

## Squamous Cell Carcinoma of the Oral Tongue in Young Non-Smokers Is Genomically Similar to Tumors in Older Smokers

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### Abstract

**Purpose:** Epidemiologic studies have identified an increasing incidence of squamous cell carcinoma of the oral tongue (SCCOT) in younger patients.

**Experimental Design:** DNA isolated from tongue tumors of young (<45 years, non-smokers) and old (>45 years) patients at was subjected to whole-exome sequencing and copy-number analysis. These data were compared with data from similar patients in the TCGA (The Cancer Genome Atlas) project.

**Results:** In this study, we found that gene-specific mutation and copy-number alteration frequencies were similar between young and old patients with SCCOT in two independent cohorts. Likewise, the types of base changes observed in the young cohort were similar to those in the old cohort even though they differed in smoking history. TCGA data also demonstrate that the genomic effects of smoking are tumor site-specific, and we find that smoking has only a minor impact on the types of mutations observed in SCCOT.

**Conclusions:** Overall, tumors from young patients with SCCOT appear genomically similar to those of older patients with SCCOT, and the cause for the increasing incidence of young SCCOT remains unknown. These data indicate that the functional impact of smoking on carcinogenesis in SCCOT is still poorly understood. *Clin Cancer Res*; 20(14); 3842–8. ©2014 AACR.

### Introduction

Epidemiologic studies have recently identified the increasing incidence of squamous cell carcinoma of the oral tongue (SCCOT) in younger patients (1–3). These tumors are not human papilloma virus (HPV)-related and are often found in women who are non-smokers (2, 4). Those observations contrast with most SCCOT cases that are seen in older men with a history of cigarette smoking (5). The causes for this increasing incidence in the young are unknown. We hypothesized that this epidemiologically distinct disease would also prove to be genomically distinct, especially with respect to alterations caused by smoking,

and that a better understanding of the differences would identify novel opportunities for treatment and/or prevention. Hence, we undertook the sequencing and integrated genomic profiling of a cohort of younger patients with SCCOT, as reported here.

### Patients and Methods

Fresh-frozen surgically resected previously untreated tumor tissue and matched nonmalignant adjacent tissue were obtained from consented patients treated for head and neck SCC (HNSCC) at The University of Texas MD Anderson Cancer Center (Houston, TX), under an Institutional Review Board-approved protocol. Young tongue (YT) patients were chosen based on oral tongue primary tumor site, age less than 46 and less than 1 pack-year smoking history. Old tongue (OT) patients were chosen based on oral tongue primary tumor site and age older than 45. Patient characteristics are shown in Supplementary Table S1. Exome DNA was captured with Nimblegen reagents (Nimblegen) and sequenced on a SOLiD or Illumina platform as described previously (6). SNP analysis was conducted on SNP6.0 or CytoscanHD arrays (Affymetrix), and the data were analyzed with Partek (v6.6; Partek Inc.), ASCAT (v2.1), GISTIC (v2.0.12), and R software, as described previously (6). We have previously reported sequencing and copy-number data from some of these

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### Translational Relevance

Genomic alterations do not explain the increasing incidence of oral tongue cancer in young patients, and smoking does not dramatically alter the genome of tongue cancer at any age. Therefore, the causes of tongue cancer are still largely unknown, and their identification could provide novel avenues for therapeutic intervention.

patients (6, 7). Mutation data can be found in Supplementary Table S3.

A similar cohort of patients was identified from within the TCGA HNSCC project. YT patients were chosen based on oral tongue site, age less than 46 and lifelong non-smoker or reformed smoker with unknown pack-year history. OT patients were chosen based on oral tongue site and age older than 45. Patient characteristics are shown in Supplementary Table S1. Mutation and copy-number data were obtained from the TCGA pan-cancer project (8) and the Synapse website ([www.synapse.org](http://www.synapse.org); doi:10.7303/syn300013; ref. 9).

The statistical significance of mutation and copy-number frequencies was determined by the Fisher exact test. Mutation-type frequencies were determined for each patient, excluding indels and multiple-base mutations. Group frequencies were determined by averaging the individual patient frequencies: this prevented skewing of the data by patients with large numbers of mutations. Mutation-type frequencies were compared by using the *t* test on arcsine transformed data.

