Genomic Profiling of Advanced-Stage Oral Cancers Reveals Chromosome 11q Alterations as Markers of Poor Clinical Outcome

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Abstract

Identifying oral cancer lesions associated with high risk of relapse and predicting clinical outcome remain challenging questions in clinical practice. Genomic alterations may add prognostic information and indicate biological aggressiveness thereby emphasizing the need for genome-wide profiling of oral cancers. High-resolution array comparative genomic hybridization was performed to delineate the genomic alterations in clinically annotated primary gingivo-buccal complex and tongue cancers (n = 60). The specific genomic alterations so identified were evaluated for their potential clinical relevance. Copy-number changes were observed on chromosomal arms with most frequent gains on 3q (60%), 5p (50%), 7p (50%), 8q (73%), 11q13 (47%), 14q11.2 (47%), and 19p13.3 (58%) and losses on 3p14.2 (55%) and 8p (83%). Univariate statistical analysis with correction for multiple testing revealed chromosomal gain of region 11q22.1–q22.2 and losses of 17p13.3 and 11q23–q25 to be associated with loco-regional recurrence (P = 0.004, P = 0.003, and P = 0.0003) and shorter survival (P = 0.009, P = 0.003, and P = 0.0001) respectively. The gain of 11q22 and loss of 11q23-q25 were validated by interphase fluorescent in situ hybridization (I-FISH). This study identifies a tractable number of genomic alterations with few underlying genes that may potentially be utilized as biological markers for prognosis and treatment decisions in oral cancers.

Introduction

Oral squamous cell carcinoma (OSCC) is a major cause of morbidity and mortality worldwide, accounting for more than 275,000 new cases and over 120,000 deaths every year [1]. Although there have been improvements in the therapeutic modalities, OSCC-associated morbidity and mortality remain high and have not changed in over three decades [2]. This lack of improvement in survival indicates that tumor size, lymph node involvement and stage, which are considered as markers of disease aggressiveness, do not sufficiently account for the observed variability in clinical outcomes [3]. Therefore, a comprehensive understanding of the pathological mechanisms of OSCC is needed to complement the existing paradigms in assessing disease aggressiveness and prognosis.

Chromosomal abnormalities are a characteristic attribute of cancer cells and they have been used to define specific disease entities. The advent of genome-wide screening methods such as comparative genomic hybridization (CGH), and, more recently, array CGH (aCGH), have opened up new possibilities to catalogue chromosomal aberrations at high resolution [4,5]. Many chromosomal aberrations that may harbor oncogenes or tumor suppressor genes have emerged as predictive and prognostic markers for tumors [3,6]. OSCC is reported to arise through the accumulation of numerous specific chromosomal alterations [2]. Gains mapped on chromosomal arms 3q, 6q, 8q, 9p, 9q, 11p, 11q, 14q, 17q, and 20q and losses mapped on 3p, 4q, 9p, and 18q have suggested putative oncogenes and tumor suppressor genes associated with oral cancer [7–15]. Molecular profiles of oral cancers vary throughout the world and are influenced by both aetiological factors and ethnicity, yet no conclusive studies have been reported to date [16,17]. Most genome-wide studies on OSCC have been carried out on various intra-oral sites that are associated with different aetiological agents. Apart from tobacco and alcohol, human papilloma virus (HPV) infection is a known risk factor for OSCC. HPV-infected oropharyngeal tumors comprise a distinct
molecular, clinical, and pathological disease entity with distinct genetic alterations and better prognosis [18–20].

Previous studies have revealed certain over- and under-expressed genes in oral cancer. Based on the existing literature, we compiled a list of genes associated with oral cancer, which may be useful in identifying and functionally validating driver genes in the underlying regions of alteration. To date, only one study has examined the clinicopathological association of genomic alterations in a small set of OSCC (n = 8) [9]. Therefore, the present study aims at delineating genome-wide copy number alterations (CNAs) in oral cancer and to understand whether these genetic alterations are associated with clinical characteristics and prognosis. The study focuses on advanced-stage cancers of the gingivobuccal complex and tongue, which are associated with tobacco use and were found to be unrelated to HPV infection. We demonstrate the potential of high-resolution genome-wide aCGH for calling chromosomal alterations and identifying genomic lesions associated with high risk of relapse and decreased survival time.

Materials and Methods

Tissue sample collection and tumor micro-dissection

The study was approved by the human ethics committee of the Tata Memorial Hospital. Neo-primary tumor samples were obtained from 60 patients undergoing surgery for oral cavity cancers at the Head and Neck Unit and were collected from the tumor tissue repository at Tata Memorial Centre, Mumbai. Patients received neither radiation nor chemotherapy before the surgery. The tumor content in the tissues was assessed on a Hematoxylin and Eosin (H&E)-stained section independently by two pathologists. Tissues with more than 70% tumor content were processed for aCGH. Informed written consent was obtained from all participants of the study.

DNA Isolation from tissues

DNA was extracted following a standard phenol/chloroform protocol. DNA quantification was performed on NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware) and the quality was assessed by electrophoresis on 0.8% agarose gel. A pool of ethnicity and gender-matched normal DNA was isolated from the peripheral blood lymphocytes of healthy donors (n = 10) which was used as reference for aCGH.

HPV Typing

HPV presence was determined by polymerase chain reaction (PCR) using GP5+/6+ primers [21] following confirmation of amplifiable DNA by Beta-Globin PCR [22]. SiHa DNA for HPV16, HeLa DNA for HPV18 (positive controls), and C33A DNA, SCC074 (negative controls) were included while performing all PCR reactions.

Array CGH Hybridization

Whole-genome copy number profiling was performed on 105K CGH oligonucleotide arrays (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. Briefly, 4.5 μg of tumor and pooled gender-matched reference DNA were labeled with fluorochromes Cy3 and Cy5, respectively. Labeled samples were purified using the genomic DNA purification module (Agilent Technologies), combined, mixed with human Cot-1 DNA, and denatured at 95°C (Oligo aCGH hybridization kit, Agilent Technologies). The mixture was applied to microarrays and hybridization was performed at 65°C for 40 hours. After hybridization, the microarrays were washed with Oligo aCGH wash buffer followed by drying of slides. After drying, the arrays were scanned using an Agilent Scanner (Agilent Technologies), and log2-intensities were extracted from raw microarray image files using the Agilent feature extraction software version 9.0 (Agilent Technologies). The raw aCGH data have been submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE23831.

