Multiple pathways in the FGF signaling network are frequently deregulated by gene amplification in oral dysplasias

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Abstract

Genetic alteration in oral premalignant lesions (OPLs), the precursors of oral squamous cell carcinomas (OSCCs), may represent key changes in disease initiation and development. We ask if DNA amplification occurs at this early stage of cancer development and which oncogenic pathways are disrupted in OPLs. Here we evaluated 50 high-grade dysplasias and low-grade dysplasias that later progressed to cancer for gene dosage aberrations using tiling-path DNA microarrays. Early occurrences of DNA amplification and homozygous deletion were frequently detected, with 40% (20/50) of these early lesions exhibiting such features. Expression for 88 genes in seven recurrent amplicons were evaluated in five independent head and neck cancer datasets, with 40 candidates found to be overexpressed relative to normal tissues. These genes were significantly enriched in the canonical ERK/MAPK, FGF, p53, PTEN, and PI3K/AKT signaling pathways ($P = 8.95 \times 10^{-3}$--3.18×10^{-2}). These identified pathways share interactions in one signaling network, and amplification-mediated deregulation of this network was found in 30.0% of these preinvasive lesions. No such alterations were found in 14 low-grade dysplasias that did not progress, while 43.5% (10/23) of OSCCs were found to have altered genes within the pathways with DNA amplification. Multi-target FISH showed that amplification of *EGFR* and *CCND1* can co-exist in single cells of an oral dysplasia, suggesting the dependence on multiple oncogenes for OPL progression. Taken together, these findings identify a critical biological network that is frequently disrupted in high-risk OPLs, with different specific genes disrupted in different individuals.

Keywords

oral premalignant lesion; gene amplification; homozygous deletion; DNA microarray; signaling pathway

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common head and neck neoplasm, with more than 30,000 cases identified in the United States each year.[1] Despite advances in treatment, the 5-year survival rate of all stages and advanced stage (stage III and IV) remains at less than 50% and 25%, respectively, for the past three decades.[2] The poor survival rate is mainly because most patients were diagnosed at the advanced stages of the disease. Early detection and treatment at the premalignant stages with intensified follow-up would improve patient survival.[2, 3]

Oral cancer is believed to progress from hyperplasia, through various extent of dysplastic changes, carcinoma *in situ* (CIS), and finally breaking through the basement membrane at the invasive SCC stage.[4] In oral premalignant lesions (OPLs), the presence and degree of epithelial dysplasia is used to assess the risk for progression to malignancy. By definition, dysplasia is characterized by cellular atypia and loss of normal maturation and stratification and has no evidence of invasion.[5] High-grade preinvasive lesion, including severe dysplasia and CIS, is associated with the strongest risk for malignant transformation.[3, 6-9] Thus, patients with these lesions are treated in British Columbia to prevent further development into invasive SCC.[3] However, the majority of the low-grade dysplasia, mild and/or moderate dysplasia, will not progress to cancer. Previous studies have started integrating imaging and molecular analysis with histopathologic evaluation for improving the ability to predict progression risk in OPLs. [10-15] An understanding of the molecular mechanisms that govern the promotion of OPLs into cancer would be very relevant for clinical practice in the identification of genes suitable for therapeutic targeting, prognostic and risk predictive markers.

In oral cancer, the accumulation of genetic alterations is associated with the progression of OPL to invasiveness.[16, 17] Molecular analysis of OPLs is necessary to identify key changes in disease initiation and progression. However, studies of OPLs are rare and have not been attempted on a genome-wide scale, owing mostly to the minute amount of DNA obtainable from primary OPLs.[16] Changes in gene dosage occur frequently in cancer genomes. Gains and losses of genomic regions may contain proto-oncogenes and tumor suppressor genes, which may lead to aberrant expression useful for malignant transformation. Low-level copy number changes involving large regions with many genes have been frequently observed in OSCCs, but their effect on gene expression remains ambiguous.[18-21] High-level copy number changes, DNA amplification or homozygous deletion, encompass focal changes and often lead to the discovery of cancer-causing genes. Amplicons are defined as DNA segments less than 20 megabase pairs (Mbps) of which at least five copies exist in a single cell.[22] They are useful for oncogene discovery because these unstable regions are under relentless selection and thus harbor genes advantageous for tumor growth.[23-25] Amplified oncogenes are also clinically useful for therapeutic development.[22, 23] Furthermore, amplification of *EGFR* with more than 12 copies per cell in head and neck SCC (HNSCC) is associated with poor survival.[26] On the other hand, biallelic loss such as homozygous deletion contributes to functional inactivation, facilitating the localization of tumor suppressors.[27, 28] High-resolution whole genome DNA microarrays have been useful in exploring genetic alterations in formalin-fixed paraffin-embedded (FFPE) specimens without the need for sample amplification.[18, 29] Indeed this technology has a functional resolution of 50 kbp, improving the localization of regions with gene amplification and homozygous deletion.[29, 30]

Oral high-grade dysplasias are known to have a high likelihood of cancer progression, whereas low-grade lesions have a low probability of progression and can even regress.[14] We evaluated genome wide gene dosage alteration in 50 manually microdissected FFPE high-risk OPLs, which included 43 high-grade dysplasias and seven low-grade dysplasias that are known to have later progressed to cancer. We focused on the identification of high-level DNA

amplification and homozygous deletions instead of low-level copy number change. This type of analysis has never been undertaken for such early stage lesions. We also compared these findings to 23 OSCC and 14 low-grade dysplasias that never progressed. Expression of gene candidates within recurrent amplicons in OPLs were analyzed in public datasets with 188 HNSCCs and further confirmed in 61 oral cancers. Taken together, our analysis suggests that a common signaling network involving the ERK/MAPK, FGF, p53, PTEN, and PI3K/AKT signaling pathways is frequently deregulated in high-risk OPLs.

