Detection of ERBB2 (Her2/neu) and P16 (INK4A) genes in oral squamous cell carcinoma using fluorescent in situ hybridization (FISH)

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ABSTRACT

Background Head and neck cancers account for approximately 5% of all carcinomas in industrialized countries, with a worldwide incidence of 500,000 new cases annually. Nearly all head and neck cancers (90%) are squamous cell carcinomas (SCCs), and >50% of tumors arise in the oral cavity. It is important to know what prognostic factors can facilitate diagnosis, optimize therapeutic decisions, and improve the survival of these patients. A member of the epidermal growth factor receptor (EGFR) family, HER2/neu, has received much attention because of its therapeutic implications. The p16 gene produces P16 protein, which in turn inhibits phosphorylation of Rb, thus inhibiting the Rb-induced release of transcription factor EF1 and cell cycle progression. Genetic aberration analyzed by fluorescence (FISH) to measure the gene copy number. The aims of the present study are to detect HER2/neu amplification and P16 deletion in oral squamous cell carcinoma and correlate them with various clinicopathological parameters (age, sex, clinical presentation, tumor site, tumor stage, tumor grade).

Materials and Methods Thirty formalin-fixed paraffin embedded tissue blocks of oral squamous cell carcinoma which were collected from laboratories archives included in this study. H&E stain was done for each block for reassessment of histological examination. DNA probes were used to detect copy numbers of the HER2/neu and P16 genes using fluorescent in situ hybridization technique (FISH).

Results FISH evaluation showed that HER2/neu gene amplification was found in 12 cases (40%), while 18 cases (60%) showed no amplification. Among the cases in which amplification was not found, 8 cases (44.45%) showed polysomy of chromosome 17.P16 gene deletion was found in 20 cases (66.7%) while 10 cases (33.3%) showed no deletion.

Conclusions: HER2/neu amplification and P16 deletion were observed in studied oral squamous cell carcinoma samples using FISH, however, statistically non significant correlation with all clinicopathological findings (age, sex, clinical presentation, tumor site, tumor stage, tumor grade) and also between both genes were found in the present study. It is premature to conclude that HER-2/*neu* and P16 alterations may have prognostic significance, but it is also too early to dismiss that possibility without a larger, perhaps multicenter study.

Key words: Oral squamous cell carcinoma using fluorescent. (J Bagh Coll Dentistry 2012; 24(4):46-51).

INTRODUCTION

Oral cancer is a major public health problem worldwide; it remains a highly lethal and disfiguring disease [(1)]. Squamous cell carcinoma is the most common intraoral malignancy which comprised about 4% of all malignancies in United States. This corresponds to an estimated 17 per 100,000 persons with newly diagnosed SCC of the head and neck per year [(2)].

Oral squamous cell carcinoma is the eleventh most common cancer world-wide, with an especially high incidence reported in Indian subcontinent, Australia, France, Brazil and southern Africa. The average annual incidence and mortality rates however, vary considerably between different races, genders, and age groups [(3)]. In Iraq oral cancer remains a highly lethal and disfiguring disease. Patient at their fifth decade of life were the most commonly affected with male to female ratio 2:1 [(4)].

Carcinogenesis is a multistep process at both phenotypic and genetic level [(5)]. Each characterized by the sequential stimulation of additional genetic defects, followed by clonal expansion.

The genetic alterations observed in head and neck cancer are mainly due to oncogen activation and tumor suppressor gene inactivation, leading to de-regulation of cell proliferation and death. These genetic alterations, include gene amplification and overexpression of oncogenes such as c-myc, HER2/neu, Epidermal Growth Factor Receptor (EGFR), cyclinD1 and mutations, deletions and hypermethylation leading to p16 and p53 tumor suppressor gene inactivation [(6)].

Human epidermal growth factor Receptor2 proto-oncogene (HER2/neu, also known as c-erbB2, ERBB2) belongs to the HER gene family that regulates cell growth, survival, differentiation and migration [(7,8)]. It is located on chromosome 17 and encodes an 185kD transmembrane protein with intrinsic tyrosine kinase activity that mediates the signal transduction pathway [(9)].

