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Squamousness: Next-generation sequencing reveals shared molecular features across squamous tumor types

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In order to gain a better understanding of the underlying biology of squamous cell carcinoma (SCC), we tested the hypothesis that SCC originating from different organs may possess common molecular alterations. SCC samples (N = 361) were examined using clinical-grade targeted next-generation sequencing (NGS). The most frequent SCC tumor types were head and neck, lung, cutaneous, gastrointestinal and gynecologic cancers. The most common gene alterations were *TP53* (64.5% of patients), *PIK3CA* (28.5%), *CDKN2A* (24.4%), *SOX2* (17.7%), and *CCND1* (15.8%). By comparing NGS results of our SCC cohort to a non-SCC cohort (N = 277), we found that *CDKN2A*, *SOX2*, *NOTCH1*, *TP53*, *PIK3CA*, *CCND1*, and *FBXW7* were significantly more frequently altered, unlike *KRAS*, which was less frequently altered in SCC specimens (all $P < 0.05$; multivariable analysis). Therefore, we identified “squamousness” gene signatures (*TP53*, *PIK3CA*, *CCND1*, *CDKN2A*, *SOX2*, *NOTCH 1*, and *FBXW7* aberrations, and absence of *KRAS* alterations) that were significantly more frequent in SCC versus non-SCC histologies. A multivariable co-alteration analysis established 2 SCC subgroups: (i) patients in whom *TP53* and cyclin pathway (*CDKN2A* and *CCND1*) alterations strongly correlated but in whom *PIK3CA* aberrations were less frequent; and (ii) patients with *PIK3CA* alterations in whom *TP53* mutations were less frequent (all $P \leq 0.001$, multivariable analysis). In conclusion, we identified a set of 8 genes altered with significantly different frequencies when SCC and non-SCC were compared, suggesting the existence of patterns for “squamousness.” Targeting the PI3K-AKT-mTOR and/or cyclin pathway components in SCC may be warranted.

Introduction

Squamous cell carcinomas (SCCs) occur in tissues normally covered with squamous epithelium, and may involve many different organs, although 4 major sites include head and neck, cutaneous, esophageal, and lung SCCs.¹ SCCs, other than those in the skin, commonly metastasize,^{2,3} and the survival rates have not improved in decades. A better understanding of the underlying biology of SCCs is just beginning to be elucidated,⁴ but would be enhanced by the characterization of their common molecular alterations.

Defining the genetic landscape of SCCs would also facilitate the use of targeted therapies, which have been generally lacking for SCCs. For example, while targeted therapy employing EGFR inhibitors is commonly used to treat lung adenocarcinomas harboring *EGFR* alterations, no effective agents have been

specifically developed for squamous cell carcinomas of the lung. Indeed, *EGFR* mutations are very rare in their squamous cell counterpart.⁵ Interestingly, it has been suggested that head and neck SCCs may share patterns of molecular alterations with SCCs of the lung.^{6–9} Further, esophageal and head and neck SCCs may also share some pathogenic mechanisms.¹⁰ Examining genetic aberrations of SCCs may shed light on additional common pathways underlying SCC tumorigenesis.

We hypothesized that squamous cell carcinomas may possess common biologic/molecular alterations, regardless of organ of origin. Herein, 361 tumor samples of patients with diverse squamous cell carcinomas including, but not limited to, lung, head and neck, cutaneous, gastrointestinal, and gynecologic/genitourinary SCCs, were analyzed using targeted next-generation sequencing (NGS) in order to determine if a pattern of “squamousness” alterations could be discerned.

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Table 1. Histologies breakdown of 361 squamous cell carcinomas

| Squamous Cell Carcinomas (N = 361) | |
|--|-------------|
| Histology | Number (%) |
| Head and neck | 107 (29.6%) |
| Lung | 92 (25.5%) |
| Cutaneous | 36 (10.0%) |
| Gynecologic/Genitourinary ^a | 40 (11.1%) |
| Gastrointestinal (GI) ^b | 51 (14.1%) |
| Unknown origin | 35 (9.7%) |
| TOTAL | 361 (100%) |

^aIncluded cervical, n = 22; vagina, n = 5; vulva, n = 3; penile, n = 6; bladder, n = 2; and urethra, n = 2.

^bIncluded esophageal, n = 21; anal, n = 21; rectal, n = 7; and gallbladder, n = 2.

