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NOTCH1 in Cutaneous Squamous Cell Carcinoma Arising in Immunosuppressed Patients: A Systematic Review and Quantitative Analysis

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Abstract

Immunosuppression is a strong risk factor for cutaneous squamous cell carcinoma (cSCC). Immunosuppression is also associated with unique mutagenic stressors that likely contribute to cSCC pathogenesis. However, it is unknown whether these stressors contribute to a distinct mutation profile that may drive disease progression. This review was conducted to assess the mutational landscape of cSCC arising in immunosuppressed hosts. Specifically, we sought to determine gene mutation frequencies in immunosuppressed cSCC. An electronic search was performed in PubMed, Embase, Scopus, and Cochrane databases. Studies performing DNA sequencing or genotyping of cSCC were identified. Studies were excluded if the immune status of each tumor was not available. Eighteen studies met inclusion criteria. Due to study heterogeneity a meta-analysis was unable to be performed. However, statistical analysis was performed on the most frequently reported genes. NOTCH1 was the most frequently mutated gene in immunosuppressed cSCC, and was significantly higher than immunocompetent cSCC after multiple comparison adjustment (77.7 versus 58.1%, OR 2.50, 95% CI 1.40-4.46, p=0.002). No other statistically significant differences were observed. Our results suggest that NOTCH1 mutations are more common in cSCC arising in immunosuppressed hosts. Several prior observations reviewed here further support a role for NOTCH1 in immunosuppressed cSCC, however larger studies are needed to confirm our findings.

Introduction

Cutaneous squamous cell carcinoma (cSCC) is the second most common malignancy in Caucasians with an increasing incidence worldwide [1]. While surgery is curative in most cases, locally advanced and metastatic disease is associated with significant morbidity and mortality [2]. High-risk clinical and histologic features correlate with prognosis and have been incorporated into staging criteria [3]. However, the genetic predictors of advanced disease remain poorly understood.

These genetic predictors are difficult to investigate as a result of the number and types of mutations present. At 50 mutations per megabase pair of coding DNA, the tumor mutation burden in cSCC is significantly higher than any solid organ or hematologic malignancy [4,5]. Mutations affect diverse pathways involving keratinocyte differentiation, cell-cycle regulation, cellular proliferation, and chromatin maintenance. Additionally, histologically normal skin from sun-exposed areas has a mutation rate equal to most human cancers, making identification of cSCC driver mutations particularly difficult [6]. Gross chromosomal aberrations and widespread epigenetic dysfunction due to DNA methylation, mutations in noncoding DNA, and variable expression of noncoding RNA molecules further complicate the genomic landscape of cSCC [7-10].

An important risk factor for cSCC is immunosuppression, with the highest risk observed in organ transplant recipients (OTR). In OTR, the risk of developing cSCC is 65 to 100-fold higher than the general population [11]. Cutaneous SCC in OTR also have greater propensity for aggressive subclinical extension, local recurrence, metastasis, and death [12-15]. The clinical and histologic features predictive of poor outcome in immunosuppressed (IS) cSCC are similar to those in immunocompetent (IC) cSCC [16]. Importantly, immunosuppression is associated with unique mutagenic stressors that likely contribute to genetic instability leading to cutaneous oncogenesis. Despite these unique stressors, few studies have directly compared the mutation profiles of IS and IC cSCC.

Therefore, the purpose of this review is to provide a quantitative summary of mutation frequencies in IS and IC cSCC. By doing so, we hope to aid in the discovery of differentially mutated genes that may contribute to the more aggressive phenotype observed in IS hosts.

Methods

Search Strategy and Study Selection

A systematic search of PubMed, Embase, Scopus, and Cochrane databases was completed by two authors (ADM, MLC) from each database's earliest inception to April, 2020. Search terms included "cutaneous squamous cell carcinoma", "genetics", and "mutation". Bibliographies of articles were reviewed for additional relevant studies. Studies were initially screened by article title and abstract. Studies deemed relevant based on screening criteria were reviewed in full to establish a final set of studies.

