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Clinicopathological and prognostic implications of genetic alterations in oral cancers

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Abstract. This study evaluated the clinicopathological and prognostic implications of genetic alterations characterizing oral squamous cell carcinoma (OSCC). Comparative genomic hybridization was used to identify chromosomal alterations present in primary OSCCs obtained from 97 patients. In this population, tobacco use was a significant risk factor for OSCC. By contrast, the 97 samples were negative for human papillomavirus (HPV) DNA integration, ~~which is~~ another known risk factor for OSCC in certain populations. Results of the Fisher's exact test, followed by the Benjamini-Hochberg correction for multiple testing, showed a correlation of 7p gain and 8p loss with node-positive OSCC ($p \leq 0.04$ for both genetic alterations) and an association of 11q13 gain with high-grade OSCC ($p \leq 0.05$). Univariate Cox-proportional hazard models, also corrected for multiple testing, showed a significant association of 11q13 gain and 18q loss with decreased survival ($p \leq 0.05$). The findings were supported by multivariate analysis, which revealed that 11q13 gain and 18q loss together serve as a strong bivariate predictor of poor prognosis. In conclusion, our study ~~has~~ identified genetic alterations that correlate significantly with nodal status, grade and the poor survival status of OSCC. These potential biomarkers may aid the current tumor node metastasis system for better prediction of clinical outcome.

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Key words: oral cancer, comparative genomic hybridization, prognosis, survival analysis, gain of 11q13, loss of 18q

Introduction

Oral cancer is the sixth most common malignancy worldwide (1). India has a higher incidence compared to developed countries due to the habits of tobacco chewing and bidi smoking (2,3). Despite improved treatment modalities, the 5-year survival rates have not changed during the past few decades. The tumor node metastasis (TNM) staging system used for treatment decisions should be complemented with genomic biomarkers, which may aid in overcoming its limitations (4). Genomic biomarkers may predict the biological variability in tumors that alters their clinical behavior.

To obtain biomarkers, a better understanding of the molecular basis of oral carcinogenesis is required, which can be achieved via a genomic method of analysis, such as comparative genomic hybridization (CGH). CGH has revealed a recurrent pattern of genetic alterations characterizing oral squamous cell carcinomas (OSCC) (5-14). The clinical and prognostic evaluation of these alterations is crucial in order to determine their clinical utility as biomarkers. Studies investigating chromosomal CGH of OSCC are descriptive in nature and their patient cohort is limited, rendering it difficult to find biomarkers. A comprehensive clinicopathological evaluation of genomic alterations associated with OSCC is lacking.

The existence of divergent pathways of oral cancer progression has recently been demonstrated using oncogenetic tree models (15). The distinct sets of genetic alterations underlying the progression of node-negative (pN_0) and node-positive (pN^+) OSCC indicated that specific alterations characterize subtypes of oral cancers. To elaborate on these findings, clinicopathological associations are required to define genetic biomarkers that may aid in predicting the aggressive behavior of primary oral cancers.

In this study, genetic alterations were correlated with clinicopathological parameters, such as site, size, stage, lymph node involvement and tumor grade. The prognostic significance of these alterations was also determined for

those patients for whom complete follow-up information was available (n=40). To obtain candidate genetic biomarkers, stringent statistical methods for data analysis were applied.

Results of previous studies showed that human papillomavirus (HPV) infection is associated with a subset of head and neck squamous cell carcinoma (HNSCC). HPV-positive HNSCC constitutes a distinct molecular and clinicopathological disease entity and has better prognosis than HPV-negative HNSCC (16-18). Thus, the presence of HPV is considered to be an additional factor in the sub-grouping of tumors. This study therefore aimed to evaluate the HPV status in all tumors profiled by CGH.

Materials and methods

Study population and sample collection of the primary data set. Fresh frozen tumor samples were collected from 97 patients with primary oral carcinomas treated at the Tata Memorial Hospital, India, between 2001 and 2005. Written informed consent was obtained from all 97 participants prior to the collection of tissues. The tumor tissues used for CGH analysis were microdissected and only those with >70% of tumor cells were included for analysis. The clinicopathological characteristics of the cases were as previously described (15). Staging and differentiation were determined according to the joint TNM and American Joint Committee on Cancer classification of 2002, and the World Health Organization international histological classification of tumors, respectively. Follow-up information was available for 40 (41%) patients. The overall survival time was defined as the time from the date of surgery to the date of the last follow-up visit. Of the 41 patients, 27 were alive and remained disease-free throughout the time of follow-up, with a median follow-up of 24.4 months (range 4.9-75).

