

Fluorescent in Situ Hybridization Evaluation of Epidermal Growth Factor Receptor and Cyclin D1 Genes in Oral Squamous Cell Carcinoma

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ABSTRACT

BACKGROUND: Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the oral cavity, and one of the tenth most common causes of death. Although there are advances in both surgical and non-surgical treatments of oral cancer, the overall survival and mortality rates have not improved. Therefore, it is necessary to define molecular markers to allow early diagnosis, and to identify new therapeutic targets. Epidermal growth factor receptor (EGFR) is a transmembrane protein kinase which consists of extra and intracellular domains. Activation of the EGFR pathway promotes tumor cell proliferation, angiogenesis, metastasis and decreases apoptosis. Cyclin D1 (CCND1) regulates G1 to S phase transition of cell cycle. Its overexpression may lead to disturbance in the normal cell cycle control and tumor formation.

METHODS: Thirty formalin-fixed paraffin-embedded tissue blocks of OSCC were included in this study. Diagnostic confirmation was performed through an examination of hematoxylin and eosin (H&E) sections. Fluorescent in situ hybridization method was used to evaluate EGFR and CCND1 gene copy number.

RESULTS: Fluorescent in situ hybridization evaluation showed that the EGFR amplification was present in 70% of the cases while CCND1 amplification was present in 43% of the cases. Statistically there was no significant correlation with clinic pathological findings.

CONCLUSION: We did not find a statistically significant relationship between clinic pathological data and gene amplification.

Keywords: EGFR; CCND1; Oral Squamous Cell Carcinoma; Fluorescent in Situ Hybridization

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a malignant neoplasm of oral stratified squamous epithelium that is capable of locally destructive growth and distant metastasis [1]. It's the sixth most common malignancy, constitutes a major health problem, and is associated with severe morbidity and reduced survival. More than 300,000 new cases of OSCC are being diagnosed each year worldwide, but the poor prognosis of oral cancer has not improved significantly over the last four decades [2]. To improve the long-term survival of patients, early detection and diagnosis of disease with discovery of more accurate prognostic markers that can identify patients with a high risk of recurrence and that lead to more effective treatment is needed [3, 4].

Epidermal growth factor receptor (EGFR) is a transmembrane protein kinase which consists of extra and intracellular domains. The intracellular domain serves as the site of protein kinase activity and has important role in the cancer cell proliferation, apoptosis, angiogenesis and metastasis [5]. EGFR gene is located on chromosome 7 (7p11) and its product is a 170 kDa protein [6, 7]. EGFR expression and abnormal gene copy number is known to play an important role in the head and neck squamous cell carcinoma (HNSCC) development, radiation resistance and poor prognosis [8].

Cyclin D1 (CCND1) is an important regulator of cell cycle G1 to S-phase transition in numerous cell types from diverse tissues. CCND1 is located on chromosome 11 (11q13) in humans. This region is commonly amplified in several types of

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cancer including squamous cell carcinoma (SCC) [9]. In HNSCC, CCND1 amplification and overexpression are seen in approximately 20-40% and 40-80% of cases, respectively. These abnormalities have been associated with cancer development, progression, aggressiveness, poor prognosis and metastasis [10]. Fluorescence in situ hybridization (FISH) is a powerful technique that detects chromosomal changes in tumor cells and is often used in the study of structural cytology of the cell nucleus. It provides a reliable means for studying the genetic composition of cells in mitosis as well as in interphase. The technique has reached high detection sensitivity, (i.e. individual genes can be detected), and high multiplicity (i.e. several probes can be applied to the same nucleus) [11].

METHODS

This was a retrospective study which was performed on thirty formalin fixed paraffin embedded blocks of OSCC, collected from laboratory archives of oral and maxilla facial pathology department, College of Dentistry, Baghdad University and Histopathological laboratory, Surgical Specialties Hospital. Clinical staging, presentation and grading were obtained from medical records (2003- 2011). The normal mucosa was considered as normal control which was taken from an individual who needed surgical removal of impacted wisdom teeth while lung carcinoma samples were taken as positive control. This study was reviewed and approved by ethics review committee at the Iraqi Ministry of Health. All samples were processed using 4% neutral formalin for fixation, graded alcohol for dehydration, xylene for clearing and finally paraffin infiltration and sectioning. Each paraffin block had serial sections, one section for haematoxylin and eosin staining (H&E) and the other two sections for FISH detection. Histopathological examination was performed on all collected samples by two experimental pathologists to confirm the diagnosis.

