Common Oncogenic Mutations Are Infrequent in Oral Squamous Cell Carcinoma of Asian Origin

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Abstract

Objectives: The frequency of common oncogenic mutations and TP53 was determined in Asian oral squamous cell carcinoma (OSCC).

Materials and Methods: The OncoCarta™ panel v1.0 assay was used to characterize oncogenic mutations. In addition, exons 4-11 of the TP53 gene were sequenced. Statistical analyses were conducted to identify associations between mutations and selected clinico-pathological characteristics and risk habits.

Results: Oncogenic mutations were detected in PIK3CA (5.7%) and HRAS (2.4%). Mutations in TP53 were observed in 27.7% (31/112) of the OSCC specimens. Oncogenic mutations were found more frequently in non-smokers (p = 0.049) and TP53 truncating mutations were more common in patients with no risk habits (p = 0.019). Patients with mutations had worse overall survival compared to those with absence of mutations; and patients who harbored DNA binding domain (DBD) and L2/L3/LSH mutations showed a worse survival probability compared to those patients with wild type TP53. The majority of the oncogenic and TP53 mutations were G:C > A:T and A:T > G:C base transitions, regardless of the different risk habits.

Conclusion: Hotspot oncogenic mutations which are frequently present in common solid tumors are exceedingly rare in OSCC. Despite differences in risk habit exposure, the mutation frequency of PIK3CA and HRAS in Asian OSCC were similar to that reported in OSCC among Caucasians, whereas TP53 mutations rates were significantly lower. The lack of actionable hotspot mutations argue strongly for the need to comprehensively characterize gene mutations associated with OSCC for the development of new diagnostic and therapeutic tools.

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Introduction

Oral squamous cell carcinoma (OSCC), a subset of head and neck squamous cell carcinoma (HNSCC), is one of the most common malignancies with more than 400,000 of new cases diagnosed annually worldwide [1]. Particularly in South East Asia, the disease is reaching epidemic proportions with age-standardized rates (ASR) of 6.7 compared to 4.3 and 4.0 in Europe and America respectively [2]. The disease has significant physical and psychological morbidity and a survival rate of approximately 50% over 5 years, a figure that reflects the stage of the tumour at presentation and the development of loco-regional recurrences, distant metastases and second primary tumours. Survival rates have not improved for decades and taken together, the findings argue strongly for the need to develop new therapeutic strategies.

Cancer occurs due to the progressive accumulation of abnormalities in cellular DNA which, in turn, provide a selective
growth advantage to cancer cells and facilitate metastatic dissemination [3]. Dysregulation of certain signaling pathways, together with chromosomal abnormalities, have been identified in HNSCC [4] and more recently, TP53, CDKN2A, PIK3CA, PTEN and HRAS, together with FBXW7, NOTCH1, IRF6 and TP63, have been shown to play fundamental roles in the pathogenesis of HNSCC [5-7]. Further, the nature of gene mutation is thought to reflect the exposure to specific risk factors, with G > T transversions at non-CpG sites being characteristic of tobacco exposure [6,8]. However, these and other studies [5,9,10] have been undertaken using tissue specimens and cell lines from Caucasian populations where smoking and excessive alcohol consumption are primary risk factors. By contrast, very little is known about the spectrum of gene mutations in OSCC of Asian origin where the disease is most prevalent [1] and where the practice of betel quid chewing, with or without smoking has been shown to be associated with the increase risk to oral cancer in about 50% of the patients [11-13].

Mutations in genes that play fundamental roles in driving cancer development have defined treatment protocols in a diverse group of tumor types [14,15], but information regarding oral squamous cell carcinoma is limited. In the present study, we used high-throughput mutational profiling to determine the prevalence of mutations at 238 sites across 19 oncogenes in Asian OSCC as well as TP53 in 107 tissues and 16 cell lines. We demonstrate lower levels of TP53 mutations but similar mutational frequencies in HRAS and PIK3CA in Asian OSCC compared to Caucasian OSCC. Most notably, we show that mutations in the 19 oncogenes are exceedingly low compared to other solid cancers including lung cancer where the etiological factors are similar to that of OSCC. The findings suggest that mutations other than those commonly seen in solid cancers may play an important role in driving OSCC and argue strongly for further comprehensive analysis of gene mutations in this tumor type.

Materials and Methods

Ethics Statement

All of the clinical samples were obtained from patients with written informed consent, and this study was approved by the Institutional Review Board of the Faculty of Dentistry, University of Malaya (Medical Ethics Number: DF OS1002/0008/L).

The 16 cell lines that were used in this study were established in our laboratory and have been described previously [16]. These were established from tissues that were collected with written informed consent and were approved by the Institutional Review Board of the Faculty of Dentistry, University of Malaya (Medical Ethics Number: DP OP0306/0018/L).

Clinical samples and cell lines

One hundred and thirty genomic DNA (gDNA) samples from 107 fresh frozen OSCC tissues, 16 oral squamous cell carcinoma (OSCC) cell lines and 7 control cell lines positive for specific mutations were included in this study. gDNA from OSCC tissues that had a minimum of 70% tumor coverage and the data associated with these specimens were obtained from the Malaysian Oral Cancer Database & Tissue Bank System (MOCDBTS) [17]. Information pertaining to the tissue specimens is shown in Table 1. Sixteen OSCC cell lines (Table S1 in File S1) were established from primary explant cultures in our laboratory, as described previously [16]. With the exception of ORL-156, all of the cell lines have been authenticated to tissues and/or blood samples. ORL-156 has a suspicious identity with a 60% match to the original tumor tissue. gDNA from seven cell lines which contained mutations in specific genes were kind gifts from Dr. Ramsi Haddad, Laboratory of Translational Oncogenomics, Karmanos Cancer Institute, Wayne State University, USA (Table S2 in File S1). Five of these lines originated from breast carcinomas [18,19], one was from an ovarian cancer [20] and another was from an ovarian cancer mouse xenograft. All gDNA extraction was performed using the QiAamp DNA mini kit (Qiagen, Germany), according to manufacturer’s recommendation and the quantity and quality of gDNA was determined using the NanoDrop ND1000 Spectrophotometer and gel agarose electrophoresis.

