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ORIGINAL PAPER

Her-2 Immunohistochemical Expression in Oral Squamous Cell Carcinomas is Associated with Polysomy of Chromosome 17, Not *Her-2* Amplification

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Abstract Based on the prognostic role of *Her-2* amplification and protein overexpression in breast cancer, various studies have been performed in oral squamous cell carcinomas (OSCC) with inconsistent results. As in invasive breast carcinomas Her-2 overexpression has been related to an increased number of chromosome 17 copies, a common chromosomal alteration in OSCC, we evaluated the association between polysomy 17 and Her-2 protein expression in a series of primary OSCC. Forty-one incisional biopsies of primary OSCC were included in the study. Protein expression was evaluated immunochistochemically with CB11 mouse monoclonal anti-human antibody. The reaction was arbitrarily characterized as absent, faint, moderate, and strong, and staining pattern as cytoplasmic and membranous. Positive cases were analyzed by chromogenic in situ hybridisation (CISH) to access *Her-2* status. The association between polysomy 17 and Her-2 expression was checked by Fisher's exact test. Four cases were negative and 37 cases were positive for Her-2. Staining was faint in 15 cases and moderate in 22 cases. CISH showed that all cases with faint staining were diploid, while from the cases with moderate staining 10 were diploid and 12 polysomic for chromosome 17. Thirteen cases showed purely cytoplasmic staining, while in 24 there were areas of both cytoplasmic and membranous staining. There was a statistically significant correlation between intensity of the reaction and polysomy 17 ($P = 0.0036$), in particular for cases with both cytoplasmic and membranous staining ($P = 0.0128$). In some OSCC Her-2 immunohistochemical expression may be associated with chromosome 17 polysomy and not *Her-2* amplification.

Keywords Her-2 Gene · Genetic abnormalities · Oral squamous cell carcinoma · Immunohistochemistry · Hybridization in situ

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Introduction

Human epidermal growth factor Receptor 2 proto-oncogene (*HER-2*, also known as c-*erbB2*, *ERBB2*) is the human homologue of the rat neuroglioblastoma oncogene (*neu*). *Her-2/neu* belongs to the *HER* gene family that regulates cell growth, survival, differentiation and migration [1, 2]. It is located on chromosome 17 (q12–q21) and encodes an 185 kD transmembrane protein with intrinsic tyrosine kinase activity that mediates the signal transduction pathway [1]. Her-2 is an orphan receptor, as no specific, high-affinity ligand to the extracellular domain has been identified [3]. It is hypothesized that its extracellular

domain dimerises with other Her receptors upon ligand binding to them, probably resulting in inter-receptor activation and synergetic signal transduction [4].

Her-2/neu gene amplification and protein overexpression is one of the most common genetic alterations in invasive breast carcinomas, associated with poor prognosis and response of the tumor to the Her-2 monoclonal antibody trastuzumab [5]. Correlations of Her-2 to unfavorable prognosis have been found in a diverse array of human malignancies, including gliomas, and carcinomas of the ovaries, lung, colon, bladder, endometrium, pancreas, stomach, and salivary glands [3].

Her-2 expression in oral squamous cell carcinoma (OSCC) has been usually studied within the heterogenous group of head and neck carcinomas (HNSCC). Numerous immunohistochemical studies have shown protein expression from 2.5 to 88% of the cases examined [6–24], a variation attributed to differences in the methodology utilized, i.e. tissue fixation procedure, antibodies sensitivity, and scoring criteria [24–26]. Correlation of Her-2 expression with prognostic clinicopathologic parameters remains inconclusive. Positive correlations have been found with variables, such as stage, metastasis, or overall survival in some [8–10, 14, 20, 22, 27–29], but not all of the studies [6, 11, 13, 14, 17, 19, 24], while simultaneous expression of multiple ERBB receptors has been suggested as a better indicator of decreased survival [8, 10]. Loss of Her-2 immunostaining has been considered as an indicator of neoplastic transformation potential in premalignant lesions [18, 30–32], although there are reports to the contrary [32, 33].

Studies of *Her-2* amplification in OSCC/HNSCC are limited and usually show Her-2 expression in the absence of *Her-2* amplification [7, 15, 17, 24, 34–38]. In invasive breast carcinomas, where in approximately 95% of the cases overexpression of Her-2 protein results from *Her-2* amplification [39], overexpression in non-amplified tumors has been related to an increased number of chromosome 17 copies, i.e. polysomy 17 [40–42]. It has been suggested that this genetic aberration may result in a significant increase of *Her-2* gene copies in the tumor cells and an increased Her-2 protein production to the level that could be demonstrated by immunohistochemistry as overexpressed [5, 42].

