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ABSTRACT

Subgroups of patients with oral pre-malignant lesions (OPLs) are at extremely high risk for developing invasive cancer in spite of surgical excision. The objective of this study was to evaluate the utility of specific genes and their associated centromeres as markers to stratify OPLs for their cancer risk. Samples used in this study included 35 oral dysplasia with known outcome and 20 normal oral mucosa. Of the dysplasias, 20 were from an ongoing longitudinal study showing progression. The remaining 15 cases (2 of which progressed) were chosen from the population-based, provincial BC Oral Biopsy Service (OBS). Copy number alterations at *EGFR*, *CEP7*, *CCND1*, and *CEP11* were evaluated by fluorescent *in situ* hybridization (FISH). There was no significant difference in demographics between progressors and non-progressors. Specific FISH profiles at these genes and their corresponding centromeres were associated with progression. High gene gain of *CCND1* was associated with an 8-fold elevated risk of progression compared with those with no gain in time-to-progression analysis. Numerical alterations of *EGFR* and *CCND1* and their centromeres might be an effective means for identifying OPLs at risk. Future studies will expand on this analysis and set the stage for application of this approach in routine clinical practice.

KEY WORDS: oral dysplasia, progression, *EGFR*, *CCND1*, fluorescent *in situ* hybridization, chromosomal instability.

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Unique FISH Patterns Associated with Cancer Progression of Oral Dysplasia

INTRODUCTION

Oral squamous cell carcinoma (SCC) is the 6th most common cancer in the world (Warnakulasuriya, 2009). It is believed to progress through various stages of oral pre-malignant lesions (OPL), with or without dysplasia, to invasive cancer. Once cancer has developed, prognosis is poor, with 5-year survival rates of ~ 50% (Epstein *et al.*, 2008). Even when OPLs are successfully treated, survivors frequently have serious cosmetic and/or functional compromise. These dismal mortality rates accentuate the need to prevent the formation of invasive lesions through early diagnosis and effective treatment of OPLs.

The current 'gold standard' in OPL risk assessment is the presence and degree of dysplasia. However, the determination of progression risk in OPLs with no or low-grade (mild/moderate) dysplasia remains challenging, since the majority of these lesions will not progress (Waldron and Shafer, 1975; Schepman *et al.*, 1998). This inability to define aggressive behavior in OPLs underscores the need for markers predicting this behavior. Advances in molecular technologies have provided useful tools in developing markers to characterize "high-risk" OPLs (Mao *et al.*, 1994; Rosin *et al.*, 2000; Garnis *et al.*, 2009; Tsui *et al.*, 2009).

Previously, we applied whole-genome analysis to a panel of high-grade lesions and identified chromosomal loci 7p11.2 and 11q13 as the most frequently occurring regions of amplification (Tsui *et al.*, 2009). The 7p11.2 region spans *EGFR*, the well-characterized tyrosine kinase that has been found to be over-expressed in a number of cancers, including oral cancers (Garnis *et al.*, 2004, 2009; Temam *et al.*, 2007; Ryott *et al.*, 2009; Sheu *et al.*, 2009; Tsui *et al.*, 2009). *EGFR* is currently viewed as a promising molecular target for cancer therapy and chemoprevention, with multiple inhibitory strategies being developed that will target either the receptor itself or its downstream signaling pathway (Langer, 2008; Egloff and Grandis, 2009). Over-expression of *EGFR* has been found in a wide variety of solid tumors and may relate to poor prognosis. Over-expression of *Cyclin D1* (*CCND1*) – which maps to the chromosome 11q13 region – has been well-documented in oral cancer and has been reported to be associated with disease progression and poor prognosis (Michalides *et al.*, 1997; Kuo *et al.*, 1999; Shintani *et al.*, 2002). Whole-genome analysis of tumors also reported high frequency of amplification for both *EGFR* and *CCND1* in oral cancers, indicating the ongoing importance of these alterations in later-stage disease (Garnis *et al.*, 2004). Numerical aberration of the *CCND1* gene has been linked to failure of regional control with neck metastasis in early-staged oral SCC (Myo *et al.*, 2005).

