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Abstract
Cutaneous squamous cell carcinoma is the second most prevalent malignancy, most frequently occurring in the head and neck (head and neck cutaneous squamous cell carcinoma). Treatment of locally advanced or metastatic disease is associated with functional morbidity and disfigurement. Underlying genetic mechanisms are poorly understood. Targeted sequencing of 48 clinically relevant genes was performed on DNA extracted from formalinfixed and paraffin-embedded high-risk primary head and neck cutaneous squamous cell carcinomas that remained non-metastatic at minimum follow-up of 24 months. Associations of somatic mutations with clinicopathologic characteristics were evaluated and compared with those described in the literature for metastatic disease. Alterations in 44 cancer-associated genes were identified. TP53 was mutated in 100% of cases; APC, ATM, ERBB4, GNAQ, KIT, RB1 and ABL1 were altered in 60% of cases. FGFR2 mutations (40%) were exclusively seen in patients with perineural invasion. MLH1 mutations were exclusively seen in the two younger patients (<45>years). Lower incidences of NOTCH1 mutations were observed compared with that described in metastatic head and neck cutaneous squamous cell carcinoma in the literature. Somatic mutations susceptible to EGFR inhibitors, and other small molecular targeted therapeutics were seen in 60% of cases. This study provides insights into somatic mutations in non-metastatic, high-risk head and neck cutaneous squamous cell carcinoma and identifies potential therapeutic targets. Alterations in FGFR2 and NOTCH1 may have roles in local and distant disease progression.

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Analysis of Clinically Relevant Somatic Mutations in High Risk Head and Neck Cutaneous Squamous Cell Carcinoma

Somatic Mutation Analysis of HNcSCC

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Abstract

Cutaneous Squamous Cell Carcinoma is the second most prevalent malignancy, most frequently occurring in the head and neck (Head and Neck cutaneous squamous cell carcinoma). Treatment of locally advanced or metastatic disease is associated with functional morbidity and disfigurement. Underlying genetic mechanisms are poorly understood. Targeted sequencing of 48 clinically relevant genes was performed on DNA extracted from formalin fixed and paraffin embedded high risk primary Head and Neck cutaneous squamous cell carcinomas that remained non-metastatic at minimum follow up of 24 months. Associations of somatic mutations with clinicopathologic characteristics were evaluated and compared to those described in the literature for metastatic disease. Alterations in 44 cancer-associated genes were identified. *TP53* was mutated in 100% of cases; *APC, ATM, ERBB4, GNAQ, KIT, RB1* and *ABL1* were altered in 60% of cases. *FGFR2* mutations (40%) were exclusively seen in patients with peri-neural invasion. *MLH1* mutations were exclusively seen in the 2 younger patients (<45 years). Lower incidences of *NOTCH1* mutations were observed compared with that described in metastatic Head and Neck cutaneous squamous cell carcinoma in the literature. Somatic mutations susceptible to EGFR inhibitors, and other small molecular targeted therapeutics were seen in 60% of cases. This study provides insights into somatic mutations in non-metastatic, high risk Head and Neck cutaneous squamous cell carcinoma and identifies potential therapeutic targets. Alterations in *FGFR2* and *NOTCH1* may play roles in local and distant disease progression.

Cutaneous squamous cell carcinoma is the second most common malignancy in the world and most frequently occurs in the head and neck (1). Disease incidence is rising in countries with a high fair skin population and solar ultraviolet index, and with the aging population (2). The National Comprehensive Cancer Network (https://www.nccn.org/) guidelines indicate that nearly 14% of Head and Neck cutaneous squamous cell carcinomas seen in a Dermatology department are 'high-risk' lesions due to presence of perineural invasion and/or invasion of the subcutaneous tissues (3).

Surgical resection forms the mainstay of treatment and is followed in selected cases by adjuvant radiotherapy, unfortunately a significant proportion of Head and Neck cutaneous squamous cell carcinomas develop local recurrences and approximately 14% of high risk lesions develop regional metastases (3) . Predicting which patients will develop recurrence or metastases is not possible as the currently available clinical and histologic prognostic indicators are unreliable (4). Due to lack of effective second line therapies, over 30% of patients with advanced nodal disease and 89% of patients with distant metastases die from Head and Neck cutaneous squamous cell carcinoma, and in those cured by radical surgery the quality of life remains poor, highlighting the need for other therapeutic options (5).

The recent advances in massive parallel sequencing technologies have significantly transformed the treatment and survival in several lethal malignancies such as melanoma and pulmonary adenocarcinoma (6, 7). The utility of these techniques remains relatively unexplored in Head and Neck cutaneous squamous cell carcinoma. Herein, we evaluate the somatic mutations in high risk tumors using a clinically relevant, commercially available, targeted cancer gene panel to ensure universal applicability of our findings. A cohort of patients with high risk Head and Neck cutaneous squamous cell carcinoma that did not

develop metastases after a minimum follow up of 24 months was selected for this study to enable comparison with the cohorts of metastatic cutaneous squamous cell carcinoma described in the literature. The primary aims of this study were to identify somatic mutations associated with adverse histopathological features in Head and Neck cutaneous squamous cell carcinoma and to investigate the differences in somatic mutations observed in this non-metastatic cohort with those described in metastatic Head and Neck cutaneous squamous cell carcinoma cohorts in the literature. The secondary aim was to identify somatic mutations amenable to currently available clinical and preclinical targeted therapeutic agents.

Materials and Methods

Following institutional Human Research Ethics committee approval, patients with high risk Head and Neck cutaneous squamous cell carcinoma treated with curative intent between 2008 and 2014 were identified from the prospectively collected database held at the Sydney Head and Neck Cancer Institute. A representative example of the type of Head and Neck cutaneous squamous cell carcinoma included in this study is depicted in **Figure 1**. High risk disease was defined as per the criteria provided in the 7th edition of American Joint Commission on Cancer Staging Manual (8). Only patients who had either undergone concurrent sentinel node biopsy or neck dissection with at least 24 months of follow up and complete clinical data were included. Patients with histopathologically positive sentinel lymph node biopsies or concurrent neck dissections and those who developed nodal metastases during follow up were excluded. In total, 24 cases of high risk Head and Neck cutaneous squamous cell carcinoma met the selection criteria.

