi-square statistic to calculate the likelihood that the inactivating to non-activating ratio for a specific gene is significantly higher than expected by chance. This chi-square method identified a list of 24 candidate tumor suppressor genes (Supplemental Table S9), including five well-known tumor suppressors that were among the top 10 most significant genes detected (Table 1). Although the chi-square approach can identify mutated genes with an inactivation bias, it is possible that certain genes are more predisposed to nonsense mutations because of their specific codon usage and the abnormally high rate of C>T (or G>A) mutations due to the UV signature. We therefore developed an additional method that takes into account the gene-specific codon usage and impact of UV signature to detect inactivation bias, using a different statistic based upon a multinomial probability model. Interestingly, the multinomial method largely identified a subset of the same genes found by the chi-square analysis (Table 1 and Supplemental Table S10). The results of MutSig, IntOGen, chi-square and multinomial analysis appear in Table 1, which includes genes identified as significant by MutSig (i.e., the gold standard) or by at least two of the other methods. TP53, CDKN2A, NOTCH2, NOTCH1, and AJUBA were identified as significant by all four methods, whereas SNX25, EIF2D, and PARD3 were significant by the three alternative methods but not by MutSig. Furthermore, the known tumor suppressor genes FAT1 and KMT2C (MLL3) and a putative tumor suppressor RASA1 (22, 23) were significant by the two methods detecting inactivation
bias. The distribution for these mutations among the cohort can be seen in Figure 3 along with the frequency of inactivating, missense, and silent mutations.

Interestingly, eight of our top 23 genes were also found to be significantly mutated in HNSCC (Table 1)(24). Only four genes were significant in lung squamous cell carcinoma (LUSC) and two genes were significant in melanoma (SKCM)(24). The genes common to cSCC and HNSCC are TP53, CDKN2A, NOTCH1, HRAS, CASP8, AJUBA, RASA1, FAT1, and KMT2D. The presence of moderately frequent mutations in eight common genes suggests that the biology of cSCC may be similar to that of HNSCC,

In both cSCC and HNSCC, NOTCH1 alterations appear to be inactivating (Figure 4) because the missense mutations cluster in the EGF-like repeats responsible for ligand binding, and the truncating mutations are distributed throughout the gene but not clustered in the C-terminal PEST domain, in contrast to what is found for T-cell Acute Lymphoblastic Leukemia. NOTCH1 and NOTCH2 were similarly mutated in >50% of cSCC cases and more than 30% of the mutations are inactivating. We recently showed that NOTCH1 is a tumor suppressor gene in HNSCC (25) but the role of NOTCH2 in cancer is poorly understood. NOTCH2 mutations are not statistically significant in HNSCC by MutSig, but do have a high inactivating mutation ratio in this cancer as well (Figure 4).

We next compared a few genes significantly mutated in related tumor types but not in cSCC. Mutations in the oxidative stress gene NFE2L2 were first described in LUSC (15% of cases) (26) and later found in HNSCC (7%)(24). No mutations in NFE2L2 were found in our cSCC cohort.
PIK3CA is also significantly mutated in LUSC (16%) and HNSCC (19%), but was mutated only five times in four cSCC patients (10%) and was not statistically significant (Table 1). Additionally, the mutations included 2 inactivating mutations and no mutations in the classical hotspots (E545, H1047) (Supplemental Table S5).

Melanoma is characterized by frequent hotspot mutations in BRAF and NRAS (14). No hotspot mutations in BRAF or NRAS were observed in cSCC. However, hotspot mutations in RAC1 and STK19 have been reported in melanoma and were found in our cohort. We observed 1 P29S mutation in RAC1, and 5 mutations around D89 in STK19. These included 3 D89N, 1 E88K, and 1 P90S mutations (Supplemental Table S5).

We also identified two new candidate tumor suppressor genes for cSCC. PARD3 and RASA1 were mutated in 31% and 13% of cSCC patients (Table 1), with 33% and 66% of their mutations predicted to truncate or eliminate the proteins (Figure 3), respectively. RASA1 was identified in pan-cancer analyses and HNSCC as a candidate tumor suppressor gene because of its high inactivation mutation ratio (22, 24).