Results

Genetic alterations identified in SCCs

Our squamous cell carcinomas (SCCs) cohort included 361 samples, the majority of them being SCCs of the head and neck (N = 107, 29.6%) and lung (N = 92, 25.5%), **Table 1**. The most frequent genetic alterations found were *TP53* (64.5%), *PIK3CA* (28.5%), *CDKN2A* (24.4%), *SOX2* (17.7%), and *CCND1* (15.8%) (**Fig. 1A**). Most of the alterations were mutations (61.2%) or amplifications (30.5%) (**Fig. 1B**). A total of 1,382 alterations were identified in 132 different genes (775 distinct alterations), **Table S1**. Patients had a median number of 4 (range 1–11) abnormalities identified, **Figure 1C**.

Specificity of the alterations portfolio identified in SCC specimens

We next investigated if we could identify a particular set of genes whose alteration frequencies were different compared to non-SCC samples. For this purpose, we compared the alterations found in 361 SCC samples (SCCs cohort) to a separate cohort including 277 patients' samples with diverse malignancies, but none with SCCs (non-SCCs cohort), that had undergone similar next-generation sequencing testing. Organ of origin breakdown was as follows for the non-squamous tumors: head and neck, n = 32; lung, n = 30; cutaneous, n = 38; gynecologic/genitourinary (included n = 29 gynecologic cases), n = 37; gastrointestinal, n = 117; other, n = 23. We first performed a univariable analysis that highlighted 8 genes whose alteration frequency was statistically different between SCC vs. non-SCC specimens. Because there were imbalances in the number of patients for some tumor types (i.e. organs of origin) between the SCC and non-SCC cohorts (gastrointestinal, head and neck, and lung, all $P < 0.05$) we included these tumor types and the 8 genes identified in the univariable analysis as variables for a multivariable model. The results of the multivariable confirmed that 8 genes had statistically different alteration frequencies. More specifically, 7 genes were found to be significantly more altered in SCC specimens compared to non-SCCs, as follows: *CDKN2A* (24% versus 8%, $P = 0.003$), *SOX2* (18% vs. 1%, $P = 0.001$), *NOTCH1* (12% versus 1%, $P = 0.0002$), *TP53* (65% vs. 45%, $P = 0$