Genome mapping and Human structural variation

Genomic coordinates were standardized to the NCBI build 36 (hg18) assembly of the human genome. Loci of structural and copy number variants were obtained from the Database of Genomic Variants (DGV) version 9 at The Centre for Applied Genomics (TCAG, http://projects.tcag.ca/variation/) [23].

Data Analysis

Raw aCGH intensity values were normalized using the R package snapCGH [24] and segmented with the circular binary segmentation (CBS) algorithm [25]. Recurrent copy number alterations (CNAs) were called using the RAE method [26]. This algorithm identifies significantly recurring CNAs using a background model of genomic variability and computes an empirical false discovery rate (q-value) for each candidate CNA. Within CNAs, the RAE algorithm also identifies subintervals called “focal regions” that were more common. Thresholds for losses/deletions and gains/amplifications were set adaptively from the distribution of segment heights obtained by the CBS algorithm [25]; the thresholds for deletions and amplifications were more stringent than for losses and gains. RAE distinguishes between a “gain” of at least a single copy and an “amplification” by two or more copies. Similarly, RAE defines a “loss” of a single copy and a homozygous “deletion” of both copies [26].

CNAs were considered significant, if their q-value was smaller than 0.1. Recurrent CNAs were further distinguished from known copy number variants (CNVs) present in DGV. For survival analysis, Cox proportional hazards models were calculated with corresponding p-values from the Wald test. Relapse and death from disease were considered as events for recurrence-free and disease-specific survival, respectively. Associations of CNAs with clinical parameters were tested with Fisher’s exact test. All statistical computations were performed in R (www.r-project.org).

Validation of array CGH results using Fluorescence In-situ Hybridization (FISH)

Chromosome 11q alterations associated with recurrence-free and disease-specific survival revealed by aCGH were confirmed by interphase FISH (I-FISH) using a dual color procedure. The probes were prepared by differentially labeling the region and centromere-specific bacterial artificial chromosome (BAC) clones obtained from the Children’s Hospital Oakland Research Institute, BACPAC Resources Center. The specificity of all the BAC clones was confirmed on metaphase target slides (Vysis, CA, USA) before hybridizations. BAC clone RP11-135H4 was used as centromere-specific probe for all FISH experiments on chromosome 11, and served as a hybridization control. The copy number status of chromosomal regions 11q22.1-q22.2 and 11q24.1 was determined by applying probes prepared from clones RP11-90M3 and RP11-69G13 respectively and comparing them with the centromeric control. In addition, gain of 11q12 and amplification of 11q13 were validated using locus-specific BAC clones RP11-339F13 and RP11-300F16, respectively. BAC clone RP11-74J15 was used as centromere probe for chromosome 7. FISH images
were captured under a fluorescence microscope (Axioskop II, Carl Zeiss, Germany) and analyzed using the ISIS imaging software (Metasystems, Germany).

Comparison of the identified copy number alterations to published data. Using Entrez PubMed, PubMedCentral, and the Science Citation Index, we compiled a list of studies that reported either gene expression changes or copy number changes associated with oral cancer. We also did a focused search for studies suggesting roles for microRNAs in oral cancer, as this has been a topic of increasing interest recently. Wherever possible, gene names were standardized to the name approved by the HUGO Nomenclature Committee (www.genenames.org) as of June 2010.

Results

Demographic and clinicopathological characteristics

Array CGH profiling was done for 60 OSCC patient samples. All patients in the cohort were tobacco habitues and were found to be HPV-negative (Table 1, Figure S1). The mean age of the study cohort was 53 years (range, 31–80 years) with a higher proportion of males (80%). Tumors were predominantly moderately differentiated (60%) and were of locally advanced stages III and IV (92%). The cohort had equal representation of node-positive and node-negative groups. The median follow-up period of patients was 22.7 months. Detailed demographic and clinicopathological data of the study cohort is represented in Table S1.

Genomic Aberrations

RAE analysis was performed to identify recurring disease-associated chromosomal aberrations and segregate them from neutral ones. At a false discovery rate of q = 0.1, a total of 93 distinct CNAs were found by the RAE algorithm (Figure 1; Figure S2), seven of which in the centromeric regions; for 13 CNAs an additional localized peak region was detected (Table S2). Non-centromeric chromosomal aberrations occurring in more than 20% of the cases are presented in Tables 2 and 3. A large fraction of samples show gross whole chromosome-level alterations (Figure 1). Overall, the number of chromosomal losses (n = 61, including 7 centromeric) was higher than the number of gains (n = 32), but the difference was smaller for high-frequency CNAs (n = 35 versus n = 30). The detailed list of “candidate genes” for all the regions found altered is presented in Table S2.

Copy number losses

The most frequently occurring losses were identified on chromosomal regions 3p (62%), 5q (37%), 8p (83%), 9p (28%), 10p (35%), 11q (20%), 13p13 (32%), 18q (30%), and 19p12 (13%) as presented in Table S2. The previously unreported focal loss of 9p23-p24.1 (PTPRD) in oral cancer is shown in Figure 2.

Copy number gains

The most frequent aberrations included gain of chromosomal regions 3q (60%), 5p (50%), 7p (50%), 8q (73%), 9q (40%), 11q13 (47%), 14q (38%), 19p13.3 (58%) and 20q (40%) as represented in Table 3. Focal regions of amplification included 1q21.2 (NCKAP5), 3p12.1 (PTPRG), 3p25.2–p26.3 (CHLI, GRM7, RAD18, SRGAP3), 3q25.2 (MTN1RA1, FAT1), 6p21.3 (HLA-DRA, HLA-DRB5, HLA-DRB6, HLA-DRB7, HLA-DQB1), 8p23.1 (CMMMD1, HLA-B, PON1, APOE, 9p21 (MTA1, CH3f53, CDKN2A, CDKN2B, KMT2D, 17p13.3 (RAD51, MGC70870), and 22q13.1 (APOBEC3A, APOBEC3B) and is presented in Table S2. The previously unreported focal loss of 9p23-p24.1 (PTPRD) in oral cancer is shown in Figure 2.