Inclusion and Exclusion Criteria

Original studies in which DNA sequencing or genotyping of cSCC was performed were included in the analysis. Upon screening, studies were excluded for any of the following: (1) studies consisting exclusively of actinic keratoses, squamous cell carcinoma in situ, keratoacanthoma, and/or non-cutaneous SCC; (2) studies in subjects with predisposing genetic conditions; (3) studies of cSCC arising secondary to treatments based on BRAF-inhibition, psoralen and ultraviolet A, radiation, or arising in chronic wounds; (4) studies

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utilizing human cell lines; (5) studies with indiscernible immune status; and (6) studies utilizing techniques other than DNA sequencing, small nucleotide polymorphism (SNP) microarray, or microsatellite analysis (e.g., single strand conformational polymorphism analysis).

Data Collection, Quality Assessment, and Risk of Bias

The mutation status of each tumor was recorded as a binary outcome (mutated/wild-type) for the genes reported in four or more studies. Limitations of each study were sought and disclosed. The limitations affecting study quality and contributing to potential bias include: (1) small sample sizes, (2) unequal group sizes, (3) varying definitions of immunosuppression, and (4) varying methods and assays used for mutation analysis.

Statistical Analysis

We initially sought to perform a meta-analysis. However, there was considerable interstudy variability in terms of methods for gene analysis and how immunosuppression was defined. Additionally, four studies included either IS or IC samples, but not both. Therefore, appropriate statistical analysis using a random effects model was not possible. Instead, data from studies were combined to calculate a single mutation frequency and odds ratio for each gene reported in at least six separate studies. Fisher's exact test was used to assess statistical significance between the two groups,and an unweighted odds ratio was used to estimate effect size. Multiple comparisons were adjusted using the Bonferroni correction with statistical significance set at $p < 0.003$ (type I error rate, α, of 0.05 with 17 separate gene comparisons).

Results

Search Results

A flowchart of our selection process is depicted in Figure 1. A total of 763 articles resulted from our literature search and 13 articles were identified through bibliography review. After screening by title and abstract, 712 studies were excluded due to ineligibility or duplication. The remaining 64 studies were reviewed in full text, and 18 studies were ultimately included in our analysis [4,17-33].

Studies and Genes

The study characteristics are shown in Supplementary Table 1. The number of genes analyzed in each study ranged from a single gene to whole exome analysis. DNA sequencing was performed in all studies, and the types of mutations detected varied depending on the methods used. Additionally, genotyping was performed in four studies using SNP or microsatellite analysis to detect gene-specific loss-of-heterozygosity (LOH), copy number alterations, and microdeletions. The 18 studies include a total of 601 cSCC: 264 from IS and 337 from IC subjects. Mutation status was recorded for the 136 genes analyzed in at least four studies. The number of cSCC for which mutation status was available varied depending on the genes included in each study: the range was 37-156 tumors per gene for IS and 69-232 tumors per gene for IC cSCC.

Quantitative and Statistical Analysis

The mutation frequencies of the 136 genes reported in four or more studies are shown in Supplementary Table 2. The mutation frequencies with corresponding p-values and odds ratios for the 17 genes reported in six or more studies are shown in Figure 2. NOTCH1 was the most frequently mutated gene in IS cSCC (77.7%) and overall (65.4%). Additionally, the frequency of NOTCH1 mutations was significantly higher in IS versus IC cSCC (77.7 versus 58.1%, OR 2.50, 95% CI 1.40-4.46, p=0.002).TP53 was more frequently mutated in IC versus IS cSCC (64.7 versus 50.6%), however this was not statistically significant after multiple comparison adjustment (p=0.006).No other statistically significant differences were observed.