Cyclin-dependent kinase inhibitor 2A, (CDKN2A, $p16^{Ink4A}$) also known as multiple tumor suppressor1 (MTS-1), is a tumor

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suppressor protein, that in humans is encoded by the *CDKN2A* (P16) gene [10,11].

The p16 gene (located at 9p21) produces p16 protein, which in turn inhibits phosphorylation of Rb, thus inhibiting the Rb-induced release of transcription factor EF1 and cell cycle progression [12].

Fluorescent in situ hybridization (FISH) is a technique in which single-stranded nucleic acids (usually DNA, but RNA may also be used) are permitted to interact so that complexes, or hybrids, are formed by molecules with sufficiently similar, complementary sequences [13].

FISH has a large number of applications in molecular biology and medical science, including gene mapping, diagnosis of chromosomal abnormalities, and studies of cellular structure and function [13].

MATERIALS AND METHODS

Prospective and retrospective study performed on thirty formalin- fixed, paraffin-embedded blocks of oral squamous cell carcinoma (from 2003 to 2011) which were retrieved from the archives of Oral Pathology laboratory, College of Dentistry-Baghdad University and the histopathological laboratory in Al-shaheed Ghazi hospital for specialized surgeries. The clinicopathological information regarding age, sex, tumor sites, clinical presentation , tumor size , lymph node involvement (if present), were obtained from the case sheets presented with the tumor specimens. The normal tissue control was four samples for HER2/neu and ten samples for P16 of normal oral mucosa were obtained from patients who need surgical removal of impacted teeth,while the Positive tissue control which is a specimen shown by previous works and according to the manufacture data sheets,the positive tissue control in this study was lung carcinoma.

FISH

FISH analyses were performed using the KEREATECH HER2/neu and P16 DNA ProbeS kit. The Her2/neu 17q12 specific DNA prob is optimized to detect copy numbers of (Her2/neu) ERBB2 gene region at region 17q12. The probe is direct labeled with Platnium Bright 550 (red region). The chromosome 17 satellite enumeration (SE) probe is included to facilitate chromosome identification, the probe is direct labeled with Platnium Bright 495 (green region) The Her2/neu (17q12) specific DNA probe is designed as dual colour assay to detect ampification at 17q12. Amplification involving the Her2/neu gene region at (17q12) will show several red signals, while the control at the chromosome 17 centromere region will provide 2 signals. Two signal colored red and green will identify the normal chromosome 17. The P16 (9p21) specific DNA probe is optimized to detect copy number of the P16 (INK4A) gene region at region 9p21.The probe is direct labeled with Platnium Bright550 (red region), The 9q21 specific region probe is included to facilitate chromosome identification, the probe is direct labeled with Platnium Bright 495 (green region), [14].The P16 (9p21) probe is designed as a dual-color assay to detect deletions at 9p21. Deletions involving the P16 gene region at 9p21will show one red signals, while the control at the chromosome 9q12 region will provide 2 signals in hemizygous deletion. No red signal, but 2 green signals for 9q12 will be visible in homozygous deletions of 9p21. Tow signals color red and green signals will identify the normal chromosome 9. Quantification of different molecular markers in situ hybridization signal was evaluated under fluorescent microscopy at low power X40 and X100 (X4, X10, and X opjective and X10 eye piece respectively), whereas the counting of positive cells was performed at oil immersion (X1000). The cases were considered to be amplified when the average copy number ratio, HER2/CEN17, was $\geq$ 2.0 in all nuclei evaluated or when the HER2 signals formed a tight gene cluster. Among the cases in which the gene was not amplified, samples showing more than four copies of the HER2 gene and more than four CEN17 in more than 10% of the tumor cells were considered to be polysomic for chromosome 17[15]

For the identification of *p16* deletion, the average signals per nucleus for the chromosome 9 centromere and *p16* probes were calculated for 100 non overlapping nuclei from each tumor specimen. Similarly, dual-color FISH was performed on 10 samples of normal tissue as control. For each normal tissue sample, the average signal per nucleus for the chromosome 9 centromere and *p16* probes was calculated by counting 100 nuclei. To quantify for *p16* gene deletions in tumors, cutoffs were established by using mean ± 3 standard deviations (SD) from normal controls for the average signal ratios and the mean *p16* signal per nucleu s **(16, 17)** (Because of the fact that tissue sectioning results in the truncation of nuclear material and leads to an under-representation of chromosome and gene copy number). In this study cut off point was 23%.