Principle of FISH test identifies or labels target genomic sequence so that their location can be studied. The procedure was performed according to kit manufacture instruction taking the following steps: Paraffin embedded tissue sections of 4-6 μm thickness were mounted on positively charged slide and baked for 16 hours at 56 $^{\circ}\text{C}$, deparaffinized by soaking in xylene for 10 min, rehydrated by soaking in ethanol series 3 min for each and washed with distilled H_2O (dH_2O) for 3min at room temperature. Slides were then

pre-treated with 0.2 M HCl for 20 min followed by washing in dH_2O for 3min at room temperature. Slides were placed in 8% sodium thiocyanate in dH_2O at 80 $^{\circ}\text{C}$ for 30 min then rinsed in 2x saline sodium citrate for 3min at room temperature. Digestion was performed using 0.025% pepsin in 0.01 M HCl at 37 $^{\circ}\text{C}$ for 5-45 min. The slides were washed in dH_2O for 1 min and in 2 x saline sodium citrate for 5 min at room temperature followed by dehydration by soaking in ethanol series for 1 min each and air-dried. Ten μL of probe was applied per 22x22 mm field then covered with coverslip and sealed with fix gum. Sample and probe were denatured on a hot plate at 80 $^{\circ}\text{C}$ for 5 min and incubated overnight at 37 $^{\circ}\text{C}$ in humidified chamber. Coverslips were removed then washed in 1x post-wash buffer for 2 min at 72 $^{\circ}\text{C}$ ($\pm 1^{\circ}\text{C}$) followed by another wash-buffer for 1 min at room temperature (37 $^{\circ}\text{C}$) without agitation. Dehydration was done in ethanol series for 1 min each. Finally, slides were air dried at room temperature and 15 μL of diamidino-2-phenylindole (DAPI) counter stain was applied and glass coverslips were applied

In this study two types of probes were used, first, Poseidon TM EGFR was optimized to detect copy number of EGFR gene at 7p11 region and Poseidon TM CCND1 was optimized to detect copy number of CCND1 gene at 11p13 region. Both probes were designed as dual color assay; the red signal indicated the gene and the green signal the centromere.

The slides were viewed using fluorescence microscope equipped with custom optical filters at 100x objective. For EGFR gene FISH slides at least 100 cells were viewed using fluorescence microscope. EGFR FISH patterns were classified into four different groups: disomy (consist of \leq two genes copies in more than 90% of the cells), trisomy (three genes copies in more than 10% of cells and \geq four gene copies in less than 15% of cells), low-level gains (consist of \geq four gene copies in $\geq 15\%$ of cells but less than 30% of cells) and high-level gains (\geq four gene copies in $\geq 30\%$ of cells). Tumors classified as disomy and trisomy was considered FISH negative. Tumors showing low-level gains and high-level gains were considered to be FISH positive [12]. The CCND1 (11q13) specific DNA probe is optimized to detect copy numbers of the CCND1 gene at region 11q13. According to the criteria described by Mahdy et al [13], enumeration of fluorescent signal of CCND1 gene was performed in at least 200 nuclei per slide using fluorescence microscope. The cases were

considered to be amplified when the average copy number ratio, cyclin D1/CEP 11, was >2.0 in all nuclei evaluated. In all, multiple comparisons significant p-value was at ($p < 0.05$).

RESULTS

FISH evaluation of EGFR amplification: All study cases showed red signals of EGFR genes, which could be suitable for evaluating the EGFR gene amplifications (Figure 1). 70% (21 out of 30) samples showed positive EGFR gene amplification. In the positive group, there were 6 tumors with high-level gains and 15 tumors with low-level gains. 30% (9 out of 30) of samples showed negative gene amplification and can be considered trisomy. There was no significant correlation of EGFR gene amplifications with clinic pathological findings (Table 1).

FISH evaluation of CCND1 amplification: All study cases showed red signals of CCND1 gene, which could be suitable for evaluating the CCND1 gene amplifications (Figure 2). 43.3% percent (13 out of 30) of samples showed positive CCND1 gene amplification while 56.6% (17 out of 30) of samples showed negative gene amplification. There was no significant correlation of CCND1 gene amplifications with clinic pathological findings (Table 1).

Correlation of EGFR and CCND1 Genes: The correlations between EGFR and CCND1 amplification with all clinic pathological findings in patient with OSCCs, was statistically non-significant.

DISCUSSION

We did not find a statistically significant association between FISH pattern of EGFR or CCND1 genes and clinical pathological parameters. Our findings are consistent with the most previous studies that have also reported no association [8, 12, 14]. On the other hand, our results are in contrast to the studies that have reported an association [15-18]. According to EGFR gene assessment, we found that 50% of cases showed low-level gain FISH pattern, 20% of cases with high-level gain FISH pattern and 30% cases with trisomy FISH pattern. Chung et al stated that 58% of OSCCs demonstrated positive EGFR amplification [14], while Ryott et al stated that 54% of oral tongue squamous carcinoma showed positive EGFR amplification [20]. While assessment of CCND1 gene showed

Figure 1: FISH Evaluation of EGFR signals in OSCC. Red signals indicated the EGFR gene and green signals indicate centromere region in chromosome 7