Materials and Methods

Tissue Samples

This study involves 87 (64 dysplasias and 23 OSCCs) archival FFPE specimens obtained from the British Columbia Oral Biopsy Service (Supp. Table S1). The group of "high-risk OPLs" includes 43 high-grade preinvasive lesions (22 severe dysplasia and 21 CIS) and seven low-grade lesions (one hyperplasia and six mild and/or moderate dysplasias) that later progressed to high-grade dysplasias or OSCCs. These lesions represent 86 patients, all with no prior history of cancer. Samples from one patient with a severe dysplasia on the lower lip (sample Oral7) and an OSCC on the tongue (sample Oral80) are both included in the study as they are from distinct anatomical sites. For low-grade lesions, patients were followed up with a median duration of eight years in a longitudinal study established at the BC Oral Cancer Prevention Program. Low-grade lesions that did not progress between 1985 and 2009 consisted of one hyperplasia and 13 mild and moderate dysplasias. All diagnoses were confirmed by the study pathologist (LZ) using criteria established by the World Health Organization (WHO).[5] Areas of dysplasia were identified using hematoxylin and eosin (H&E) stained sections cut from FFPE tissues. Epithelial cells in these areas were meticulously dissected from adjacent non-epithelium tissue under an inverted microscope using a 23G needle. DNA was extracted as previously described.[18]

Whole genome DNA microarray analysis

Tiling-path genomic arrays (SMRT v.1 and v.2) were obtained from the BC Cancer Research Centre Array Laboratory.[29] The whole genome is represented as 26,819 overlapping bacterial artificial chromosome (BAC) clones spotted in duplicate with complete coverage of the human genome, allowing breakpoint detection at a resolution of 50 kbp.[29, 30] Briefly, each sample DNA and normal reference pooled male genomic DNA (Novagen, Mississauga, ON, Canada) (250 ng each) were random prime labeled with cyanine-3 and cyanine-5 dCTP, respectively, mixed with 100 µg of human Cot-1 DNA, purified and hybridized to the array at 45°C for 36 hours before washing. Hybridized arrays were scanned as previously described.[31, 32]

Data analysis

Array images were analyzed using SoftWoRx Tracker Spot Analysis software (Applied Precision, Issaquah, WA). A three-step normalization procedure, including LOWESS fitting, spatial, and median normalization, was used to remove systematic biases.[33] *SeeGH* software was used to display $\log_2$ signal intensity ratios in relation to genomic locations in the hg17 assembly (NCBI Build 35).[34] Data points with standard deviation >0.075 and signal to noise ratio <3 in either channel were removed. None of the 87 genomic profiles contain technical artefacts of wavy pattern which are often observed from FFPE samples.[35] All profiles has been deposited to Gene Expression Omnibus (GEO) database at NCBI, series accession number GSE9193.[36]

High-level DNA amplifications and presumptive homozygous deletions were identified by a moving-average based algorithm as previously described.[31] The threshold for high copy number was set to $\log_2$ signal intensity ratio > 0.8 for amplification or < -0.8 for homozygous

deletions. Only those alterations containing ≥ 3 overlapping clones were identified in order to avoid false-positives due to hybridization artifacts. Recurrent minimal altered regions of amplification were identified by the presence of a given amplicon in at least two high-risk OPLs. Genes within such altered regions were mapped according to the RefSeq Genes track release 25. As copy number variation-associated BAC clones are often associated with amplification and DNA rearrangement, such BACs are included in the analysis.[31, 37]

Transcript expression analysis

Independent transcript analyses of genes mapped within minimal altered regions of amplification were performed using the Oncomine database.[38] Five studies within Oncomine analyzed expression patterns between head and neck tumors (N = 188) and normal tissues (N = 38) (Supp. Table S2).[39-43] In Oncomine, Student's *t* test was performed to reflect the significance of differential expression observed in tumors compared to normal tissue in each study. Furthermore, two public GEO datasets (*GSE10121* and *GSE9844*) containing 61 oral tumors and 18 normal samples were downloaded.[41, 43, 44] The normalized data from each dataset were extracted. Two-sided student's *t*-test along with a Benjamini-Hochberg multiple testing correction was performed comparing the oral tumors with the normal samples per study. The significance threshold was set at *P*<0.05.

Real-time polymerase chain reaction

Total RNA from eight OSCCs and nine normal oral mucosal tissue from different healthy individuals were extracted using TRIzol (Invitrogen, Carlsbad, CA). Five hundred nanograms of total RNA from each sample were converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a total volume of 20 μL. Real-time PCR using TaqMan Universal PCR master mix was performed to analyze *TLN1* and *CREB3* expression levels with Applied Biosystems Fast Real-Time PCR System. TaqMan gene expression assays (Assay ID) of *TLN1* (Hs00196775_m1), *CREB3* (Hs00197255_m1), and *18S rRNA* (Hs99999901_s1) were purchased from Applied Biosystems. Reactions were performed in triplicate and according to manufacturer's protocol. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression values using the average of cycle thresholds of target genes and 18S rRNA, and the value of 1 was arbitrarily assigned to one normal sample. Expression levels between OSCCs and normal samples were compared by a two-sided Wilcoxon-rank sum test.

Biological functions and pathway analysis

To define the biological functions of all the genes mapped within recurrent amplicons in OPLs, we interrogated the *Biological Function Analysis* in Ingenuity Pathway Analysis (IPA) (version 7.0) (Ingenuity® Systems, www.ingenuity.com). *Biological Function Analysis* identifies biological functions and diseases that are significantly enriched in the data relative to chance alone by Fisher's exact test. In addition, genes in recurrent amplicons in high-risk OPLs and associated with transcript overexpression in at least one HNSCC dataset were further explored using the Canonical Pathways Analysis. Canonical Pathways Analysis explores 48 well-characterized metabolic and signaling pathways for the significant enrichment of the dataset in these pathways, again using Fisher's exact test to calculate the probability that the association between genes in the dataset and the canonical pathway is explained by chance alone.

Fluorescence in situ hybridization (FISH)

FISH assays were performed as described in Romeo *et al.*,[45] except with the modifications of using a lower concentration of pepsin (0.032%) and longer digestion time (80-90 minutes). Two sets of dual colored probe (Vysis, Downers Grove, IL) were sequentially performed on the same tissue section according to manufacturer's instruction, which included the pair of *CEP11* (centromere 11p11.11-q11, SpectrumGreen)/ *CCND1* (11q13, SpectrumOrange), and

CEP7 (7p11.1-q11.1, SpectrumGreen)/ *EGFR* (7p12, SpectrumOrange). Signals were captured and imaged using Olympus BX61 and ImagePro Plus 5.1.