Statistical Analysis

Descriptive statistics: Numerical values were used in this study for describing the variables which includes: No. mean, SD, minimum, maximum and percents.

Inferential statistics: Chi-square test used to test the association between clinical data with FISH results. Pearson correlation coefficient of correlation (r) was used to find the relation between two markers. P value from 2 sided tests was used to determine significance, with a P value < 0.05 indicating statistical significance.

RESULT

The study sample consisted of 20 males (66.7%) and 10 females (33.3%), with an age range between 25-75 years of age, with an average of (50.1) and male to female ratio was (2/1). Endophytic clinical presentation was the most predominant (53.34%).The majority of the cases (36.67%) were located in the tongue. Most of the cases (53.3%) appeared in stage IV, and moderately differentiated carcinoma was the most common histopathological type (63.34%).

FISH evalauation of HER2/neu amplification

All 30 cases of OSCC showed presence of gene signal.Gene amplification was found in 12 cases (40%), and 18 cases (60%) showed no amplification. (Among the cases in which amplification was not found, 8 cases (44.45%) had polysomy of chromosome 17). Table and fig

Table 1: HER2/neu Amplification

HER2/neu	No.	%
Amplification	12	40
Polysomy	8	26.67
Not amplified	10	33.34
Total	30	100

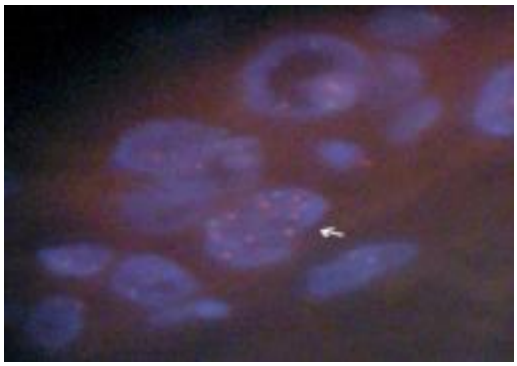

Figure 1: Positive HER2/neu amplification signal in well differentiated OSCC. (100X) Arrow: 2green dots and multiple red dots for the amplification.

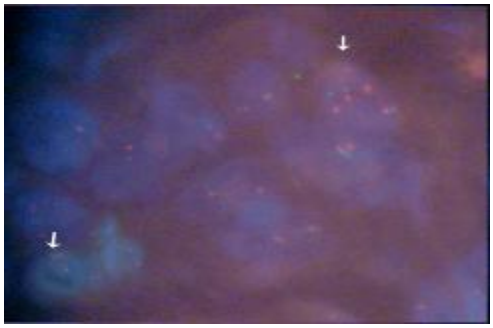

Figure 2: Positive HER2/neu amplification signal in moderately differentiated OSCC.(100X). Arrows: 2green dots and multiple red dots for the amplification

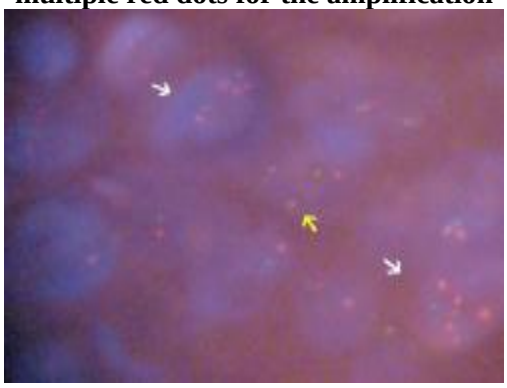

Figure 3: Positive HER2/neu amplification in poorly differentiated OSCC(100X) (white arrow +ve amplification[2green dots and multiple red dots],yellow arrow normal cell[2 green dots and 2 red dots]).

FISH Evalauation of P16 Deletion

All 30 cases of OSCC showed presence of gene signal. Gene deletion was found in 20 cases (66.7%) while 10 cases (33.3%) showed no deletion. Table 2 & fig (4,5,6)

Table 2: P16 Deletion.