### Results

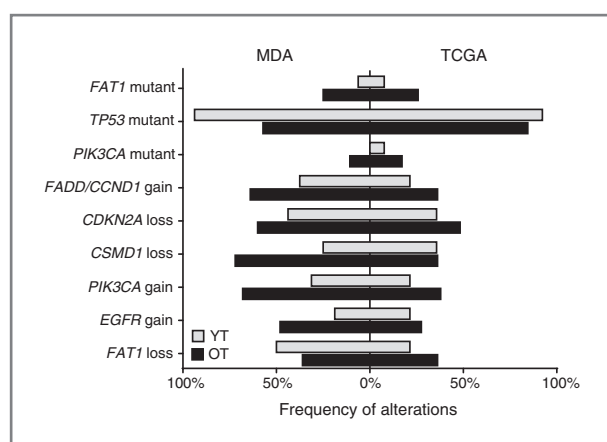
DNA from 16 YT and 28 OT patients treated at the MD Anderson Cancer Center (MDA) was subjected to whole-exome sequencing (Supplementary Table S1). A median of

29 and 83 mutations was identified in the YT and OT tumors, respectively. The elevated number of mutations in the OT is not surprising, because mutation number is known to be related to both age and smoking (10). This finding was validated in The Cancer Genome Atlas (TCGA) cohort, in which the median number of mutations increased from 63 in YT to 112 in OT. Supplementary Fig. S1 shows the relationships between mutation number and age. We next asked whether any specific genes were mutated at a different frequency in the YT cohort. Because of the low sample size, we limited our initial search to the most significantly mutated genes identified by MutSig in the entire TCGA HNSCC project (HNSC). *TP53* (unadjusted  $P = 0.015$ ) showed a slight increase in mutation frequency in the MDA YT cohort, but it was not statistically significant when adjusted for multiple testing (Table 1; Fig. 1). Comparable analysis was then performed on the TCGA cohort. The *TP53* mutation frequency was also elevated in the TCGA YT patients (Table 1; Fig. 1). To increase statistical power the two cohorts were combined. Three genes showed trends toward statistical significance; *FAT1*, *TP53*, and *PIK3CA* (Table 1; Fig. 1). However, none of those genes showed a statistically significant difference between the combined YT and OT patient cohorts. The trend of increased *TP53* mutations in YT is provocative because the YT lacks exposure to cigarette smoke, which has been associated with *TP53* mutations. *FAT1* and *PIK3CA* showed a lower mutation frequency in the YT cohort. Mutation frequencies for HPV<sup>+</sup> tumors and the entire TCGA cohort are shown for comparison (Table 1). An analysis of mutation frequencies in all genes in the combined cohorts was also performed, but no genes were found to be significantly different. Additional subset analysis for really YTs (<30 years), OT smokers, and OT non-smokers are shown in Supplementary Table S2.

Whole-genome copy-number analysis was also performed. We compared the number and size of copy-number

**Table 1.** Mutation frequencies

Cohort (N)	MDA		TCGA				Total		Unadjusted P value
	YT (16)	OT (28)	YT (13)	OT (58)	HPV <sup>+</sup> (35)	All (279)	YT (29)	OT (86)	
Median number of mutations (range)	28.5 (10–81)	83 (11–251)	63 (9–136)	112 (11–268)					
<i>FAT1</i>	6.3%	25.0%	7.7%	25.9%	0.0%	22.9%	6.9% (2)	25.6% (22)	0.0355
<i>TP53</i>	93.8%	57.1%	92.3%	84.5%	2.9%	73.1%	93.1% (27)	75.6% (65)	0.0583
<i>PIK3CA</i>	0.0%	10.7%	7.7%	17.2%	37.1%	20.8%	3.4% (1)	15.1% (13)	0.1140
<i>CDKN2A</i>	6.3%	3.6%	30.8%	32.8%	0.0%	22.6%			
<i>CASP8</i>	6.3%	10.7%	7.7%	6.9%	0.0%	8.6%			
<i>AJUBA</i>	0.0%	0.0%	0.0%	5.2%	0.0%	6.1%			
<i>NOTCH1</i>	25.0%	17.9%	7.7%	22.4%	8.6%	18.6%			
<i>MLL2</i>	0.0%	7.1%	7.7%	8.6%	17.1%	17.6%			
<i>NSD1</i>	0.0%	3.6%	0.0%	6.9%	8.6%	10.4%			
<i>HLA-A</i>	0.0%	14.3%	0.0%	1.7%	0.0%	3.2%			
<i>TGFBR2</i>	0.0%	0.0%	0.0%	1.7%	2.9%	3.6%			