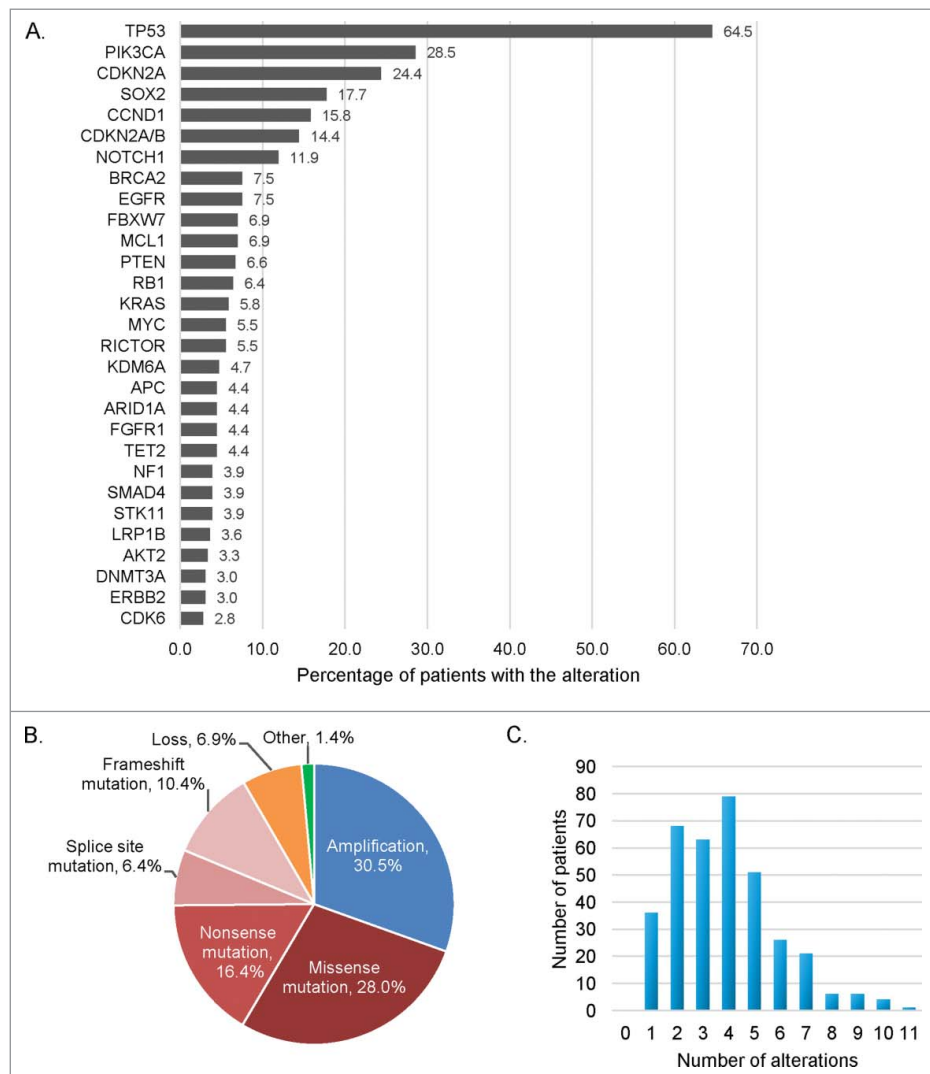


Figure 1. Frequency and type of molecular alterations identified in 361 patients with diverse squamous cell carcinomas. **(A)** Bar graph representing the alteration frequency. Only the more frequent alterations are represented (at least 10 patients with the alteration), and some patients had multiple alterations in the same gene. **(B)** Pie chart displaying the different type of alterations. Other refers to truncation (n = 11), fusion (n = 3), rearrangement (n = 3), deletion (n = 2), and duplication (n = 1). **(C)** Bar graph representing the number of patients by number of molecular alterations. Patients had a median of 4 alterations, (range 1–11).

Table 2. Alteration frequency differences in squamous versus non-squamous cell carcinomas (multivariable analysis)

| | Squamous cases (N = 361) | Non-Squamous cases (N = 277) | Wald statistic | Odds Ratio | CI 95% | P-Value |
|-------------|--------------------------|------------------------------|----------------|------------|-----------|---------|
| Alterations | | | | | | |
| TP53 | 233 (65%) | 125 (45%) | 8.4 | 1.82 | 1.22–2.74 | 0.004 |
| PIK3CA | 103 (29%) | 26 (9%) | 20.7 | 3.86 | 2.16–6.91 | <0.0001 |
| CDKN2A | 88 (24%) | 23 (8%) | 8.8 | 2.44 | 1.35–4.39 | 0.003 |
| SOX2 | 64 (18%) | 3 (1%) | 10.8 | 7.94 | 2.31–27.3 | 0.001 |
| CCND1 | 57 (16%) | 12 (4%) | 10.7 | 3.57 | 1.67–7.65 | 0.001 |
| NOTCH1 | 43 (12%) | 4 (1%) | 13.9 | 8.38 | 2.74–25.6 | 0.0002 |
| FBXW7 | 25 (7%) | 7 (3%) | 3.9 | 2.70 | 1.01–7.20 | 0.048 |
| KRAS | 21 (6%) | 66 (24%) | 11.8 | 0.33 | 0.18–0.62 | 0.001 |

For all the included variables, the P-values in the univariable analysis were <0.001. To account for imbalances of number of cases in certain tumor types, lung, gastrointestinal, and head and neck histologies were also computed in the multivariable. All P-values remained significant in the multivariable analysis. Further, a bootstrapping analysis (5,000 bootstrap samples) confirmed the results, with all P-values ≤ 0.005 , except FBXW7, with $P = 0.029$. Genes altered in more than 20 SCC cases were included.

.004), *PIK3CA* (29% versus 9%, $P < 0.0001$), *CCND1* (16% vs. 4%, $P = 0.001$), and *FBXW7* (7% versus 3%, $P = 0.048$), **Table 2 and Figure 2.** Of note, the only gene that was statistically less frequently altered in SCC specimens was *KRAS* (6% in SCC vs. 15% in non-SCC cases, $P = 0.001$).