High-throughput somatic mutation detection and analysis

The OncoCarta™ Panel v1.0 assay (Sequenom, San Diego, CA, USA) was used for the detection of somatic mutations because it is a rapid and cost effective method of identifying key cancer driving mutations also known as “actionable mutations” across a large number of samples. Two key advantages of using the Sequenom platform, which detects mutations based on the mass of the sequence, are 1) it has the ability to simultaneously profile multiple mutations in several genes in an large number of samples through multiplexing and 2) it can provide a 3-fold increase in mutation detection limit (as low as 5-10% of the mutant allele) compared to sequencing. In order to analyze these hotspot mutations, multiplex reactions were prepared, spotted on the SpectroChipII using the MassARRAY® Nanodispenser, resolved by MALDI-TOF on the Compact Mass Spectrometer (Sequenom, San Diego, CA, USA) and analyzed using the MassARRAY® Typer Analyzer software 4.0.22 where an OncoMutation™ report to indicate mutant specimens by comparing the ratios of the wild type allele peak to those of suspected mutant allele peak is automatically generated, as described by others [21,22]. The hotspot mutations that were included in this assay are tabulated in Table S3 in File S1.

Polymerase Chain Reaction (PCR) and direct DNA sequencing

All of the mutations that were detected by the OncoCarta™ Panel v1.0 assay (Sequenom, San Diego, CA, USA) were validated by direct sequencing. The PIK3CA, BRAF, EGFR, HRAS, KRAS, NRAS and MET oncogenes were also sequenced in the 16 oral cancer lines to ensure concordance between the OncoCarta™ Panel v1.0 assay and direct sequencing. The chosen genes were selected for their high mutation frequency in HNSCC according to the Catalogue of Somatic Mutations in Cancer (COSMIC) v60 information.
Table 1. Demographics and clinico-pathological characteristics of patients included in the study.

| Variable                   | n=107 % |
|----------------------------|---------|
| Gender                     | Male 43 / 40.2 |
|                           | Female 63 / 58.9 |
| Information unavailable    | 1 / 0.9 |
| Age                        | Mean 58 / -- |
|                           | Range 58 / -- |
| Risk Habits                | Exclusively smokers 12 / 11.2 |
|                           | Exclusively betel quid chewers 35 / 32.7 |
|                           | Exclusively alcohol drinkers 3 / 2.8 |
| Two Habits                 | Chewing * Smoking 4 / 3.7 |
|                           | Chewing * Drinking 7 / 6.5 |
|                           | Smoking * Drinking 12 / 11.2 |
|                           | All 3 Habits 7 / 6.5 |
|                           | None 23 / 21.5 |
| Information unavailable    | 4 / 3.7 |
| Tumor Site                 | Buccal 41 / 38.3 |
|                           | Tongue 34 / 31.8 |
|                           | Gum 17 / 15.9 |
|                           | FOM & palate 6 / 5.6 |
|                           | Information unavailable 9 / 8.4 |
| Tumor Size                 | Tis, T1 & T2 40 / 37.3 |
|                           | T3 & T4 51 / 47.7 |
| Information unavailable    | 16 / 15.0 |
| Lymph Node Metastasis      | Negative 47 / 43.9 |
|                           | Positive 44 / 41.1 |
| Information unavailable    | 16 / 15.0 |
| TNM Stage                  | Early (I & II) 31 / 29.0 |
|                           | Late (III & IV) 60 / 56.0 |
| Information unavailable    | 16 / 15.0 |
| Tumor Differentiation      | Well 42 / 39.3 |
|                           | Moderate/poor 48 / 44.9 |
|                           | Information unavailable 17 / 15.9 |
| Overall survival           | Range (months) 1-91 / -- |
|                           | Median 18 / -- |
|                           | Mean 22.8 / -- |

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Detection of TP53 somatic mutations in OSCC

The mutational status of TP53 was determined in 112 OSCC samples that were used in the OncoCarta™ Panel v1.0 assay. The positive control cell lines with oncogenic mutations (n=7) and 11 OSCC samples with insufficient DNA were excluded. Mutation detection was conducted by direct sequencing of exon 4 to exon 11 where more than 85% of TP53 mutations have been reported [27]. The procedures of PCR, purification, sequencing and analysis have been described previously [16]. The primer sequences for TP53 are tabulated in Table S4 in File S1. The TP53 mutations found in this study were compared to those reported in the IARC version R15 (http://www-p53.iarc.fr/) [28]. Mutations were classified into five groups: DNA binding domain (DBD), L2/L3/LSH hotspot, disruptive and truncating, and based on functional consequences, as described by others [29-31].

Statistical Analysis

All statistical analyses were performed using the SPSS software (SPSS for Windows, version 16.0 (Chicago, IL) to determine statistical associations of mutations with risk habits and pathological parameters. Survival probability differences were compared by the log-rank test using Kaplan-Meier survival analysis. A p-value of <0.05 was considered statistically significant.

Results

Mutations in OSCC

Of the 123 specimens (107 OSCC tissues, 16 OSCC cell lines), 38 (30.9%) had at least one mutation taking into account both oncogenic mutations and TP53 mutations (Table S5 in File S1). Ten oncogenic mutations were detected in eight specimens (7 OSCC tissues and 1 OSCC cell line; 6.5%) and these mutations were found in the PIK3CA and HRAS genes. Two of the OSCC tissues had mutations in both genes (06-0005-10 and 01-002-10). The majority of oncogenic mutations were detected via the OncoCarta™ Panel v1.0 assay whilst others were detected via direct sequencing, as described in detail below. Of the oncogenic mutations that were identified, all but one was base transitions (Table 2). Notably, no mutations were detected in the remaining 17 oncogenes.

Mutations in the PIK3CA gene were detected in 7/123 (5.7%) specimens. Mutations at H1047R, E545K, Q546R, E542K, and M1043I were found in six OSCC tissues and one cell line, and the mutated allele frequency ranged from 17-50% (Table 2). The Q546R mutation, not present in the OncoCarta™ Panel, was detected in sample ORL150T by direct sequencing. HRAS was the only other gene that was mutated and mutations were detected in 3/123 (2.4%) of specimens. Mutations at G12S and G12D were detected in three OSCC tissues, with mutation allele frequencies of 23-82%; no mutations were detected in the cell lines (Table 2). We used seven cell lines from various tissue types as positive controls in the OncoCarta™ Panel v1.0 assay and all of the mutations that were harbored in these cell lines have been documented in Table S2 in File S1. The concordance between the
Table 2. Oncogenic mutations in OSCC.