Results

Early occurrence of DNA amplification and homozygous deletion in OPLs

It is evident that copy number alterations are more frequent among OSCCs relative to high-grade dysplasias across the whole genome (Supp. Figure S1). High-level copy number alteration, including DNA amplification and homozygous deletion, is a frequent event in high-risk OPLs. In total, 40% of high-risk OPLs (19/43 high-grade dysplasias and 1/7 progressing low-grade dysplasias) exhibited at least one region of high-level copy number change. A frequency of 65.2% (15/23) was found in OSCCs. No such changes were detected in low-grade lesions that did not progress. A whole genome karyogram of one high-grade dysplasia Oral42 with seven regions of gene amplification is illustrated in Figure 1.

Recurrent amplicons and rare regions of homozygous deletion harbor known and novel candidate cancer genes

To distinguish genetic events at the premalignant stage from late-stage events, high-level copy number changes were identified separately in 50 high-risk OPLs and 23 OSCCs. In the 20/50 high-risk OPLs with high-level copy number alteration, 43 incidents of gene amplification and six regions of homozygous deletion were detected. At a similar level, 46 occurrences of amplification and two regions of homozygous deletion were identified in 15/23 OSCCs (Supp. Table S3). Many of these lesions have at least two regions of high-level copy number alteration, including 11/20 of the high-risk OPLs and 9/15 of the OSCCs. In addition, many of the detected amplicons overlap, suggesting that these regions do not occur by chance.

Homozygous deletions were seen less frequently than DNA amplification, occurring in five high-grade dysplasias and one OSCC (Fig. 2). The eight identified regions of homozygous deletion do not overlap (Table 1), but two regions on 9p22.3 (in samples Oral12 and Oral88) are separated by 1.2 Mbp. In total, 44 genes were identified as homozygously deleted, including known tumor suppressors *CDKN2A* (9p21.3), *CDKN2B* (9p21.3), *MTAP* (9p21.3), and *WWOX* (16q23.1). Genes bounded by the novel regions of homozygous deletion that we identified (9p21.1-p21.2, 9p22.3, 9p22.3-p23, 9q33.1, 9q33.1-q33.2, and 15q15.1) may represent tumor suppressors driving oral carcinogenesis (Supp. Table S4).

DNA amplifications occurring in OPLs may activate genes that facilitate the development of oral cancer. Seven recurrent amplicons, ranging from 0.45 Mbp to 2.26 Mbp in size, were identified in our dataset of 50 high-risk OPLs (Table 2). Minimal altered regions of these recurrent amplicons contain 88 unique genes, 15 of which are known to be involved in cancer as determined by *IPA Functional Analysis* ($P = 1.31 \times 10^{-5}$—1.41×10^{-2}) (Table 3, Supp. Table S5). Except for chromosomal loci at 2q11.2 and 8q22.3, all amplicons harbor at least one cancer-related gene according to the IPA knowledgebase (though literature searches for genes in the outstanding regions identified potential cancer genes) (Table 2). This further substantiates the need to investigate the genes within these recurrent amplicons in OPLs.

Transcript analysis of independent HNSCC datasets

Candidate oncogenes within amplicons are likely to have increased mRNA expression in cancer specimens. Thus, we evaluated transcript levels of genes in the recurrent amplicons to refine the gene list using five independent studies of HNSCC.[39-43] Of the 88 genes identified within recurrent amplicons, 40 were overexpressed in at least one dataset of HNSCC relative to normal tissues. This validation at the transcript level in independent datasets suggests the potential importance of the 40 candidate genes in cancer development.

Frequent oncogenic activation of a common signaling network in OPLs

We hypothesize that genes with elevated copy number in recurrent OPLs and increased mRNA levels in HNSCC are important for oral cancer development. To understand the signaling defects in OPLs, we interrogated 48 well-characterized signaling pathways in the IPA canonical pathway database to examine which pathways are significantly enriched with the candidate genes. The top five deregulated canonical pathways include the ERK/MAPK, FGF, p53, PTEN, and PI3K/AKT signaling pathways (Table 4, $P = 8.95 \times 10^{-3}$, 1.63×10^{-2}, 1.96×10^{-2}, 1.96×10^{-2}, and 3.18×10^{-2} respectively). Graphical representation of each canonical pathway is provided in Supp. Figures S2, S3, S4, S5, S6.

It is important to note that several genes within recurrent amplicons, including the *CREB3*, *CCND1*, and *YWHAZ*, participate in multiple canonical pathways. In addition, amplified genes including the *FGFR* and *EGFR* could both activate the ERK/MAPK and the PI3K/AKT pathways. Furthermore, 14 amplified genes share direct and indirect relationships in one network (Supp. Fig. S7). As multiple pathways could contribute to cancer development, the interactions among the top significantly over-represented canonical pathways were considered as a network (Fig. 3). Seven candidate genes (*FGF3*, *EGFR*, *TLN1*, *YWHAZ*, *CCND1*, *CREB3* and *SNAI2*) within recurrent amplicons in high-risk OPLs participate in this signaling network. Of these seven candidate genes, *FGF3*, *EGFR*, and *CCND1* have been frequently associated with gene amplifications in OSCCs,[46-48] and protein expression of *YWHAZ* has been detected in oral dysplasias.[49]; while *SNAI2* is often described as an important regulator for epithelialmesenchymal transition.[50] We validated expression levels of *TLN1* and *CREB3* by quantitative PCR in eight OSCCs and nine normal oral mucosa tissues. Expression of *TLN1* and *CREB3* both showed increased expression in the OSCC group relative to normal tissues ($p = 4.53 \times 10^{-3}$, 8.14×10^{-3}, respectively, Wilcoxon rank-sum test) (Supp. Fig. S8). Genes within this signaling network not identified in recurrent amplicons include *PAK4*, *FGFR1*, *and EIF4EBP1*. *PAK4* is amplified in one high-grade dysplasia and one OSCC, while *FGFR1* and *EIF4EBP1*were amplified in one high-grade dysplasia. Taken together, gene amplification of at least one of these 10 genes within this signaling network was found in 30.0% (15/50) of high-risk OPLs and 43.5% (10/23) of OSCCs.