The histopathology slides and paraffin blocks were retrieved from the archives of the Department of Tissue Pathology and Diagnostic Oncology at Royal Prince Alfred Hospital,

Sydney, New South Wales, Australia. A complete histopathology review was performed and the tumor size, depth of invasion, lympho-vascular and perineural invasion, bone involvement and margins of resection were recorded. Highly cellular areas of the tumor with a neoplastic cell content of 30-90% and without necrosis, keratin, inflammatory infiltrate or hemorrhage were identified.

Malignant tissue selected as described above was macro-dissected from the blocks for deoxyribonucleic acid (DNA) extraction. Case matched normal tissue from the benign neck lymph nodes was used as germline control for mutation filtering. DNA extraction was performed using truXTRAC® formalin fixed paraffin embedded DNA microTUBE kit (Covaris, Woburn, MA, USA) as per the manufacturer's instructions.

Samples with sufficient DNA that passed the quality control checks using Illumina formalin fixed paraffin embedded quality control Kit were available in 10 cases. Thus, the final cohort used for library preparation included 10 patients. The TruSeq Amplification Cancer Panel® (Illumina, San Diego, USA) was utilized to assess 48 clinically relevant genes: *ABL1, ALK, AKT1, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL.* The reads were aligned to the human reference genome (GRCh37) using Isis Smith-Waterman-Gotoh (v2.6). Illumina Somatic Variant Caller (v4.0) Illumina variant studio (v2.3) was used for variant calling and annotation, respectively. Somatic variants were identified after deducting the normal/germline variants observed in the matched normal samples from those observed in the tumor samples. Variant positions with a coverage of 500x read depth or more, and those variant alleles observed at a >5% frequency were included in

further analysis. A single patient (case 1) did not have a matched normal, so variants known to be common in ExAC (MAF > 0.1%) were filtered for this individual.

In-silico analysis using PolyPhen 2, (9) and SIFT (10) was performed. Catalogue of Somatic Mutations in Cancer (COSMIC) databases (11) were used for functional annotation of the identified variants and to understand their potential interactions in the relevant signal transduction pathways. Functional Analysis through Hidden Markov Models (FATHMM) (http://fathmm.biocompute.org.uk/cancer.html) were used to understand the pathogenicity of the identified somatic alterations (12).

All functionally characterized mutations that have been shown to promote carcinogenesis in other malignant neoplasms, in animal models and in cell cultures, and all truncations and deletions in tumor suppressor genes were selected for further analysis.

Literature searches using PubMed for English language literature were performed to identify studies providing the details of somatic mutations in metastatic Head and Neck squamous cell carcinoma and somatic alterations amenable to currently available therapies.

Results

Clinicopathologic characteristics of the high-risk, non-metastatic cSCC cohort

The final cohort of 10 patients included 8 men and 2 women with a median age of 71 years (range 38-92 years) at the time of surgery. The median tumor diameter was 25mm (range 7-160mm) with a median depth of invasion of 10.5mm (range 2-70mm) and 4 (40%) patients exhibited peri-neural invasion (**Table 1**).

Overview of somatic mutations

Targeted sequencing of 48 cancer-associated genes to a mean coverage of 6907 ± 2044 (n=9) in the normal samples and 9544 ± 6486 (n=10) in the tumor samples was performed.

A total of 1313 mutations were identified across the 10 patient samples. These included 488 mutations in the coding region and 12 essential splice site variations, and 813 mutations in the non-coding region. Of the coding region and splice site mutations 464 somatic mutations were found to be functionally significant across 44 of the targeted genes using COSMIC database, FATHMM scores, literature review and including those mutations leading to truncations or deletions of genes. A mean of 46 (range 3-146) significant somatic mutations were observed per patient. Whilst the unpaired sample had a higher than average number of mutations, it did not demonstrate the greatest mutation burden (53 putative somatic variants vs range of 3-146). C/T and G/A transitions, characteristic of UV mediated DNA damage (13) were the dominant substitution comprising 72% of the mutation spectrum. 90% of samples contained at least one C/T transition.

The most frequently observed non-synonymous somatic mutations were in *TP53* (N=10,100%), *ATM* (N=6, 60%), *APC* (N=6, 60%), *ERBB4* (N=6, 60%), *GNAQ* (N=6, 60%), *ABL1* (N=6, 60%), *KIT* (N=5, 50%), *PIK3CA* (N=5, 50%), *AKT1 (*(N=5, 50%) and *RB1* (N=5, 50%). An overview of mutated genes and types of alterations identified is provided in **Figure 2**.

Table 2 list the validated loss of function mutations in tumor suppressor genes. Eleven mutually exclusive, functionally relevant *TP53* mutations were identified in 9 cases - 9 of which were within the DNA-binding domain of the gene (**Figure 3A**). *TP53* R282W has been described in cutaneous SCC (14) and basal cell carcinoma (15) while the others have been described in breast, colorectal and other carcinomas (**Table 2**).

Functionally significant alterations in the *APC* gene were observed in 6 patients. These

occurred within the mutational cluster region of the gene (codons 1030-1700 containing domains integral to β-catenin regulation). Two truncating mutations at positions R1114* and Q1328* in the *APC* gene that are well annotated in colorectal carcinoma (16, 17) were also observed (**Figure 3B**).