**Figure 1.** Frequency of common genomic alterations in YT and OT. The frequency of each event in the MDA cohort is shown by a bar to the left of center and the frequency in the TCGA cohort is shown by a bar to the right of center.

alterations (CNA) between the YT and OT cohorts, identifying an average of 129 CNAs in YT samples and 72 in the OT samples from the MDA cohort (Table 2), and 62 and 64 CNAs in the YT and OT samples from the TCGA cohort, respectively (Table 2). These differences were not statistically significant, although the YT had a smaller mean segment length for copy-number gains ( $P = 0.00817$ ; Table 2) in the MDA cohort. There was no difference in segment length of losses in between YT and OT in either cohort (Table 2). The mean copy number of gains or losses was also not different for gains or losses in either cohort (Table 2). Finally, no specific genomic regions were found to be significantly different between the YT and OT patients in either the MDA cohort or the TCGA cohort. Analysis of some genes frequently gained or lost in HNSCC revealed modest differences in frequency between YT and OT (Fig. 1). For example, gains in *FADD* and *PIK3CA* were less frequent in YT, but the difference was not significant (Fig.

1). Overall, the CNAs were very similar between the YT and OT cohorts, and the regions of copy-number change were similar to those reported previously (6).

Because smoking is known to leave its mark on the genome by causing certain types of mutations, we compared mutation types in the YT and OT patients. Taking into account directional redundancy, six types of mutations can be distinguished. The frequencies of these six types of mutations have been shown to vary across tumor types (8, 11), but we found no significant difference in that respect for YT and OT patients in either the MDA or TCGA cohort (Fig. 2A). The profiles resembled that of all head and neck tumors in the TCGA project. The profile, however, was distinct from that of HPV<sup>+</sup> tumors or laryngeal tumors (Fig. 2A). HPV<sup>+</sup> tumors show an increase in C>T mutations ( $P < 0.0001$ ) and decreases in C>A, A>T (both  $P < 0.0001$ ), and A>G ( $P = 0.0074$ ) mutations when compared with HPV<sup>-</sup> HNSC tumors. Laryngeal tumors show a decrease in C>T mutations ( $P < 0.0001$ ) and increases in C>A and A>T mutations (both  $P < 0.0001$ ) when compared with non-laryngeal tumors (Fig. 2A). It was expected that OT tumors would exhibit a mutation signature related to cigarette smoking when compared with the YT tumors from non-smokers. The similarity between YT and OT mutation signatures could indicate either the presence of a smoking signature in the YT tumors or a lack of a smoking signature in the OT tumors. To address those alternative possibilities, we investigated the smoking signatures in other tumor sites from the TCGA project.