To further substantiate the importance of this signaling network, we investigated two genetic features in all 87 oral lesions: 1) the presence of multiple amplicons harboring genes of this signaling network within a single specimen, and 2) the presence of overexpression in members of this signaling network that are not deregulated by gene amplification. We found that three high-grade dysplasias and one OSCC maintained multiple regions of gene amplification of different genes of this network. For example, one high-grade dysplasia (Oral22), exhibited three regions of high-level amplification on 7p11 (*EGFR*), 11q13 (*CCND1*, *FGF3*), and 19q13.2 (*PAK4*). The presence of multiple amplicons targeting a common network illustrates the potential importance for the disruption of multiple components within single samples. Next, we evaluated mRNA overexpression among 47 genes of this network not having DNA amplification in five independent HNSCC datasets. Among them, *AKT1, AKT3, NRAS*, *PIK3CA, PIK3CB, PIK3CG, PRKACB, PRKAR1A, PRKCA, PRKCB1, PRKCE, PRKCI*, *RRAS*, and *RRAS2* were significantly overexpressed in at least two of the five datasets. This further demonstrates the frequent deregulation of this signaling network in the development of HNSCC.

Validation of mRNA levels in OSCCs

It has been previously suggested that different anatomical sites of cancers could affect mRNA expression profiles. However, the availability of OSCC expression datasets deposited in GEO database is limited, thus hindering sub-group analysis from different sites in the oral cavity or the head and neck region. Focusing on oral cancer, we analyzed expression levels of two oral

cancer-specific studies (N = 61) to validate the importance of the 88 genes in recurrent amplicons for oral carcinogenesis.[41, 43, 44] Of the 88 genes, 46 genes were overexpressed in at least one OSCC dataset, including 28 of the 40 overexpressed genes in HNSCC datasets (Supp. Table S6). Components of the signaling network, including the *FGF3*, *FGF4*, *FGF19*, *EGFR*, *CCND1*, *CREB3*, and *YWHAZ*, were also found to be significantly overexpressed in OSCC datasets. Thus, the expression pattern identified from oral cancer datasets was found to be similar to that of HNSCC.

Single cells of oral dysplasia exhibit co-amplification of EGFR and CCND1

Co-amplification of at least two regions of the genome exists in 11 high-risk OPLs and 8 invasive SCC, constituting 55.9% (19/34) of samples with DNA amplification. We ask if the observed co-amplification is a manifestation of intra-lesion heterogeneity or if synchronous gene amplifications exist in single cells of the lesion. We performed FISH using probes spanning genomic regions of *EGFR*, *CCND1*, and the centromere of chromosome 11 on high-grade dysplasia Oral22 to examine this question (Fig. 4). Gene amplifications of *EGFR* and *CCND1* were observed to co-exist in single cells of high-grade dysplasia, demonstrating that both of these changes can occur synchronously at the premalignant stage during oral cancer development.

Discussion

Genetic alterations of DNA amplification and homozygous deletion have long been recognized as chromosomal regions that contain genes important for cancer development.[23, 31, 51-57] As genetic alterations accumulate to produce a neoplastic phenotype, we focus on preinvasive stages of high-grade and low-grade lesions that are known to further develop into cancer, aiming to identify early genetic events during cancer development. Amplicons recurrently present in OPLs were identified, and genes that are overexpressed in HNSCC datasets were further distinguished. The purpose of this study includes: identifying early genetic events in cancer development, understanding the underlying pathways governing the progression of OPLs, and discovering gene candidates that might have therapeutic value for the prevention and treatment of oral cancer patients.

Amplifier phenotype in oral high-grade dysplasias

Gene amplification is a major mechanism of oncogene activation and has been associated as poor prognostic indicator in human cancers.[22] Here, we detected frequent events of gene amplification in OPLs. Previous studies have reported gene amplifications in OSCC and oral cancer cell lines, with some studies evaluating known oncogenes or using interval-marker CGH.[18, 20, 21, 58-62] Here, using an unbiased genome-wide approach we show that the early stages of oral lesions already suffer from increased genomic complexity. Seven amplicons were found to be recurrently present in oral dysplasias, ranging in size from 0.45 Mbp to 2.26 Mbp. Previously, Snijders *et al.* examined 89 oral invasive tumors by interval-marker array and identified nine amplicons smaller than 3 Mbp.[21] The similar number of amplicons detected in our set of 50 high-risk OPLs was interesting, as it is believed that genetic alterations accumulate as oral dysplasias progress to invasiveness. Although tiling-path array provided increased genomic coverage compared with interval-marker, parallel analysis of our dataset of 23 OSCCs also detected similar level of genomic complexities between high-risk OPLs and invasive carcinomas. In addition, none of the 14 non-progressing low-grade lesions harbor region of DNA amplification. All the identified amplicons in oral dysplasias contain at least one cancer-related gene, supporting the concept that DNA amplification is likely to activate oncogene and contributes to OPL progression. Taken together, these results show that an amplifier phenotype exists in OPLs, and these amplicons might directly contribute to oral carcinogenesis. As gene amplification is readily detectable in clinical specimen, and our data showed that amplification

frequently exists in high-risk OPLs, it might be plausible for gene amplification to serve as marker in OPLs predictive for aggressive progression to malignancy. However, a larger sample size of low-grade lesions with clinical outcome would be required to address this hypothesis.

Disruption of multiple components of a signaling network in oral dysplasias

It is crucial to understand the deregulated molecular pathways that govern the progression of oral premalignancy. By evaluating OPLs for gene amplification and examining genes with transcript overexpression in HNSCC and OSCC datasets, we identified genes that are involved in the ERK/MAPK, FGF, p53, PTEN, and PI3K/AKT signaling pathways. The FGF signaling pathway regulates developmental processes and angiogenesis, and has been an important therapeutic target in human cancers.[63, 64] FGF signaling can activate the PI3K/AKT signaling cascade, leading to an induction of epithelial-mesenchymal transition and cell migration;[65] while the FGF-stimulated ERK/MAPK signaling pathway is implicated in cell differentiation, proliferation, and survival.[66] Activation of Akt by phosphorylation has been shown as an early event in oral preneoplastic lesions, and its expression is correlated with poor outcome in oral cancer patients.[67] By examining the molecular interactions among the most significantly deregulated pathways as a single network (Fig. 3), we demonstrated the diverse mechanisms for the activation of this network, emphasizing the need for molecular targeted therapies of multiple signaling pathways.