| Gene | Mutation | Mutation type | Sample | Mutant allele frequency | Site | pT | pN | pM | Stage | Habit |
|------|----------|---------------|--------|------------------------|------|----|----|----|-------|-------|
| HRAS | G12S     | G>C > A:T     | 03-0004-04 | n/a                    | information unavailable | Information unavailable | 4  | 0  | 0  | IV    | BQ chewing |
| HRAS | G12D     | G>C > A:T     | 01-0002-10 | 23%                    | Buccal | 2  | 0  | 0  | II    | BQ chewing & Alcohol Drinking |
| HRAS | G12D     | G>C > A:T     | 06-0005-10 | 82%                    | Buccal | 2  | 0  | 0  | II    | BQ chewing |
| PIK3CA | H1047R   | A>T > G:C     | 01-0016-07 | 17%                    | Buccal | 1  | 0  | 0  | I     | BQ chewing |
| PIK3CA | H1047R   | A>T > G:C     | 04-0005-04 | 45%                    | Buccal | 4  | 0  | 0  | IV    | BQ chewing & Alcohol Drinking |
| E545K | G>C > A:T | 01-0025-07 | 50% | Tongue | 3  | 0  | 1  | IV    | None |
| E545K | G>C > A:T | 01-0002-10 | 30% | Buccal | 4  | 0  | 0  | IV    | BQ chewing |
| E542K | G>C > A:T | 01-0011-10 | 24% | Buccal | 4  | 1  | 0  | IV    | BQ chewing |
| Q546R | A>T > G:C | 150T       | n/a | Tongue | 1  | 0  | X  | I     | Alcohol Drinking |
| M1043I | G>C > T:A | 06-0005-10 | 32% | Buccal | 2  | 0  | 0  | II    | BQ chewing & Alcohol Drinking |

a. Mutations were detected only through direct DNA sequencing
b. Pathological characteristic
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OncoCarta™ Panel v1.0 assay and direct sequencing was 99.9% (data not shown).

Thirty three TP53 mutations were found in 31/112 specimens (27.7%). The cell lines ORL48T and ORL195T had two TP53 mutations respectively (Table 3). The majority of the mutations were base transitions (60.6%) with G:C to A:T being by far the most common alteration (48.5%; Table 3). Most of the mutations occurred within the DDB (81.8%), 63.6% occurred in L2/L3/LSH domain, 24.2% were hotspot mutations and 48.5% and 27.3% were disruptive and truncating mutations, respectively. Notably, the missense mutation M237K and mutations (Figure 1a). However, the presence of any mutation was not an independent factor for poor survival (Table 4).

The mutational frequencies of TP53 in patients with the different risk habits were similar (Table 6). Regardless of the nature of the risk habits, base transitions were the most common mutations (Table S7 in File S1). Truncating mutations were significantly enriched in OSCC patients with no risk habits (23.8%) compared to 4.6% in patients with at least one risk factor (p = 0.019). All types of TP53 mutations were enriched significantly in OSCC cell lines compared to OSCC tissues (Table 7). In addition, patients who harbored DBD and L2/L3/LSH mutations showed a worse survival probability compared to patients who had wild type TP53 (Figure 1b, 1c, 1d) but the Cox-Regression analysis showed that TP53 mutations were not a significant independent factor in modulating survival (Table 4).

Discussion

The comprehensive profiling of cancer mutations in tumor samples has led to the detection of key perturbations that promote tumorogenesis in many types of cancers. Further, with the advent of next generation sequencing, the genomes of many types of cancers can be comprehensively characterized [32]. Such technology, however, is limited by the cost of characterizing large numbers of samples. For example, next generation sequencing data on OSCC are still limited [5-7,33] and comprehensive mutational information on OSCC amongst Asians, where the incidence is most prevalent is still unavailable. High-throughput analysis of key cancer driving mutations using mass-spectrometry remains a cost effective and efficient way of analyzing multiple mutations across a large number of samples, particularly when these are known and could influence clinical management of patients [22].

In this study, we examined the spectrum of oncogenic mutations across ABL1, AKT1, AKT2, BRAF, CDK4, EGFR, ERBB2, FGFR1, FGFR3, FLT3, HRAS, JAK2, KIT, KRAS, MET, NRAS, PDGFRα, PIK3CA and RET in a broad spectrum of tissues and cell lines derived from Asian OSCC. The mutation sites that were included in the OncoCarta™ Panel v1.0 assay are those that are frequently seen in many different types of solid tumors and are clinically actionable. Information concerning 12 of the 19 oncogenes investigated by the OncoCarta™ Panel v1.0 assay is either limited or absent in COSMIC for OSCC. In this study, PIK3CA and HRAS were the only two oncogenes mutated. Notably, only 6.5% of OSCC patients harbored at least one PIK3CA and HRAS mutation, whereas, these oncogenic mutations occur in 30-70% of solid tumours, including colorectal, ovarian, endometrial, lung, melanoma and breast cancer (Table S8 in File S1) [22,34].
| # | Exon | CDS Mutation | Amino Acid Mutation | Mutation Type | Sample | Site | Pathological characteristic | Habit | Characterisation | DBD | L2/L3/LSH | hotspot | Disruptive | Truncating |
|---|------|-------------|---------------------|--------------|--------|------|-----------------------------|--------|------------------|-----|-----------|---------|------------|-----------|
| 4 | 336 | 336_338delCTT | F113del | deletion | 115T | Gingiva | 4 x 0 IV | BQ chewing | Y N N N N |
| 5 | 454 | C124R | G:C > A:T | 06-0051-05 | Floor of Mouth | 1 2 0 IV | Alcohol Drinking & Smoking | Y N N N N |
| 6 | 524 | R175H | G:C > A:T | 01-0005-06 | Gingiva | 4 0 0 IV | BQ chewing | Y Y Y N N |
| 7 | 536 | R175H | G:C > A:T | 01-0005-06 | Gingiva | 4 0 0 IV | BQ chewing | Y Y Y N N |
| 8 | 701 | Y234C | G:C > A:T | 06-0021-09 | Gingiva | 4 0 0 IV | BQ chewing | Y N N N N |
| 9 | 731 | R248Q | G:C > A:T | 06-0032-08 | Gingiva | 4 0 0 IV | BQ chewing | Y Y Y N N |
| 10 | 817 | R273C | G:C > A:T | 04-0012-10 | Buccal | 2 0 0 II | none | Y Y Y N N |
| 11 | 832 | R282W | G:C > A:T | 215T | Tongue | 4 2 x 0 | IV | BQ chewing | Y Y Y N N |
| 12 | 856 | R296H | G:C > A:T | 06-0005-10 | Buccal | 2 0 0 II | BQ chewing | Y Y N Y N |
| 13 | 860 | E294K | G:C > A:T | 06-0005-10 | Buccal | 2 0 0 II | BQ chewing | Y Y N Y N |
| 14 | 871 | E294K | G:C > A:T | 06-0005-10 | Buccal | 4 2 x 0 | IV | BQ chewing & Alcohol Drinking | Y Y Y N N |
| 15 | 1013 | E294K | G:C > A:T | 06-0005-10 | Buccal | 4 2 x 0 | IV | BQ chewing & Alcohol Drinking | Y Y Y N N |
| 16 | 1024 | E294K | G:C > A:T | 06-0005-10 | Buccal | 4 2 x 0 | IV | BQ chewing & Alcohol Drinking | Y Y Y N N |