Detection of HPV by general primer PCR. The integrity and quality of the DNA extracted was checked by 0.8% agarose gel electrophoresis and DNA quantification was performed on a Nanodrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). To screen oral cancer samples for HPV integration into the human genome, a general HPV PCR was carried out using 250-300 ng of sample DNA and the primer pair $GP5^+/GP6^+$ for L1 consensus sequence (19). The CaSki cell line was used as a positive control. Samples with bands obtained at 150 bp were considered to be positive. To determine the quality of amplification of the extracted tumor DNA, β-globin PCR was performed (20).

CGH and digital image analysis. CGH was performed as previously described (15). The digital image analysis was carried out using the ISIS software (Metasystems, Altlussheim, Germany). Altered chromosomal regions were determined as the ratio of green to red (G/R) fluorescence intensities. G/R thresholds were set to 1.25 and 0.75 to determine gains and losses, respectively (21,22).

Interphase FISH. To validate the CGH predicted gains of 11q13, interphase fluorescence *in situ* hybridization (FISH) was performed on archival tumor samples with known CGH results. Dual-colored FISH was performed on 4-μm sections of archival OSCC samples (n=32) and the corresponding normal oral mucosal tissues (n=3) as previously described (15).

Statistical analysis. Statistical analysis was performed using the R software package (www.r-project.org). The frequency of copy number alterations (CNAs) was determined and only those alterations occurring in at least 30% of the 97 cases were considered for further analysis. The threshold of 30% was selected to limit multiple testing. The Fisher's exact test was used to determine the association of CNAs with various clinicopathological parameters. To correct for multiple testing, p-values were adjusted using the Benjamini-Hochberg method (23). A corrected p-value of ≤0.05 was considered to be statistically significant for all analyses. Survival analysis was performed using the Cox proportional hazards model (24). The risk of death is shown by the hazard function, $h(t) = h_0(t) \exp(\sum_i \beta_i \chi_i)$, where $h_0(t)$ is the baseline hazard. The co-efficient β_i determines the relative contribution of the clinical factors and CNAs χ_i determines the contribution to the overall risk. The prognostic value of each CNA and each clinicopathological parameter was initially analyzed in a univariate model. P-values were obtained from a likelihood ratio test. Stepwise model

Table I. Frequencies of CNAs among 97 oral cancer patients.

Genetic alteration	Frequency (%)
+8q	74.2
-3p	49.5
-8p	47.4
+9q	41.2
+11q13	41.2
+7p	37.1
+3q	35.1
-18q	34.0
+20q	33.0
+20p	27.8
+14q	25.8
+5p	24.7
-11q14-qter	20.6

CNAs, copy number alterations.

Table II. Concordance of CGH and FISH results for region 11q13.

	11q13		
	Gains	No gains	Total concordant (n)
CGH	13	19	32
FISH	10	15	25

For 11q13, two samples showed no signals in fluorescene *in situ* hybridization (FISH), so the concordance rate was 25/30 (83.33%). CGH, comparative genomic hybridization.

Table III. Correlation of CNAs with nodal status.

Genetic alteration	Nodal status		Fisher	BH
	N_0 (n=43)	N^+ (n=54)	p-value[a]	p-value[b]
+3q	13 (30.2)	21 (38.9)	0.250	0.28
+11q13	16 (37.2)	24 (44.4)	0.310	0.31
+20q	12 (27.9)	20 (37.0)	0.230	0.28
+7p	10 (23.0)	26 (48.1)	0.010	0.04
-8p	14 (32.5)	32 (59.3)	0.008	0.04
+9q	13 (30.2)	27(50.0)	0.040	0.09
-18q	10 (23.0)	23 (42.6)	0.040	0.09
+8q	28 (65.1)	44 (81.5)	0.060	0.10
-3p	19 (44.1)	29 (53.7)	0.230	0.28

Number and percentage (in parentheses) of cases are shown. [a]One-sided Fisher's exact test, p<0.05 is significant. [b]BH, Benjamini-Hochberg (1995) method of adjusting for multiple tests. CNAs, copy number alterations.