There were 3 nonsense and 2 deletion mutations in the *ATM* gene, including two cases with truncations at R2443* previously reported in Mantle Cell Lymphoma (18) (**Figure 3C**). Two patients with functionally relevant *ATM* mutations also showed loss of function mutations in the *MLH1* gene (Q407*, Q426*, R423*). These are novel *MLH1* mutations, resulting in loss of the C-terminal dimerization domain necessary for normal function of the mismatch repair protein (19).

The 3 cases with *SMAD4* mutations include; two deletions (c.533delC, S178*) and (c.1186delG, p. D396Mfs*19), and 2 truncating mutations, K122* and Q448*. *SMAD4* deletion in head and neck epithelia has been described to result in spontaneous generation of Head and Neck cutaneous squamous cell carcinoma in mice (20).

Loss of function mutations in *VHL* were identified in 3 cases, including R113* and Q132*; both of which are well characterized in renal cell carcinoma. Loss of function mutations in *PTEN* gene have been associated with disease progression and resistance to radiotherapy in head and neck and pulmonary squamous cell carcinoma (21, 22). A validated loss of function mutation in *PTEN*, Q17*, was observed in only a single patient. We noted 3 functionally significant mutations in *FBXW7* including W446* and W486* in 2 patients leading to premature truncation of the WD2 repeat domain. Both are well described in colorectal carcinoma (**Table 2**).

CDKN2A mutations are well characterized in cutaneous squamous cell carcinoma and in melanoma. 3 cases had mutations in this gene, 2 of these are the known truncating variants within the Ankyrin-repeat containing domain, R58* and E61*, described in cutaneous squamous cell carcinoma and melanoma (23).

Known gain of function mutations in oncogenes were present in 60% of samples. A total of 12 functionally relevant oncogenic gain of function mutations were present across 10 genes (**Table 3**). Mutations in receptor tyrosine kinases resulting in constitutive tyrosine kinase activity and elevated downstream signalling were seen in this cohort. Two mutations, *HRAS* G13D and *BRAF* G464R have been described in a metastatic cutaneous squamous cell carcinoma cohort (24). The *BRAF* mutation, and alterations in *KDR* Q472H, *KIT* T670I and *GNAQ* R183Q have been previously detected in cutaneous and uveal melanoma samples (25, 26). *EGFR* A864T is a rare activating variant (27) was also seen in this cohort**.** An activating *JAK3* A572T mutation, previously detected in T-cell acute lymphoblastic leukemia at a location shown to promote hematological malignancy in mice was identified**.** Two alterations in *PTPN11* (D61Y, T73I), characteristically found in hematological malignancies (28), were also identified in 2 patients.

Association with Clinicopathologic Characteristics:

Novel somatic mutations in *MLH1* (Q407*, Q426*, R423*) leading to premature truncation and loss of the C-terminal dimerization domain were seen in 2 patients, both younger than 45 years of age.

Somatic missense mutations in the receptor tyrosine kinase *FGFR2* were exclusively seen in patients with histologic evidence of perineural invasion. Of these, *FGFR2* N549K and *FGFR2* M536I are validated activating mutations, conferring constitutive tyrosine kinase activation facilitating tumorigenesis in cell culture (29). *FGFR2* N549K is well documented in endometrial carcinoma (30). In addition, 2 novel mutations, *FGFR2* A380D and D528N leading to changes within the transmembrane domain and protein tyrosine kinase domain, respectively were also observed in this cohort. Of the 4 patients with evidence of perineural invasion, 2 had histologic evidence of lymphovascular invasion. There were no unique findings in this sub-group. Additionally, there were no differences in mutations according to histologic differentiation.

NOTCH1 alterations were observed in one patient in this non-metastatic cohort using the targeted panel covering 43 commonly mutated amino acid residues in positions 1562 to 1600 and 1674 to 1678 in the NOTCH1 protein. The *NOTCH1* L1569P mutation occurs within a functionally significant negative regulatory region of the gene, however the functional significance, if any, of this mutation has not been described in the literature.

Somatic mutations in HNcSCC and targeted therapies

Table 4 lists 10 activating mutations in oncogenes which demonstrate susceptibility to currently clinically available targeted therapeutics or to small molecule drugs undergoing clinical trials or preclinical investigations. 60% of the cases demonstrated at least one alteration amenable to small molecule therapy, though recurrent alterations were rare. Of these, 3 cases showed mutually exclusive mutations (*EGFR* A864T, *KIT* T670I) with sensitivity to approved therapies (Erlotinib/Gefitinib – EGFR inhibitors, Sorafenib – broad tyrosine kinase inhibitor, respectively) that are used in other malignancies. One of the

patients showed a *KDR* Q472H mutation which is susceptible to the anti-VEGF drug Bevacizumab that is approved for the treatment of metastatic cervical, colorectal, breast and renal cancers (31). *HRAS* G13D that demonstrates in vitro susceptibility to combined MET and MEK inhibition, and to novel Ras inhibitors (32, 33) was seen in one patient, and was mutually exclusive to the patient with as *EGFR* activating mutation. The *SMO* L412F mutation, previously detected in Basal cell carcinoma, shows resistance to Vismodegib (34), but may be susceptible to a new class of compounds, the GLI inhibitors that are under investigation for Hedgehog pathway blockade (34).

Discussion

The current cohort of 10 patients is unique in that it represents a group of high-risk Head and Neck cutaneous squamous cell carcinoma tumors with a median depth of invasion of 10.5mm and without evidence of metastatic disease. In addition to confirming the presence of the typical UV signature of C/T transitions and the high incidence of tumor suppressor mutations including *TP53*, the analysis puts forth several findings. These include; presence of somatic mutations in *MLH1* in young patients with cSCC, presence of *FGFR2* mutations exclusively in patients with perineural invasion, lower incidence *NOTCH1* mutations in this non-metastatic cohort as compared to metastatic cohorts described in the literature, and the presence of actionable mutations with targeted therapeutic agents approved for other common malignancies in nearly 60% of cases.