**Y = Yes; N = No; * Stop codon**

**. Patient has 2 oncogenic mutation: G:C > A:T transition in *HRAS* gene and G:C > T:A transversion in *PIK3CA* gene**

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Further, mutations in 5 of 19 genes identified by the OncoCarta™ Panel v1.0 assay are typically seen in many of these cancers [22,34]. With respect to lung cancer, for example, which shares similar risk factors to OSCC, mutations of PIK3CA, HRAS, NRAS, Kras, BRAF, EGFR, ERBB2, PDGFRα and RET are seen in some 30% of patients [34]. Whole exome sequencing reported by Stransky et al. (2011) and Agrawal et al. (2011) indeed have provided us with comprehensive information on the mutation spectrum in HNSCC but their work has been confined to Caucasian samples. Interestingly, the results of the present study are similar to those reported for OSCC in patients of Caucasian origin with low mutation frequencies in ERBB2 (1/32 patients), FLt3 (1/38 patients) and EGFR (1/38 patients) [5,6]. More recently, a similar comprehensive integrative genetic analysis reported by Pickering et al. (2013) also revealed that aberrations in OSCC are commonly confined to mitogenic signaling pathway which mostly involves genes such as PI3K and RAS [7]. The results suggest that mutations within this spectrum of oncogenes appear not to be a characteristic of OSCC and, most probably, are unrelated to risk factors such as tobacco, alcohol and betel quid chewing that are historically associated with OSCC.

Deregulation of HRAS is known to activate two major signaling pathways, namely, PI3K/AKT and MAPK [35,36]. In this study, only some 3% of samples contained HRAS mutations, findings that were surprising in view of the fact that studies in India have reported higher HRAS mutation frequencies [37-39] whereas those relating to Caucasian patients with OSCC range from 4-8% [5,6,40,41]. Historically, the high prevalence of HRAS mutations in the Indian subcontinent has been attributed to betel quid chewing [37] but...
the patients used in the present study were also betel quid chewers suggesting that the low mutational frequency of *HRAS* in this study was due to factors other than risk factor exposure. Other up- or down-stream proteins within the RAS pathway such as activation or over-expression of EGFR [42], and/or loss of PTEN [43] can result in the activation of the RAS signaling pathway, and may be a reason for the lack of RAS mutations in the present study.

*PIK3CA* mutations occur frequently in many cancers including colorectal, breast, brain, gastric, ovarian and lung and 75% of these occur in exons 9 and 20 [34,44]. Hotspot mutations at these sites (E545K, E542K and H1047R) increase kinase activity and induce transformation, tumour cell proliferation, invasion and metastasis [45-47] resulting in over-activated PI3K pathway as shown in *in vitro* and *in vivo* models [48,49]. Oncogenic activation of this pathway is one of the most commonly de-regulated pathway implicated in HNSCC [50]. In the present study, hotspot *PIK3CA* mutations were found in 5.7% of OSCC specimens, findings that confirm previous observations in both Asian [51,52] and Caucasian populations [5,6,9].

Importantly, the fact that oncogenic mutations occur in a small subset of OSCC patients suggests that they may benefit from targeted therapy as opposed to the conventional treatment modalities. While only a small percentage of patients may have such mutations, this translates to significant patient numbers when the global incidence of the disease is considered. *PIK3CA* mutations, for example, have been demonstrated to modulate response to mTOR- and EGFR-targeted therapy [53-55]. New generation of drugs targeting PI3K are currently being tested clinically (NCT01690871, NCT01219699, and NCT01501604) on patients with and without *PIK3CA* mutations, and results from these trials should provide further information on the role of these mutations in modulating drug response. Although the inhibition of RAS genes was relatively unsuccessful in previous studies, the activation of *HRAS* in a subset of HNSCC suggests that this

Table 5. Oncogenic mutations in association with risk habits and pathological characteristics.

| Risk Habits/Pathological Characteristic | Patients (n) | Wildtype | Oncogenic mutations | p-value | Odds Ratio | 95% confidence |
|----------------------------------------|-------------|----------|---------------------|---------|------------|---------------|
| Overall Habit                          |             |          |                     |         |            |               |
| Any habit                              | 94          | 86 (92.5%)| 7 (7.5%)            | 0.682   | 2.01       | (0.24-17.13)  |
| No habit                               | 26          | 26 (96.3%)| 1 (3.7%)            |         |            |               |
| Smoking                                |             |          |                     |         |            |               |
| Ever smokers                            | 43          | 42 (100%) | 0 (0%)              | 0.049   | -          | -             |
| non-smokers                            | 77          | 70 (89.7%)| 8 (10.3%)           |         |            |               |
| Betel Quid chewing                     |             |          |                     |         |            |               |
| Ever chewers                            | 60          | 54 (90%)  | 6 (10%)             | 0.272   | 3.22       | (0.62-16.66)  |
| non-chewers                            | 60          | 58 (96.7%)| 2 (3.3%)            |         |            |               |
| Alcohol drinking                       |             |          |                     |         |            |               |
| Ever drinkers                           | 35          | 32 (91.4%)| 3 (8.6%)            | 0.690   | 1.50       | (0.34-6.65)   |
| non-drinkers                           | 85          | 80 (94.1%)| 5 (5.9%)            |         |            |               |
| Lymph Node Metastasis                   |             |          |                     |         |            |               |
| Negative                               | 54          | 46 (88.5%)| 6 (11.5%)           | 0.056   |            |               |
| Positive                               | 54          | 54 (98.2%)| 1 (1.9%)            |         |            |               |
| TNM stage                              |             |          |                     |         |            |               |
| Early (G, I, II)                       | 36          | 32 (91.4%)| 3 (8.6%)            | 0.679   |            |               |
| Late (III & IV)                        | 72          | 69 (94.5%)| 4 (5.5%)            |         |            |               |