Chromosomal instability (CIN), resulting in gains and losses of parts of or whole chromosomes, has been recognized as one of the critical driving forces for carcinogenesis (Weaver and Cleveland, 2006). Imbalance of chromosome copy number is responsible for large-scale gene dysregulation and is observed in various cancers, including oral cancers (Squire *et al.*, 2002). In tumors, FISH-detected chromosomal instability in cells at resection margins has been reported as a strong indicator of recurrence (Bergshoeff *et al.*, 2008).

In this study, we investigated the chromosome imbalances and the numerical alterations of specific genes in OPLs and the association of these changes with disease progression following surgical intervention. Analyses were undertaken on a set of invaluable OPLs with extensive clinical follow-up, including rigorously collected temporal clinicopathological data. Alterations identified as significantly associated with aggressiveness by this analysis could have immediate clinical utility, since they were evaluated by an established clinical technique.

MATERIALS & METHODS

Sample Selection

Fifty-five archival tissue blocks from patients were collected from the province-wide population-based BCOBS, including 35 dysplasia with no prior cancer history and 20 normal oral mucosa. The histological diagnoses were determined by study pathologists (LZ, KWB) using criteria from the World Health Organization (Axéll *et al.*, 1996). Among patients seen in the ongoing longitudinal study in 2007, 20 cases progressed into carcinoma *in situ* or invasive squamous cell carcinoma. The initial biopsies showing dysplasia that had enough tissue left on the tissue block were used for analysis. The remaining 15 dysplasia cases were randomly chosen from the BCOBS to match the former group by the year of the initial biopsy. When cross-checked with the pathology database and the BC Cancer Agency Registry, where all cancer cases in the province are documented, two of these cases were identified as showing progression. As a control, we also included 20 normal oral mucosa samples (amalgam tattoo with minimal inflammation). This study was approved by the Research Ethics Board of the University of British Columbia.

Fluorescent *in situ* Hybridization (FISH) and Scoring

The protocol used was a modification of Romeo *et al.* (2003) with a lower concentration of pepsin (0.032%) and a longer digestion time (80-90 min) (Tsui *et al.*, 2009). In brief, 4- μ m sections on charged slides were incubated in 2X SSC at 37°C for 30 min, dehydrated in ethanol series, and incubated in 70% glacial acetic acid for 1 min. The specimens were then digested in 0.032% pepsin at 37°C for 80 to 90 min, and fixed in 1% neutral buffered formaldehyde at room temperature for 10 min. Regions for analysis were selected based on our previous analysis of genomic alterations in oral tumors and a review of the literature, which indicated that these are significantly altered regions in oral cancer (Garnis *et al.*, 2004). Two sets of dual-colored probes (Vysis, Downers Grove, IL, USA) were performed on the sequential sections according to the manufacturer's instructions, which included

the pairs of *CEP7* (7p11.1-q11.1, SpectrumGreen)/*EGFR* (7p12, SpectrumOrange) and *CEP11* (centromere 11p11.11-q11, SpectrumGreen)/*CCND1* (11q13, SpectrumOrange). The probe set was applied, and the hybridization area was sealed and co-denatured at 80°C for 8 min, followed by incubation at 37°C for 24 hrs in a humidified chamber. Post-hybridization washes were performed consecutively in 50% formamide/2X SSC, 2X SSC, and 2X SSC/0.1% NP-40, each at 46°C for 6 min. DAPI in Vectashield antifade was applied as chromatin counterstain.

Signals were captured and imaged with Olympus BX61 and ImagePro Plus 5.1. At least 200 non-overlapping intact nuclei were scored. Samples with > 90% nuclei showing signals were considered informative.

The sample was scored and classified according to the frequency of nuclei with specific numbers of copies of the chromosome centromeres or genes, in a protocol modified from Hirsch *et al.* (2003) and Cappuzzo *et al.* (2005): for gene dosage change, (1) no gain, ≤ 2 copies of gene in >90% of nuclei, (2) low gain, 3 copies of gene in $\geq 10\%$ and ≥ 4 copies of gene in < 10% of nuclei, and (3) high gain, ≥ 4 copies of gene in > 10% of nuclei, a ratio of gene to centromere ≥ 2 , or ≥ 15 copies of gene *per* nuclei in $\geq 10\%$ of nuclei analyzed; and for numerical change of centromere, (1) disomy, ≤ 2 copies in > 90% of nuclei, (2) trisomy, 3 copies in > 10% and ≥ 4 copies < 10% of nuclei, and (3) polysomy, ≥ 4 copies in > 10% of nuclei analyzed.