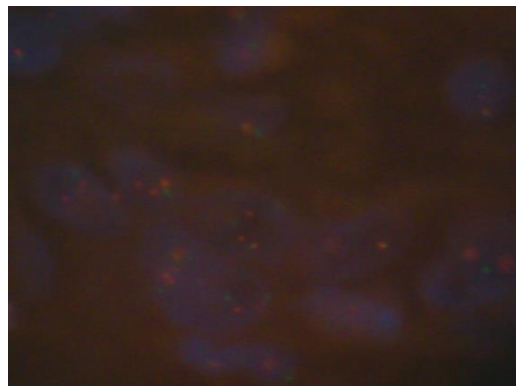
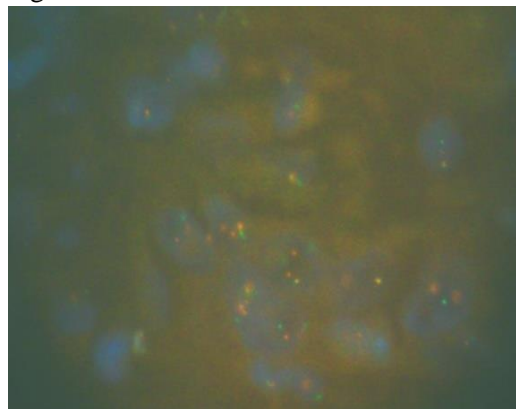


Figure 2: FISH Evaluation of CCND1 signals in OSCC. Red signals indicated the EGFR gene and green signals indicate centromeric region in chromosome 11



43.3%, only 3% of cases were positive. The discrepancy in positive EGFR & CCND1 amplification in the present study and other studies may be due to inclusion of all sub sites of SCC of the oral cavity, variations in scoring system and differences in the quality of observation [8, 18, 19-21].

Recently, several studies have suggested that CCND1 plays a critical role in EGFR-driven tumorigenesis and that deregulated CCND1 overexpression may be significantly associated with resistance of HNSCC to EGFR inhibitors [22]. This suggests that CCND1 is a pivotal down-stream target gene in the EGFR pathway, so that to predict the prognosis of patients with OSCCs and the therapeutic effectiveness of EGFR inhibitors we need to investigate the genetic status of not only EGFR but also CCND1. We therefore performed a simultaneous

Table 1: Association of EGFR & CCND1 amplification and clinic pathological in OSCC cases

Clinical Characteristic	EGFR Amplification			CCND1 Amplification		
	Positive (N=21)	Negative (N=9)	P value	Positive (N=13)	Negative (N=17)	P value
Male	14 (46.7%)	6 (20%)	P=0.9	8 (26.6%)	12 (39.9%)	P=0.9
Clinical site						
Tongue	7 (24.1%)	4 (13.7%)	P=0.9	5 (17.2%)	6 (20.6%)	P=0.9
Buccal mucosa	7 (24.1%)	2 (6.9%)		3 (10.3%)	5 (17.2%)	
maxilla	3 (10.3%)	1 (3.4%)		2 (6.9%)	3 (10.3%)	
Mandibular	4 (13.8%)	1 (3.4%)		2 (6.9%)	3 (10.3%)	
Clinical presentation						
Exophytic	7 (23.3%)	3 (10%)	P=1.0	4 (13.3%)	6 (20.0%)	P=0.9
Endophytic	11 (36.7%)	5 (16.7%)		7 (23.3%)	9 (30.0%)	
leukoplakia	3 (10%)	1 (3.3%)		2 (6.7%)	2 (6.7%)	
Clinical staging						
Stage I	2 (6.7%)	3 (10%)	P=0.2	1 (3.3%)	4 (13.3%)	P=0.5
Stage II	2 (6.7%)	2 (6.7%)		1 (3.3%)	3 (10.0%)	
Stage III	4 (13.3%)	1 (3.3%)		3 (10.0%)	2 (6.6%)	
Stage IV	13 (43.3%)	3 (10%)		8 (26.6%)	8 (26.6%)	
Grading						
Well	4 (13.3%)	3 (10%)	P=0.8	3 (10.0%)	4 (13.3%)	P=0.9
Moderate	15 (50%)	5 (16.7%)		9 (30.0%)	11 (36.6%)	
Poor	2 (6.7%)	1 (3.3%)		1 (3.3%)	2 (10.0%)	

assessment of CCND1 and EGFR genetic status to define subgroups of patients at increased risk of disease recurrence and poor prognosis [4].

Concerning the correlation between EGFR and CCND1 amplification, the results of the present study showed no significant association between the two. These results are in contrast to what were reported by Takashashi et al, who found a significant association between CCND1 and EGFR by FISH analysis of 85 OSCCs samples [4]. Riessmann et al stated that in cohort of 298 non-small-lung-cancer (NSCLC) specimens, amplification of the CCND1 occurs frequently in conjunction with amplification of the EGFR [23]. Another study on vulvar carcinoma stated that there was a highly significant association between EGFR copy number increase and CCND1 amplifications as well as the total number of gene amplifications [24]. Our smaller study sample size may have failed to pick up the association reported in these other studies with larger sample sizes.

CONCLUSION

EGFR amplification was positive in 70% of OSCC cases and CCND1 amplification was positive in 43% of OSCC cases. No significant association between EGFR or CCND1 amplification and clinic pathological characteristic of studying sample, as well as the correlation between EGFR and CCND1 amplification was reported in our study.

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