| Multivariate analysis | p value | risk ratio (95% CI) |
|-----------------------|---------|---------------------|
| (A) Oncogenic + TP53 mutation (Wild type vs mutation) | 0.144 | 1.551 (0.861 - 2.794) |
| Age group (≤ 58 vs > 58) | 0.030 | 1.873 (1.062 - 3.301) |
| Lymph Nodes Metastasis (Positive vs Negative) | <0.001 | 4.849 (2.102 - 11.183) |
| Staging (Early vs Late) | 0.719 | 0.85 (0.350 - 2.060) |
| (B) Overall TP53 mutation (Wild type vs mutation) | 0.319 | 1.416 (0.715 - 2.803) |
| Age group (≤ 58 vs > 58) | 0.037 | 1.906 (1.039 - 3.497) |
| Lymph Nodes Metastasis (Positive vs Negative) | <0.001 | 5.748 (2.238 - 14.76) |
| Staging (Early vs Late) | 0.444 | 0.687 (0.262 - 1.798) |
| (C) L2L3/LSH mutation (Wild type vs mutation) | 0.128 | 1.801 (0.844-3.841) |
| Age group (≤ 58 vs > 58) | 0.026 | 2.073 (1.093 - 3.930) |
| Lymph Nodes Metastasis (Positive vs Negative) | 0.001 | 5.202 (2.053 - 13.183) |
| Staging (Early vs Late) | 0.476 | 0.711 (0.279 - 1.815) |
| (D) DNA Binding Domain mutation (Wild type vs mutation) | 0.294 | 1.442 (0.728 - 2.859) |
| Age group (≤ 58 vs > 58) | 0.041 | 1.883 (1.026 - 3.454) |
| Lymph Nodes Metastasis (Positive vs Negative) | <0.001 | 5.628 (2.195 - 14.435) |
| Staging (Early vs Late) | 0.429 | 0.68 (0.261 - 1.769) |

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the patients used in the present study were also betel quid chewers suggesting that the low mutational frequency of *HRAS* in this study was due to factors other than risk factor exposure. Other up- or down-stream proteins within the RAS pathway such as activation or over-expression of EGFR [42], and/or loss of PTEN [43] can result in the activation of the RAS signaling pathway, and may be a reason for the lack of RAS mutations in the present study.

*PIK3CA* mutations occur frequently in many cancers including colorectal, breast, brain, gastric, ovarian and lung and 75% of these occur in exons 9 and 20 [34,44]. Hotspot mutations at these sites (E545K, E542K and H1047R) increase kinase activity and induce transformation, tumour cell proliferation, invasion and metastasis [45-47] resulting in over-activated PI3K pathway as shown in *in vitro* and *in vivo* models [48,49]. Oncogenic activation of this pathway is one of the most commonly de-regulated pathway implicated in HNSCC [50]. In the present study, hotspot *PIK3CA* mutations were found in 5.7% of OSCC specimens, findings that confirm previous observations in both Asian [51,52] and Caucasian populations [5,6,9].

Importantly, the fact that oncogenic mutations occur in a small subset of OSCC patients suggests that they may benefit from targeted therapy as opposed to the conventional treatment modalities. While only a small percentage of patients may have such mutations, this translates to significant patient numbers when the global incidence of the disease is considered. *PIK3CA* mutations, for example, have been demonstrated to modulate response to mTOR- and EGFR-targeted therapy [53-55]. New generation of drugs targeting PI3K are currently being tested clinically (NCT01690871, NCT01219699, and NCT01501604) on patients with and without *PIK3CA* mutations, and results from these trials should provide further information on the role of these mutations in modulating drug response. Although the inhibition of RAS genes was relatively unsuccessful in previous studies, the activation of *HRAS* in a subset of HNSCC suggests that this
| Risk Habits/Pathological Characteristic | Patients (n) | Wild Type | overall TP53 mutations | p-value | odds ratio | 95% confidence intervals | Patients (n) | Wild Type | DBD mutations | p-value | odds ratio | 95% confidence intervals |
|---------------------------------------|-------------|-----------|------------------------|---------|-----------|--------------------------|-------------|-----------|---------------|---------|-----------|--------------------------|
| Overall Habit                         | Any habit   | 86        | 62 (72.1%)             | 24 (27.9%) | 0.605 | 0.774 | 0.293 | 2.044 | 84 | 62 | (73.8%) | 0.823 | 1.135 | 0.372 | 3.465 |
|                                       | No habit    | 24        | 16 (66.7%)             | 8 (33.3%) | 21 | | | | 16 | (76.2%) | 5 (23.8%) |
| Smoking                               | Ever smokers | 40        | 29 (72.5%)             | 11 (27.5%) | 0.781 | 0.885 | 0.374 | 2.096 | 39 | 29 | (74.4%) | 0.989 | 0.994 | 0.402 | 2.460 |
|                                       | non-smokers | 70        | 49 (70.0%)             | 21 (30.0%) | 66 | | | | 49 | (74.2%) | 17 (25.8%) |
| Betel Quid Chewing                    | Ever chewers | 55        | 39 (70.9%)             | 16 (29.1%) | 1.000 | 1.000 | 0.439 | 2.277 | 54 | 39 | (72.2%) | 0.619 | 1.250 | 0.519 | 3.012 |
|                                       | non-chewers | 55        | 39 (70.9%)             | 16 (29.1%) | 51 | | | | 39 | (76.5%) | 12 (23.5%) |
| Alcohol drinking                      | Ever drinkers | 34        | 22 (64.7%)             | 12 (35.3%) | 0.338 | 1.527 | 0.640 | 3.642 | 32 | 22 | (68.8%) | 0.390 | 1.497 | 0.594 | 3.771 |
|                                       | non-drinkers | 76        | 56 (73.7%)             | 20 (26.3%) | 73 | | | | 56 | (76.7%) | 17 (23.3%) |
| Lymph Node Metastasis                 | Negative    | 46        | 35 (76.1%)             | 11 (23.9%) | 0.139 | | | | 35 | (76.1%) | 0.427 |
|                                       | Positive    | 53        | 33 (62.3%)             | 20 (37.7%) | 48 | | | | 33 | (68.8%) | 15 (31.2%) |
| TNM stage                             | Early (I, II) | 31        | 20 (64.5%)             | 11 (35.5%) | 0.617 | | | | 20 | (64.5%) | 11 (35.5%) | 0.288 |
|                                       | Late (III & IV) | 69        | 48 (69.6%)             | 21 (30.4%) | 64 | | | | 48 | (75.0%) | 16 (25.0%) |
| Risk Habits/Pathological Characteristic | Patients (n) | Wild Type | Hotspot mutations | p-value | odds ratio | 95% confidence intervals | Patients (n) | Wild Type | DBD mutations | p-value | odds ratio | 95% confidence intervals |
| Overall Habit                         | Any habit   | 68        | 62 (91.2%)             | 6 (8.8%) | 0.671 | 0.774 | 0.143 | 4.204 | 72 | 62 | (86.1%) | 0.316 | 0.516 | 0.155 | 1.724 |
|                                       | No habit    | 18        | 16 (88.9%)             | 2 (11.1%) | 21 | | | | 16 | (76.2%) | 5 (23.8%) |
| Smoking                               | Ever smokers | 33        | 29 (87.9%)             | 4 (12.1%) | 0.476 | 1.69 | 0.392 | 7.276 | 32 | 29 | (90.6%) | 3.94 | 0.422 | 0.110 | 1.623 |
|                                       | non-smokers | 53        | 49 (92.5%)             | 4 (7.5%) | 61 | | | | 49 | (80.3%) | 12 (19.7%) |
| Betel Quid Chewing                    | Ever chewers | 43        | 39 (90.7%)             | 4 (9.3%) | 1.000 | 1.000 | 0.233 | 4.286 | 47 | 39 | (83.0%) | 0.813 | 1.143 | 0.378 | 3.458 |
|                                       | non-chewers | 43        | 39 (90.7%)             | 4 (9.3%) | 46 | | | | 39 | (84.8%) | 7 (15.2%) |
| Alcohol drinking                      | Ever drinkers | 24        | 22 (91.7%)             | 2 (8.3%) | 1.000 | 0.848 | 0.159 | 4.528 | 26 | 22 | (84.6%) | 1.454 | 0.902 | 0.266 | 3.218 |
|                                       | non-drinkers | 62        | 56 (90.3%)             | 6 (9.7%) | 67 | | | | 56 | (83.6%) | 11 (16.4%) |
| Lymph Node Metastasis                 | Negative    | 37        | 35 (94.6%)             | 2 (5.4%) | 0.263 | | | | 35 | (87.5%) | 5 (12.5%) | 0.203 |
|                                       | Positive    | 39        | 33 (84.6%)             | 6 (15.4%) | 43 | | | | 33 | (76.7%) | 10 (23.3%) |
| TNM stage                             | Early (I, II) | 21        | 20 (95.2%)             | 1 (4.8%) | 0.432 | | | | 20 | (76.9%) | 6 (23.1%) | 0.540 |