Our study identified the genes that are amplified early during carcinogenesis. These genes were targeted towards one signaling network, and gene amplification frequently disrupted this signaling network as early as the low-grade dysplasia. Moreover, mRNA overexpression was frequently found in members of this network not activated by gene amplification in HNSCC datasets. The HNSCC datasets were chosen because of their large sample size, public availability, and could potentially broaden the scope of this oral cancer specific study to other HNSCCs. Nevertheless, expression analysis of oral cancer samples was also performed using two public datasets and similar results were found. In conclusion, our data highlights the early and frequent activation of one signaling network in OPLs. This also gives important insights into therapeutic strategies as different genes are mechanistically altered in different individuals.

Pathway addiction in oral dysplasias

The term "oncogene addiction" was first coined to describe the physiological dependence of cancer cells on a single activated oncogene for the maintenance of their malignant phenotype. [68] In contrast to this phenomenon, a majority of our samples with amplification harbor more than one amplicon, suggesting the dependence on multiple oncogenes for OPL progression. As amplicons are known to be unstable regions, the maintenance of multiple amplicons must allow them to gain a selective advantage for clonal growth.[23, 25] Interestingly, four oral lesions, including three high-grade dysplasias and one invasive carcinoma, exhibited multiple amplicons harboring genes from the same signaling pathways, suggesting the phenomenon of "pathway addiction" in these early stage lesions.[69] Although intra-lesional heterogeneity might exist in dysplastic cells, which could contribute to the detection of various amplicons in different clonal populations, we observed co-amplification of two genes in this signaling network, *EGFR* and *CCND1*, could occur in single cells of a preinvasive lesion. This demonstrates the occurrence of an amplifier phenotype in single cells of oral dysplasia, and that multiple oncogenes are potentially important for overgrowth of such cells to form cancer. Whether the FGF signaling network is causative in oral cancer development needs to be investigated in further studies.

The potential "pathway addiction" to this signaling network yields important insight for drug development. Targeting a receptor tyrosine kinase might not be sufficient if downstream targets

are also disrupted. In addition, genetic testing of multiple components of this network, possibly by FISH assays to detect amplification hotspots, might enhance therapeutic efficacy.[70,71]

Summary

This is the first comprehensive examination of DNA amplification and homozygous deletion in OPLs. We identified one signaling network that is frequently deregulated at different components in oral dysplasias, reflecting diverse mechanisms but common underlying biology governing the progression from oral premalignancy into invasiveness. This study suggests the importance of multiple signaling pathways in the early stage of oral carcinogenesis. Combined targeting of these oncogenic pathways might be effective for treatment of oral cancer patients since several genes of this network could be activated in a single specimen. Furthermore, since different targets are altered in different specimens, it is important to identify multiple markers to stratify patients that may benefit from personalized therapies for oral cancer.[71]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations

OPL, oral premalignant lesion; OSCC, oral squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; CIS, carcinoma *in situ*; FFPE, formalin-fixed paraffin-embedded; BAC, bacterial artificial chromosome; IPA, Ingenuity Pathway Analysis; FISH, fluorescence *in situ* hybridization.