| Table 1. Clinicopathological characteristics of sixty oral cancer patients. |
|-----------------------------------------------|
| Characteristics | No. of patients (%) |
| Male | 48 (80.0) |
| Female | 12 (20.0) |
| Mean Age | 53 (range 31–80) |
| Tumor sites | |
| GBC* | 53 (88.3) |
| Tongue | 7 (11.7) |
| Habit Profile | |
| Exclusive chewers | 44 (73.3) |
| Exclusive smokers | 2 (3.3) |
| Exclusive drinkers | 0 (0.0) |
| Mix habitués† | 14 (23.3) |
| Pathological Grade | |
| Well | 2 (3.3) |
| Moderate | 36 (60.0) |
| Poor | 22 (36.7) |
| Pathological Cervical Lymph Node involvement | |
| Negative (N0) | 30 (50.0) |
| Positive (N+) | 30 (50.0) |
| Pathological Stage | |
| I & II | 5 (8.3) |
| III & IV | 55 (91.7) |
| Treatment | |
| Surgery only | 6 (10.0) |
| Surgery + RT§ | 43 (71.7) |
| Surgery + RT+ CT¶ | 11 (18.3) |
| Recurrence Status | |
| No recurrence | 26 (43.3) |
| Recurrence | 25 (41.7) |
| Lost to follow-up* /unknown | 9 (15.0) |
| Clinical outcome | |
| Alive with no evidence of disease | 25 (41.7) |
| Dead of disease | 22 (36.7) |
| Alive with disease | 3 (5.0) |
| Dead of other cause | 1 (1.7) |
| Lost to follow-up* /unknown | 9 (15.0) |

GBC: Gingivobuccal complex;  †Mix habitués: Patients with at least two of the habits smoking, chewing, and drinking;  §RT: Radiation Therapy;  ¶CT: Chemotherapy;  *Lost to follow-up: Patients who did not attend the clinical check-up sessions after primary treatment and as a result their clinical status (recurrence and survival) could not be ascertained.

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Clinicopathological association of chromosomal aberrations

Chromosomal aberrations were analyzed to understand their relevance and associations with clinicopathological parameters like nodal status, grade and clinical outcome. We did not find any significant association of chromosomal aberrations with nodal status or grade. Using the Cox proportional hazards model, we find, at a corrected p-value <0.1, \( n = 11 \) CNAs associated with recurrence-free and \( n = 12 \) alterations associated with disease-specific survival (Table 4). Whereas the gains of chromosomal regions 11q12.2-q14.1 (\( P = 0.06 \)) and 11q22.1-q22.2 (\( P = 0.009 \)) were associated with poor clinical outcome, gain of 19p13.3 (\( P = 0.04 \)) was associated with better survival. Chromosomal losses of 3p25.3-p26.3 (\( P = 0.08 \)), 6p25.3 (\( P = 0.07 \)), 17p13.3 (\( P = 0.003 \)), 11q23-q25 (\( P = 0.0001 \)) and 18p11.1-p11.21 (\( P = 0.04 \)) were associated with poor clinical outcome, while loss of 4q13.2 (\( P = 0.05 \)) was associated with better survival (Table 4).

Figure 1. Radial heatmap of recurring copy-number alterations (CNAs) in OSCC. Shown in the inner heatmap are copy number gains/amplifications (blue) and losses/deletions (red), where tumors are stacked radially. Significantly recurring alterations (RAE q-value <0.1) are displayed between the outermost chromosome ideograms and the inner heat map (red: losses, blue: gains). Open circles denote known copy-number variants (CNVs) that span more than 50% with recurring CNAs. Chromosome numbers are shown in bold at periphery of chromosome ideograms with genomic coordinates in megabases.

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Loss of 11q23–q25 (P=0.0001) and gain of 11q22.1–q22.2 (P=0.009) were found as the strongest predictors of poor clinical outcome in terms of recurrence and survival (Table 4). Kaplan-Meier survival curves for loss of distal 11q and gain of 11q22.1–q22.2 are shown in Figures 3A and 4A. The chromosomal interval 11q23–q25 was subdivided by RAE into four non-overlapping intervals. The p-values for the four association tests were almost identical, but the subdivision suggests that there were multiple pertinent genes on 11q23–q25, at least one gene per subinterval.