TP53 loss of function is hypothesized to occur early in cutaneous squamous cell carcinoma pathogenesis (23). The loss of heterozygosity is associated with a sharp increase in mutation burden (23). Further evidence supporting early mutation of *TP53* in skin comes from studies of physiologically normal, sun-exposed skin with clones of *TP53* mutant cells (35, 36). Of the 11 functionally validated *TP53* mutations in the current cohort, 8 affected the DNA binding domain of the gene. Location of gene mutation may have predictive roles in disease

prognosis. While this has not been explored in cutaneous SCC; *TP53* mutations within the DNA binding domain have been found to be an independent predictor of poor disease specific survival in oral squamous cell carcinoma (37).

Mutations in *ATM* were detected in 60% of tumor samples. *ATM* is an integral player in the DNA damage response pathway, orchestrating signalling following UV damage (38). In vitro changes to phosphorylated *ATM* localization (and hence signalling) have been identified in normal, sun-exposed, pre-malignant and cutaneous squamous cell carcinoma cell lines (39). Interestingly, the two youngest patients (less than 45 years) showed functionally significant somatic mutations in both *ATM* and *MLH1*. These patients also showed relatively high total mutation burdens (198 and 502). Familial *MLH1* alterations are characteristic of Hereditary Non-Polyposis Colon Cancer syndrome. Sebaceous carcinoma is a well-known component of the Muir-Torre syndrome, however, there is no information regarding cutaneous squamous cell carcinoma as a component of Hereditary Non-Polyposis Colon Cancer syndrome (40). The role of somatic mutations in *MLH1* and its association with earlier age of onset of cutaneous squamous cell carcinoma is not well studied.

FGFR2 is a receptor tyrosine kinase mediating cell division, growth and differentiation signalling. Immunohistochemical over-expression of *FGFR2* has been described to be associated with perineural invasion (41), advanced tumor stage and shorter survival in patients receiving neoadjuvant chemotherapy for rectal cancers (42). Amplification of *FGFR2* in gastric cancer is related to regional lymph node metastases and subsequent poor prognosis (43). Interestingly, *FGFR2* mutations in our patient cohort were seen only in those with histologic evidence of perineural invasion. However, the role of missense mutations in

the protein tyrosine kinase domain of *FGFR2* and its role in perineural invasion is not documented.

In the current non-metastatic cohort, we detected a single example of a mutation in the *NOTCH1* gene. The functional significance of this alteration appears to be limited. This contrasts with information obtained from the DNA analysis of metastatic tumors which demonstrated *NOTCH1* mutations in 69% of the cohort (24). In cutaneous squamous cell carcinomas from immunocompromised patients *NOTCH1/2* was altered in 89% of samples (44), and Pickering et. al described 30% prevalence of *NOTCH1* inactivating mutations (14). A direct comparison of the various studies evaluating *NOTCH* changes in cutaneous squamous cell carcinoma is primarily limited by the wide variety of the testing methods used such as whole exome sequencing and capture panel analysis. The Illumina TruSeq Amplicon Cancer Panel® used in the current study covers approximately 35Kb (exons 26 AA 1562-1600; exon 27 AA 1674-1678) of *NOTCH1*. Additionally, combinations of primary and metastatic cohorts have been used in the literature without further information regarding the distribution of NOTCH mutations in primary or metastatic tissues. Also, the details of the quality control checks while using formalin fixed paraffin embedded samples and the functional significance of the various alterations described in these studies are not readily available. For instance, the incidence of *NOTCH1* mutations drops from 69% to only 24% in the metastatic cohort described by Li et al when only functionally significant mutations are considered (24). *NOTCH1* plays multifaceted roles in carcinogenesis. It has been proposed that loss of *NOTCH1* is not an initiator of disease, but acts more as a cancer promoting event (45). Thus, the role of this gene in regional and distant progression of Head and Neck cutaneous squamous cell carcinoma bears further investigation in well-designed cohorts

using comprehensive DNA and expression analysis. This is particularly significant currently, as therapeutic targets modulating *NOTCH* activity are under development (46).

There is an unmet need for effective medical treatment of invasive and metastatic cutaneous squamous cell carcinoma. It has been hypothesized that this disease is largely tumor suppressor driven in etiology (14), which, combined with the consistently reported high mutation burden of cSCC, has been a barrier to the development of targeted therapies. Our findings indicate mutations in oncogenes such as *EGFR*, *KIT, KDR*, *GNAQ* and *ERBB4,* though these were largely mutually exclusive non-recurrent events in this cohort. Similar findings have also been described by Li et al and Al-Rohil et al (24, 47). 10 somatic mutations identified in 6 patients in this cohort may potentially be susceptible to currently approved therapies or to small molecule drugs under development. This finding merits further investigations, particularly as a phase 2 study evaluating use of gefitinib in aggressive cutaneous squamous cell carcinoma demonstrated favorable survival outcomes (48). Cetuximab has been trialled in a small cohort of patients, achieving a 69% disease control rate after 6 weeks of treatment including 8 partial and 2 complete responses (49).

The targeted panel used in this study is highly biased towards receptor tyrosine kinase genes involved in MAPK, PI3K and mTOR signalling pathways, and nearly 90% of cases contained alterations in genes belonging to PI3K/mTOR pathway. Reduced rates of cutaneous squamous cell carcinoma development have been observed in organ transplant recipients receiving mTOR inhibitors (50). Al-Rohil et. al have also recently described a clinical response in a patient with cSCC with *PIK3CA* P471L mutation, treated with an mTOR inhibitor temsirolimus (47). While 5 of our patients showed *PIK3CA* mutations, no known targetable mutations were identified in this gene.