P16 deletion	No.	%
+ve Deletion	20	66.7%
-ve Deletion	10	33.3%

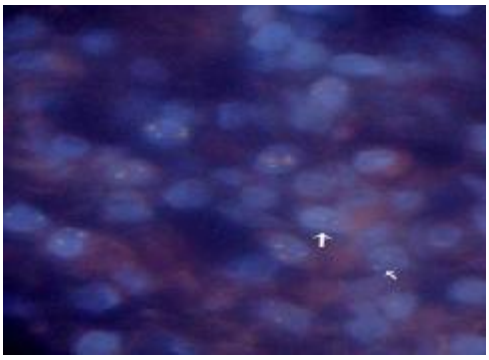

Figure 4: P16 deletion signal in well differentiated OSCC.(100X) Arrows:2green dots and 1 red dot (hemizygous deletion), 2 green dots without red dot (homozygous deletion)

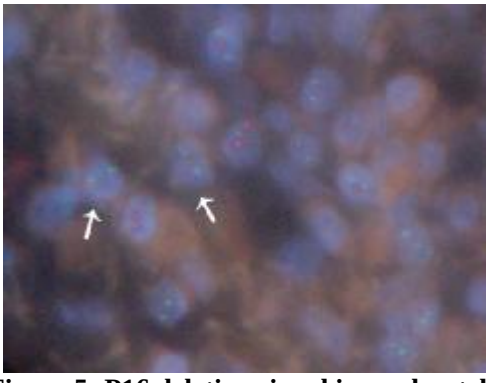

Figure 5: P16 deletion signal in moderately differentiated OSCC.(100X) Arrows:2green dots and 1 red dot (hemizygous deletion), 2 green dots without red dot (homozygous deletion)

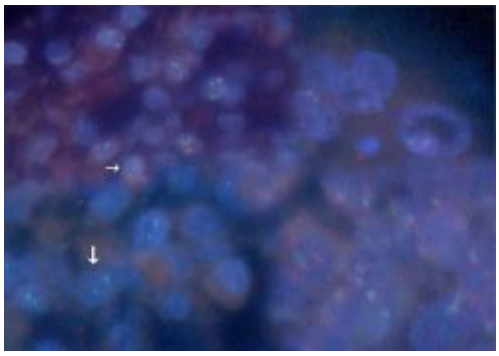

Figure 6: P16 deletion signal in poorly differentiated OSCC. .(100X) Arrows:2green dots and 1 red dot (hemizygous deletion), 2 green dots without red dot (homozygous deletion)

The results showed that there were no significant correlations among these genes and clinicopathological findings including tumor grade and stage and among each other. (Tables 3-7)

Table 3: Correlation between HER2/neu amplification and Clinical staging

HER2/neu Amplfication	Clinical staging				Total		P-value
	StageI	StageII	StageIII	StageIV	No.	%	
+ve Amplfication	1	2	4	5	12	40	0.184
-ve Amplfication	4	2	1	11	18	60	
Total	5	4	5	16	30	100	

Table 4: Correlation between HER/2neu amplification and tumor grade

HER2/neu Amplification	Tumor grade			Total		P-value
	WD.	MD.	Poor	No.	%	
+ve Ampilification	3	8	1	12	40	0.805
-ve Ampilification	4	11	3	18	60	
Total	7	19	4	30	100	

Table 5: Correlation between P16 Deletion and Clinical staging.

P16 Deletion	Clinical staging				Total		P-value
	StageI	StageII	StageIII	StageIV	No.	%	
+ve Deletion	4	3	4	9	12	40	0.636
-ve Deletion	1	1	1	7	18	60	
Total	5	4	5	16	30	100	

Table 6: Correlation between P16 Deletion and tumor grade.

P16 Deletion	Clinical			Total		P-value
	MD.	WD.	Poor	No.	%	
+ve Deletion	14	4	2	20	66.7	0.547
-ve Deletion	5	3	2	10	33.3	
Total	19	7	4	30	100	

Table 7: Correlation between HER2/neu amplification and P16 deletion.