The role of chromosome 17 polysomy in Her-2 expression, in the absence of *Her-2* amplification, has not been specifically addressed in OSCC, although chromosome 17 polysomy is a common chromosomal alteration in those tumors [43, 44]. In the present study we evaluated the association between polysomy 17 and Her-2 protein expression in a series of primary OSCC, utilizing immunohistochemistry and chromogenic in situ hybridization (CISH), the most commonly applied methods for evaluating *Her-2* status in diagnostic pathology.

Materials and Methods

Specimens

This is a retrospective analysis of 41 non-consecutive patients diagnosed with primary OSCC at the Department of Oral Pathology and Surgery. All cases represent incisional biopsies that were fixed in 10% buffered formalin and embedded in paraffin wax (FFPE). Age, sex and site of the tumor were obtained from patients' files and pathologic reports. Twenty patients were male (age range 43–91 years, average 61.72 years) and 21 female (age range 40–88 years, average 62.56 years). Most lesions were located on the tongue ($n = 18$), followed by the buccal mucosa ($n = 7$), gingiva ($n = 7$), lower lip mucosa ($n = 4$), floor of mouth ($n = 3$), and palate ($n = 1$). History of smoking and alcohol consumption were not available. Tumors were graded by the WHO classification of histological differentiation into well ($n = 20$), moderately ($n = 16$), and poorly ($n = 5$) differentiated. Due to the nature of our material (incisional biopsies) stage and survival data were not available.

Immunohistochemistry

Immunohistochemistry was performed on 4–5 μm-thick FFPE tissue sections with the mouse monoclonal anti-human antibody CB11 (NCL-L-CB11, Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK), diluted at 1:80 in TBS, following the EnVision protocol (DAKO, Glostrup, Denmark) in the Ventana Benchmark^{XT} automated slide staining system (Ventana Medical Systems, AZ, USA). CB11 reacts with the internal domain of *HER2* oncoprotein. Reaction was assessed as follows: absent (no staining is observed or focal staining is observed in less than 10% of the tumor cells), faint (a faint/barely perceptible staining is detected in more than 10% of the tumor cells), moderate (a weak to moderate complete staining is observed in more than 10% of the tumor cells), and strong (a strong complete staining is observed in more than 10% of the tumor cells), and staining pattern as cytoplasmic and membranous. The scoring system follows the rational of the HercepTest^{TM} (Dako A/S, Glostrup, Denmark) that is routinely applied for the diagnostic assessment of breast carcinomas, but evaluates only membrane staining. In accordance to this system, only cases with strong reaction were considered as overexpressing Her-2. Omission of the primary antibody served as the negative control and a Her-2 3+ breast carcinoma as the positive control.

CISH Analysis

CISH analysis was performed with the Zymed SPoT-Light® HER-2 CISH Kit (Zymed Laboratories, CA, USA) on FFPE

tissue sections of CB11 positive cases, according to the manufacturer's instructions. The kit contains a digoxigenin labeled probe that specifically binds to the *Her-2* gene locus on chromosome 17q12–21. FFPE positive control slides were included in the kit. Briefly, the slides were deparaffinized in xylene, soaked in 100% ethanol, and washed in dH_2O. Then they were heated at 98°C for 15 min in Heat Pretreatment Solution (Zymed Laboratories), enzyme digested for 15 min and dehydrated in graded ethanol series. 15 μl of HER-2 probe were applied to each slide that was covered with coverslips. Denaturation and hybridisation were performed using a PCR machine, set at 95°C for 5 min for denaturation and at 37°C for 12 h for hybridization. The slides were subsequently washed in SCC RT/75°C for 5 min. After immunodetection, the slides were counterstained with hematoxylin.

CISH staining was evaluated according to Zymed SPoT-Light® HER-2 CISH Kit manufacturer's instructions. Specifically, specimens with 1–2 HER-2 signals per nucleus in >50% of tumor cells, in the chosen area for enumeration, were considered to be normal diploid; 3–5 signals in >50% of tumor cells were indicative of polysomy for chromosome 17; and ≥6 signals in >50% of tumor cells were scored as *Her-2* amplification.

Statistical Analysis

The association between polysomy 17 and Her-2 overexpression was checked by Fisher's exact test.