The chief limitations of this study include the small cohort size, use of formalin fixed paraffin embedded samples and use of a targeted panel. Although, cutaneous squamous cell carcinoma is a common malignancy, locally advanced high risk tumors that have not developed metastases at a minimum follow up of 2 years are extremely rare, particularly if complete clinicopathologic data and follow up are also required. Furthermore, stringent quality control checks were applied to ensure that only those cases with high quality DNA were included in analysis. Extraction of high quality DNA from archival material is inherently difficult leading to further shrinkage of the cohort. The TruSeq Amplification Cancer Panel is suitable for the fragmented DNA obtained from formalin fixed paraffin embedded samples and allows for cost effective data analysis in a clinically relevant time frame that can be replicated in other centers with DNA sequencing facilities. Furthermore, our data shows several of the alterations identified by more comprehensive techniques requiring fresh tissue (14, 44). Thus, we believe that our findings are likely to be reproduced in other study cohorts of non-metastatic, high risk disease.

In conclusion, we have performed targeted sequencing of 48 cancer-associated genes on a unique cohort of 10 high-risk, non-metastatic Head and Neck cutaneous squamous cell carcinoma cases to a mean coverage of 6907. Our results confirm the presence of a UV DNA damage signature, a high mutation burden and the predominance of *TP53* mutations in disease pathogenesis. In addition, we describe several novel findings including - somatic mutations in *MLH1* in younger patients with Head and Neck cutaneous squamous cell carcinoma, *FGFR2* mutations in patients with perineural invasion and a low incidence of *NOTCH*1 mutations in this cohort, all of which open further avenues of study. Our data also indicate the presence of targetable mutations in a significant proportion of tumors suggesting

further treatment options for Head and Neck cutaneous squamous cell carcinoma, an under-researched disease with significant morbidity and mortality in the fair skinned population.

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Figure 1: 1A. Excision of HNcSCC involving the nasal alae with subcutaneous extension onto the right cheek. The entire tumor including the ulcerated component and the subcutaneous extension measure 55mm in maximum dimension. **1B.** Moderately differentiated squamous cell carcinoma, infiltrating through the dermis and the subcutaneous tissue to a depth of 15mm, Clark level 5 (Haematoxylin and Eosin X 20). **1C.** Perineural invasion of a large nerve bundle at the deep margin of the specimen (high risk) (Haematoxylin and Eosin X 40); inset: squamous cell carcinoma is seen within the perineurium, surrounding approximately two thirds of the circumference of the nerve (Haematoxylin and Eosin X 100).

Table 1. Patient Clinicopathological data.

Figure 2: Genomic overview of mutations detected by targeted gene sequencing of 48-cancer associated genes in 10 high-risk cases of non-metastatic HNcSCC. Missense variants (meeting at least one of – MutSig significance, FATHMM significance or known COSMIC mutation), nonsense mutations, insertions/deletions and splice variants illustrated in the right-hand plot. Left-hand plot demonstrates the percentage of samples with alterations detected in each given gene.

Table 2. All functionally validated or likely significant mutations in tumor suppressor genes.

Figure 3: Visual representation of location of detected missense variants, nonsense mutations, insertions and deletions in **2A.** ***TP53*****, 2B.** ***APC*** and **2C,** ***ATM*****.** Green – missense variants; Purple - nonsense mutations and gene deletions. Red highlights X-axis represent areas covered by TruSeq amplicon panel.

Table 3. All functionally validated mutations in oncogenes.

Table 4. All functionally validated mutations in oncogenes susceptible to currently approved or preclinical small molecule targeted therapies.

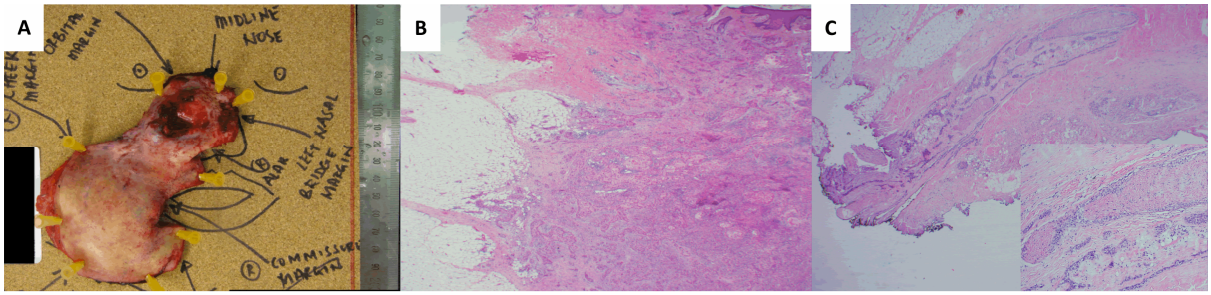
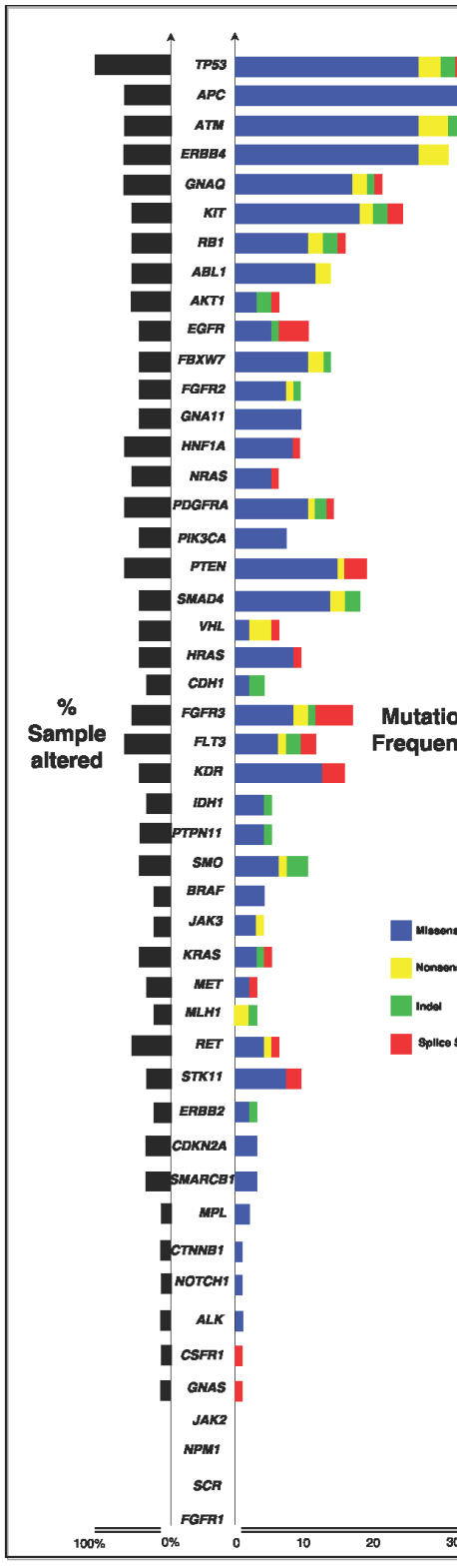