### Table 2. Genome-wide alterations in OSCC: Losses & Deletions.

| Cytoband | Position | Start (Mb) | Size (Mb) | q value | % Frequency | Losses | Deletions |
|----------|----------|------------|-----------|---------|-------------|--------|-----------|
| 1q11.1-q21.1 | 120982663 | 144003083 | 23.02 | 8.00E-06 | 30 | 5 |
| 1p36.3-p11.1 | 147134204 | 147499075 | 0.36 | 0.000231 | 23.3 | 5 |
| 1q44 | 167493797 | 167507911 | 0.01 | 0.00029 | 21.7 | 13.3 |
| 2q11.2 | 246713386 | 246852155 | 0.14 | 0.023854 | 20 | 10 |
| 3p26.3–q25.3 | 39095 | 9150490 | 9.11 | 8.00E-06 | 45 | 0 |
| 3p14.2 | 60331268 | 61710321 | 1.38 | 8.00E-06 | 55 | 18.3 |
| 3p26.3-p11.2 | 39095 | 95211866 | 94.98 | 8.00E-06 | 61.7 | 25 |
| 3q26.3 | 16391201 | 164138371 | 0.2 | 8.00E-06 | 35 | 30 |
| 4q13.2 | 68901239 | 69688431 | 0.79 | 8.00E-06 | 43.3 | 30 |
| 4q13.3 | 70188483 | 70296201 | 0.11 | 8.00E-06 | 33.3 | 25 |
| 4q35.2 | 187570439 | 18826746 | 0.65 | 0.026377 | 20 | 0 |
| 4q35.2 | 190706301 | 191176358 | 0.47 | 0.000458 | 25 | 1.7 |
| 5q11.1-q14.3 | 49759749 | 88018996 | 38.26 | 8.00E-06 | 36.7 | 13.3 |
| 6p21.34 | 29962878 | 29981959 | 0.02 | 8.00E-06 | 26.7 | 23.3 |
| 6p21.33 | 29962878 | 29981959 | 0.02 | 8.00E-06 | 26.7 | 23.3 |
| 6p21.32 | 32605329 | 3263715 | 0.03 | 8.00E-06 | 43.3 | 43.3 |
| 6p21.32 | 32519664 | 32673012 | 0.15 | 8.00E-06 | 43.3 | 43.3 |
| 8p23.2 | 3400925 | 4000623 | 0.6 | 8.00E-06 | 65 | 25 |
| 8p11.23 | 39378080 | 39464066 | 0.09 | 8.00E-06 | 68.3 | 36.7 |
| 8p23.3-p11.1 | 6328 | 44740040 | 47.68 | 8.00E-06 | 83.3 | 65 |
| 9p24.1-p23 | 8924024 | 10013871 | 1.09 | 2.32E-05 | 23.3 | 6.7 |
| 9p13.1-q21.1 | 38612224 | 70225195 | 31.61 | 8.00E-06 | 36.7 | 6.7 |
| 10p15.3-p11.1 | 138235 | 42150788 | 42.01 | 8.00E-06 | 35 | 10 |
| 10p11.21 | 37475290 | 37508431 | 0.03 | 8.00E-06 | 26.7 | 3.3 |
| 10q11.22 | 46386192 | 46516611 | 0.12 | 2.83E-05 | 25 | 5 |
| 11q23.3-q23.1 | 102611683 | 110151240 | 7.54 | 0.018658 | 20 | 0 |
| 11q23.3-q25 | 119044683 | 13316524 | 14.27 | 0.0113 | 20 | 0 |
| 13p13 | 6035 | 44752 | 0.04 | 8.00E-06 | 31.7 | 23.3 |
| 15q11.2 | 18741744 | 19059898 | 1.06 | 2.83E-05 | 23.3 | 8.3 |
| 18q12.1-q23 | 24990733 | 76110993 | 51.12 | 8.00E-06 | 30 | 5 |
| 19p13.3 | 8275 | 236876 | 0.23 | 8.00E-06 | 36.7 | 11.7 |
| 22p13-p11 | 134684 | 14797037 | 14.66 | 0.00964 | 21.7 | 5 |
| 22q13 | 37689087 | 37715408 | 0.03 | 8.00E-06 | 25 | 18.3 |

The Thresholds for losses of a single copy and homozygous deletions are set adaptively by the RAE method.

Fluorescence in situ hybridization (FISH) analysis

Array CGH results were validated using I-FISH analysis. The samples were selected randomly from the cohort of 60 samples with known array CGH-based copy number alterations. The centremer- (RP11-I153H6) and region-specific (RP11-90M3, RP11-69q13) probes hybridized to their target loci showed no cross reactivity (Figure S3). We validated loss of 11q23-q25 (Figure 3B and 3C) and gain of 11q22.1–q22.2 (Figure 4B and 4C) associated with poor clinical outcome and found a concurrence of 70% and 82% respectively with array CGH data. In addition we...
validated the focal gains 11q13.3 (CCND1, ORAOV1, MYEOV, FG3, FG4, PPFIA1, CTTN) and 7p12 (EGFR) by I-FISH.

Results were found to be concordant in 70% and 75% of the samples (Figure S4).

Comparison of the identified copy number alterations to published data. We compared the intervals found by RAE (Table S2) to the locations of genes previously suggested in other oral cancer studies. The genes overlapping with each interval are shown in the column ‘OSCC genes’ in Table S2. The functional role of representative candidate genes is discussed.

Discussion

In this study, we characterized genome-wide alterations in locally advanced, tobacco-associated OSCC to identify markers of poor prognosis for OSCC risk stratification. To our knowledge, this is the first study of OSCC aCGH profiling from the Indian subcontinent. Previous CGH studies of OSCC revealed gains of 8q followed by 3q, 9q, 11q13, 14q, and 20q and losses of 3p followed by 4q, 5q, 8p, 9p, 10q, 11q, 18q, and 21q as the most frequent alterations [7–13]. Our study not only validated the previous reports but also revealed novel focal alterations previously not described in oral cancers. Using aCGH, we observed focal gains on chromosomal regions 3q27.1, 5p15.33, 14q11.2 and 19p13.3 and losses on 3p25.2–p26.3 and 9p23-p24.3 (PTPRD), which were not reported previously in genome-wide studies of OSCC. The focal alteration of 3q27.1 spans various proto-oncogenes including ABCC5, ALG3, EIF4G1, and EPHB3. We identified a frequently altered small region on 5p15.33 spanning twenty-four potential oncogenes. These genes, however, do not include TERT and TRIO that have been proposed in the literature. One of the new candidate gene on this locus is PDCD6 which has been shown to contribute towards tumor development and expansion [27].

Table 3. Genome-wide alterations in OSCC: Gains & Amplifications.