Results

In 4 out of 41 tumors (9.75%) Her-2 immunostaining was characterized as absent. Of the 37 positive cases, 15 (40.54%) showed faint staining (Fig. 1a–c) and 22 (59.45%) moderate staining (Fig. 1d–f). No case with strong staining (Her-2 overexpression) was seen. Cases with moderate staining usually showed more positive cells than cases with faint staining. CISH showed that all cases with faint staining were diploid, while from the cases with moderate staining 10 were diploid and 12 polysomic for

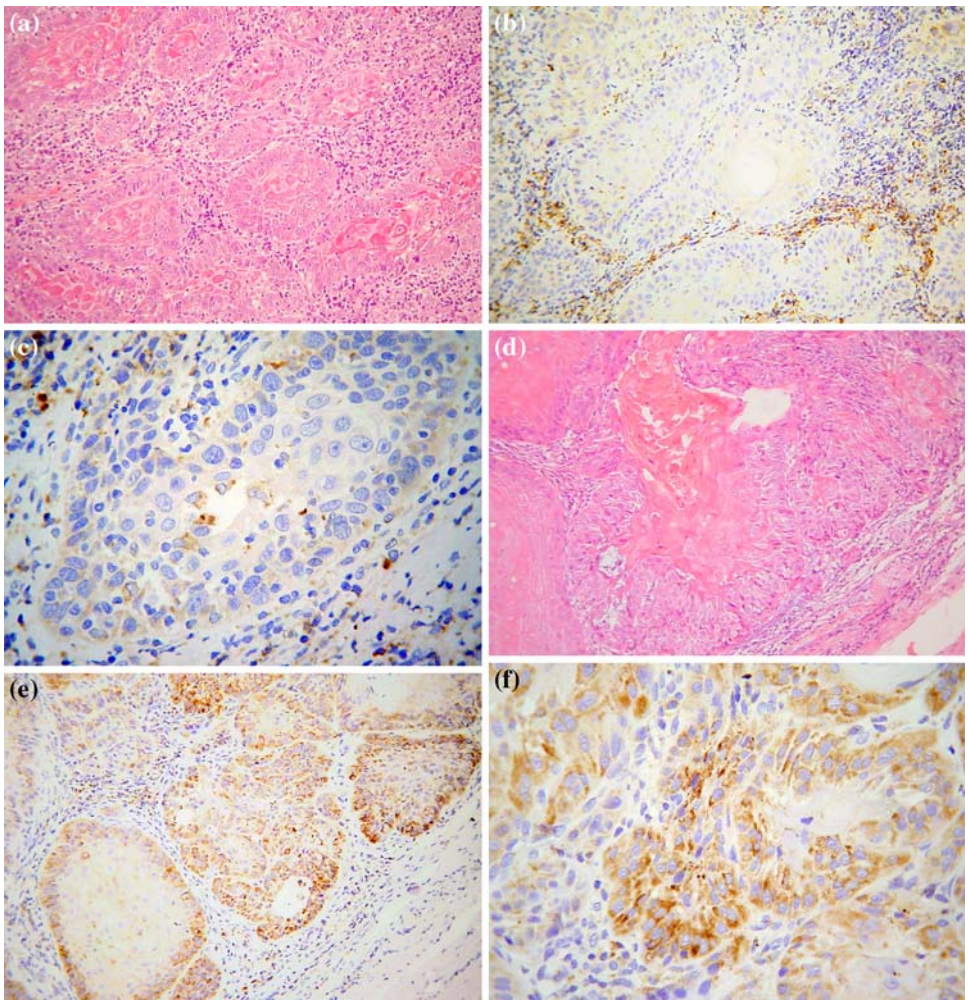

Fig. 1 Representative immunohistochemical staining for the expression of HER-2 with CB-11 in a well-differentiated OSCCs: **a** H&E, **b**, **c** Her-2 faint staining, **d** H&E, **e**, **f** Her-2 moderate staining (**a**, **b**, **d**, **e**: original magnification ×200; **c**, **f**: original magnification ×400)