A
B
C
MIDLINE NOSE

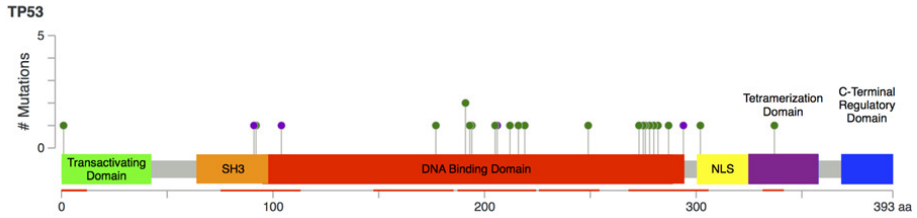
Table 1. Patient Clinicopathological Data		
Variable	N.	%
Age, years		
≤55	2	20
56 to 65	2	20
≥66	6	60
Sex		
Male	8	80
Female	2	20
Location		
Ear	2	20
Forehead/scalp	4	40
Lip	2	20
Nose	1	10
Pre-auricular	1	10
Differentiation		
Well	1	10
Moderate	7	70
Poor	2	20
Depth of invasion		
<5mm	3	30
5-10mm	2	20
>10mm	5	50
Pattern of invasion		
Pushing	0	0
Infiltrative	10	100
Margins		
Clear	4	40
Close	1	10
Involved	5	50
Lympho-vascular invasion		
Yes	2	20
Perineural invasion		
Yes	4	40
Pathological T category		
T1	2	20
T2	6	60
T3	0	0
T4	1	10
Pathological N category		
N0	10	100
Treatment		
Surgery alone	7	70
Surgery + radiotherapy	3	30



Case Number	Gene Symbol	Mutation	COSMIC ID	Location	Evidence in other cancers
1	*TP53*	c.273G>A, p. W91*	COSM44492	Premature truncation within Proline-rich domain, loss of DNA Binding Domain (DBD)	Biliary Tract, Breast, Endometrium, Esophagus (Carcinoma), Lung (NSCLC – Adenocarcinoma)
1	*TP53*	c. 880G>T, p E294*	COSM10856	Premature truncation	Colorectal, Breast, Endometrium, Lymphoid (DLBCL)
1	*APC*	c.2752_2753delGA, p.R919Kfs*4	N/A	Frameshift deletion with Armadillo-associated region, Predicted premature truncation with loss of downstream functional domains	N/A
1	*ATM*	c. 3991C>T, p. Q1331*	N/A	Premature truncation, loss of downstream functional domains	N/A
1	*VHL*	c. 394C>T, p. Q132*	COSM14356	Premature truncation within VHL beta-domain responsible for complex formation	Kidney (Clear Cell), Pancreas, Soft Tissue (Disseminated Cerebellar Hemangioblastoma)
1	*PTEN*	c. 49C>T, p. Q17*	COSM5153	Premature truncation, loss of downstream functional domains	Breast, Endometrium, Lymphoid, Lung (Adenocarcinoma)
3	*TP53*	c. 655C>T, p. P219S	COSM44076	Missense mutation within DBD	Esophagus (SCC), Lymphoid (T-cell), Aerodigestive tract (HNSCC), Leiomyosarcoma, Tumor growth in cell culture models
3	*APC*	c. 3982C>T, p. Q1328*	COSM18859	Premature truncation, loss of downstream Ctnnb1 binding sites	Colorectal, Endometrial
3	*APC*	c.4034delA, p. E1345Dfs*70	N/A	Frameshift deletion, leading to premature truncation and loss of downstream Ctnnb1 binding sites	N/A
3	*SMAD4*	c.364A>T, p. K122*	N/A	Premature truncation within MAD homology (MH1) domain involved in nuclear import and protein interactions	N/A
3	*SMAD4*	c.533delC, p.S178*	N/A	Frameshift deletion, leading to premature truncation, loss of downstream MH2 functional domain	N/A
3	*RB1*	c. 1735C>T, p. R579*	COSM892	Premature truncation, loss of downstream functional domains	Biliary Tract, Lung (SCLC), Melanoma, Retinoblastoma, Lymphoid (COSMIC unknown), BCC
1, 3	*ATM*	c. 7327C>T, p. R2443*	COSM21678	Premature truncation within Focal Adhesion Targeting (FAT) domain	Adrenal Cortex (Adenoma), Lymphoid (Mantle cell)
5	*TP53*	c. 818G>A, p. R273H	COSM10660	Missense mutation within DBD	Biliary Tract, Bone (Chondrosarcoma, Ewing's sarcoma, Osteosarcoma), Increased cell migration