We then used the 8 genes found to be significantly differentially altered in squamous cases versus non-squamous cases (more

frequent in SCC: *TP53*, *PIK3CA*, *CCND1*, *CDKN2A*, *SOX2*, *NOTCH1*, *FBXW7*; less frequent in SCC: *KRAS*) as a “signature” set of altered genes (“squamousness” signature). To confirm the specificity of this signature in SCCs, we scored all tumor samples by allocating one point for each gene of the signature altered and for each patient. As *KRAS* alterations were found less frequently in squamous cases, it was the absence of *KRAS* that was given 1 point (as opposed to all the other genes of the signature for which the presence of the alteration gave 1 point). Since the signature comprised 8 genes, each patient could cumulate a maximum of 8 points. Overall, SCC cases had a median of 3 points vs. only 1 for non-SCC specimens ($P < 0.0001$). The overall population (SCC and non-SCC cases) had a median of 2 points. We found that the

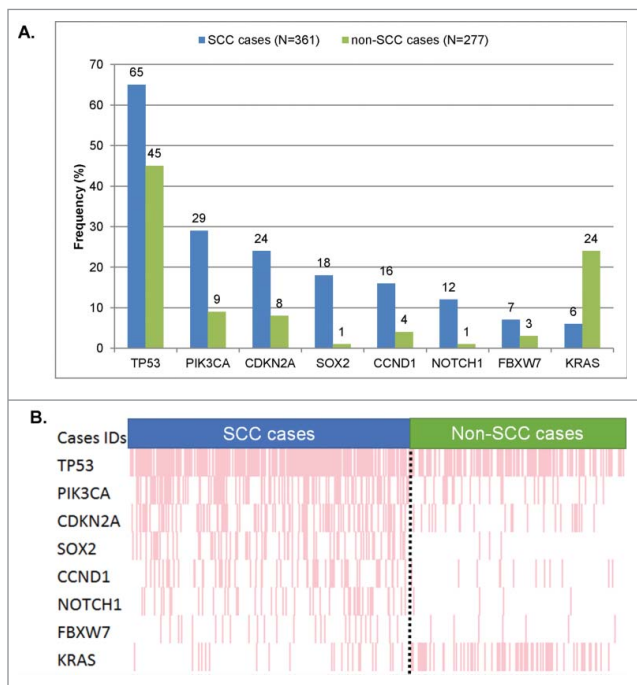


Figure 2. Significant differences in frequency of molecular alterations between squamous vs. non-squamous cases. Only genes that were found to be aberrant at statistically different rates (**Table 2**) in squamous versus non-squamous cancers were included. **(A)** Bar graph comparing the alteration frequencies between squamous vs. non-squamous cases. **(B)** Raw data on each patient in a “reverse array” fashion. Each pink bar corresponds to an alteration in the designated gene in one patient. Squamous cases have an over-representation of alterations in all the genes included, except *KRAS* (whose alterations are under-represented in SCCs). All the P-values were ≤ 0.001 , except for *FBXW7* ($P = 0.048$). All detailed P-values can be found in **Table 2**.

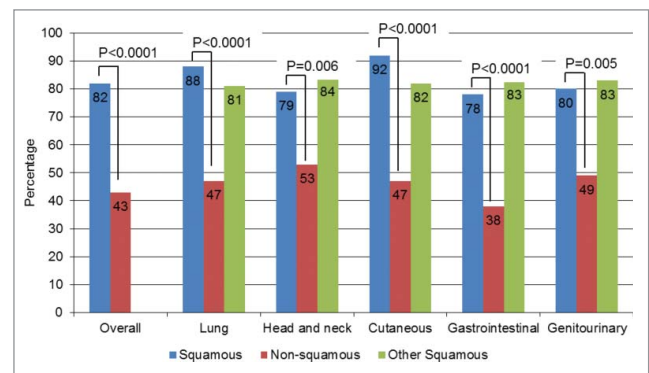


Figure 3. Squamousness signature frequency: comparison by histology. For this analysis, one point (cumulative) was given for each of the genes where abnormalities were more prevalent in squamous tumors—*TP53*, *PIK3CA*, *CCND1*, *CDKN2A*, *SOX2*, *NOTCH1*, and *FBXW7*—each time they were present. Because *KRAS* aberrations were significantly less prevalent in squamous tumors, 1 point was also assigned for the absence of *KRAS*. The numbers were then added up for each case. Overall, squamous cases had a median of 3 points versus only 1 point for non-squamous cases, $P < 0.0001$. In this bar graph, we represented the percentages of patients with ≥ 2 points (2 points was the median for the overall population (squamous and non-squamous cases)). All the P-values comparing squamous (blue bars) vs. non-squamous cases (red bars) were ≤ 0.006 . When comparing squamous tumors of a particular histology with all other squamous tumors (blue versus green bars), no significant differences were seen. For details on numbers and P-values refer to **Table S2**.