Kappa analysis was performed to identify correlations between the mutations in each gene (Supplemental Table S11). HRAS was highly correlated with AJUBA (0.423, p=0.008) and inversely associated with TP53 (-0.107, p=0.004). TP53 and HRAS are also inversely correlated in HNSCC.
Because 4 tumors lacked a UV signature we repeated the MutSig analysis while excluding those tumors. The top 12 most significant genes remained at the top of the list, however one new gene was added as the 10th most significant gene, RIPK4 (q=0.053). This gene is quite interesting because it encodes a serine/threonine kinase essential for keratinocyte differentiation (27). RIPK4 was mutated in 28% of the tumors with a UV signature, with all mutations clustering in either exon 2 or exon 8 which encode the kinase and ankyrin repeat domains, respectively. There was also a high ratio of nonsense, frameshift, and splice mutations (35%) that was nearly significant by the two methods detecting inactivation bias, suggesting selection for inactivation of the gene in cSCC.

**Copy Number Alterations**

Copy number values were calculated from the exome sequencing coverage data and adjusted for purity and ploidy by using the ABSOLUTE algorithm. Large regions of copy number gain were frequently detected (in > 25% of samples) on chromosomes 7, 8q, 9q, 14, and 20, and regions of loss were detected on 3p, 4, 5q, 8p, 9p, 11, 17p, 18, 19, and 21 (Supplemental Figure S2 and Table S12). The CCND1 region of chromosome 11q was also focally amplified. In the copy number data, as with the mutation data, there are many similarities between cSCC and HNSCC. For example, both tumor types have losses in 3p, 5q, 8p, 18, and 21 and gains in 3q, 5p, 8q, 14, and 20 (25).

**Clinical Significance**

To begin addressing the importance of genetic alterations in cSCC, the top candidate genes along with the total number of mutations per patient were analyzed for clinicopathological...
associations. This analysis correlated 29 different clinical ordinal characteristics, three continuous clinical variables, and eight different measurable parameters related to time intervals or patient status (Supplemental Table S2). Although all of the patients in our cohort were selected based upon a clinical diagnosis of cSCC, in order to maintain clinical uniformity the four nasal cases with ambiguous site of origin were removed from the clinical analyses. As these studies were exploratory in nature, multiple testing corrections were not applied so that sensitivity could be maximized. A summary matrix of all p-values obtained for the clinical parameters tested by gene appears in Supplemental Table S13. The total number of mutations observed per tumor did not correlate with any of the genes or clinicopathological parameters examined except for histological subtype (p=0.02). Tumors classified as acantholytic had a median number of mutations (3589) that was roughly 1.5 times greater than tumors with no specific histologic subtype (2295) and more than triple the median number of mutations (1033) in tumors with sarcomatoid or adenosquamous histology.

Tumors with AJUBA mutations were positively correlated with depth of invasion (p = 0.02), and on average invaded with a depth (16.0 ± 6.4 mm) almost twice that of tumors lacking the mutation (8.4 ± 5.6 mm). The presence of PNI, a known aggressive feature, was positively associated with mutations in NOTCH2 (p=0.04) (Figure 5A). Approximately 70% of patients with NOTCH2 mutations had PNI present compared to just 33% of patients with no NOTCH2 mutation. Interestingly, NOTCH2 mutations were also associated with primary tumor site (p=0.04), as the presence of NOTCH2 mutation was more common in cSCCs arising in the scalp or periorbital regions compared to the ear (Figure 5A). The increased tendency for tumors with
NOTCH2 mutations to have PNI may be independent from tumor site, as there was no significant association between tumor site and PNI (p=0.19).

There was a highly significant positive association between bone invasion and KMT2C mutations (p=0.008). Only 10% of patients with wild type KMT2C had bone invasion, compared to 53% of patients with KMT2C mutation (Figure 5A). Consistent with the positive association between KMT2C and bone invasion, patients with KMT2C mutation had significantly shorter recurrent free survival times (p=0.003) with a median recurrent survival of 21.6 months compared to 167.5 months for patients with wild type KMT2C (Figure 5B). The hazard ratio for recurrence or death for patients with KMT2C mutation was 5.16 (1.55 to 17.18, 95% CI) compared to those whose tumors were wild type. Similarly, patients with tumors harboring KMT2C mutation had trends towards shorter time to disease recurrence (p=0.07), and shorter overall survival (p=0.09). Poor prognosis of patients with KMT2C mutation appeared to be independent from bone invasion, as patients with bone invasion did not have shortened recurrent free survival times (p=0.98).