Table 6. TP53 mutations in association with risk habits and pathological characteristics.
could be an opportunity for the revival of drugs such as farnesyltransferase inhibitors. One sample in this study had both PIK3CA and HRAS activating mutations implying the significant synergistic signals of PI3K and RAS pathway critical for oral carcinogenesis may converge to activate a single downstream target that would be critical for tumorigenesis [56]. Interestingly, a recent in vitro study has shown that cells containing coexistence PIK3CA and RAS mutations were resistant to PI3K inhibitors [57] suggesting that coexistence of these mutations may be a predictive biomarker for resistance to PI3K inhibitors.

In the present study, TP53 mutations occurred in 27.7% of OSCC specimens, which is very similar to that reported in the Indian subcontinent [58,59]. It is very apparent that the TP53 mutational frequency of OSCC patients from Asia (17-21%) [58,59] differs dramatically from those reported from the West (53-80%) [5,6,29]. The lack of TP53 mutations in these samples were not due to involvement of HPV as only 2.7% of the samples were positive for HPV. Further, these specimens

Table 6 (continued).

| Risk Habits/Pathological Characteristic | Patients (n) | Wild Type | overall TP53 mutations | p-value | odds ratio | 95% confidence intervals | Patients (n) | Wild Type | DBD mutations | p-value | odds ratio | 95% confidence intervals |
|----------------------------------------|-------------|-----------|------------------------|---------|------------|--------------------------|-------------|-----------|---------------|---------|------------|--------------------------|
| Late (III & IV)                        | 55          | 48 (87.3%)| 7 (12.7%)              |         |            |                          | 57          | 48 (84.2%)| 9 (15.8%)     |         |            |                          |
| Smoking                                |             |           |                        |         |            |                          |             |           |               |         |            |                          |
| Ever smokers                           | 36          | 29 (80.6%)| 7 (19.4%)              | 0.745   | 0.845      | 0.306 - 2.336            | 30          | 29 (96.7%)| 1 (3.3%)     | 0.252   | 0.241      | 0.028 - 2.062          |
| non-smokers                            | 63          | 49 (77.8%)| 14 (22.2%)             |         |            |                          | 56          | 49 (87.5%)| 7 (12.5%)    |         |            |                          |
| Betel Quid Chewing                     |             |           |                        |         |            |                          |             |           |               |         |            |                          |
| Ever chewers                           | 52          | 39 (75.0%)| 13 (25.0%)             | 0.332   | 1.625      | 0.806 - 4.357            | 41          | 39 (95.1%)| 2 (4.9%)     | 0.270   | 0.333      | 0.063 - 1.754          |
| non-chewers                            | 47          | 39 (83.0%)| 8 (17.0%)              |         |            |                          | 45          | 39 (86.7%)| 6 (13.3%)    |         |            |                          |
| Alcohol drinking                       |             |           |                        |         |            |                          |             |           |               |         |            |                          |
| Ever drinkers                          | 30          | 22 (73.3%)| 8 (26.7%)              | 0.381   | 1.566      | 0.571 - 4.298            | 24          | 22 (91.7%)| 2 (8.3%)     | 1.000   | 0.848      | 0.159 - 4.528          |
| non-drinkers                           | 69          | 56 (81.2%)| 13 (18.8%)             |         |            |                          | 62          | 56 (90.3%)| 6 (9.7%)     |         |            |                          |
| Lymph Node Metastasis                  |             |           |                        |         |            |                          |             |           |               |         |            |                          |
| Negative                               | 44          | 35 (79.5%)| 9 (20.5%)              | 0.490   |            |                          | 37          | 35 (94.6%)| 2 (5.4%)     | 0.263   |            |                          |
| Positive                               | 45          | 33 (73.3%)| 12 (26.7%)             |         |            |                          | 39          | 33 (84.6%)| 6 (15.4%)    |         |            |                          |
| TNM stage                              |             |           |                        |         |            |                          |             |           |               |         |            |                          |
| Early (I, II)                          | 29          | 20 (69.0%)| 9 (31.0%)              | 0.251   |            |                          | 22          | 20 (90.9%)| 2 (9.1%)     | 1.000   |            |                          |
| Late (III & IV)                        | 60          | 48 (80.0%)| 12 (20.0%)             |         |            |                          | 54          | 48 (88.9%)| 6 (11.1%)    |         |            |                          |