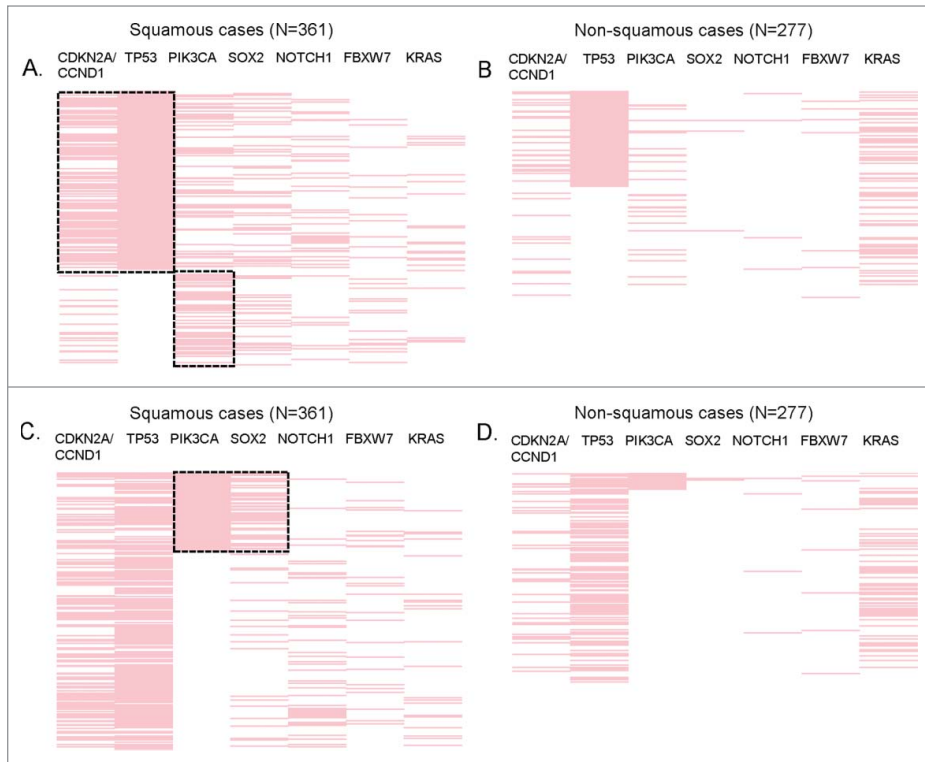


Figure 4. Significant co-alterations in patients with SCCs. Reverse array plot displaying raw data. Each pink bar corresponds to an alteration in the designated gene in one patient (total N = 361 SCC and N = 277 non-SCC specimens). **(A)** (squamous cases) and **(B)** (non-squamous cases) have been sorted according to the presence of *TP53* mutations. Panel A shows that within squamous tumors, *TP53* is co-altered with *CDKN2A/CCND1*, $P < 0.0001$ (P -values were also ≤ 0.001 if *CDKN2A* and *CCND1* were considered separately). There also was a negative association between *TP53* and *PIK3CA* alteration, $P = 0.001$. **(B)** shows that within non-squamous tumors, *TP53* alterations were also associated with *CDKN2A/CCND1*, $P = 0.003$ (P -value = 0.008 for *CCND1* and $P = 0.129$ for *CDKN2A* when considered separately). However, the co-alteration frequency of *TP53* and *CDKN2A/CCND1* was significantly higher in squamous tumors (31% vs. 9%, $P < 0.0001$). No association (positive or negative) was found between *TP53* and *PIK3CA* in non-squamous cases. **(C)** (squamous cases) and **(D)** (non-squamous cases) display the same data as panel A and B, but sorted by the presence of *PIK3CA* alteration. Within squamous tumors, *PIK3CA* is co-altered with *SOX2* ($P < 0.0001$). **(D)** *PIK3CA* alterations are not significantly associated with *SOX2* in non-squamous tumors.

percentage of number of specimens with ≥ 2 points was significantly higher in squamous cases than in non-squamous cases (82% versus 43%, $P < 0.0001$). These results were also statistically significant within all primary site of origins (all $P \leq 0.006$), **Figure 3**. Further, when comparing squamous tumors of a particular site of origin to all other squamous tumors, no significant differences were discerned (**Fig. 3** and **Table S2**). These results demonstrate that even though the tumor specimens share the same tissue location, their genetic characteristics were statistically different depending on their status as a squamous tumor or not.