| Cytoband | Position | Size (Mb*) | q value | % Frequency | Gains | Amplifications |
|----------|----------|------------|---------|-------------|-------|----------------|
| 1q31.3   | 195026732| 195104236  | 0.08    | 1.04E-05    | 35.0  | 28.3           |
| 1q31.3   | 195026732| 195048237  | 0.02    | 1.04E-05    | 35.0  | 28.3           |
| 2q23.3   | 242501268| 242717042  | 0.22    | 1.81E-05    | 33.3  | 21.7           |
| 3q13.33-q24 | 121355348 | 144551988 | 23.20   | 0.001810    | 21.7  | 1.7            |
| 3q27.1   | 185019156| 185896384  | 0.88    | 1.04E-05    | 48.3  | 1.7            |
| 3q24-q29 | 145873432| 193379595  | 54.19   | 1.04E-05    | 60.0  | 20.0           |
| 4q13.2   | 69085413 | 69165843   | 0.08    | 1.04E-05    | 35.0  | 26.7           |
| 5p15.33-p11 | 7517     | 46136094   | 46.06   | 1.04E-05    | 50.0  | 20.0           |
| 5p15.33  | 7517     | 942987     | 0.87    | 1.04E-05    | 48.3  | 18.3           |
| 6p21.33-p21.32 | 31524806 | 32225578 | 0.70    | 0.014905    | 21.7  | 3.3            |
| 6p21.32  | 32519964 | 32673012   | 0.15    | 4.59E-05    | 30.0  | 18.3           |
| 7p22.3-p11.1 | 149297   | 57562112   | 57.41   | 1.04E-05    | 50.0  | 20.0           |
| 8q24.13-q24.3 | 123102996 | 146250794 | 23.15   | 1.04E-05    | 71.7  | 10.0           |
| 8q11.1-q24.4 | 43452765 | 146250794 | 102.80  | 1.04E-05    | 73.3  | 13.3           |
| 9p24.3-p21.3 | 153160   | 21931457   | 21.78   | 1.04E-05    | 33.3  | 10.0           |
| 9p21.3-p13.1 | 21980551 | 3924358    | 17.26   | 1.81E-05    | 38.3  | 6.7            |
| 9q13-q34.3 | 70238468 | 140241905  | 70.00   | 1.04E-05    | 40.0  | 11.7           |
| 11q12.2-q14.2 | 60970713 | 78077527   | 17.11   | 1.04E-05    | 53.3  | 26.7           |
| 11q13.2-q13.3 | 68554476 | 70150073   | 1.50    | 1.04E-05    | 46.7  | 26.7           |
| 11q22.1-q22.2 | 101407278| 102165885  | 0.76    | 0.021750    | 20.0  | 10.0           |
| 14q11.2   | 19560721 | 22142166   | 2.58    | 1.04E-05    | 46.7  | 5.0            |
| 14q11.2   | 21538460 | 22005864   | 0.47    | 1.04E-05    | 45.0  | 5.0            |
| 14q21.3-q31.1 | 48265939 | 80627188   | 32.36   | 0.000386    | 28.3  | 3.3            |
| 14q33.2-q32.33 | 87604117 | 106349785  | 18.75   | 9.60E-05    | 38.3  | 20.0           |
| 15q11.2   | 18741744 | 20060990   | 1.32    | 1.04E-05    | 35.0  | 13.3           |
| 17q25.3   | 77385789 | 78462808   | 1.08    | 1.04E-05    | 28.3  | 15.0           |
| 19p13.3   | 232109   | 258746     | 0.03    | 1.04E-05    | 58.3  | 31.7           |
| 20p13-p12.3 | 18609    | 5869816    | 5.85    | 0.022988    | 23.3  | 0.0            |
| 20p11.21  | 24728243 | 25680524   | 0.95    | 0.025337    | 20.0  | 0.0            |
| 20q11.21-q13.33 | 29436566 | 62363603   | 32.93   | 1.04E-05    | 40.0  | 8.3            |

The thresholds for gains of a single copy and amplifications by two or more copies were set adaptively by the RAE method.

aMb: mega base pair.

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11q Alterations: Marker of Poor Prognosis in OSCC
focal loss of RAD18 on chromosome band 3p25.3. RAD18 is an E3 ligase which is reported to play an important role in homologous recombination and repairs double strand break (dsb)[28]. We speculate that loss of RAD18 may lead to the impaired DNA repair and genomic instability. Our study also reports loss of PTPRG on 3p14.2. PTPRG encodes receptor-type tyrosine-protein phosphatase gamma acting in growth control by suppressing cyclin D1. A tumor suppressive function of PTPRG has been reported in breast cancer [29] and PTPRG was one of the earliest suggested oral cancer genes [30] but has not been reported as lost in recent CGH studies. A related member, tyrosine-protein phosphatase delta (PTPRD), present on chromosome 9p23-p24.3, was suggested to be gained by Snijders et al. [7], but was not selected as a driver gene for oral cancer. PTPRD is a known tumor suppressor for lung cancer [31] and glioblastoma [32]. It antagonizes growth stimulating signaling pathways that are also altered in oral cancers. In our cohort, PTPRD had a complex pattern of gains and losses; PTPRD was present in a small interval that was lost in 23% of the cases (Table S2), but also in a larger interval of 9p that was gained in 33% of the cases (Table S2). Due to its anti-proliferative function we hypothesize that tumors with PTPRD loss may be amenable to therapeutic intervention using growth factor inhibitors.

HPV-related OSCCs are characterized by 16q loss and better clinical outcome. Whereas HPV-unrelated tumors, such as those studied here, had gains of 11q13 and more losses at 3p, 5q, 9p, 15q, and 18q with poor clinical outcome [18]. Array CGH revealed that the samples in this study exhibit a genome-wide
profile similar to previously published HPV-unrelated OSCC specimens from other parts of the world, substantiating the presence of a distinct genomic profile of HPV-free OSCCs.

Most OSCC patients report with locally advanced disease at the time of diagnosis [www.seer.cancer.gov/statfacts/html/oralcav.html#survival]. The overall survival of these patients is generally poor as the majority of patients develop recurrent disease with chemoresistance. Patients at similar stages of OSCC, however, do not have identical course of disease and often differ in their clinical outcome. Hence, we analyzed advanced-stage OSCC samples to delineate the genomic alterations that could identify subsets of tumors differing with respect to recurrence and survival. We note that the gain of chromosomal region 11q22.1–q22.2 ($P=0.009$), losses of 17p13.3 ($P=0.003$) and 11q23–q25 ($P=0.0001$) are associated with poor clinical outcome. These regions were also found to be significantly associated with recurrence-free survival ($P=0.004$, $P=0.003$, and $P=0.0003$, respectively). Although the association of these chromosomal loci with poor clinical outcome is novel, the loci have been previously reported altered in OSCC [7,8,33]. In our study, clinical outcome was strongly associated with specific genomic aberrations detected by aCGH, however, there was no significant association with clinicopathological markers such as nodal status, grade or stage. This finding emphasizes the usefulness of genomic alterations as independent markers of prognosis.

Amplification of 11q13 is reported in about 45% of HNSCC [34,35]. We find the amplification in 47% of OSCC samples, similar to the earlier reports. The amplification of the 11q13 region was validated using I-FISH. Contradictory reports exist on the association of 11q13 alterations with clinical outcome [36–38]. We did not find any significant association of 11q13 with clinicopathological parameters or survival. In the breakage-fusion-bridge (BFB) cycle model of 11q13 amplification, distal 11q loss precedes 11q13 amplification and is therefore considered an early event in HNSCC progression [39]. Jin et al. reported that, in addition to 11q13 amplification, loss of distal 11q may be important for biological aggressiveness of head and neck carcinomas [40]. Further, they found that tumors with 11q loss had concomitant 11q13 amplification. In our cohort only one case (1.7%) had loss of distal 11q without the presence of 11q13 gain (Table S3).