Discussion

This study aimed to review the literature and calculate gene

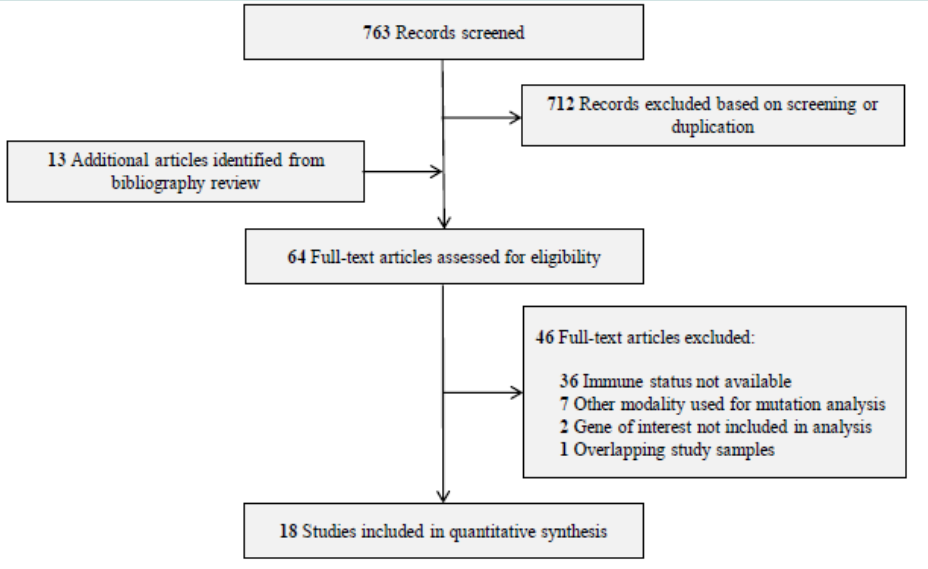

Figure 1: Flowchart for the Literature Search Results.

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A

Gene	Studies (n)	IS (% mutated)	IC (% mutated)	OR (95% CI)	P Value
NOTCH1	8	73/94 (77.7)	93/160 (58.1)	2.50 (1.40, 4.46)	0.002*
FAT1	6	27/40 (67.5)	46/95 (48.4)	2.21 (1.02, 4.80)	0.042
APC	6	14/41 (34.2)	24/99 (24.2)	1.75 (0.79, 3.86)	0.230
EGFR	7	10/74 (13.5)	12/145 (8.3)	1.73 (0.71, 4.22)	0.223
KRAS	7	9/108 (8.3)	8/150 (5.3)	1.61 (0.60, 4.33)	0.338
NOTCH2	8	56/94 (59.6)	77/160 (48.1)	1.59 (0.95, 2.66)	0.078
HRAS	8	20/109 (18.4)	21/169 (12.4)	1.58 (0.81, 3.08)	0.174
IGF1R	6	8/49 (16.3)	12/106 (11.3)	1.53 (0.58, 4.02)	0.136
NRAS	6	4/76 (5.3)	5/134 (3.7)	1.43 (0.37, 5.51)	0.387
KDR	6	11/41 (26.8)	33/111 (29.7)	1.30 (0.56, 3.01)	0.726
EP300	6	14/42 (33.3)	33/113 (29.2)	1.21 (0.57, 2.59)	0.619
MLL3	6	18/39 (46.2)	44/104 (42.3)	1.17 (0.56, 2.45)	0.679
NF1	7	14/41 (34.2)	37/118 (31.4)	1.14 (0.53, 2.41)	0.742
MLL2	6	22/40 (55.0)	63/111 (56.8)	0.93 (0.45, 1.93)	0.848
ARID1A	6	7/41 (17.1)	23/111 (20.7)	0.79 (0.31, 2.00)	0.616
CDKN2A	9	40/127 (31.5)	65/164 (39.6)	0.70 (0.43, 1.14)	0.152
TP53	12	79/156 (50.6)	150/232 (64.7)	0.56 (0.37-0.85)	0.006