Statistical Analyses

Differences and associations between progressors (N = 22) and non-progressors (N = 13) to carcinoma *in situ* or invasive squamous cell carcinoma and clinical parameters were examined by either Fisher's exact test for categorical variables or unpaired *t* tests for continuous variables. Correspondence analyses were used to produce 2-dimensional displays of similarities of relative frequency among groups, by SAS9.1.2. Time-to-progression curves were estimated by the Kaplan-Meier method, and comparisons were performed by log-rank tests. Relative risks were determined by Cox regression analysis. All tests were two-sided. Any $p < 0.05$ was considered to be statistically significant.

RESULTS

Characteristics of Progressors and Non-progressors

There were no differences in gender, age, and smoking status between the 2 groups (Appendix Table 1). Lesions that progressed were more commonly located at high-risk anatomical sites (*i.e.*, those from the lateroventral tongue, floor of mouth, or soft palate, $p = 0.05$). The average time to outcome or last follow-up was 36 ± 19 mos. When follow-up times were compared based on progression status, non-progressors had twice the length of follow-up time compared with progressors (52 ± 11 and 26 ± 15 mos, $p < 0.0001$).

Gene Dosage Change and Clinical Outcome

While absence of *EGFR* gain was more commonly associated with non-progressors (84%) and normal oral tissue (100%), 82% of progressors showed either low or high gene gain ($p < 0.0001$).

Table. Numerical Change of Gene^a and Centromere^b in Progressor, Non-progressor, and Normal Groups

	Progressor	Non-progressor	Normal	p			
Case number	22	13	20	All groups	Progressor vs. Non-progressor	Progressor vs. Normal	Non-progressor vs. Normal
EGFR							
No gain (%)	4 (18%)	11 (84%)	20 (100%)	< 0.0001	0.0006	< 0.0001	0.19
Low gain (%)	13 (59%)	1 (8%)	0				
High gain (%)	5 (23%)	1 (8%)	0				
CCND1							
No gain (%)	1 (4%)	9 (70%)	20 (100%)	< 0.0001	0.0002	< 0.0001	0.03
Low gain (%)	7 (32%)	2 (15%)	0				
High gain (%)	14 (64%)	2 (15%)	0				
CEP7							
Disomy (%)	7 (32%)	11 (84%)	20 (100%)	< 0.0001	0.009	< 0.0001	0.19
Trisomy (%)	12 (54%)	1 (8%)	0				
Polysomy (%)	3 (14%)	1 (8%)	0				
CEP11							
Disomy (%)	5 (23%)	10 (77%)	20 (100%)	< 0.0001	0.007	< 0.0001	0.08
Trisomy (%)	9 (41%)	1 (8%)	0				
Polysomy (%)	8 (36%)	2 (15%)	0				

^aNo gain: gene signal ≤ 2 in $> 90\%$ of nuclei counted; low gain: gene signal ≥ 3 in $\geq 10\%$ nuclei counted and gene signal ≥ 4 in $< 10\%$ nuclei counted; high gain: gene signal ≥ 4 in $\geq 10\%$ of nuclei counted or Gene/CEP ratio ≥ 2 or tight clusters of gene signal ≥ 15 in $\geq 10\%$ of nuclei counted.

^bDisomy: centromere signal ≤ 2 in $> 90\%$ of nuclei counted; Trisomy: centromere signal $= 3$ in $\geq 10\%$ and centromere signal ≥ 4 in $< 10\%$ nuclei counted; Polysomy: centromere signal ≥ 4 in $\geq 10\%$ of nuclei counted.

A similar trend was observed for *CCND1* ($p < 0.0001$). The increases in *EGFR* and *CCND1* gene dosage (i.e., low or high gene gain) were significantly associated with progression ($p = 0.0006$ and 0.0002 , respectively). The frequency of high gene gain of *CCND1* was significantly higher in progressors than in non-progressors (64% vs. 15%, $p = 0.01$). Any level of *EGFR* gain was strongly associated with progressors ($p = 0.0002$). One progressor in each group showed clumps of gene amplifications for *EGFR* and *CCND1*, with ~20-30 gene signals clumped together (see Fig. 1).