Data included OSCC tissues and cell lines and analyzed by Pearson's Chi-Square Test and Fisher Exact Test doi: 10.1371/journal.pone.0080229.t006

Table 7. Comparison of TP53 mutations between OSCC tissues and cell lines.

| TP53 mutation type | OSCC tissue samples; n=96 | OSCC cell line samples; n=16 | p-value* |
|--------------------|--------------------------|------------------------------|----------|
| overall            | 21 (21.88%)              | 12 (75.0%)                   | <0.001   |
| DBD                | 20 (20.63%)              | 7 (43.75%)                   | 0.017    |
| L2/L3/LSH          | 15 (15.63%)              | 6 (37.5%)                    | 0.016    |
| hotspot            | 5 (5.21%)                | 3 (18.75%)                   | 0.032    |
| disruptive          | 8 (8.33%)                | 8 (50.0%)                    | <0.001   |
| truncating          | 3 (3.13%)                | 6 (37.5%)                    | <0.001   |

* Data were analyzed using Fisher Exact Test doi: 10.1371/journal.pone.0080229.t007
had TP53 mutations reiterating the fact that HPV and TP53 mutations are not mutually exclusive events in OSCC [60]. Although both TP53 mutation and lymph node metastasis are associated with overall survival (Table 4), there was no significant association between TP53 mutation and lymph node metastasis (Table 6). The association between TP53 mutations and survival in the univariate analysis may reflect other functions of mutant TP53 that is independent of metastasis. For example, mutant TP53 have been shown to interfere with mechanisms that maintain genome integrity including DNA damage response pathways resulting in genomic instability which is a major driver of cancer development and a hallmark of cancer [61,62]. After considering other prognostic factors in the multivariate analysis, lymph node metastasis was the only significant factor associated with poor survival indicating that lymph node metastasis is a stronger driving factor in comparison to TP53 mutations, in determining the probability of poor overall survival. Interestingly, TP53 mutations were more prevalent in cell lines compared to OSCC tissues suggesting that they may confer an advantage during the establishment and propagation of the keratinocyte cultures. The results are consistent with previous observations where TP53 mutations facilitate the establishment of human myeloid cell lines [63] and enhance tumor implantation in vivo [64]. Interestingly, the diversity of TP53 point mutations makes this gene informative for the identification of tumor- and exposure-specific mutation patterns [65]. In the present study, 60.6% of TP53 mutations were base transitions with G:C to A:T being the most common alteration (48.5%; Table S7 in File S1). Similarly, G:C to A:T transitions have been reported as the most predominant TP53 mutation in OSCC in Taiwan where risk habits include the use of betel quid and tobacco [66]. However, truncating mutations in the present study were found more frequently in OSCC patients with absence of risk habits suggesting that inactivation of TP53 may be important in the pathogenesis of OSCC. Notably, one OSCC patient in this study has three concurrent mutations in PIK3CA, HRAS and TP53. The prognostic significance of this remains unclear as this was only observed in one particular patient. In summary, we show low mutation frequencies in Asian OSCC compared to a broad spectrum of solid tumours. We demonstrate that HRAS and PIK3CA mutations in Asian OSCC are uncommon but comparable to that seen in the West. TP53 mutations, however, are significantly less common in Asian compared to Caucasian OSCC. The findings may reflect tumour heterogeneity and the diversity of risk factors between the West and India and South East Asia, but this requires verification. In the present study, the presence of actionable mutations within a few key genes may ultimately be important in clinical management. However, the data also demonstrate the urgent need for a comprehensive genetic analysis of Asian OSCC where the disease is most prevalent and where risk factors differ from those seen in the West.

Supporting Information

File S1. File includes Tables S1-S8. Table S1: Demographics and clinico-pathological characteristics of patients from which the cell lines used in this study were derived. Table S2: Positive control samples for the OncoCarta™ Panel v1.0 Assay. Table S3: Mutations in the OncoCarta™ Panel v1.0 Assay. Table S4: Primer sequences that were used for PCR and sequencing. Table S5: Mutation data across 123 samples on 19 oncogenes and TP53. Table S6: The presence of any mutations in relation with risk habits and pathological characterization. Table S7: Frequency of the different base changes in TP53 in patients with different risk habits. Table S8: Oncogenic mutations across common solid tumors.

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Author Contributions

Conceived and designed the experiments: SCC SNSZ. Performed the experiments: SNSZ SYH PSY YHK. Analyzed the data: SNSZ SYH PSY SCC SSP. Contributed reagents/materials/analysis tools: WMNWAG RBZ ZAAR WMWM. Wrote the manuscript: SNSZ PSY SCC SSP.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E et al. (2011) Global cancer statistics. CA Cancer J Clin 61: 69-90. doi:10.3322/caac.20107. PubMed: 21296855.
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C et al. (2010) GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 Lyon, France. International Agency for Research on Cancer.
3. Vogelstein B, Kinzler KW (2004) Cancer genes and the pathways they control. Nat Med 10: 789-799. doi:10.1038/nrn1087. PubMed: 15286780.
4. Leemans CR, Braakhuis BJ, Brakenhoff RH (2011) The molecular biology of head and neck cancer. Nat Rev Cancer 11: 9-22. doi:10.1038/nrc2982. PubMed: 21160525.
5. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K et al. (2011) Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science 333: 1154-1157. doi:10.1126/science.1206923. PubMed: 21798897.
6. Stranksy N, Egloff AM, Tward AD, Kostic AD, Cibulskis K et al. (2011) The mutational landscape of head and neck squamous cell carcinoma. Science 333: 1157-1160. doi:10.1126/science.1208130. PubMed: 21798893.
7. Pickering CR, Zhang J, Yoo SY, Bengtsson L, Moorthy S et al. (2013) Integrative genomic characterization of oral squamous cell carcinoma identifies frequent somatic drivers. Cancer Discov, 3: 770–81. doi: 10.1158/2159-8290.CD-12-0537 PubMed: 23619168.
8. Le Calvez F, Mukeria A, Hunt JD, Kelm O, Huang RJ et al. (2005) TP53 and KRAS mutation load and types in lung cancers in relation to
Hum Genet Chapter 10: Unit 10.11: Unit 10. PubMed: 18428421

S0022-2836(05)80360-2. PubMed: 2231712.