Loss of chromosomal region 11q23-25 was significantly associated with poor clinical outcome. The results so obtained were confirmed by I-FISH. Parikh et al. reported the loss of distal region of 11q in HNSCC cell lines encompassing several DNA damage response encoding genes (MRE11, ATM, H2AFX) and found that this leads to compromised DNA damage response and reduced sensitivity to ionizing radiation [33]. Henson et al. reported a decreased expression of microRNAs miR-125b and miR-100 present on distal 11q in OSCC cell lines and showed their role in the development and progression of disease [41]. These microRNAs were regulated in a copy number dependent fashion as well as via decreased expression of ATM [41]. Parikh et al. predicted direct translational relevance for HNSCC patients, as patients with distal 11q loss did not benefit from aggressive radiation therapy [33]. Since in our study tumors with distal 11q loss were found to be a subset of tumors with 11q13 gain, we hypothesize that the distal 11q loss may be used as a risk marker to identify patients who do not benefit from aggressive radiation therapy, but could alternatively benefit from CCND1 inhibitors.

Another predictor of poor survival is the gain of 11q22.1–q22.2. Snijders et al. reported the presence of this rare amplicon in 5.6% of OSCC cases [7]. TAPI, BIRC2 and MMP7 genes present in this region were proposed as the candidate driver genes based on their role in apoptosis, cell adhesion and migration; BIRC3 was also mentioned, but not identified as a driver gene. Baldwin et al. reported the copy number gain of 11q22.2–q22.3 amplicon at a higher frequency (15%) and identified two gene clusters with nine matrix metalloproteinase (MMP) genes and two baculoviral IAP repeat-containing protein (BIRC) genes [8]. In our study, the 11q22.1–q22.2 amplicon encompassing TRPC6, ANGPTL5 and TAPI was associated with poor clinical outcome. The frequency of this alteration was 20%, similar to the frequency reported by Baldwin et al. [8].

TAPI can itself promote proliferation and transformation or it can act as a transcriptional cofactor by regulating the expression of

| Cytoband        | Aberration | Recurrence-free survival | BH Corrected p-value* | CPH coeff. | Disease-specific survival | BH Corrected p-value* | CPH coeff. |
|-----------------|------------|--------------------------|-----------------------|------------|--------------------------|-----------------------|------------|
| 3p25.3-p26.3    | Loss       | >0.1                     | -                     | 0.08       | 0.79                     | -                     | -          |
| 4q13.2          | Loss       | >0.1                     | -                     | 0.05       | -0.55                    | -                     | -          |
| 6p25.3          | Loss       | 0.09                     | 0.52                  | 0.07       | 0.55                     | -                     | -          |
| 11q12.2-q14.1   | Gain       | 0.06                     | 0.46                  | 0.06       | 0.48                     | -                     | -          |
| 11q22.1-q22.2   | Gain       | 0.004                    | 0.87                  | 0.009      | 0.80                     | -                     | -          |
| 11q22.2-q22.3   | Loss       | 0.005                    | 1.25                  | 0.001      | 1.46                     | -                     | -          |
| 11q23.1-q23.3   | Loss       | 0.004                    | 1.35                  | 0.001      | 1.51                     | -                     | -          |
| 11q23.3-q25     | Loss       | 0.0003                   | 1.60                  | 0.0001     | 1.76                     | -                     | -          |
| 11q25           | Loss       | 0.001                    | 1.46                  | 0.0004     | 1.62                     | -                     | -          |
| 17p13.3         | Loss       | 0.003                    | 1.06                  | 0.003      | 1.08                     | -                     | -          |
| 18p11.1-p11.21  | Loss       | 0.09                     | 0.78                  | 0.04       | 0.93                     | -                     | -          |
| 19p13.3         | Gain       | 0.06                     | —0.51                 | 0.04       | —0.59                    | -                     | -          |
| 20q11.21-q13.33 | Gain       | 0.07                     | 0.57                  | >0.1       | -                        | -                     | -          |

*Benjamini-Hochberg [49] method of adjusting for multiple tests.

CPH coeff.: Cox Proportional Hazard coefficient.

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various transcription factors including RUNX2, SMAD7, p73, p53BP2 and the TEA domain (TEAD) transcription factor family members [42]. YAP1 can induce anchorage-independent growth, epithelial mesenchymal transition, growth factor-independent proliferation, inhibit apoptosis and activate AKT and ERK pathways [43]. Another candidate gene on 11q22 is TRPC6, as suggested by two recent studies which reported overexpression of TRPC6 in glioma and glioblastoma multiforme (GBM) and analyzed its functional importance in cell growth, proliferation and increased radioresistance [44,45]. Although the relevance of TRPC6 function in OSCC needs to be further explored, our data indicate that TRPC6 may be one of the key genes responsible for radioresistance and poor clinical outcome in OSCC.

We report chromosomal loss of 17p13.3 in 13% of oral cancer samples analyzed. No previous study of oral cancer has identified the loss of this locus. Losses of 17p13.3 have been reported in many solid tumors including lung cancers [46]. The only known gene in the precise region chr17:118,535–134,424 is RPH3AL, but there exists no conclusive evidence for a tumor suppressive role [47,48] despite the fact that loss of 17p13.3 is strongly associated with poor recurrence-free and disease-specific survival.

In summary, our study reports genome-wide alterations in tobacco-associated, HPV-unrelated oral cancers. The study revealed genomic lesions on chromosome arms 11q and 17p13.3 associated with a high risk of relapse and decreased survival. These genomic alterations can potentially help in risk stratification of oral cancer patients beyond the currently used clinical paradigms. Our findings demonstrate the use of genetic alterations for predicting disease outcome, which may be helpful in developing accurate and objective markers for the prognosis of oral cancers.

Figure 3. Patient survival curves and FISH validation of 11q23-q25 loss. A) Kaplan-Meier survival estimates of patient groups with and without loss of chromosome 11q23–q25; survival in months (x-axis) is plotted against the fraction of samples alive (y-axis). Interphase FISH analysis detecting the chromosome 11 centromere (red) and the 11q24.1 region (green), B) A case without 11q24.1 loss and C) A case of 11q24.1 loss are shown. doi:10.1371/journal.pone.0017250.g003
Supporting Information

**Figure S1** Screening of HPV DNA in tumor samples. Representative gel picture of HPV general primer pair (GP5+/6+) PCR. Beta-globin PCR was done to check the genomic integrity in oral cancer samples. CaSki (HPV-16) and HeLa (HPV-18) cervical cell lines were used as positive controls, while C-33A and SCC074 (HPV negative) cervical and oral cell lines were used as negative controls. M: 50 bp marker.