The association of gene dosage change and clinical aggressive behavior can be easily visualized by correspondence analysis. High gene gain of *CCND1* was located very close to the progressors in a 2-dimensional display, demonstrating the similarity of their relative frequencies (Fig. 2a). Any level of *EGFR* gain was even more strongly tied to the progressors (Fig. 2b).

Chromosomal Instability with the Presence of Trisomy and Polysomy and Outcome

When centromere number was analyzed, trisomy or polysomy was more frequently observed in the progressors ($p < 0.0001$ in *CEP7* and *CEP11*, Table) than in the non-progressors and normal mucosa. When the diploid status and outcome were considered, the non-progressors were more commonly disomy for both *CEP7* (84%) and *CEP11* (77%) compared with the progressors (32%, $p = 0.005$; 23%, $p = 0.004$, respectively). This lends further weight to the idea that chromosomal instability might be one of the driving forces for oral carcinogenesis.

Time-to-Progression Risk

Specific numerical change in gene dosage for *EGFR* and *CCND1*, and the centromeres of chromosomes 7 and 11, were examined for their association with disease progression by the Kaplan-Meier method. Time-to progression curves were plotted as a function of *EGFR* gain, *CCND1* gain, and the frequency of disomy, trisomy, and polysomy of *CEP7* and *CEP11*. When these 4 markers were considered independently, gene gains were significantly associated with time to progression for *EGFR* ($p = 0.004$) and *CCND1* ($p = 0.002$; Fig. 3), but not with changes in *CEP7* and *CEP11*. When *EGFR* and *CCND1* were considered together in a Cox proportional hazards model, *CCND1* appeared to be significantly associated with time to progression ($p = 0.05$, adjusted for *EGFR*) but not *EGFR* ($p = 0.2$, adjusted for *CCND1*). This is possibly due to some association/interaction between *CCND1* and *EGFR*. Using a logistic regression model to evaluate the performance of gene gain at *CCND1*, *EGFR*, *CCND1*+*EGFR*, and the interactive term of *CCND1*+*EGFR* for their sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the area under curve (AUC), we found that all models performed well according to these criteria (Appendix Table 2). The model with the interaction term of *CCND1* and *EGFR* as predictor fitted the data best (AUC = 0.95). Furthermore, the cancer risk of high gene gain of *CCND1* is estimated to be eight times that of no gain, and the cancer risk of low gene gain of *CCND1* is estimated to be three times that of no gain, both adjusted for *EGFR*. The frequencies of disomy, trisomy, and polysomy of *CEP7* and *CEP11* did not seem to be significantly associated with time to progression.

DISCUSSION

There are two main challenges for the development of effective markers for risk prediction in OPLs. One challenge surrounds the rarity of acquiring lesions for analysis, particularly lesions for which outcome information has been accrued. The other challenge has been deriving biomarkers that can be easily adopted for use in a standard histology laboratory.

In British Columbia, we have addressed the first challenge by making use of cases included in an ongoing longitudinal study since 1998. This unique cohort is made possible by our centralized population-based OBS (~ 60,000 archived cases in over 30 years' history).

To address the second challenge described above, we have restricted our molecular assays to those already in routine use in the clinic. Our application of FISH in the present work could be readily translated for use in a clinical lab setting, since this approach has already been standardized for other genes and in other tissues at many pathology laboratories. Probes used to detect *EGFR*/CEP7 and *CCND1*/CEP11 are currently commercially available.

Amplifications of *EGFR* and *CCND1* Are Associated with Aggressive Clinical Behavior

EGFR activation has previously been implicated in several cancer types, including oral cancer (Grandis, 2006; Karamouzis *et al.*, 2007). It has been demonstrated to play a role in gene transcription, cell proliferation, angiogenesis, apoptosis inhibition, and tissue invasion. Activation of *CCND1* has also been implicated in tumorigenesis and contributes to oral tumor phenotypes (Todd *et al.*, 2002; Brinkman and Wong, 2006). Analysis of our data indicates that increased gene gain of *EGFR* and *CCND1* is a recurring event in OPLs, confirming findings from earlier whole-genome analyses performed by our group (Garnis *et al.*, 2004; Tsui *et al.*, 2009).