Table IV. Correlation of CNAs with tumor grade.

Genetic alteration	Grade			Fisher	BH
	Well (n=14)	Moderate (n=53)	Poor (n=30)	p-value[a]	p-value[b]
+3q	1 (7.10)	24 (45.3)	9 (30.0)	0.020	0.091
+11q13	1 (7.10)	22 (41.5)	17 (56.7)	0.005	0.050
+20q	2 (14.3)	16 (30.2)	14 (46.7)	0.088	0.260
+7p	3 (21.3)	21 (40.0)	12 (40.0)	0.500	0.740
-8p	7 (50.0)	25 (47.2)	14 (46.7)	1.000	1.000
+9q	6 (42.3)	21 (40.0)	13 (43.3)	0.960	1.000
-18q	4 (28.6)	20 (37.8)	9 (30.0)	0.760	0.970
+8q	8 (57.1)	40 (75.4)	24 (80.0)	0.270	0.500
-3p	4 (28.6)	28 (52.8)	18 (60.0)	0.280	0.500

Number and percentage (in parentheses) of cases are shown. [a]One-sided Fisher's exact test, p<0.05 is significant. [b]BH, Benjamini-Hochberg (1995) method of adjusting for multiple tests. CNAs, copy number alterations.

selection was performed for combinations of prognostic factors in a multivariate Cox model based on the Bayesian information criterion (BIC) (25). In each step, the factor reducing the BIC score most was added to (or removed from) the Cox model. This procedure was repeated until no further BIC improvement was possible. Additionally, parameter selection was performed using the least absolute shrinkage and selection operator (LASSO) method (26,27). In this method, the size of the risk co-efficients is constrained by a shrinkage parameter *s*, such that $\sum_i |\beta_i| \leq s$. Since a number of co-efficients vanish for small values of *s*, parameter selection is achieved by selecting an appropriate value of *s*, via generalized cross validation.

Results

CNAs detected by CGH in 97 primary OSCC. The frequencies of chromosomal aberrations detected by CGH are shown in Table I. The average number of total CNAs was 9.9 (range 1-30), including 6.73 gains and 3.05 losses on average. For further analysis, we considered alterations occurring in at least 30% of samples. The bandless CGH data are available at ftp://ftp.ncbi.nlm.nih.gov/pub/oral_cancer (chromosomal gains are denoted as '+' and chromosomal losses as '-').

HPV integration. The 97 tumor samples screened for HPV were found to be HPV-negative, indicating that HPV is not causally associated with the tumors profiled in this study.

Confirmation of CGH results with interphase FISH. To further assess some of the CNAs detected by CGH on chromosome arm 11q, FISH was performed on 32 samples and sub-band 11q13, which is known to be frequently gained, was evaluated (11,12,14). The results obtained from tumor samples were compared to three normal oral mucosal samples. For

Table V. Univariate Cox proportional hazards regression analysis of single predictors for overall survival for 40 patients.

Factor	p-value[a]	BH[b]
+11q13	0.005	0.05
-18q	0.007	0.05
+3q	0.220	0.41
-3p	0.100	0.30
+7p	0.630	0.86
+8q	0.500	0.75
-8p	0.170	0.36
+9q	0.170	0.36
+20q	0.920	0.92
Age	0.080	0.30
Nodal status	0.090	0.30
Grade	0.300	0.50
TNM stage	0.880	0.92
Size	0.840	0.92
Site	0.760	0.92

[a]Log-ratio test. [b]Benjamini-Hochberg (1995) method of adjusting for multiple tests.

all samples for which FISH signals were obtained, the concordance rate between CGH and FISH was high (83.33%, Table II). For 11q13, the five discrepancies comprised two non-informative tissues and three cases where FISH detected a gain that was not found by CGH.