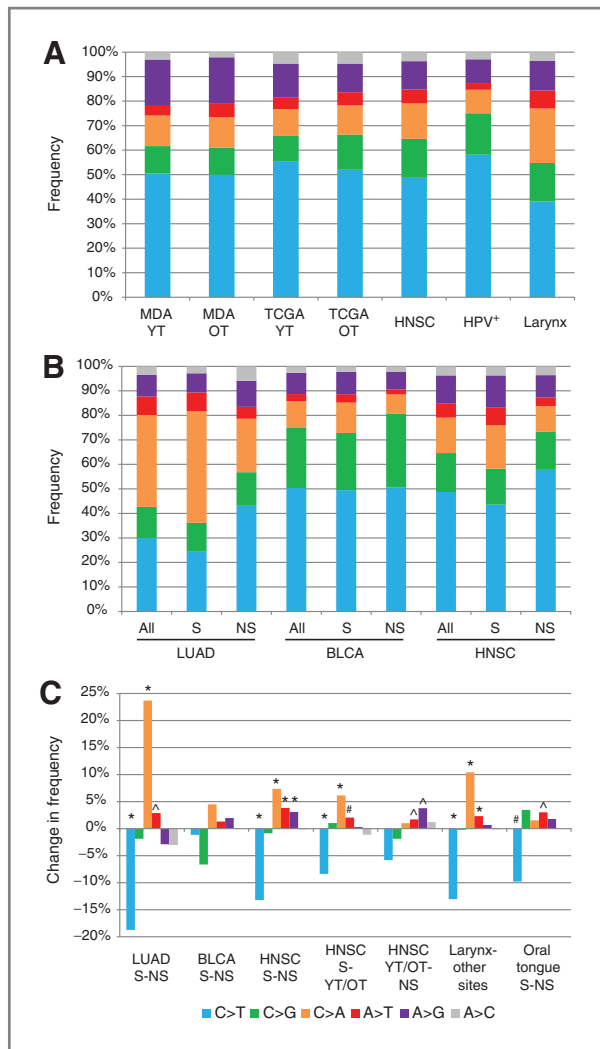
The mutational events linked to smoking are traditionally reported as an increase in C>A mutations and a decrease in C>T mutations. TCGA collected smoking history information for lung adenocarcinoma (LUAD), bladder urothelial carcinoma (BLCA), and HNSC. Each of these tumor types was analyzed for the presence of a smoking mutation signature. In LUAD tumors C>A mutations increased 23.7 percentage points in smokers when compared with non-smokers, and C>T mutation decreased

**Table 2.** Copy-number alterations

	MDA			TCGA		
	YT	OT	P	YT	OT	P
Mean number						
All CNAs	129.00	71.56	NS	61.93	63.78	NS
Gains	57.94	36.52	NS	29.21	24.91	NS
Losses	71.06	35.04	NS	32.71	38.86	NS
Mean copy number <sup>a, b</sup>						
Gains	3.45	3.09	NS	0.77	0.85	NS
Losses	-1.14	-1.41	NS	-1.19	-1.21	NS
Mean segment length (Mb)						
Gains	7.37	20.50	0.00817	5.61	8.27	NS
Losses	11.39	15.24	NS	3.61	5.11	NS

<sup>a</sup>MDA values are normalized absolute copy numbers.

<sup>b</sup>TCGA values are log<sub>2</sub> centered at 0.



**Figure 2.** Analysis of mutation profiles. A, the frequency of each type of single-base substitution is indicated by a different color in each annotated HNSC cohort. B, frequency distributions by smoking status and tissue site. C, differences in frequency between two cohorts. The sample name indicates which samples were compared. For example, S-NS indicates that the frequency in the NS cohort was subtracted from the frequency in the S cohort; therefore, a positive result is a higher frequency in the S cohort; \*,  $P < 0.0001$ ; #,  $P < 0.001$ ; ^,  $P < 0.01$ . Abbreviations: NS, non-smokers; S, smokers; YT/OT, combined TCGA YT and OT cohorts.

18.7 percentage points (Fig. 2B and C). A>T mutations were also found to increase 2.9 percentage points in smokers (Fig. 2B and C).

BLCA showed a very different profile, indicating the effect of tissue of origin. BLCA shows a much higher frequency of C>T and C>G mutations and a lower frequency of C>A when compared with LUAD (Fig. 2B). In BLCA the impact of smoking is quite different from that in LUAD. The largest change with smoking in BLCA is a decrease in C>G of 6.6 percentage points (Fig. 2B and C). C>A is also increased 4.5 percentage points, but neither change is statistically significant (Fig. 2B and C).

We next examined the impact of smoking on the mutation profile of HNSC. The changes in HNSC were generally similar to those in LUAD but of lower magnitude. We found a decrease in C>T of 13.2 percentage points and an increase in C>A of 7.3 percentage points that were consistent with the changes in LUAD (Fig. 2B and C). HNSC also demonstrated increases in A>G and A>T of 3.8 and 3.1 percentage points, respectively (Fig. 2B and C and Supplementary S2A).