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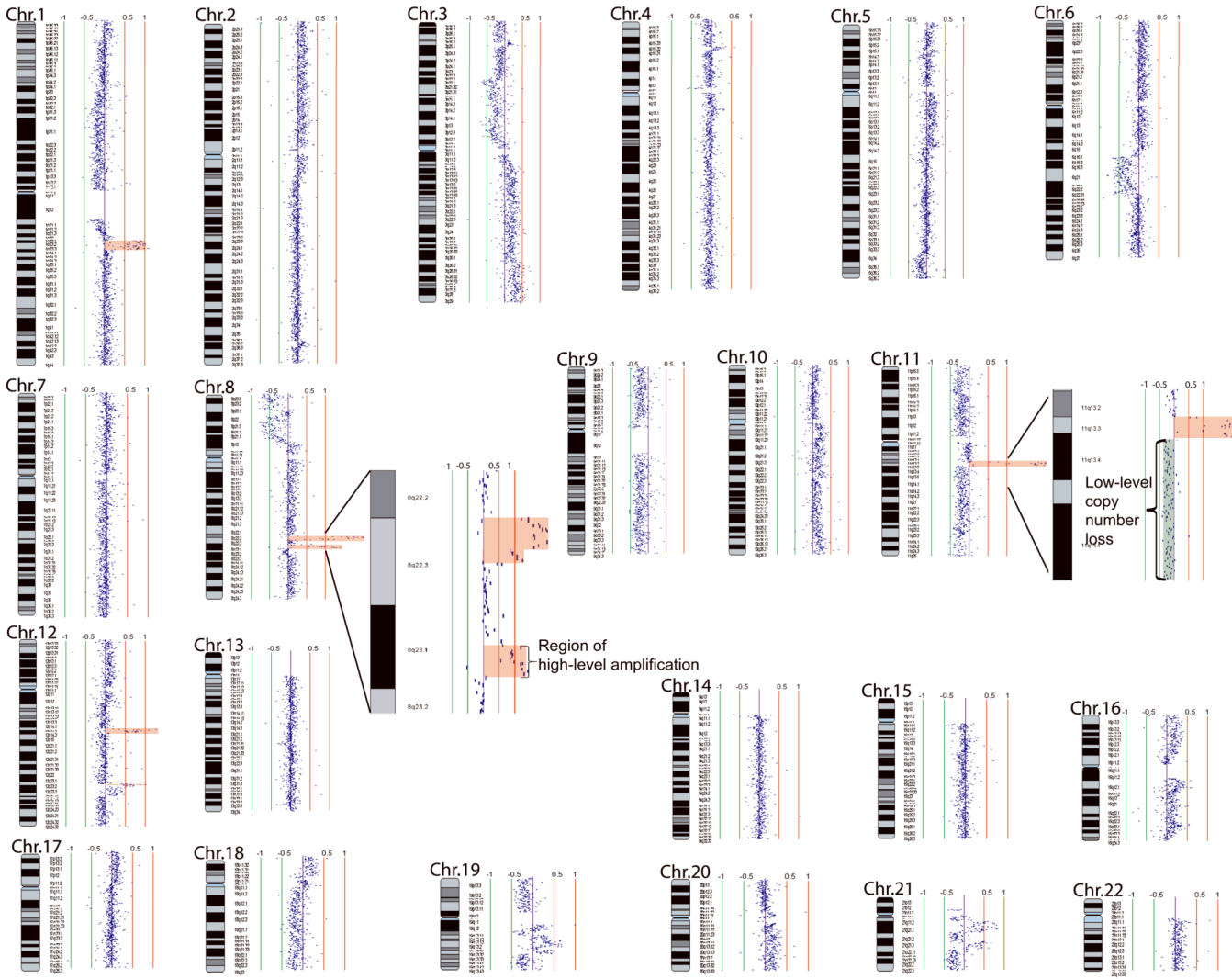
Figure 1.
Whole genome tiling-path array profile of a carcinoma *in situ* (CIS) Oral42. Each data point represents one BAC-derived segment on the array. The log$_2$ signal intensity ratios of a competitive hybridization with pooled male genomic DNA are plotted by *SeeGH* software.[34] The red and green bar lines are positive and negative log$_2$ signal intensity ratio lines scaled by an increment of 0.5. Data points to the left and right of the centre line represent DNA copy number losses and gains, respectively. Specifically, seven regions of gene amplification on 1q23.2-q23.3, 1q23.3-q24.1, 8q22.2-q22.3, 8q23.1, 11q13.3-q13.4, 12q14.3, and 12q23.2-q23.3 are shaded red. Magnified views of the two amplicons on chromosome 8, and a region of low-level copy number loss and a region of gene amplification on chromosome 11 are shown in two insets.
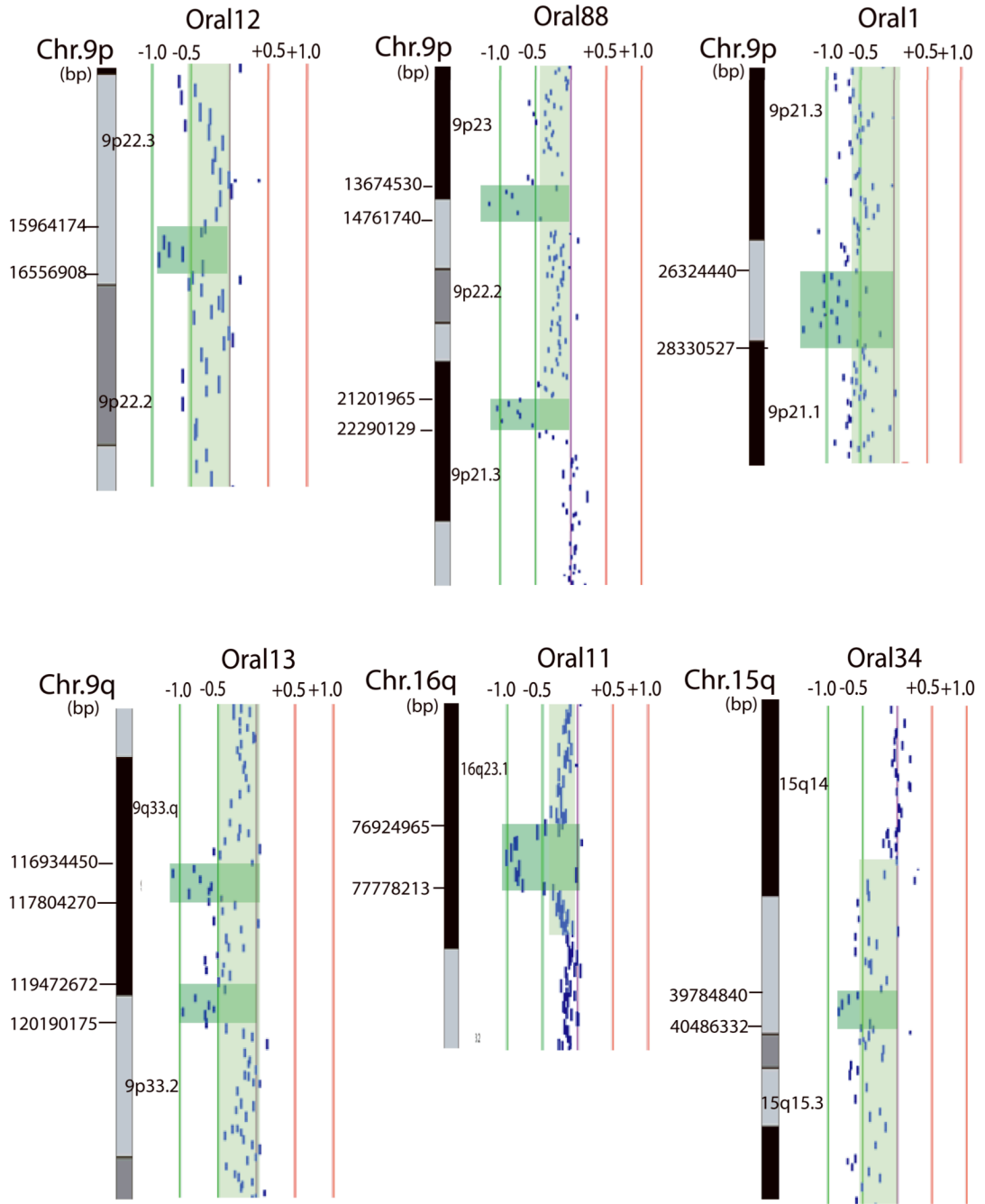
Figure 2.
Graphical representations of region of homozygous deletion in oral lesions. Each data point
represents one BAC-derived segment on the array. The detected region of homozygous deletion
in each sample is shaded in dark green, whereas single copy loss is shaded in pale green on the
corresponding data points. The red and green lines are positive and negative ratio lines scaled
by an increment of $\log_2$ signal ratios of 0.5.
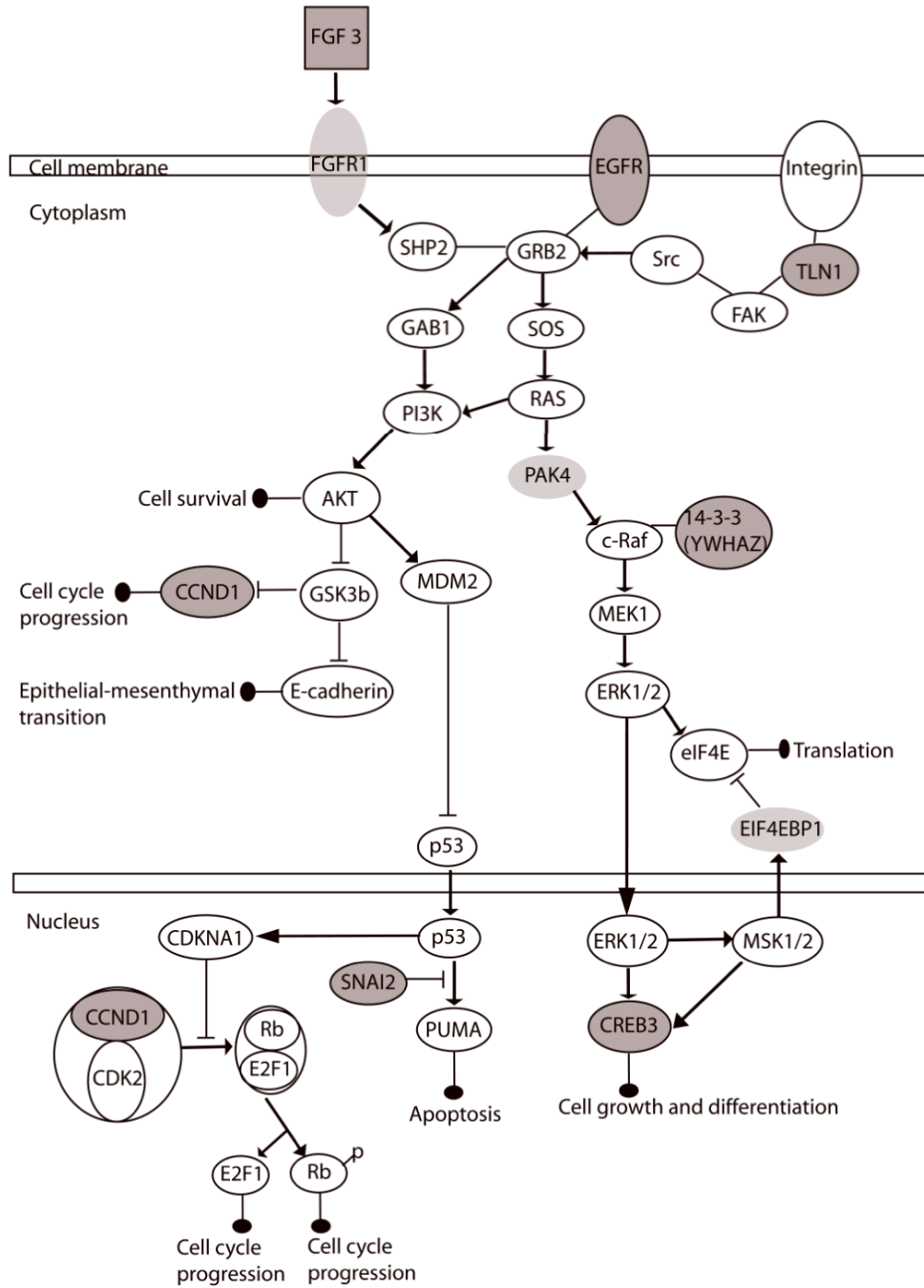
Figure 3.
Multiple disruptions in a single network driven by the mechanism of gene amplification. The top significantly deregulated canonical pathways in oral dysplasias are the ERK/MAPK, FGF, p53, PTEN, and PI3K/AKT signaling pathways, which share common nodes and interplay as a single network. Genes colored in grey with outlined circle were recurrently deregulated by gene amplification in oral dysplasias and significantly overexpressed in independent head and neck cancer datasets, whereas those colored in light grey were found to be amplified only in one preinvasive lesion. Altogether, 25 oral lesions (one progressing low-grade lesions, 14 high-grade dysplasias, and ten OSCCs) exhibited high-level gene amplification of different genes