S1368-8375(99)00058-5. PubMed: 10889924.

Mutational Profiling in Oral Cancer
48. Bader AG, Kang S, Vogt PK (2006) Cancer-specific mutations in PIK3CA are oncogenic in vivo. Proc Natl Acad Sci U S A 103: 1474-1479. doi: 10.1073/pnas.0510857103. PubMed: 16432179.

49. Zhao JJ, Liu Z, Wang L, Shin E, Loda MF et al. (2005) The oncogenic properties of mutant p110alpha and p110beta phosphatidylinositol 3-kinases in human mammary epithelial cells. Proc Natl Acad Sci U S A 102: 18443-18448. doi: 10.1073/pnas.0508988102. PubMed: 16339315.

50. Molinolo AA, Amorphimotham P, Squarize CH, Castilho RM, Patel V et al. (2009) Dysregulated molecular networks in head and neck carcinogenesis. Oral Oncol 45: 324-334. doi: 10.1016/j.oraloncology.2008.07.011. PubMed: 18805044.

51. Kozaki K, Imoto I, Pimkhaokham A, Hasegawa S, Tsuda H et al. (2006) PIK3CA mutation is an oncogenic aberration at advanced stages of oral squamous cell carcinoma. Cancer Sci 97: 1351-1358. doi: 10.1111/j.1349-7006.2006.00343.x. PubMed: 17052259.

52. Murugan AK, Hong NT, Fukui Y, Munirajan AK, Tsuchida N (2008) Oncogenic mutations of the PIK3CA gene in head and neck squamous cell carcinomas. Int J Oncol 32: 101-111. PubMed: 18097548.

53. Di Nicolantonio F, Arena S, Tabernero J, Grosso S, Molinari F et al. (2010) Deregulation of the PI3K and KRAS signaling pathways in human cancer cells determines their response to everolimus. J Clin Invest 120: 2858-2868. doi: 10.1172/JCI37539. PubMed: 20684172.

54. Sartore-Bianchi A, Martini M, Molinari F, Veronese S, Nichelatti M et al. (2009) PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. Cancer Res 69: 1851-1857. doi: 10.1158/0008-5472.CAN-08-2468. PubMed: 1923544.

55. Janku F, Wheler JJ, Naing A, Falchook GS, Hong DS et al. (2013) PIK3CA mutation H1047R is associated with response to PI3K/AKT/mTOR signaling pathway inhibitors in early-phase clinical trials. Cancer Res 73: 276-284. doi: 10.1158/0008-5472.CAN-12-1726. PubMed: 23066039.

56. Kennedy AL, Morton JP, Manoharan I, Nelson DM, Jamieson NB et al. (2011) Activation of the PIK3CA/AKT pathway suppresses senescence induced by an activated RAS oncogene to promote tumorigenesis. Mol Cell 42: 36-49. doi: 10.1016/j.molcel.2011.02.020. PubMed: 21474066.

57. Ihle NT, Lemos R Jr., Wipf P, Yacoub A, Mitchell C et al. (2009) Mutations in the phosphatidylinositol-3-kinase pathway predict for antitumor activity of the inhibitor PX-866 whereas oncogenic Ras is a dominant predictor for resistance. Cancer Res 69: 143-150. doi: 10.1158/0008-5472.CAN-07-6656. PubMed: 19117997.

58. Munirajan AK, Tsutsumi-Ishi Y, Mohanprasad BK, Hirano Y, Munakata N et al. (1996) p53 gene mutations in oral carcinomas from India. Int J Cancer 66: 299-300. doi: 10.1002/(SICI)1097-0215(19960503)66:3. PubMed: 8821246.

59. Rahman R, Agarwal S, Nath N, Mathur M, Wasfyk B et al. (2001) Correlation between p53 gene mutations and circulating antibodies in betel- and tobacco-consuming North Indian population. Oral Oncol 37: 243-250. doi: 10.1016/S1368-8375(00)00092-0. PubMed: 11287278.

60. Lechner M, Frampton GM, Fenton T, Feber A, Palmer G et al. (2013) Targeted next-generation sequencing of head and neck squamous cell carcinoma identifies novel genetic alterations in HPV+ and HPV-tumors. Genome Med 5: 49. doi: 11.1086/gm453. PubMed: 23718628.

61. Gualberto A, Aldape K, Czekajewicz K, Tlsty TD (1998) An oncogenic form of p53 confers a dominant, gain-of-function phenotype that disrupts spindle checkpoint control. Proc Natl Acad Sci U S A 95: 5169-5171. doi: 10.1073/pnas.95.9.5169. PubMed: 9560247.

62. Song H, Hollstein M, Xu Y (2007) p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. Nat Cell Biol 9: 573-580. doi: 10.1038/nvc/b1517. PubMed: 17417627.

63. Sugimoto K, Toyoshima H, Sakai R, Miyagawa K, Hagiwara K et al. (1992) Frequent mutations in the p53 gene in human myeloid leukemia cell lines. Blood 79: 2378-2383. PubMed: 1571549.

64. Sano D, Xie TX, Ow TJ, Zhao M, Pickering CR et al. (2011) Disruptive TP53 mutation is associated with aggressive disease characteristics in an orthotopic murine model of oral tongue cancer. Clin Cancer Res 17: 6658-6670. doi: 10.1158/1078-0432.CCR-11-0046. PubMed: 21903770.

65. Hsiaieh LL, Wang PF, Chen IH, Liao CT, Wang HM et al. (2001) Characteristics of mutations in the p53 gene in oral squamous cell carcinoma associated with betel quid chewing and cigarette smoking in Taiwanese. Carcinogenesis 22: 1497-1503. doi: 10.1093/carcin/22.9.1497. PubMed: 11532872.