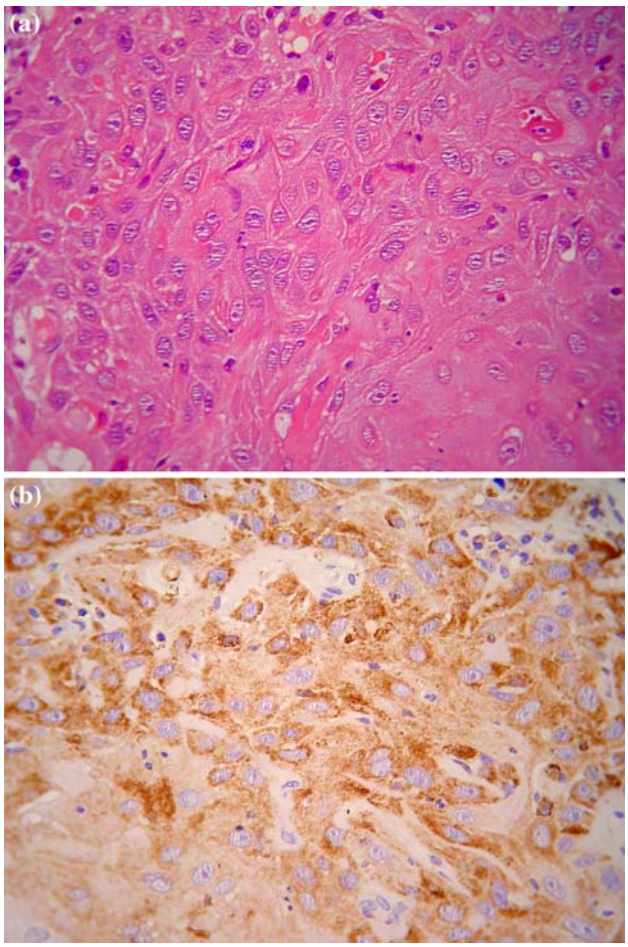

Fig. 2 Moderate cytoplasmic staining pattern in a well-differentiated OSCC: **a** H&E and **b** HER-2 staining with CB-11 (original magnification ×400)

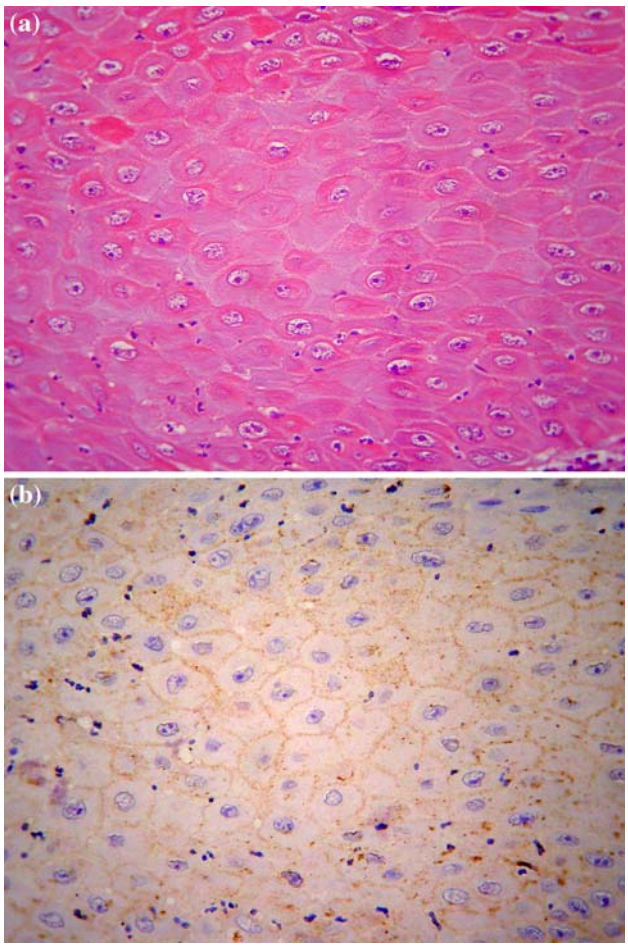

Fig. 3 Membranous staining pattern in a well-differentiated OSCC: **a** H&E and **b** HER-2 staining with CB-11 (original magnification ×400)

chromosome 17. No case with Her-2 amplification was found.

Considering the staining pattern, 13 (31.13%) showed purely cytoplasmic staining (Fig. 2), while in 24 (64.86%) there were areas of both cytoplasmic and membranous staining (Fig. 3). From the 13 cases with purely cytoplasmic expression, three with faint staining were normal diploid, 2 with moderate staining were polysomic and 8 with moderate staining were normal diploid. Regarding the 24 cases with both cytoplasmic and membranous staining, 8 with faint staining were normal diploid, 10 with moderate staining were polysomic for chromosome 17 (Fig. 4), and 6 with moderate staining were normal diploid (Fig. 5).

In the adjacent epithelium, usually showing varying degrees of epithelial dysplasia, faint and diffuse cytoplasmic reaction was seen in the prickle layer of positive cases.