HER2/neu Amplification	P16 deletion		Total		P-value
	+ve	-ve	No.	%	
+ve	9	2	12	40	0.122
-ve	11	7	18	60	
Total	20	9	30	100	

DISCUSSION

Assessment of HER2/neu Amplification

In this study, all 30 cases of OSCC showed presence of gene signal. The criterion for gene

amplification was a HER-2/*neu* /centromere 17 signal ratio of >2, which corrects for polysomy-related increases in gene copy [15]. Using this criterion, 12(40%) of the 30 cases analyzed by FISH were found to be HER-2/*neu* amplified, while 8 cases from the negative cases showed chromosome polysomy (more than four copies of the HER2 gene and more than four CEN17 in more than 10% of the tumor cells).Utilizing FISH, the reported frequencies of HER-2/neu amplification in OSCC vary from 2 to 33% of cases [18,19, 20].This variability could be due to differences in tissue preparation, probes, and the methods used to evaluate the alterations. In the present study gene amplification was demonstrated in 40% of cases, higher than any previously reported frequency and that could be attributed to small size of sample, random collection (different site of investigation), different scoring system and also the preservation type of paraffin embedded tissue in the labs.Successful FISH analysis depends on a pretreatment protocol that is consistent in the use of digesting agents (sodium thiocyanate, pepsin or proteinase K) as well as appropriate conditions (concentration, temperature and time of incubation) to unmask the target DNA and optimize hybridization.The current study showed no significant correlation between HER2/neu amplification and clinicopathological parameters. These finding are in line with [18, 15]

From the studying findings it was apparent that HER2/neu has no significant correlation with clinicopathological parameters which may be due to small samples and random collection.The small sample size of the current study is considered as a main factor for limitation of these data in determining diagnostic significance and clinical implication. Although the role of this oncogene in breast cancer is well known, still there is a controversy and confusion surrounding its role in HNSCC. It is premature to conclude that HER-2/*neu* alterations may have prognostic significance, but it is also too early to dismiss that possibility without a larger, perhaps multicenter study.

Assessment of P16 (INK14) deletion:

The current study tried to evaluate *p16* deletion using FISH in paraffin embedded biopsy samples from primary oral SCCs, all 30 cases of OSCC showed presence of gene signal, [considering lesions positive for loss of 9p21 if the percentage of conspicuous cells exceeded the threefold standard deviation of normal control tissue samples, that because of the fact that tissue sectioning results in the truncation of nuclear material and leads to an under-representation of chromosome and gene copy number [16,17] so the cut-off point for P16 deletion was 23%] and observed p16 deletion in 20 of 30 samples (66.7%).From reviewing literature it has been found that there are previous studies regarding OSCC in relation to p 16 except: Narikazu et al who used FISH to evaluate *p16* deletion on FNA samples of OSCCs and found that 22 out of 57 sample (38.6%) were positive for gene deletion, this deletion was correlated significantly with cellular differentiation and local and regional recurrence. The discrepancy between this study and Narikazu et al study may be attributed to the type of sample collected (FNA biopsy from fresh primary tumors vs paraffin-embedded tissues), and size of the sample and different scoring system.Namazie et al studied P16 gene deletion by FISH in head and neck cancer and detected *p16* deletion in 52% of their samples. This discrepancy may have been caused by differences in the primary cancers investigated (OSCCs vs. HNSCCs) and different scorring system. Moreover, Namazie et al suggested that there was a significant association between *p16* deletion and the development of distant metastasis, while this study didn't find any significant correlation with clinicopathological parameter, this difference may attributed to the difference in sample size and the primary tumor investigated. In contrast, under expression of p16 is correlated significantly with poor survival in patients with prostate cancer [22] and neuroblastoma [23]. In HNSCCs, including oral cancers, the correlation between p16 expression level and prognosis remains controversial [17, 24, 25]. Therefore, this issue remains a matter of controversy, and additional investigations are required to determine the clinical and prognostic significance of *p16* genetic and protein status in the carcinogenetic pathway of OSCC.

Correlation between HER2/neu amplification and P16 deletion:

The results of the current study showed that there was no significant correlation between the two genes. To the best of our knowledge, this is the first study in Iraq assessing the correlation between HER2/neu amplification and P16 deletion in OSCC. Since this is a pioneer research in assessing that correlation, it's difficult to establish a comparison with other studies, however conclusive remarks can be withdraw from other studies using p16 with other genetic markers. Narikazu et al found that it is possible to identify patients with a higher risk of recurrence, metastasis, and mortality by combined analysis of P16 deletion and the CCND1 genetic status

compared with the analysis of either marker alone.

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