Figure S2 Manhattan plot for statistically significant genomic alterations in OSCC. The false discovery rates (q-value; y-axis) for gains (blue) and losses (red) are plotted against the 22 autosomes (x-axis). The threshold for significance (q≤0.1) is indicated by a dotted line. Stars (*) indicate known copy-number variants (CNVs) according to the database of genomic variants (DGV).

Figure S3 Metaphase FISH confirming the specificity of BAC clones. A representative FISH image of metaphase plates with A) FISH probes confirming the specificity of loci 11q22.1-q22.2 (Green signals), B) 11q24.1 (Green signals) and chromosome 11 centromere (Panels A and B, Red signals).

**Figure 4** Patient survival curves and FISH validation of 11q22.1-q22.2 gain. A) Kaplan-Meier survival estimates of patient groups with and without gain of chromosome 11q22.1–q22.2: survival in months (x-axis) is plotted against the fraction of samples alive (y-axis). Interphase FISH analysis detecting the chromosome 11 centromere (red) and the 11q22.1–q22.2 region (green), B) A case without 11q22.1-q22.2 gain and C) A case of 11q22.1–q22.2 gain are shown.

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Figure S4 Interphase FISH validating the gain of 11q13.3 and 7p12 revealed by array CGH. A) Validation of 11q13.3 amplification by interphase FISH using centromere-specific (Cy3, Red) and locus-specific (FITC, Green) probe. B) Validation of 7p12 amplification by interphase FISH using centromere-specific (Cy3, Red) and locus-specific (FITC, Green) probe. (TIF)

Table S1 Detailed demographic and clinicopathological data for the study group. (DOC)

Table S2 A compendium of chromosomal alterations in 60 oral tumors with gene list and known candidate genes cited in the literature. (XLS)

Table S3 Case-wise chromosomal aberrations at 11q in oral cancer patients. (DOC)

References

1. Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. CA Cancer J Clin 55: 74–108.
2. Lippman SM, Hong WK (2001) Molecular markers of the risk of oral cancer. N Engl J Med 344: 1323–1326.
3. Akervall J (2006) Genomic screening of head and neck cancer and its implications for therapy planning. Eur Arch Otorhinolaryngol 263: 297–304.
4. Sollins-Toldo S, Lampel S, Sulgenbauer S, Nickolodjo J, Benner A, et al. (1997) Matrix-based comparative genomic hybridization: biases to screen for genomic imbalances. Genes Chromosomes Cancer 20: 399–407.
5. Kallioniemi A (2008) CGH microarrays and cancer. Curr Opin Biotechnol 19: 36–40.
6. Frohling S, Dohner H (2008) Chromosomal abnormalities in cancer. N Engl J Med 358: 722–734.
7. Snijders AM, Schmidt BL, Fridlyand J, Dekker N, Pinkel D, et al. (2005) Rare amplifications implicate frequent deregulation of cell fate specification pathways in oral squamous cell carcinoma. Oncogene 24: 4232–4242.
8. Baldwin G, Garnis G, Zhang L, Resin MP, Lam WL (2005) Multiple microsatellite alterations detected at high frequency in cancer. Cancer Res 65: 7561–7567.
9. Liu CJ, Lin SC, Chen YJ, Chang KM, Chang KW (2006) Array-comparative genomic hybridization to detect genome-wide copy number changes in microdissected primary and metastatic oral squamous cell carcinomas. Mol Carcinog 45: 721–731.
10. Nakamura K, Kozakai K, Tsuda H, Suzuki E, Pinkhaokham A, et al. (2008) Frequent silencing of a putative tumor suppressor gene melatonin receptor 1 A (MTNR1A) in oral squamous-cell carcinomas. Cancer Sci 99: 1390–1400.
11. Nakayama K, Yamagata HD, Arita N, Nakashiro KI, Nose M, et al. (2007) Identification of homozgyous deletions of tumor suppressor gene FAT in oral cancer using CGH-array. Oncogene 26: 5300–5308.
12. Suzuki E, Imoto I, Pinkhaokham A, Nakagawa T, Kamata N, et al. (2007) PRTDTC1, a possible tumor-suppressor gene, is frequently silenced in oral squamous-cell carcinomas by aberrant promoter hypermethylation. Oncogene 26: 7921–7932.
13. Rekant K, Knoepfel K, Flechtemacher C, Pungs S, Devens F, et al. (2010) Recurrent copy number gain of transcription factor SOX2 and corresponding high protein expression in oral squamous cell carcinoma. Genes Chromosomes Cancer 49: 9–16.
14. Garnis C, Coe BP, Zhang L, Resin MP, Lam WL (2004) Overexpression of LRFP12, a gene contained within an 8q22 amplicon identified by high-resolution array CGH analysis of oral squamous cell carcinomas. Oncogene 23: 2562–2566.
15. Jarvinen AK, Auto R, Kilpinen S, Saarela M, Leivo I, et al. (2008) High-resolution copy number and gene expression microarray analyses of head and neck squamous cell carcinoma cell lines of tongue and larynx. Genes Chromosomes Cancer 47: 500–509.
16. Paterson K, Evenson JW, Prime SS (1996) Molecular changes in oral cancer may reflect alleiotypic and ethnic origin. Eur J Cancer B Oral Oncol 32B: 150–153.
17. Shah JP, Singh B (2006) Keynote comment: why the lack of progress for oral cancer? Lancet Oncol 7: 356–357.
18. Klussmann JP, Mooren JJ, Lehen M, Claessen SM, Stemmer M, et al. (2009) Genetic signatures of HPV-related and unrelated oropharyngeal carcinoma and their prognostic implications. Clin Cancer Res 15: 1779–1786.
19. Gillison MI (2004) Human papillomavirus-associated head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst 100: 261–269.
20. de Roda Huamán AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders P (1995) The use of general primers GP-I and GP-II elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 76(Pt 4): 1057–1062.
21. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, et al. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350–1354.
22. Iadrate AJ, Feink L, Rivera MN, Listewnik ML, Donahoe PK, et al. (2004) Detection of large-scale variation in the human genome. Nat Genet 36: 905–909.
23. Smith ML, Marioni JC, Hardcastle TJ, Thorne NP (2006) snapCGH: Segmentation, Normalization and Processing of aCGH Data User’s Guide, Biocomput.
24. Venkatraman ES, Olshen AB (2007) A faster circular binary segmentation algorithm for the analysis of array CGH data. Bioinformatics 23: 657–663.
25. Taylor BS, Barretina J, Socci ND, Decarolis P, Ladanyi M, et al. (2008) Functional copy-number alterations in cancer. PLoS One 3: e3179.
26. La Cour JM, Hoy BR, Mollerup J, Simons R, Sauter G, et al. (2008) The apoptosis linked gene ALG-2 is dysregulated in tumors of various origin and contributes to cancer cell viability. Mol Oncol 1: 431–439.
27. Tong L, Jun H, Junie C (2010) RAD18 lives a double life: its implication in DNA double-strand break repair. DNA Repair (Amst) 9: 1241–1248.
28. Shu ST, Sugimoto Y, Liu S, Chang HL, Ye W, et al. (2010) Function and regulatory mechanisms of the candidate tumor suppressor receptor protein tyrosine phosphatase gamma (PTPRG) in breast cancer cells. Anticancer Res 30: 1947–1956.
29. Scully C, Field JK, Tanzawa H (2000) Genetic aberrations in oral and head and neck squamous cell carcinoma 2: chromosomal aberrations. Oral Oncol 36: 311–327.
30. Kohno T, Osaka A, Girard L, Sato M, Iwakawa K, et al. (2010) A catalog of genes homozygously deleted in human lung cancer and the candidacy of PTPRD as a tumor suppressor gene. Genes Chromosomes Cancer 49: 342–352.
31. Veeriah S, Brennan C, Meng S, Singh B, Fagin JA, et al. (2009) The tyrosine phosphatase PTPRD is a tumor suppressor that is frequently inactivated and mutated in glioblastoma and other human cancers. Proc Natl Acad Sci U S A 106: 9435–9440.
32. Parikh RA, White JS, Huang X, Schoppy DW, Baysal BE, et al. (2007) Loss of distal 11q is associated with DNA repair deficiency and reduced sensitivity to ionizing radiation in head and neck squamous cell carcinoma. Genes Chromosomes Cancer 46: 761–775.
33. Gollin SM (2001) Chromosomal alterations in squamous cell carcinomas of the head and neck: window to the biology of disease. Head Neck 23: 238–253.
34. Jin C, Jin X, Gielsdson D, Weneчерberg J, Wahl T, et al. (2006) Molecular cytogenetic characterization of the 11q13 amplicon in head and neck squamous cell carcinoma. Cytogenet Genome Res 115: 99–106.
35. Akervall J, Kurumi DM, Adams M, Zhu S, Fisher SG, et al. (2004) Overexpression of cyclin D1 correlates with sensitivity to cisplatin in squamous cell carcinoma cell lines of the head and neck. Acta Oncolaryngol 124: 851–857.
36. Huang X, Gollin SM, Raja S, Godfrey TE (2002) High-resolution mapping of the 11q13 amplicon and identification of a gene, TAIQ1, that is amplified and overexpressed in oral cancer cells. Proc Natl Acad Sci U S A 99: 11369–11374.
37. Huang X, Godfrey TE, Gooding WE, McCarty KS Jr., Gollin SM (2006) Comprehensive genome and transcriptome analysis of the 11q13 amplicon in
human oral cancer and syntenoy to the 7F5 amplicon in murine oral carcinoma. Genes Chromosomes Cancer 45: 1058–1069.