Co-alteration analysis

We then conducted a multivariable co-alteration analysis for *TP53*, which was by far the most altered gene in SCCs (64.5% among the SCC cases, **Fig. 1A**). This analysis established that *TP53* alterations strongly correlated with aberrations in the cyclin pathway components *CDKN2A* and *CCND1* ($P < 0.0001$), **Figure 4A**. *TP53* and *CDKN2A/CCND1* co-alteration was more

frequently observed in squamous cases than in non-squamous cases (31% vs. 9%, $P < 0.0001$), though it was also observed in non-squamous cases ($P = 0.003$, **Fig. 4B**). More specifically, the co-alteration frequencies of *TP53* and *CDKN2A/CCND1* were as follows: 37.4% for head and neck SCC (vs. 15.6% in non-squamous head and neck cancers), 29.3% for SCC of the lung (vs. 6.7% in non-squamous lung cancers), 23.5% for gastro-intestinal SCC (vs. 9.4% for non-squamous gastro-intestinal cancers), and 7.5% for gynecologic/genitourinary SCC (vs. 0% of 37 cases in non-squamous gynecologic/genitourinary cancers).

In contrast, cases with *TP53* alterations (versus wild-type *TP53*) had less *PIK3CA* alterations in squamous cases ($P = 0.001$); this difference was not observed in non-squamous specimens, **Figure 4A**. In addition, we detected that *SOX2* alterations were statistically associated with *PIK3CA* alterations ($P < 0.0001$) in squamous cases (**Fig. 4C**), but not in non-squamous cases (**Fig. 4D**). *PIK3CA* and *SOX2* co-alterations were the most frequently observed in lung SCC (24% vs. 3.3% in lung non-SCC), followed by 11.8% in gastro-intestinal SCC (vs. 0% of 117 non-squamous gastro-intestinal cases), 10% in gynecologic/genitourinary SCC (vs. 2.7% in non-squamous gynecologic/genitourinary cancers), 9.3% in head and neck SCC (vs. 3.1% in non-squamous head and neck cancers). Of note,

none of the cutaneous cases (squamous or not) harbored *PIK3CA/SOX2* co-alterations.

Discussion

Our study examined different types of SCC specimens by targeted next-generation sequencing and demonstrated that the most commonly altered genes across squamous tumors were *TP53* (64.5%), *PIK3CA* (28.5%), *CDKN2A* (24.4%), *SOX2* (17.7%), and *CCND1* (15.8%), **Figure 1**. Furthermore, when comparing the alterations portfolio of SCC specimens with alterations found in non-SCC cases, we established that 8 genes had alteration frequencies that were statistically different when SCCs and non-SCC cases were compared in a multivariable analysis. While *TP53*, *PIK3CA*, *CDKN2A*, *CCND1*, *SOX2*, *NOTCH1* and *FBXW7* were found to be significantly more frequently altered in SCCs vs. non-SCCs, *KRAS* appeared to be less altered. In a review recently published¹¹ and focusing on head and neck

SCCs, the most commonly altered genes were *TP53*, *NOTCH1*, *HRAS*, *PIK3CA*, as well as *CDKN2A*; additional studies focusing on other pathologic diagnoses, such as esophageal,¹⁰ or lung cancer,^{12,13} reported alterations in genes overlapping with our findings. Of note, 5.6% of our head and neck specimens carried *HRAS* alterations (6/107 cases), while only one single specimen harbored a *KRAS* alteration (0.9%), consistent with what has been previously described in head and neck SCCs.^{7,8}

More than half of the specimens carried *TP53* alterations (64.5%), making this tumor suppressor gene the most commonly mutated gene in SCCs. *TP53* is the gatekeeper of the genome and has crucial function such as DNA repair, apoptosis, and cell cycle arrest. *TP53* alterations have been identified as early events in the disease and have been associated with disease progression and a poorer outcome.¹¹ Therapies targeting *TP53* loss of function are currently being explored in clinical trials, and several studies suggest that patients harboring *TP53* alterations might respond better to angiogenesis inhibitors.^{14,15} Of note, a *TP53* adenoviral-based treatment (rAd-p53; Gendicine) for patients with HNSCC has recently been approved for use in China.¹⁶

Cyclin-dependent kinase inhibitor 2A (*CDKN2A*), located at chromosome 9p21, is another well-known tumor suppressor gene involved in the regulation of cell cycle progression and was altered in 24.5% of our SCC cases. Cyclin D1 (*CCND1*), another component of this pathway, was also found highly altered in SCCs (15.8%). *CCND1* interacts with *CDK4/6* and forms complexes that play a central role in the G1/S transition of the cell cycle, acting by phosphorylating Rb.¹⁷⁻¹⁹ Several agents targeting the cyclin pathway are undergoing clinical phases. Studies of one such agent, palbociclib, have produced encouraging data in women with breast cancer²⁰ and has been recently approved by the FDA (Feb 2015). Given the high incidence of cyclin pathway alterations in SCCs, testing cyclin-pathway inhibitors in clinical trials may be warranted in patients with SCCs.