As is shown in Table 1, there is a statistically significant correlation between intensity of the reaction and polysomy 17 ($P = 0.0036$), in particular for cases with both cytoplasmic and membranous staining ($P = 0.0064$). No statistical significant correlation was found between the sex and age of the patient, or the location and grade of the

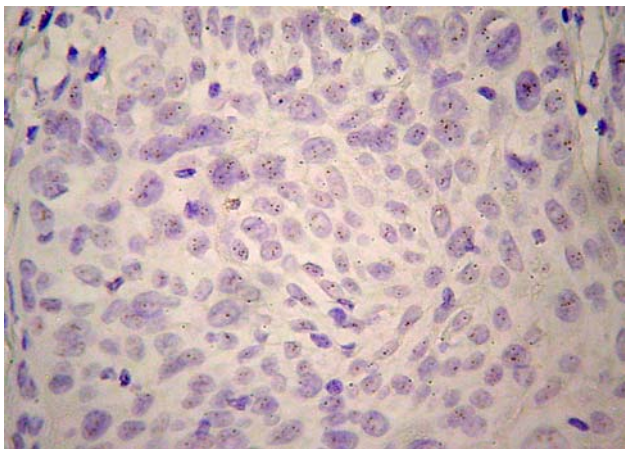

Fig. 4 CISH polysomic nuclei in a moderately stained Her-2 OSCC (Zymed SPoT-Light® HER-2 CISH Kit, original magnification ×400)

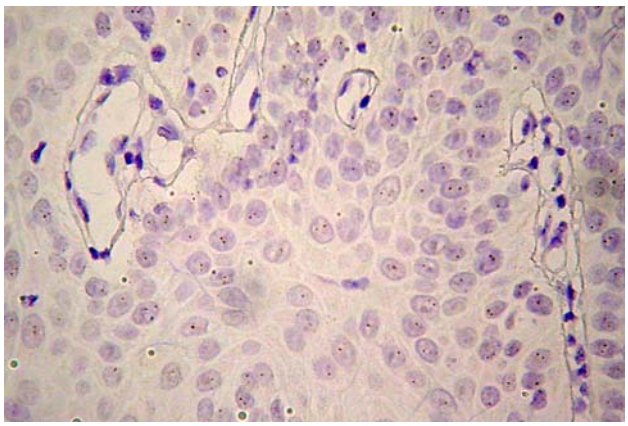

Fig. 5 CISH diploid nuclei in a faintly stained Her-2 OSCC (Zymed SPoT-Light® HER-2 CISH Kit, original magnification ×400)

tumor, and the immunohistochemical score or CISH ploidy status.

Discussion

In the present study Her-2 expression was assessed with the CB11 mouse monoclonal antibody that is highly sensitive [25], but produces significant cytoplasmic staining [24]. The importance of cytoplasmic staining and whether it may be evaluated when determining Her-2 expression in OSCC/HNSCC is controversial [24]. In various studies purely membranous [6, 12–14] or cytoplasmic [6, 11, 13, 24, 45], and mixed membranous-cytoplasmic [6, 8–10, 21, 23, 46] expression have been reported, while mixed positivity has been shown in adult oral epithelium and dysplastic lesions [23]. Some authors suggest that cytoplasmic staining is an artifact or non-specific finding due to antibody cross-reactivity [6, 47], possibly with keratin [46]. Others, however, propose that it may represent true protein overexpression [8, 48], probably due to incomplete receptor degradation [9]. The staining pattern and intensity considered as positive may account for discrepancies in the percentage of Her-2 positive cases reported in various studies [24]. In our study, membranous staining was always associated with cytoplasmic staining. Obviously, as long as the biological basis and prognostic significance of cytoplasmic staining in OSCC has not been settled, discrimination among the different staining patterns, as applied in this study, would be useful.

This study is the first to apply CISH on OSCC. CISH is a reliable alternative to FISH for assessing *Her-2* gene amplification when immunohistochemistry shows Her-2 protein overexpression, especially in cases with weak expression [49–51]. Studies have shown that there is a 96–100% concordance between CISH and FISH, the gold standard for the assessment of ERBB2 status in breast cancer [49, 52]. Apart from being faster and cheaper than FISH, CISH achieves stable signal intensity over time, and tissue and cellular features can be directly correlated with gene status.