B

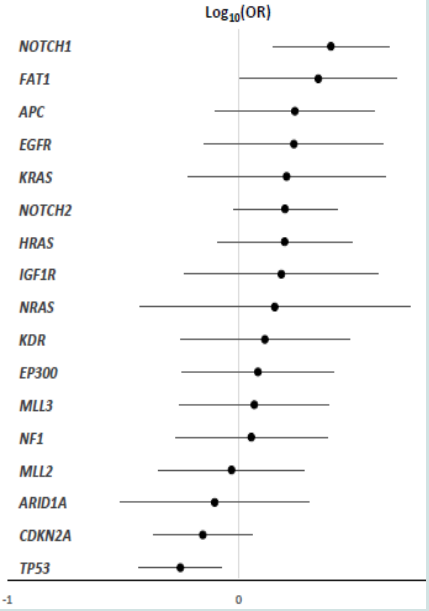

Figure 2: (A) Mutation frequencies for the 17 most frequently reported genes in IS and IC cSCC. (B) Forest plot depicting OR and 95% CI on a logarithmic scale. IS, immunosuppressed; IC, immunocompetent; OR, odds ratio; CI, confidence interval.

mutation frequencies in IS cSCC in order to better understand its genetic determinants. Despite our efforts, a meta-analysis could not be performed due to the scarcity and heterogeneity of existing data. However, our review and analysis suggest that NOTCH1 may be preferentially mutated in IS cSCC.

Ultraviolet (UV) radiation, particularly UVB, is the most important risk factor forall non melanoma and melanoma skin cancers. This is demonstrated by the predominance of C->T mutations, which are the hallmark UVB-induced mutagenesis [4].In addition to chronic UVB exposure, immunosuppression is associated with unique mutagenic stressors that may contribute to cSCC oncogenesis through distinct genetic mechanisms. These stressors may act synergistically with UV light or through UV-independent mechanism. This is supported by fewer UVB-associated mutations observed in IS cSCC [34]. Additionally, clinical evidence supporting a UV-independent mechanism is demonstrated by the observation that cSCC in OTR may predict development of subsequent non-cutaneous SCC, particularly of the oropharynx and lung, suggesting an internal carcinogenic driver [35].Through UV-dependent mechanisms, both calcineurin inhibitors and mycophenolate mofetil inhibit nucleotide excision repair enzymes leading to persistence of UVB-induced cyclopyrimidine dimers and reduced cellular apoptosis [36,37]. Conversely, the purine analog azathioprine sensitizes cells to UVA-mediated oxidative DNA damage. This mechanism is distinct from the UVB-induced mutations, as demonstrated by the unique cSCC mutation signature observed in patients receiving azathioprine [22]. Separate from the mutagenic effects of immunosuppressive medications, unique mutation profiles have been observed in head and neck SCC (HNSCC) associated with HIV and HPV infection [38,39]. These mutations may be due to insertional mutagenic events or a host defense mechanism meant to protect against retroviral infection, respectively.

Despite the unique and numerous mutagenic stressors related to immunosuppression, the overall mutation burden does not appear to be higher in IS compared to IC cSCC. While one early microsatellite analysis study observed the rate of LOH in OTR to be less than half of that in IC cSCC [40], a subsequent study using higher resolution, genome-wide SNP microarray found no difference in rate of LOH between IS and IC cSCC [41]. Instead, they demonstrated the number of chromosomal aberrations correlated with the degree of tumor differentiation, a finding that has been reproduced [22]. Similarly, targeted gene and whole-genome sequencing studies found no difference in overall mutation burden based on immune status [9,34].

To date, few studies have compared the specific genetic alterations of IS and IC cSCC. In a targeted sequencing study, no difference in mutation frequency of seven driver genes in 52 IS and 39 IC cSCC [4]. Similarly, a whole-exome sequencing study found no difference in 22 significantly mutated genes in 33 IS and 7 IC cSCC [22]. These studies suggest that cSCC share common driver mutations regardless of the underlying immune status of the host. An earlier study performed by Ridd et al., sought to characterize gene mutation status and protein expression of six receptor tyrosine kinases known to be mutated in a subset of cSCC [31]. They found that EGFR protein over expression was significantly higher in non-OTR compared to OTR. However, mutations and amplifications of the EGFR gene were exceedingly rare in both groups, suggesting posttranscriptional modifications contributing to protein over expression. Similar discordance between EGFR protein over expression and gene amplifications was reported by Cañueto et al., however no difference in protein over expression was observed this study based on immune status [42]. Mutations in the