Another highly activated pathway in SCCs appeared to be the PI3K-Akt-mTOR axis, as reflected by the high incidence of *PIK3CA* alterations. This pathway also leads to an increased translation of *CCND1* and plays a crucial role in the cell cycle,²¹⁻²³ as well as migration and invasion.²⁴ Further, alteration of this pathway components, such as *PIK3CA*, may confer resistance to agents targeting upstream constituents, such as EGFR inhibitors, as many receptor tyrosine kinases (RTKs) activate PI3K.²⁴ Of note, a case series investigating the use of an mTOR inhibitor for patients with head and neck SCCs and *PTEN* loss or *PIK3CA* mutations showed significant tumor regression in 3 of 5 patients.²⁵

SOX2 is a transcription factor encoded at 3q26.33, which can act either as a suppressor or activator of gene transcription. *SOX2* has been implicated in cellular reprogramming and normal embryogenesis mechanisms,²⁶ and may represent an important player in tumor initiation and progression of SCCs.^{27,28} We have found that 18% of our specimens harbored *SOX2* amplifications (compared to only 1% in non-squamous cases), making this gene a hallmark candidate for SCCs. Further, we found that *SOX2* and *PIK3CA* were highly co-altered ($P < 0.0001$ in multivariable analysis), which may be explained by their chromosomal co-localization at 3q26.33.²⁹

NOTCH1 is believed to play important roles in different biological processes disturbed in cancer, and *NOTCH1* alterations were identified in 12% of our SCC samples (versus 1% in non SCCs). Murine models have highlighted the importance of *NOTCH1* in squamous epithelial differentiation, and in cutaneous SCC, recent sequencing efforts also suggest loss of function mutational pattern of *NOTCH1*.³⁰⁻³² Indeed, in our subset of cutaneous SCCs, *NOTCH1* alterations were found in 33% of cases, vs. 10% in the other types of SCCs, ($P = 0.016$ in a multivariable analysis, data not shown). Interestingly, in addition to *NOTCH1* alterations, *FBXW7* alterations were identified in 7% of our SCC specimens, versus 3% in non-SCC cases ($P = 0.048$ in multivariable). *FBXW7* forms part of the ubiquitin ligase complex that can mediate *NOTCH1* degradation; consequently, *FBXW7* mutations could also be affecting the *NOTCH1* pathway,¹¹ although *FBXW7* is also known to target other oncogenic pathways by degrading cyclin E and c-myc.³³

In the recent years, human papilloma virus (HPV) has emerged as a primary etiology of oropharyngeal cancers,¹³ as well as cutaneous and lung SCCs.¹ HPV positive-associated cancers exhibit different characteristics (e.g. risk factors, epidemiology) but also a distinct clinical course. Not surprisingly, the alterations identified in these subgroups are also different. Several studies described that most HPV-negative samples carried *TP53* as well as cyclin pathway alterations,³⁴⁻³⁶ as opposed to HPV-positive samples which appeared to harbor more *PIK3CA* alterations.^{35,37} Of potential interest, another study showed that squamous-like tumors without *TP53* mutations had a higher density of *PIK3CA* mutations.³⁸

There are several limitations to our study. There may have been selection bias of the specimens, as patients/physicians may have elected to submit tissue when there were fewer standard therapeutic options left. Also, the samples came from diverse institutions and practices. However, the fact that all the samples had undergone the same next-generation sequencing test by the same laboratory improved the homogeneity of our analysis. The size of the test panel changed from 182 to 236 genes over the course of the study sample. However, the markers identified as part of the squamousness signature were all present in both the original and expanded panels. The pool of samples also originated from different tissue types, and this heterogeneity may have confounded some of the analysis, though it also was crucial to our ability to compare squamous molecular portfolios in different malignancies. The HPV status of our patients was not known. In future studies, the existence of different squamousness signatures depending on HPV status should be investigated. Finally, a confirmatory cohort of patients should be tested to confirm our results.