Her-2 amplification was reported by Beckhardt et al. [7] in 2 out of 11 cell lines of HNSCC with blotting techniques, and by O-Charoenrat et al. [53] in none of 15 cell lines of OSCC with PCR. No *Her-2* amplification in HNSCC was found by Kearsley et al. [46] in 46 cases (24 OSCC) by Southern blot, and Leonard et al. [36] in 66 cases and Rodrigo et al. [35] in 59 cases by PCR. Willmore-Payne et al. [37] did not find *Her-2* activating mutations in 24 cases by high-resolution melting amplicon analysis (HRMAA) and direct DNA sequencing. Utilizing FISH, *Her-2* amplification was found by Khan et al. [15] in 4 (5.2%) out of 77 cases of oropharyngeal SCC; by Freier et al. [54] in 8 (3.8%) out of 213 OSCC; and by Angiero et al. [24] in 1(2.5%) out of 40 tongue SCC. In contrast, Scheer et al. [17] reported Her-2 amplification in 14 (33.3%) out of 42 OSCC; 3 cases showed amplification without overexpression. We did not find *Her-2* amplification in 37 OSCC, but it should be noticed that CISH was applied only in CB11 positive cases, when this antibody may not select all *Her-2* CISH amplified tumors [25].

Thus, in the present study, a significant percentage of OSCC showed weak or moderate Her-2 expression without CISH *Her-2* amplification. In breast carcinomas, lack of amplification has been found in 3–15% of cases overexpressing Her-2, and this has been attributed to transcriptional or posttranslational activation of Her-2, artifactual high sensitivity of immunohistochemical assays,

Table 1 Staining intensity by immunohistochemistry and CISH chromosome 17 ploidy status in 37 Her-2 positive cases of oral squamous cell carcinomas

	Cytoplasmic and membarnous			Purely cytoplasmic			Both staining patterns		
	Faint	Moderate	Total	Faint	Moderate	Total	Faint	Moderate	Total
Diploid	8	6	14	7	4	11	15	10	25
Polysomic	0	10	10	0	2	2	0	12	12
Total	8	16	24	7	6	13	15	22	37
	$p = 0.006429$			$p = 0.192308$			$p = 0.0036$		

single copy overexpression of the HER-2 gene at the messenger RNA (mRNA) transcription level, or gene amplification below the level of detection of FISH assays [26]. A role for chromosome 17 polysomy has also been investigated. Lal et al. [42], Bose et al. [55], and Salido et al. [5] found that in non-amplified breast tumors polysomy 17 was associated with immunohistochemical expression of HER-2 protein, in particular borderline to weak expression. In contrast, Varshney et al. [26] reported that in cases without gene amplification chromosome 17 polysomy is not associated with weak expression of HER-2, but has a role in moderate (weakly positive or equivocal) protein expression without amplification. Sauer et al. [52] concluded that an abnormal number of chromosome 17 copies has a low impact on *Her-2* and its expression. The discordant results may be partly explained by the different criteria applied for the definition of chromosome 17 polysomy [26]. However, it seems than in none-amplified breast carcinomas cases, expression of Her-2 may be associated with polysomy 17 and those cases seem to behave more similarly to Her-2 negative tumors [5, 42].

Polysomy 17 seems to be a common chromosomal aberration in oral carcinogenesis [44], but the association of Her-2 expression with polysomy 17 has not been previously studied in OSCC. Khan et al. [15] reported 7 cases with polysomy 17 by FISH in 77 oral and oropharyngeal SCC. Four cases were strongly positive, 2 faintly positive, and 1 negative, but with intense cytoplasmic staining. We found a statistically significant correlation between polysomy for chromosome 17 and moderate Her-2 expression in cases with both cytoplasmic and membranous staining pattern, but not in those with purely cytoplasmic pattern. On the other hand, it has been shown that in OSCC polysomy 17 correlates with p53 mutation [43] or overexpression [44], while loss of normal p53 function play a potential role in multistep tumorigenesis in OSCC [43, 56, 57] Thus, the association between Her-2 expression and *p53* alterations would be interesting to be investigated.

We conclude that in some OSCC Her-2 immunohistochemical expression, in particular moderate expression, may be associated with chromosome 17 polysomy and not *Her-2* amplification. As polysomy 17 seems to correlate with oral carcinogenesis [43, 44] and *Her-2* amplification is rather uncommon in OSCC, Her-2 expression in previous studies may not reflect true amplification and this may account for the discrepant results considering prognosis. Utilization of more strict and consistent methodology in studies of OSCC, e.g. definition of positive staining, scoring according to the ASCO/CAP system [58] and application of FISH/CISH, is necessary for determining the role of Her-2 expression in OSCC and its association with polysomy 17.

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