Association of CNAs with clinicopathological parameters. The recurrent CNAs in OSCC were found to be associated with high grade and positive lymph node status (Table III). In particular, +7p and -8p significantly correlated with pN^+ as opposed to pN_0 (Table III). Furthermore, 11q13 gain was more prominent in high-grade (moderately and poorly differentiated) than in low-grade (well-differentiated) OSCC (Table IV). The CNAs did not show any correlation with clinicopathological parameters, such as size, site and stage of OSCC (data not shown).

Association of CNAs and clinicopathological parameters with prognosis. Follow-up data were available for 40 patients, including 12 patients with reduced survival (median survival time 6.1 months). Survival was tested against clinical parameters and genetic alterations. Univariate Cox proportional hazards models were used to determine the effect of clinicopathological parameters and genetic alterations on survival (Table V). The analysis revealed that clinical parameters, including TNM stage, did not show any significant association with survival. Among the genetic alterations, univariate analysis revealed correlations of +11q13 and -18q with poor survival (Table V; Fig. 1A and B).

To determine the best prognostic combination of CNAs, we used a multivariate Cox proportional hazards model. To avoid overparameterization, model selection based on the LASSO method and stepwise model selection using BIC

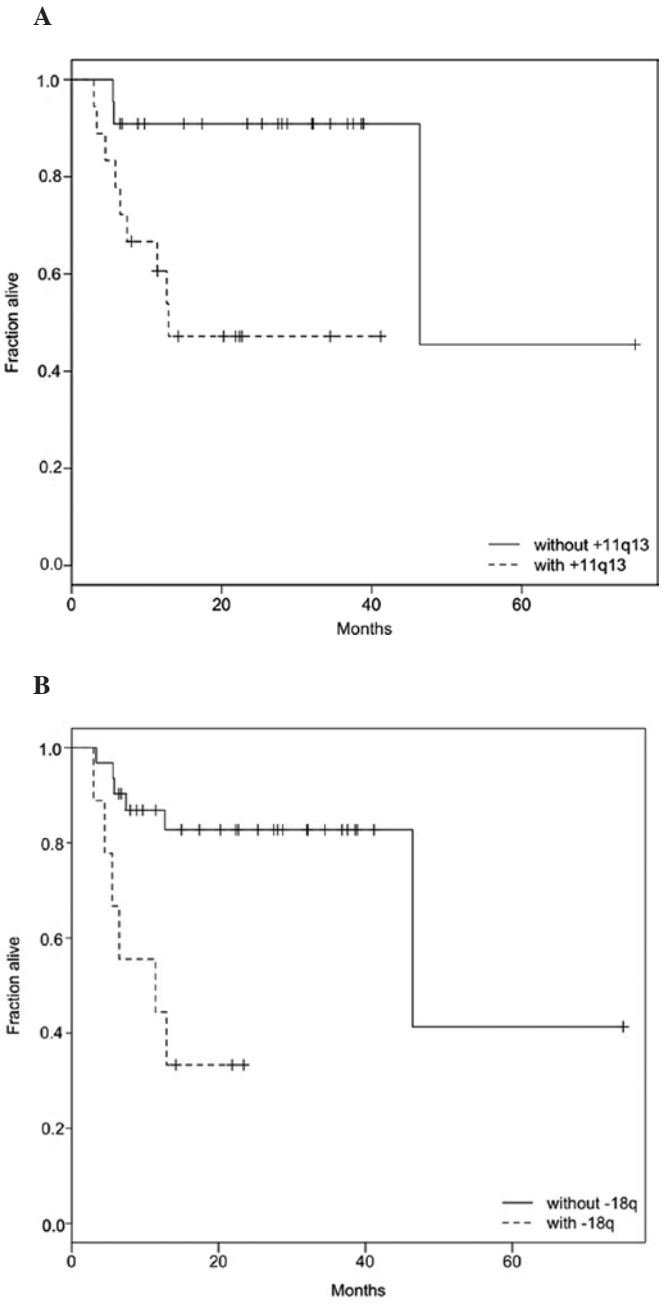

Figure 1. Kaplan-Meier estimates for (A) 11q13 gain and (B) 18q loss. In both cases, the survival rate was significantly higher in the group lacking alterations. (A) For patients with 11q13 gain, the median survival time was 12.9 months, while (B) for patients with 18q loss, it was 11.4 months. The survival time compares to a half-life of 46.4 months in the absence of either alteration.