5	*TP53*	c. 880G>T, p. R280K	COSM330620	Missense mutation within DBD	Breast, Colorectal, Ovarian, Hematopoietic (Primary Plasma Cell Leukemia), Increased activation novel targets
5	*ATM*	c. 1063C>T, p. Q355*	N/A	Premature truncation, loss of downstream functional domains	N/A
5	*ATM*	c.2617delG, p.G873Efs*7	N/A	Frameshift deletion, leading to premature truncation with loss of downstream functional domains	N/A
5	*ATM*	c.5066delA, p.Q1689Hfs*25	N/A	Frameshift deletion, leading to premature truncation, loss of downstream functional domains	N/A
5	*MLH1*	c.1219C>T, p. Q407*	N/A	Premature truncation, loss C-terminal dimerization domain	N/A
5	*MLH1*	c.1264_1268delGGCAG, p.R423*	N/A	Frameshift deletion, leading to premature truncation, loss downstream functional domains	N/A
5	*SMAD4*	c.1342C>T, p. Q448*	N/A	Premature truncation within MH2 domain involved in protein interactions	N/A
5	*VHL*	c.583C>T, p. Q195*	N/A	Premature truncation within VHL box domain	N/A
5	*CDH1*	c. 1118C>T, p. P373L	N/A	Missense mutation upstream from Cadherin 3 domain	Reduced interaction with EGFR, increased motility cell culture
5	*CDH1*	c.1240delA, p.T414Pfs*3	N/A	Frameshift deletion within Cadherin domain 3, leading to premature truncation	N/A
5	*FBXW7*	c. 1338G>A, p. W446*	COSM22986	Premature truncation within WD2 repeat domain	Colorectal
5	*FBXW7*	c.1469delC, p.T490Kfs*8	N/A	Frameshift deletion, Predicted premature truncation, loss of downstream functional domains	N/A
5	*RB1*	c.1090G>T, p. E364*	N/A	Premature truncation, loss of downstream functional domains	N/A
5	*RB1*	c.1811_1814+11delATATGTAAGCAAAAT	N/A	Frameshift deletion within Rb-associated protein B domain, predicted premature truncation	N/A
2	*TP53*	c.502_513delCACATGACGGAG, p.H168_E171delinsdel	N/A	Frameshift deletion within DBD	N/A
2	*CDH1*	c.349delA, p.N117Ifs*98	N/A	Frameshift deletion within Cadherin 2 domain, loss of downstream functional domains	N/A

4	*TP53*	c. 310C>T, p. Q104*	COSM10886	Premature truncation within DBD	Biliary Tract, Breast, Esophagus (Carcinoma), Hematopoietic (AML), Lung (NSCLC - Adenocarcinoma)
4	*VHL*	c. 337C>T, p. R113*	COSM30228	Premature truncation within VHL beta-domain responsible for complex formation	Kidney (clear cell)
4	*FBXW7*	c. 1458G>A, p. W486*	COSM87016	Premature truncation within WD3 (Tryptophan-aspartic acid) repeat domain	Colorectal, Tumor growth cell culture
6	*TP53*	c. 844C>T, p.R282W	COSM10704	Missense mutation within DBD	cSCC, BCC, Tumor growth mouse models
7	*TP53*	c. 746G>T, p. R249M	COSM43871	Missense mutation within DBD	Breast, Colorectal, Liver, Bone (Ewing Sarcoma), CNS (glioma), Lymphoid (CLL), Hematopoietic (Myelodysplasia)
7	*TP53*	c.617delT, p.L206Wfs*41	N/A	Premature truncation within DBD	N/A
7	*APC*	c. 3340C>T, p. R1114*	COSM13125	Premature truncation within unstructured region of APC, leading to loss of downstream Ctnnb1 binding sites	Colorectal, Endometrial
9	*TP53*	c. 1010G>A, p. R337H	COSM43882	Missense mutation within Tetramerization Domain	Adrenal (Carcinoma, Pheochromocytoma), Cervix Colorectal, Liver, Disrupted tetramer formation in cell culture
9	*APC*	c.4549C>T, p. Q1517*	N/A	Premature truncation, leading to downstream loss of Ctnnb1 binding sites	Loss of beta-catenin regulation
9	*MLH1*	c.1276C>T, p. Q426*	N/A	Premature truncation loss C-terminal dimerization domain	N/A
9	*SMAD4*	c.1186delG, p.D396Mfs*19	N/A	Frameshift deletion within MH2 domain, leading to premature truncation	N/A
10	*TP53*	c. 832C>T, p. P278S	COSM10939	Missense mutation within DBD	Breast, Tumor growth xenograft models


TP53
Mutations
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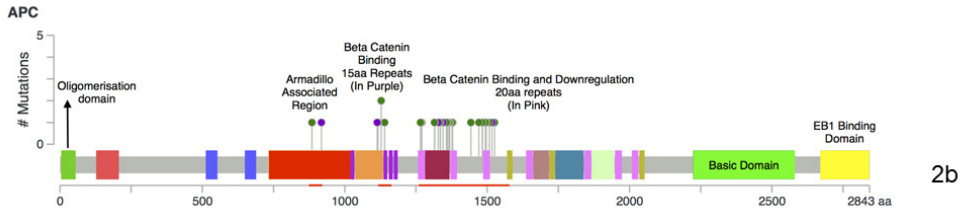
SH3
DNA Binding Domain
NLS
Tetramerization Domain
C-Terminal Regulatory Domain
0
100
200
300
393 aa

2a


APC
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Oligomerisation domain
Armadillo Associated Region
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EB1 Binding Domain
Basic Domain
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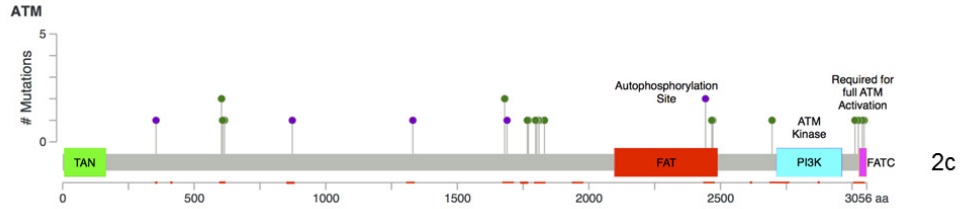
1000
1500
2000
2500
2843 aa