Discussion:
We have generated the first list of significantly mutated candidate driver genes in aggressive cSCC. The background mutation rate in cSCC is so high that more than half of all genes were mutated and 218 genes were mutated in at least half of the patients in our study. We report what are likely to be many of the most important drivers in aggressive cSCC. Currently, very few studies have comprehensively examined the mutational landscape of cSCC. These prior studies were unable to achieve statistical significance and little clinical information exists about their
cohorts. Thus, it is presently unknown whether the candidate drivers we identified are enriched in aggressive cSCC compared to cSCC with a more benign clinical course.

Six out of our top genes in aggressive cSCC, TP53, CDKN2A, NOTCH1, NOTCH2, HRAS, and FAT1 were previously reported by South et al. in a cohort comprised of 20 cSCCs derived mostly from patients with immunosuppression secondary to organ transplantation (12). It is interesting to note that cSCCs that arise in organ transplant recipients are frequently aggressive, suggesting a common biology despite differences in predisposing risk factors. Interestingly, another group has recently characterized the genomic landscape of BCC (15), and also observed frequent mutations in NOTCH1, NOTCH2, and TP53. However, PTCH1 was the only gene in BCC found to be significantly mutated. PTCH1 mutations occurred in 75% of BCC tumors and 70% of the alterations were inactivating. In our cSCC cohort, PTCH1 mutations occurred in just 17% of patients and only 2 mutations were inactivating, suggesting that PTCH1 is not a driver in aggressive cSCC.

The mutational spectrum of cSCC is quite similar to that of HNSCC. Eight of the top mutated genes are shared between these tumor types, both derived from stratified squamous epithelia. The major mutational difference between cSCC and HNSCC is the UV signature. We identified 4 tumors from the nose that lacked the UV signature. Subsequent review of those patient records identified ambiguity in the source of the tumor with respect to the skin or mucosa of the nose. Some mucosal tumors in the nose can grow through the skin and present as an apparently cutaneous lesion, especially for large tumors. Similarly, nodal or parotid metastases in the head and neck region may have uncertainty regarding whether the tumor originated in skin or mucosa.
Our data suggest that the UV signature may be able to aid clinicians in making a definitive diagnosis in these cases.

Although high frequencies of both NOTCH1 and NOTCH2 mutations in cSCC were previously reported by two groups (11, 12), we were able to show for the first time that both genes are significantly mutated in cSCC by using MutSig. Recently we demonstrated that NOTCH1 behaves functionally as an in vitro and in vivo tumor suppressor in HNSCC (25). A similar role is likely in cSCC, since conditional knockout of NOTCH1 in mouse skin predisposes animals to skin tumors (28). A role for NOTCH2 in cancers is less clear because mice with conditional knockout of NOTCH2 are not predisposed to tumors (29). However, activated NOTCH2 can arrest the growth of keratinocytes (29) in vitro and combined inactivation of both NOTCH1 and NOTCH2 can more severely alter differentiation of skin than loss of NOTCH1 alone (29). Collectively, the data suggest NOTCH2 activation may inhibit tumor growth and future functional studies are needed.

It is possible that the cell of origin for the tumor may influence the relative roles of NOTCH1 and NOTCH2. NOTCH1 and its presumed ligand JAG1 are expressed in the lower to middle epidermal layers, while expression of NOTCH2 and its presumed ligands, JAG2 and DLL1, are confined to the basal cells of skin (29). NOTCH1 and NOTCH2 may both be a barrier to carcinogenesis in some systems (human), while NOTCH1 may be the primary barrier for other systems (mouse).
An interesting and novel candidate driver gene we identified was *RIPK4*, which is known to control keratinocyte differentiation (27). Inactivating mutations in this gene are associated with a severe autosomal recessive lethal disease in humans (30) known as Popliteal Pterygium Syndrome (also Bartsocas-Papas syndrome) that affects the face, limbs, and genitalia. Knockout of *RIPK4* in mice produces a similar neonatal lethal syndrome accompanied by defective epidermal differentiation, including keratinocyte hyperplasia with expanded spinous and granular layers (27). The clustering of *RIPK4* mutations within the kinase and ankyrin repeat domains, strongly suggests the mutations were non-random and support a hypothesis that *RIPK4* is a putative tumor suppressor for aggressive cSCC.