was performed. The LASSO method revealed the bivariate combination of +11q13 and -18q as the best model (Fig. 2). For this combination, the risk is distributed relatively evenly among 11q13 (hazard: $\exp\beta_i$=4.3; p=0.08) and 18q ($\exp\beta_i$=3.1; p=0.08). A Kaplan-Meier plot with patient subgroups having none, one or both of the two CNAs is shown in Fig. 3. The results of the LASSO method were confirmed by stepwise BIC, and it was shown that a combination of +11q13, -18q, +9q and +7p is the best multivariate predictor (BIC=73.70). This model has a slightly better (i.e., lower) BIC value than the bivariate combination of +11q13 and -18q (BIC=74.58). In the best BIC model, the main effect on survival stems again

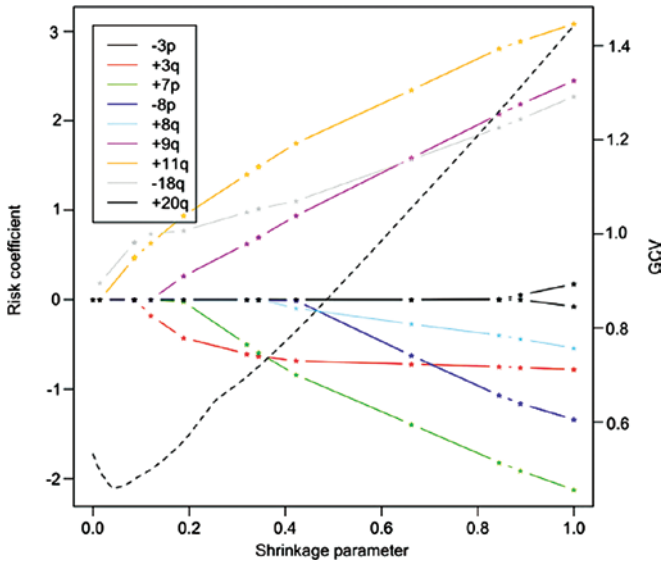

Figure 2. LASSO method estimates of the risk co-efficients in the Cox proportional hazards model. Co-efficients of high-level CNAs are shown as a function of the shrinkage parameter (straight lines). A shrinkage parameter of 1 denotes the unconstrained model. The first two non-vanishing co-efficients are those of +11q and -18q, followed by +3q and +9q. An evaluation of the generalized cross validation statistic (dashed line) reveals that it is minimal for a shrinkage parameter of 0.05. Only +11q and -18q contribute to the overall risk, making this combination an ideal multivariate marker.

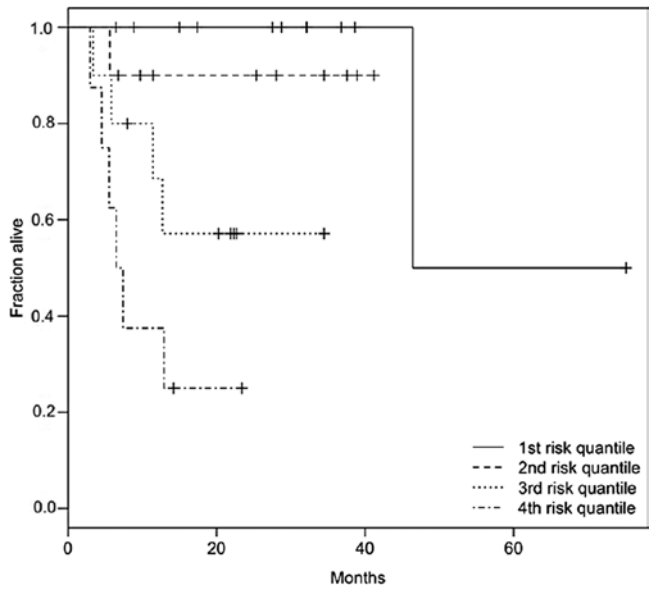

Figure 3. Kaplan-Meier plot of the multivariate predictor with +11q13, -18q, +9q and +7p as determined by BIC. Four cohorts defined by the 25, 50, 75 and 100% quantiles of the risk function of the Cox proportional hazards model are shown. As expected, survival was shortest in the high-risk group and longest in the low-risk group. With the exception of one outlier, the lowest risk quantile had complete survival, whereas in the highest risk cohort the half-life was <10 months.