Having defined the smoking signature, we could then ask whether YT and OT tumors exhibit such a signature. For this analysis, we combined the YT and OT tumors into one cohort (YT/OT) and compared it with the HNSC smoking and non-smoking cohorts. The YT/OT cohort had a lower C>A frequency and a higher C>T frequency when compared with the HNSC smoking cohort (Fig. 2C), indicating that the YT/OT tumors lack the most definitive characteristics of the smoking signature. However, the YT/OT also demonstrated increased A>G when compared with the HNSC non-smoking tumors and an intermediate level of A>T when compared with both smoking and non-smoking cohorts (Fig. 2C). Similar results were obtained when the YT and OT cohorts were separately compared with the non-smoking and smoking cohorts, although with lower statistical significance (data not shown) or when smoking and non-smoking cohorts within the OT cohort were compared with each other or YT (Supplementary Fig. S2B). Overall, the mutation profile of the YT/OT tumors seems most similar to the profile of non-smokers.

Because both YT and OT tumors lack a smoking signature, we next searched for the source of the smoking signature in HNSC. We found that tumors from the laryngeal subsite exhibit a smoking signature when compared with tumors from all other sites (Fig. 2A and C and Supplementary S2A and S2B) with an increase in C>A mutations and a decrease in C>T mutations. In addition, the smoking signature within the oral tongue subsite is less recognizable than the overall HNSC smoking signature (Fig. 2C). Those observations suggest that the smoking signature in HNSC is largely driven by the laryngeal subsite.

## Discussion

Because the incidence of YT cancer has reportedly been increasing (1–3), we hypothesized that YT and OT tumors would prove to be genomically distinct. We found, to the contrary, that the two are genomically quite similar in their CNAs, mutations and types of mutations. Those conclusions were consistent for two independent patient cohorts. Although this study is the largest genomic analysis of YT tumors, it is still limited by the small sample size. The rarity of these tumors has also prevented ideal matching of the YT and OT cohorts, which differ in a few parameters (Supplementary Table S1), and resulted in a YT cohort that is not enriched for women as some have reported (2, 4).

The mutation frequencies of *FAT1* and *TP53* showed trends toward statistical significance, but would require larger cohorts for validation (estimated 170 and 189 total

patients, respectively). *FAT1* is inactivated in a large proportion of oral cavity tumors, either by mutation or copy-number deletion (6). *FAT1* may be related to differentiation, migration, or Hippo signaling in HNSCC (6, 12–14), but its role is largely still unknown. The lower frequency of *FAT1* mutations in YT could suggest that its inactivation is not necessary for tumor formation or that it is somehow related to smoking status. However, because older smokers are expected to have more background mutations it is possible that the difference is an artifact of the increased frequency of mutations. Because *TP53* mutations are more frequent in YT patients, this result is not likely to be an artifact. *TP53* mutations are likely to be impactful on the tumor progression in YT, but a causative factor leading to increased *TP53* mutations in YT is still unknown.

We also noted that OT tumors do not exhibit a mutation signature associated with smoking. The mutation signature from smoking seems to be site-specific, with laryngeal SCC exhibiting the strongest smoking signature among the HNSCC subsites. SCCOT did not exhibit a robust mutational smoking signature, in that regard echoing the TCGA findings in urothelial bladder cancer (15). Overall, the mutational signature that has been defined by analysis of lung tumors does not seem to reflect the effect of smoking on other tissue sites. Because smoking is clearly an epidemiologic risk factor for SCCOT (5), the mechanism of carcinogenesis may differ among sites. Cigarette smoke has been shown to be both a tumor initiator and a tumor promoter (16, 17). Only the role of smoke as a tumor initiator should leave a mutational signature on the DNA. Therefore, it is clear that smoke is a tumor initiator in lung cancer and laryngeal SCC. However, it may act primarily as a tumor promoter in SCCOT and BLCA. It is also possible that epigenetic alterations are involved because we have not yet examined the DNA methylation status of these tumors.