Many of the identified genes are related to differentiation signaling. These include *NOTCH1*, *NOTCH2*, *FAT1*, *AJUBA*, *CASP8*, and *RIPK4*. Several of these genes were also identified in HNSCC and linked to differentiation there as well. This suggests a common and important barrier to tumorigenesis in these squamous epithelial tumors.

Another interesting candidate tumor suppressor gene in cSCC is *RASA1*. *RASA1* belongs to a family of RAS GTPase activating proteins, many of which have been implicated as tumor suppressors in cancer because they function to negatively regulate pro-oncogenic RAS (23). RAS GTPase family members with confirmed tumor suppressor function include *NF1*, *DAB2IP*, and *RASAL2*, which are frequently inactivated in tumors through genomic loss, mutation, or epigenetic silencing (23). Inactivation of these genes has been proposed to explain activation of the RAS pathway in tumors that do not harboring specific RAS mutations. The role of *RASA1* in
cancer has not been clearly defined, despite the fact that it is frequently inactivated by mutation in many other tumor types (22).

A primary goal of this study was to identify new targetable genes for the treatment of cSCC, because there are very few non-surgical options for patients with aggressive disease. Unfortunately, we did not identify any easily targetable events. The most frequently altered genes are nearly all tumor suppressor genes, similar to what was found in HNSCC (25). The most obvious oncogene identified is \textit{HRAS}, which has proven difficult to target. An interesting novel target is \textit{STK19}. The pattern of mutational clustering suggests that they may activate the kinase. Although little is currently known about the function of \textit{STK19} and which drugs can target it, it is hoped that this genetic characterization of cSCC will lead to identification of driver pathways that will be targetable. Even if mutated tumor suppressor genes are not directly targetable, it is possible that the pathways they define can be targetable through other co-dependent genes or that a synthetic lethality can be identified.

In our cohort of aggressive cSCC, we found frequent inactivating mutations in \textit{KMT2C}, which encodes a component of a histone methylation complex involved in transcriptional regulation. Inactivating \textit{KMT2C} mutations have been reported for a number of tumors, including cancers of the stomach (31), bladder (32), and breast (33). In the TCGA stomach cancer dataset there is a trend towards reduced overall survival in patients with \textit{KMT2C} mutation compared to patients who are wild type (median 13 vs 59 months). In our aggressive cSCC cohort, patients with \textit{KMT2C} mutations had significantly shorter recurrent free survival, shorter time to recurrence,
and were more likely to have bone invasion. The data support a role for \textit{KMT2C} in the aggressive behavior of cSCC.

One of the challenges both patients and clinicians face with aggressive head and neck cSCC is the invasive tumor behavior and the innumerable recurrences that eventually require extensive surgeries; often affecting function and form. Therefore the stakes are high in identifying the subset of cSCC that will manifest an unfavorable biology. Understanding the genomic signatures of aggressive cSCC provides an opportunity to intensify upfront therapies in order to prevent the morbid consequences of treating advanced disease. Our results set the stage for understanding and recognizing the key drivers in this disease. The goal is to use this information to explore potential biomarkers that predict aggressive cSCC and identify genomically-targeted therapies that are effective for those patients.

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\textbf{References:}


**Figure Legends:**

Figure 1: Aggressive features of cSCC. Aggressive features used to define this cohort are shown in red. Features that result in up-staging by AJCC criteria are shown in blue.
Figure 2: Mutation frequency and types in cSCC. A) Mutation frequency in cSCC compared to other tumor types. Median value is shown and indicated by the horizontal line. Non-cSCC samples are TCGA data from Lawrence et.al. (24). B) Mutation types in cSCC compared to other tumor types. Mutation type frequencies were calculated for each sample and then averaged across the cohort to eliminate bias from highly mutated samples. HNSCC and LUSC are TCGA data from Kandoth et.al. (20) Melanoma data are from Hodis et.al. (14) Abbreviations: HNSCC – head and neck squamous cell carcinoma, LUSC – lung squamous cell carcinoma, SKCM – skin cutaneous melanoma, cSCC – cutaneous squamous cell carcinoma, DNP – dinucleotide polymorphism.