from the gain of 11q13 ($\exp\beta_i$=15.2; p=0.004). The remaining risk was distributed among +9q ($\exp\beta_i$=5.3; p=0.03), -18q ($\exp\beta_i$=4.7; p=0.03), and +7p ($\exp\beta_i$=0.13; p=0.02). A Kaplan-Meier plot with four patient groups based on the calculated risk is shown in Fig. 4.

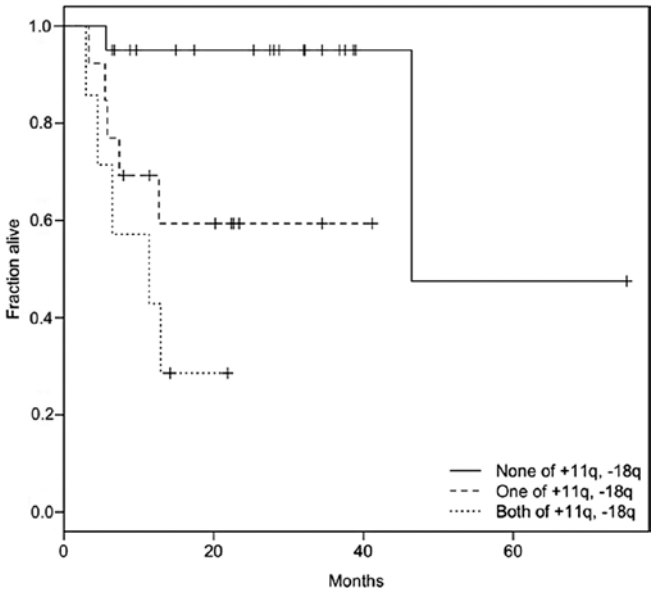

Figure 4. Bivariate predictor based on +11q13 and -18q as yielded by the LASSO method. The fraction lacking the two CNAs exhibited the lowest risk, whereas the cohort with the two alterations had the highest risk. The median survival time in the high-risk group was only 11.4 months, with 29% of the patients surviving after 24 months. By contrast, 95% of patients survived in the lowest risk group after 40 months. The presence of either of the two alterations formed an intermediate risk group with approximately 60% patient survival after 40 months.

Discussion

The chromosome-based CGH has identified recurrent chromosomal alterations in oral cancers (5-14). The recurrent chromosomal imbalances may play a significant role in the complex process of oral carcinogenesis. However, none of the 10 studies cited above has yielded genetic biomarkers for predicting the clinical outcome of oral carcinomas. The majority of these studies have not investigated the association of recurrent alterations with clinicopathological parameters and clinical outcome. In studies where the presence of prognostic CNAs has been suggested, a rigorous statistical assessment of the multivariate data is lacking. In the present study, oral cancers with CGH were analyzed and clinical associations of the recurrent alterations were evaluated by applying stringent statistical procedures, including correction for multiple testing and BIC- and LASSO-based model selection.

A recurrent CGH pattern for OSCC was obtained in this study, which is in accordance with previous CGH studies (5-14). CGH results were validated with interphase FISH for gains of region 11q13. Our statistical analysis revealed associations of specific alterations with lymph node involvement, high grade and poor clinical outcome. This result suggests that these alterations are clinically viable.

We analyzed oral cancers that are associated with tobacco use. Notably, none of the 97 samples exhibited HPV integration. This result is in agreement with former studies, in which there was little or no prevalence of HPV infection in oral cancers as compared to other HNSCC anatomic sites (28,29). Additionally HPV-related OSCCs are characterized by loss of 16q, whereas HPV-unrelated tumors, such as those of the

present study, exhibited gains/amplifications of 11q13 and more losses at 3p, 5q, 9p, 15q and 18q (30). Thus, our data support the hypothesis that HPV is an etiological factor for only a subset of HNSCC.