inside this network, contributing to a disruption of 34.2% of all the progressing low-grade dysplasias, high-grade dysplasias, and OSCCs (N = 73).
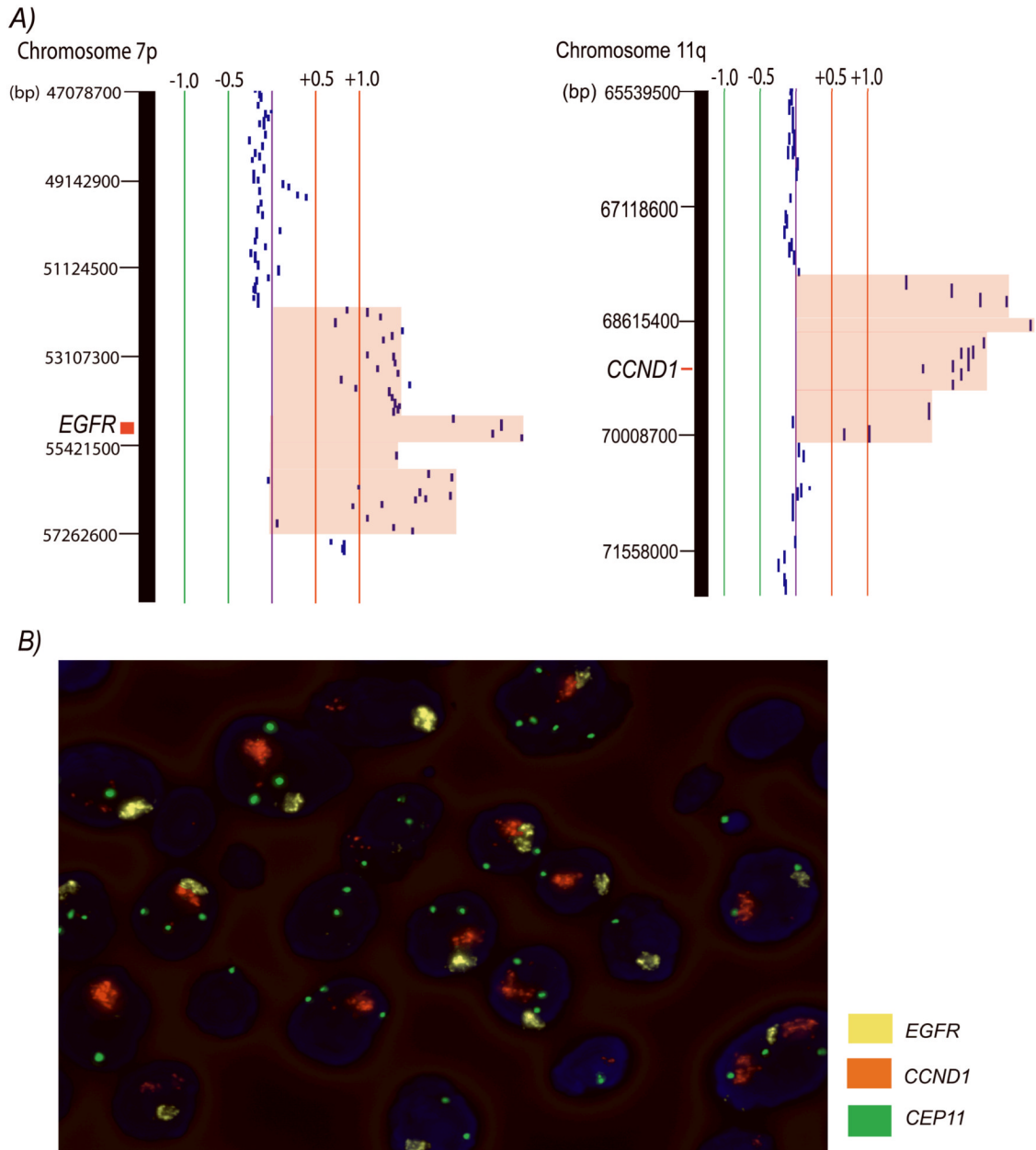
Figure 4.
Co-amplification of *EGFR* and *CCND1* in high-grade dysplasia Oral22. *A)* The left *SeeGH* profile represents amplification at 7p11.2 (*EGFR* locus), whereas the right profile represents amplification at 11q13.3 (*CCND1* locus) of sample Oral22. The red and green lines are positive and negative ratio lines scaled by an increment of $\log_2$ signal ratios of 0.5. Amplified region was shaded in red. *B)* FISH visualization of co-amplification of *EGFR* and *CCND1* in single dysplastic cells of Oral22. Sequential FISH was performed with probes mapping to *CCND1*, *EGFR*, and the centromeric region of chromosome 11, respectively displayed as orange, yellow, and green signal (original magnification, 1000x). Note the large clusters of amplification signals of *CCND1* and *EGFR* relative to the centromeric region of chromosomes 11 in the same nucleus.