In summary, we have analyzed 361 SCC specimens and highlighted a set of 8 genes whose alteration frequency was statistically different when compared to non-SCC cases ($N = 277$). Indeed, we observed an increased frequency of *TP53*, *PIK3CA*, *CDKN2A*, *CCND1*, *SOX2*, *NOTCH1*, and *FBXW7* alterations, while a decreased frequency of *KRAS* alterations was noted. These alterations were used to generate a “squamousness signature” that was significantly more prevalent in SCCs across malignancies originating in different organs compared to patients with non-squamous tumors of the same organ of origin (Fig. 3). We also

observed co-alteration patterns and report that *TP53* mutations associated positively with *CDKN2A* and *CCND1* alterations and negatively with *PIK3CA* in SCC specimens. Of interest, *KRAS*, a frequently aberrant gene in non-squamous tumors that leads to resistance to PI3K pathway inhibitors,³⁹⁻⁴¹ was significantly less often aberrant in SCCs. A multivariable co-alteration analysis identified 2 SCC subtypes: (i) patients in whom *TP53* and cyclin pathway (*CDKN2A* and *CCND1*) aberrations were associated but in whom *PIK3CA* anomalies were less common; and (ii) individuals with *PIK3CA* abnormalities, in whom *TP53* mutations were less frequent (all $P \leq 0.001$, multivariable analysis). Together, this data suggests a direction for a more precise approach for treatment of SCCs. Targeting the PI3K-AKT-mTOR and/or cyclin pathway components may represent valuable investigational avenues for clinical trials in subgroups of patients with SCCs. Future studies should concentrate on large datasets that include full phenotypic annotation. Importantly, NGS provides a potentially important diagnostic test that permits recognition of the abnormalities present in each patient, hence enabling the therapeutic decision-making process.

Patients and Methods

Patients

This study included 361 SCC samples that were tested using NGS over a 2-year period of time (from December 2011 until November 2013). All consecutive patients for whom we had data were included in the analysis. Patients were tested on physician request. This cohort was compared to 277 other patient samples (separate population) comprising individuals that had diverse cancers (other than SCCs) who were seen at the UC San Diego Moores Cancer Center. This non-SCCs cohort included the same tumor sites of origin as the SCC cohort. This study was performed in accordance with UCSD Institutional Review Board guidelines.

Next-generation sequencing

All patients (both SCC and non-SCC cohorts) had formalin-fixed, paraffin-embedded tumor samples that were submitted to Foundation Medicine (FoundationOne™, Cambridge, Massachusetts, <http://www.foundationone.com>, which is a clinical grade CLIA-approved next-generation sequencing test). Samples were reviewed by a pathologist to ensure the correct diagnosis.

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Next-generation sequencing data were collected and interpreted by N-of-One®, Inc.. (Lexington, MA, www.n-of-one.com). For this study, patients' samples were tested with a 182 or 236 gene panel; the latter sequences the entire coding sequence of 236 cancer-related genes and 47 introns from 19 genes often rearranged in cancer. The entire coding sequence of the 182 or 236 cancer-related genes was generated with an average sequencing depth of coverage greater than 250x (full list available at <http://foundationone.com/genelist1.php>). This method of sequencing allows for detection of copy number alterations, gene rearrangements, and somatic mutations with >99% specificity and >99% sensitivity for base substitutions at ≥ 5 mutant allele frequency and >95% sensitivity for copy number alterations. Foundation Medicine uses a threshold of ≥ 8 copies for gene amplification.

Statistical analysis

When appropriate, Fisher's exact tests and binary logistic regressions (univariable and multivariable) were performed. A bootstrapping analysis (5,000 bootstrap samples) was performed for internal validation of the multiple logistic regression model results used to select the genes for the "squamousness" signature. Whenever possible, 95% confidence intervals (95% CI) were reported. All statistical analysis were performed by author MS with SPSS version 22.0.

Disclosure of Potential Conflicts of Interest

Dr. Kurzrock has consultant fees from Sequenom and has an ownership interest in RScueRx Inc. Sheryl K. Elkin, Brett N. Tomson, and Jennifer Levin Carter are employees of N-of-One, Inc. Jennifer Levin Carter had a leadership position and holds ownership interest at N-Of-One, Inc. Sheryl K. Elkin holds ownership interest at N-Of-One, Inc.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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