More specifically, we found that any level gains of *EGFR* and high level of gene gain of *CCND1* were strongly associated with progression. High gene gain of *CCND1* was associated with an approximate eight-fold elevated risk of progression after adjustment for any gain of *EGFR*. These findings are intuitive, since both *EGFR* and *CCND1* govern a wide array of critical cancer cell processes.

Chromosomal Instability and Its Associated Outcome

We observed significant differences in numerical changes for CEP7 and CEP11 among progressors, non-progressors, and normal tissues, indicating that chromosomal instability might

Figure 1. Example of histology and FISH analysis on a progressor. (a) The histomicrograph shows a moderate epithelial dysplasia with a band-like lymphohistiocytic inflammatory cell infiltrate in the subtending connective tissue (H&E, original magnification, 200X). (b) FISH analysis with dual-color probe set (Vysis, Downers Grove, IL, USA) consisting of a centromeric probe for chromosome 7 (CEP7; SpectrumGreen) and a locus-specific probe for *EGFR* (7p12; SpectrumOrange). The blue line represents the basement membrane separating the epithelium (superior left half) and connective tissue (inferior right half) (original magnification, 600X). Clustered high gene gain signals (orange clumps) with normal CEP7 disomy (green signals) were noted in the epithelium [higher magnification of a dysplastic nucleus in (c), 1000X], and 2:2 signals of *EGFR* gene (orange signals) and CEP7 (green signals) were noted in the connective tissue [higher magnification of an inflammatory cell in (d), 1000X].

play a role during oral carcinogenesis. Single chromosomal imbalances have previously been proposed as a mechanism for simultaneous disruption of several genes critical for tumorigenesis (Weaver and Cleveland, 2006). The presence of this kind of broad alteration in lesion cells that linger after surgery – or in histologically ‘normal’ tissue adjacent to resection margins – could serve as a driver for disease recurrence and possibly progression. The finding that greater genomic instability exists in the progressors agrees well with previous reports demonstrating that increased genomic instability is also a marker for progression risk in oral lesions (Garnis *et al.*, 2009; Torres-Rendon *et al.*, 2009).

Taken together, our findings may have immediate clinical impact. FISH-based analysis is a common diagnostic set-up for many pathology laboratories, and tissue requirements are minimal (a 4- μ m section); hence our findings could be readily translated into clinical patient care.

In conclusion, we have shown that the presence of polysomy and gene amplification at specific genomic loci is significantly associated with progression in OPLs. Validation of these results in a larger sample set is required and is ongoing. Widespread application of this approach will stratify OPLs, identifying high-risk lesions requiring more aggressive treatment (and justifying the increased toxicity and morbidity risks associated with these treatments).

Figure 2. Correspondence analysis of gene gains of *CCND1* and *EGFR* among progressor, non-progressor, and normal groups. (a) A 2-dimensional display indicated that high gene gain of *CCND1* showed a close distance to 3, the progressors, indicating the similarity of their patterns of relative frequencies. (b) A 2-dimensional display indicated that any gene gain of *EGFR* showed an even closer distance to 3, the progressors (3): (1) normal, (2) non-progressors, (3) progressors; no gain = ≤ 2 copies of gene in $> 90\%$ of nuclei; low gain = 3 copies of gene in $\geq 10\%$ and ≥ 4 copies of gene in $< 10\%$ of nuclei; high gain = ≥ 4 copies of gene in $> 10\%$ of nuclei, a ratio of gene to centromere ≥ 2 , or ≥ 15 copies of gene per nuclei in $\geq 10\%$ of nuclei scored.

a. *EGFR* gene gain

b. *CCND1* gene gain

Figure 3. Probability of cancer progression according to gene gains of *EGFR* (a) and *CCND1* (b). Time-to-progression curves were estimated by the Kaplan-Meier method, and the comparisons were performed by log-rank tests (black line for no gain, ≤ 2 copies of gene in $> 90\%$ of nuclei; red dash line for low gain, 3 copies of gene in $\geq 10\%$ and ≥ 4 copies of gene in $< 10\%$ of nuclei; and green dotted line for high gain, ≥ 4 copies of gene in $> 10\%$ of nuclei, a ratio of gene to centromere ≥ 2 , or ≥ 15 copies of gene per nuclei in $\geq 10\%$ of nuclei analyzed).

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