39. Reshmi SC, Roychoudhury S, Yu Z, Feingold E, Potter D, et al. (2007) Inverted duplication pattern in anaphase bridges confirms the breakage-fusion-bridge (BFB) cycle model for 11q13 amplification. Cytogenet Genome Res 116: 46–52.

40. Jin Y, Hoglund M, Jin C, Martins C, Wernerberg J, et al. (1998) FISH characterization of head and neck carcinomas reveals that amplification of band 11q13 is associated with deletion of distal 11q. Genes Chromosomes Cancer 22: 312–320.

41. Henson BJ, Bhattacharjee S, O'Dee DM, Feingold E, Gollin SM (2009) Decreased expression of miR-125b and miR-100 in oral cancer cells contributes to malignancy. Genes Chromosomes Cancer 48: 569–582.

42. Saucedo LJ, Edgar BA (2007) Filling out the Hippo pathway. Nat Rev Mol Cell Biol 8: 613–621.

43. Liu AM, Xu MZ, Chen J, Poon RT, Luk JM (2010) Targeting YAP and Hippo signaling pathway in liver cancer. Expert Opin Ther Targets 14: 855–868.

44. Ding X, He Z, Zhou K, Cheng J, Yao H, et al. (2010) Essential role of TRPC6 channels in G2/M phase transition and development of human glioma. J Natl Cancer Inst 102: 1052–1068.

45. Chagurupati S, Venkataraman R, Barrera D, Naganathan A, Madan M, et al. (2010) Receptor channel TRPC6 is a key mediator of Notch-driven glioblastoma growth and invasiveness. Cancer Res 70: 418–427.

46. Konishi H, Takahashi T, Kozaki K, Yatabe Y, Mitsudomi T, et al. (1998) Detailed deletion mapping suggests the involvement of a tumor suppressor gene at 17p13.3, distal to p53, in the pathogenesis of lung cancers. Oncogene 17: 2995–2000.

47. Goi T, Takeuchi K, Katayama K, Hirose K, Yamaguchi A (2002) Mutations of rabphilin-3A-like gene in colorectal cancers. Oncol Rep 9: 1189–1192.

48. Smith JS, Tachibana I, Allen C, Chiappa SA, Lee HK, et al. (1999) Cloning of a human ortholog (RPH3AL) of (RNO)Rph3al from a candidate 17p13.3 medulloblastoma tumor suppressor locus. Genomics 59: 97–101.

49. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. J Roy Stat Soc B 57: 289–300.