Alcohol consumption is a known risk factor for HNSCC. In some patients alcohol is likely to contribute as an initiator. Ethanol is not mutagenic, but its metabolite, acetaldehyde, can lead to mutations, primarily C>T (18). Because C>T is the most common mutation in many tumor types (8, 11), other factors must also contribute to their frequency. Analysis of the TCGA HNSC data for an alcohol mutation signature resulted in a less dramatic version of the smoking signature with decreases in C>T and increases in C>A in heavy drinkers (data not shown). That observation contrasts with the expected increase in C>T that could be caused by acetaldehyde. Because many heavy drinkers are also heavy smokers, it is likely that the smoking signature masks any alcohol signature. Therefore, the impact of alcohol use is still unclear.

These findings do not lessen the importance of smoking and drinking cessation programs to reduce SCCOT incidence. However, they suggest possible new avenues for prevention or treatment of SCCOT. If an unknown tumor initiator is acting in SCCOT, its identification could lead to new prevention strategies. Our analysis of the mutation

profile does not indicate an obvious tumor initiator for SCCOT. It may, however, eliminate some candidates (e.g., many environmental mutagens) that would be expected to leave a mutation signature. It is also possible that the same initiator is acting in both YT and OT patients.

If smoking is indeed a tumor promoter in SCCOT, it will be important to determine the mechanism. In lung cancer, smoke has been shown to function as a tumor promoter by increasing inflammation (16), and that might also be the case in SCCOT. Inflammation from other causes promotes many tumor types including: esophageal, pancreatic, and colorectal (19). That connection suggests that inflammation is an important factor in non-smoking-related SCCOT, as previously reported (20, 21). Prevention strategies that include anti-inflammatory agents may prove useful. Although the results of clinical trials of COX inhibitors for prevention of SCCOT have not generally been positive (20, 22), agents that target other inflammatory pathways (such as the NF- $\kappa$ B pathway) still look very promising (20).

Another factor that could be promoting SCCOT is the oral microbiome. The oral cavity is home to a diverse population of microorganisms (23, 24), even in healthy individuals, and some of those microorganisms could also play a role in carcinogenesis (21, 25). They may produce carcinogenic compounds such as acetaldehyde, inducing local inflammation, or they may act through still-undiscovered mechanisms (21, 24, 25). Numerous microorganisms have already been associated with the development of oral cancer (21, 24, 25). Included are species of *Streptococcus*, *Neisseria*, and *Candida*. Conversely, the "normal" microbiome may protect against carcinogenesis and a disrupted microbiome may cause it to no longer be protective (23, 24). Finally, it is unlikely that microorganisms are directly oncogenic in the way that HPV is oncogenic in oropharyngeal cancer. HPV is the only virus detected in SCCOT, and it is found in only 5% of cases. In addition, we found that HPV leaves a distinct mutation signature and expect that other pathogens would also leave distinct mutation signatures. However, we detect no such signature. In sum, the oral microbiome is likely to be involved in SCCOT in some way, but very little is understood about its causal role or the specific organisms involved.

Finally, YT cancer is troubling in that it defies the stereotype of HNSCC, which generally occurs in older men with long histories of heavy smoking and drinking (5). That observation is even more disturbing because the incidence of YT cancer is increasing (1–3). Hence, there is a strong interest in identifying the cause. Clinically, the cancer-specific outcomes for YT patients are similar to those for OT patients (4, 26–29), and we have shown here that the genomic alterations in YT tumors are also similar to those in OT tumors. Therefore, the only evidence suggesting that they are a unique cohort is epidemiologic (1–3). Although statistically significant, the epidemiologic findings are based on a low number of patients, because YT tumors are still quite rare, and the apparent difference

could be a statistical anomaly. Additional epidemiologic studies will be necessary to validate the original reports. Our data indicate that the mechanistic cause of YT cancer is likely to be similar to the mechanistic cause of OT cancer. For example, if smoking causes inflammation to promote OT perhaps another factor that also induces inflammation is promoting YT, resulting in a genomically and clinically similar disease. Similarly a factor that disrupts the oral microbiome in YT could promote carcinogenesis in a way similar to the mechanism in OT. However, the identity of a causative factor in YT cancer, if it exists, is still unknown.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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