A significant correlation of +7p and -8p with pN^+ was noted in this study. This finding is consistent with the results of a previous analysis based on oncogenetic trees (15). The occurrence of +7p and -8p in pN^+ oral cancers has been reported (31-34). Genes at chromosomal regions 7p12 are known to affect the biological characteristics of OSCC; for example, activation of the oncogene EGFR on 7p12 has been shown to play a role in the nodal metastasis of OSCC. Loss of tumor suppressor genes on 8p, which contributes to the lymph node involvement of primary OSCC, remains to be determined. Our findings indicate that the two alterations confer aggressive behavior of OSCC. Thus, they may serve as biomarkers for predicting the propensity of nodal metastases in OSCC.

The correlation between genetic alterations and histological grade of OSCC is seldom studied. However, this study found a significant correlation of +11q13 with high-grade OSCC. One possible explanation for 11q13 gains affecting these characteristics may be that +11q13 is associated with an increased copy number of *CCND1* and the overexpression of its protein cyclin D1 (35). Previous studies have noted the role of cyclin D1 as a potential marker of proliferation, since the overexpression of D1 has been shown to positively correlate with other proliferation markers, such as Ki-67 and PCNA, and to inversely correlate with the expression of the anti-apoptotic gene *BCL2* (36).

In their study, Angadi *et al* showed an association between the overexpression of cyclin D1 and a high grade (poor differentiation) of oral cancers (37). A similar finding was reported by Mate *et al* in lung carcinomas. The authors speculated that differentiation, in addition to proliferation, is a major target of cyclin D1 impinging upon mechanisms of development of neoplasia (38). This raises the possibility that cyclin D1 overexpression plays a role in the inhibition of tumor cell differentiation in certain cell types (39).

Clinical follow-up data were available for a subset of patients, and univariate and multivariate Cox proportional hazards analyses were performed. Since the full multivariate model was likely to be overparameterized, key parameters were identified by means of model selection. When the prognostic impact of the clinical variables was evaluated on a univariate basis, the clinicopathological parameters, including TNM stage, did not show any prognostic association. Among the CGH-detected alterations, +11q13 and -18q were most significantly associated with poor clinical outcome. The two model selection criteria (LASSO and BIC) have shown +11q13 together with -18q to be a strong combined predictor of poor prognosis in the multivariate analysis. Moreover, the stepwise BIC selection indicates that this bivariate marker may be further improved by including genetic lesions on 9q and 7p. Our survival analysis shows that genetic aberrations define subsets of poor survival among oral cancers beyond clinical criteria.

Survival analysis of CGH data of head and neck cancers has consistently found +11q13 to be a predictor of poor prognosis (40,41). Although the chromosome was detected by interphase FISH analysis in earlier studies (42), such an association has not been found by CGH studies of oral cancers. To the best of our knowledge, the present study is the first to report an association of +11q13 with poor clinical outcome in the more homogeneous group of oral cancers. Understanding the role of different oncogenes, such as *CCND1*, *EMS1* and *FGF3/4* (located at 11q13), may aid in the development of better therapeutic strategies.

Multivariate statistical analysis identified -18q in combination with +11q13 as a predictor of poor prognosis. Loss of 18q has been associated with increased angiogenesis, recurrence and short survival of HNSCC patients (43,44). Thus, 18q loss may play a role in the aggressive behavior of oral cancers. Loss of *maspin*, a TSG on 18q, was shown to correlate with increased angiogenesis and reduced survival in OSCC (45,46). Notably, the multivariate analysis revealed the involvement of +9q and +7p as prognostic markers. The latter has been associated, not only with nodal metastasis, but also with poor prognosis. However, evidence shows 9q gain to correlate with an unfavorable outcome. Our study suggests that these alterations, if used in combination, may be useful in prognosticating OSCC patients.

The present study is the first to analyze oral cancers using rigorous statistical analysis and the first to obtain genetic alterations that are clinically relevant biomarkers. We have identified genetic alterations that correlate with clinicopathological parameters and the survival status of oral cancers. The bivariate predictor based on +11q13 and -18q may serve as a valuable biomarker for identifying patients with poor clinical outcome. Following confirmation in a larger set of samples, this candidate predictor serves as a biomarker that enhances the current TNM staging system for better prediction of the clinical behavior of oral cancers.

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