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tumor suppressor CDKN2A have also been studied with conflicting results. Brown et al.,observed CDKN2A alterations more frequently in IC cSCC [19], while Mühleisen et al., demonstrated reduced allelic imbalance at chromosome 9p21 containing CDKN2A [43]. Clearly, a knowledge gap exists in regards to the specific genetic alterations occurring in IS cSCC due to limited studies and conflicting data.

Our quantitative analysis suggests that mutations in NOTCH1 are more common in IS cSCC. The NOTCH genes encode transmembrane receptors with tissue specific function [44].In the skin NOTCH1 promotes terminal differentiation of keratinocytes, and several lines of evidence demonstrate its role as a tumor suppressor in squamous epithelium [44-46].In addition to our quantitative analysis, several prior observations implicate the NOTCH pathway specifically in IS cSCC.

First, there is a complex relationship between NOTCH and human papillomavirus (HPV).Notably, β-HPV E6 protein directly inhibits the primary cofactor of NOTCH1, MAML1, resulting in decreased expression of its target genes [47]. Similarly, E6 protein inhibits transcription of NOTCHvia p53 inhibition [48]. A transposon-mediated insertional mutagenesis protocol in mice demonstrated that HPV infection decreased the threshold of NOTCH1 loss necessary for oral SCC carcinogenesis [49]. A similar sensitizing effect may exist in cSCC arising in IS patients co-infected with HPV. Although this may predict a lower mutation frequency ofNOTCH1in IS cSCC, this assumption is an oversimplification. Specifically, NOTCH1 can play a dual role as either a tumor suppressor or oncogene in squamous epithelium depending on the overall mutational context and the presence of HPV infection [49]. In addition to HPV, calcineurin inhibitors likely contribute to IS cSCC carcinogenesis through a NOTCH-dependent mechanism. Specifically, NOTCH functions upstream of calcineurin/NFAT in an integrated pathway promoting keratinocyte terminal differentiation [50]. Lastly, it is possible that unique mutagenic stressors in IS cSCC preferentially alter regions within the NOTCH1 locus. When considering our results in the context of the above findings, there is compelling evidence supporting a role for NOTCH1 alteration in IS cSCC.

In addition to NOTCH1in IS cSCC, several other genetic domains are primed for future study. Perhaps the most intriguing are epigenetic alterations and TERT. In OTR, germline polymorphisms in MTHFR confer an increased risk for cSCC [51]. Additionally, OTR harboring these MTHFR polymorphisms were found to have higher global levels of DNA methylation in both cSCC and unaffected skin, suggesting an inherited risk due to aberrant DNA methylation and epigenetic dysfunction [52]. While our quantitative analysis did not detect differential mutation frequencies of genes involved in chromatin remodeling and repair, examination of other epigenetic determinants, including methylation signatures and noncoding RNA expression, may provide valuable insight. Additionally, mutations in the TERT promoter (TERTp) are present in 32% of IC cSCC and associated with poor outcome [53]. Interestingly, Perrem et al., found that telomeres in cSCC arising in OTR were significantly longer than those arising in non-OTR [54]. Whether activating TERTp mutations contribute to longer telomere length in IS cSCC warrants further investigation, as the studies included in this review and quantitative analysis were conducted on exonic DNA. Thus, the promoter sequence was not analyzed.

Conclusion

Despite the growing understanding of the genetic landscape of cSCC, the specific genetic determinants underlying IS cSCC pathogenesis remain poorly understood. Further investigation into this topic may help identify genetic drivers that could be targeted to better prevent and treat cSCC arising in IS patients. We propose that dysfunction in the NOTCH pathway, includingNOTCH1 mutations, is of critical importance in the pathogenesis of cSCC arising in IS patients and merits further investigation.

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