2b


ATM
Mutations
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0
Autophosphorylation Site
Required for full ATM Activation
ATM Kinase
TAN
FAT
PI3K
FATC
0
500
1000
1500
2000
2500
3056 aa

2c

Case Number	Gene Symbol	Mutation	COSMIC ID	Location and functional effect	Evidence
3	*EGFR*	c. 2590G>A, p. A864T	COSM13197	Missense mutation within Protein Tyrosine Kinase (PTK) domain	NSCLC (Large Cell), Biliary tract, Adrenal (Carcinoma), Increased Tyr kinase activity in cell culture
5	*KIT*	c. 2009C>T, p. T670I	COSM12708	Missense mutation within ATP binding pocket	GIST, Melanoma, Constitutive Tyr kinase phosphorylation cell culture, Gain of Function
1	*KDR*	c. 1416A>T, p. Q472H	COSM149673	Missense mutation; does not lie in known functional domain	Neuroblastoma, GIST, Bone, Melanoma, Lymphoid (DLBCL), Colorectal, Rhabdomyosarcoma, Increased Tyr kinase activity cell culture, Increased angiogenesis tumor samples
5	*BRAF*	c. 1390G>A, p. G464R	COSM1448615	Missense mutation within PTK domain	<1% Melanomas, cSCC, Increased Tyr kinase activity in cell culture, Gain of Function
3	*FGFR2*	c. 1608G>A, p. M536I	N/A	Missense mutations within PTK domain	Increased kinase activity and enhanced cell proliferation in the presence of ligand in culture
3	*FGFR2*	c. 1646T>A, p. N549K	N/A	Missense mutations within PTK domain	Confers a gain of function to the Fgfr2 protein, resulting in oncogenic transformation in cell-based studies
7	*GNAQ*	c. 548G>A, p. R183Q	COSM52975	Missense mutation within nucleotide binding region	Uveal Melanoma, Colorectal, Reduced GTPase activity in cell culture, Increased downstream signalling, Gain of Function
1	*HRAS*	c. 38G>A, p. G13D	COSM490	Missense mutation within the GTP nucleotide binding region	Inhibits GTPase activity leading to increased activation of downstream signalling in the absence of activation
9	*JAK3*	c. 1714G>A, p. A572T	COSM327318	Missense mutation within PTK domain	Lymphoid (T-ALL), Increased Tyr kinase activity in cell culture, Gain of Function
9	*PTPN11*	c. 181G>T, p. D61Y	COSM13011	Missense mutation within Src Homology 2 (SH2) domain	Hematopoietic (AML, ALL), neuroblastoma, Increased Tyr phosphatase activity in cell culture, Gain of Function
1	*PTPN11*	c. 218C>T, p. T73I	COSM13019	Missense mutation within SH2	Hematopoietic (myelodysplastic syndrome, CML, ALL, AML), Increased Tyr phosphatase activity in cell culture, Gain of Function
4	*SMO*	c. 1234C>T, p. L412F	COSM216037	Missense mutation within pivot region Transmembrane (TM) helix 5	Bone (Ameloblastoma), Constitutive activation of Hedgehog (HH) signalling in cell culture, Gain of Function

Case Number	Gene Symbol	Mutation	Functional Effect	Cellular Pathway	Drug Susceptibility	Evidence/use
3	*EGFR*	c. 2590G>A, p. A864T	Gain of function; constitutive kinase activity	MAPK	Erlotinib, Gefitinib	Non-Small Cell Lung Cancer
7	*KIT*	c. 2009C>T, p. T670I	Gain of function, leads to constitutive phosphorylation of KIT	MAPK	Sorafenib, resistant to Imatinib	Kidney, liver, GIST (Sorafenib) Melanoma (Preclinical)
1	*KDR*	c. 1416A>T, p. Q472H	Gain of function; increased phosphorylation	Angiogenesis	VEGFR inhibitors	Trials (NSCLC), Preclinical (Melanoma)
3	*FGFR2*	c. 1646T>A, p. I549K	Gain of function; constitutive kinase activity	MAPK	Ponatinib (resistance to dovitinib, PD173074); combination mTOR inhibitor (Ridaforolimus)	Preclinical (Endometrial cancer cell lines, BaF3 cell lines)
3	*FGFR2*	c. 1608G>A, p. M536I	Gain of function; constitutive kinase activity	MAPK	Ponatinib (decreased response to dovitinib, PD173074)	Preclinical (Endometrial cancer; BaF3 lines)
6	*GNAQ*	c. 548G>A, p. R183Q	Loss of function; reduced GTPase activity	PI3K; mTOR; PI3K	Combined PKC, MEK inhibitors	Preclinical
1	*HRAS*	c. 38G>A, p. G13D	Loss of function; inhibition GTPase activity; increased downstream signalling	MAPK; PI3K	Combined MET, MEK inhibitors (resistance to MET inhibition); Ras inhibitors	Preclinical
9	*PTPN11*	c. 181G>T, p. D61Y	Gain of function; increased Tyr phosphatase activity	MAPK	SHP2 inhibitor (actually shown in vivo)	Preclinical
1	*PTPN11*	c. 218C>T, p. T73I	Gain of function; increased Tyr phosphatase activity	MAPK	SHP2 inhibitor	Preclinical
4	*SMO*	c. 1234C>T, p. L412F	Gain of function; constitutive (Hedgehog) HH signalling	Hedgehog	GLI inhibitors; Vismodegib resistance	Preclinical