Table 1

Regions of homozygous deletion.

Sample ID	Chromosomal band	Proximal flanking clone[*]	Start bp coordinate	Distal flanking clone[*]	End bp coordinate	Size (Mbp)	Number of genes	Known tumor suppressors
Oral88	9p22.3-p23	692G11	13674530	55P10	14761740	1.09	4	-
Oral12	9p22.3	97O17	15964174	554H2	16556908	0.59	1	-
Oral88	9p21.3	380P16	21201965	145H12	22290129	1.09	14	CDKN2A, CDN2B, MTAP
Oral1	9p21.1-p21.2	802E2	26324440	133E6	28330527	2.01	10	-
Oral13	9q33.1	235P13	116934450	121P18	117804270	0.87	2	-
Oral13	9q33.1-q33.2	57K1	119472672	374B16	120190175	0.72	0	-
Oral34	15q15.1	723A20	39784840	468N2	40486332	0.70	13	-
Oral11	16q23.1	730M21	76924965	556H2	77778213	0.85	1	WWOX

[*]
All the listed human BAC clones were selected from the RPCI-11 library.

Table 2

Recurrent regions of gene amplification in OPLs. Minimal altered regions recurrent in at least two high-risk OPLs are listed.

Chromosome	Proximal flanking clone*	Start (bp)	Distal flanking clone*	End (bp)	Size (Mbp)	Frequency of amplification in OPLs (N = 50)	Frequency of amplification in OSCCs (N = 23)	Candidate Genes
2q11.2	793A22	96361719	61O17	97032268	0.67	4%	0	CIAO1
4q12	273B19	55487333	345F18	56688451	1.20	4%	0	KDR
7p11.2	164O17	54583596	708P5	55194026	0.61	8%	23.1%	EGFR
8q11.21	770E5	49653988	259D18	50105411	0.45	4%	0	SNAI2
8q22.3	302J23	102005665	375I14	102513409	0.51	6%	4.35%	YWHAZ
9p13.3	121D5	33990614	312A20	36034564	2.04	4%	0	CCL19, CCL21, CCL27, DCTN3, OPRS1, TLN1, CREB3
11q13.2-q13.4	715N9	67947069	CTD-2011L1 3	70204378	2.26	14%	26.1%	CCND1, FGF3, FGF19, GAL, FGF4

*
Unless otherwise stated, all the listed human BAC clones were selected from the RPCI-11 library.

Table 3

Cancer-related genes mapped within recurrent regions of amplicon in high-risk OPLs.

Gene Name	EntrezGene ID for human	Chromosome band
RNF103	7844	2p11.2
KDR	3791	4q11-q12
EGFR	1956	7p12
TACC1	6867	8p11
LSM1	27257	8p11.2
STAR	6770	8p11.2
WHSC1L1	54904	8p11.2
ADAM9	8754	8p11.23
FGFR1	2260	8p11.2-p11.1
EIF4EBP1	1978	8p12
SNAI2	6591	8q11
CCL19	6363	9p13
CCL21	6366	9p13
CCL27	10850	9p13
CREB3	10488	9p13.3
OPRS1	10280	9p13.3
CA9	768	9p13-p12
RECK	8434	9p13-p12
CCND1	595	11q13
FGF3	2248	11q13
FGF19	9965	11q13.1
GAL	51083	11q13.2-q13.4
FGF4	2249	11q13.3

Table 4

Disruption of canonical signaling pathways in oral premalignant lesions.

Canonical pathways identified	Overexpressed genes in head and neck SCC	*p*-values
ERK/MAPK Signaling	CREB3, TLN1, YWHAZ	8.95E-03
FGF Signaling	CREB3, FGF3	1.63E-02
p53 Signaling	CCND1, SNAI2	1.96E-02
PTEN Signaling	CCND1, EGFR	1.96E-02
PI3K/AKT pathway	CCND1, YWHAZ	3.18E-02