Figure 3: Key mutations in cSCC. Total number of mutations per patient is shown on the top. Inactivating mutations include nonsense, frame-shift, and splice site events. Mutation frequency for each gene is shown on the right.

Figure 4: Mutations in NOTCH1 and NOTCH2 appear to be inactivating in cSCC. A) The spectrum and location of NOTCH1 mutations observed in cSCC, HNSCC (TCGA data), are compared to T-cell acute lymphoblastic leukemia (COSMIC data) where NOTCH1 alterations are instead activating. In both cSCC and HNSCC, most missense mutations cluster in the N-terminal EGF-like repeats where ligand binding occurs, and truncating mutations are distributed throughout the gene; whereas, in leukemia truncating mutations are confined to the C-terminal PEST domain responsible for degradation of activated intracellular NOTCH1 and missense mutations cluster in the heterodimerization domain where they cause ligand-independent activation. B) NOTCH2 alterations in cSCC and HNSCC have a similar pattern because missense mutations are clustered in the EGF-like domains and truncating mutations are scattered throughout the gene, suggesting the alterations inactivate function. Red dots indicate truncating
mutations (splice, frameshift, or nonsense), green dots indicate missense mutations, black dots indicate inframe insertions or deletions, and purple dots indicate residues affected by different mutation types. NOTCH domains are indicated with different colors. Light green = EGF-like repeat, dark green = calcium binding EGF domain, yellow = LNR (negative regulatory) repeat, blue = heterodimerization domain (HD), orange = transmembrane (TM) region, pink = RAM domain, purple = ankyrin repeats, blue = transactivation domain (TAD), and red = Proline-Glutamic acid-Serine-Threonine rich (PEST) domain.

Figure 5: Mutation status and clinical parameters. A) Association between mutation status and various clinical parameters. B) \textit{KMT2C} mutation is associated with shorter time to recurrence. C) \textit{KMT2C} mutation is associated with worse recurrence-free survival. Censored events are indicated by a vertical bar.
Figure 1

- Invade S.C. Tissue
- PNI
- T ≥ 4cm
- Regional Lymph Nodes
- Metastasis
- Invades bone
- T > 2cm
- Poorly Differentiated
- High Risk Site
- Clark ≥ 4

Legend:
- Aggressive Feature
- AJCC Up-staging Feature
- Unknown

Site:
- Periorbital
- Scalp
- Preauricular
- Ear/lower lip
- Nose
- Other

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Figure 2

A

Somatic mutation frequency (Mb)

HNSCC (178)  |  LUSC (178)  |  Melanoma (121)  |  cSCC (39)

3.2

8.2

13.2

61.2

B

Frequency

C


A>C  |  A>G  |  A>T  |  C>A  |  C>G  |  CC>TT  |  other DNP

Research.

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Table 1

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Statistically significant genes are indicated with an *. TCGA data were collected from cBioPortal. TCGA significance was determined by MutSig in the pan-cancer analysis of Lawrence et al. ND = not determined.
Figure 3

Mutations (thousands)

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Figure 5

A

- **NOTCH2 Mutation**
  - Other: 10
  - Periorbital: 5
  - Ear/lip: 1
  - Scalp: 4
  - Periauricular: 1

- **NOTCH2 Wild Type**
  - Other: 15
  - Periorbital: 10
  - Ear/lip: 5
  - Scalp: 3
  - Periauricular: 2

- **Bone invasion**
  - No bone invasion: 20
  - Bone invasion: 10

- **PNI**
  - No PNI: 15
  - PNI: 5

- **KMT2C Wild Type**
  - Recurrence Free (%): 70%

- **KMT2C Mutation**
  - Recurrence Free (%): 30%

B

- **KMT2C Wild Type**
  - Recurrence Free Survival (%): 90%

- **KMT2C Mutation**
  - Recurrence Free Survival (%): 53%

C

- **KMT2C Wild Type**
  - Recurrence Free (%): 100%

- **KMT2C Mutation**
  - Recurrence Free (%): 90%