

# “Analysis of immunohistochemical expression of epidermal growth factor receptor in oral squamous cell carcinoma using tissue microarray technology and whole sections:” A comparative study

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## Abstract

**Background:** Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all oral cancers. Epidermal growth factor receptor (EGFR) dysregulation is associated with essentially all of the key features of cancer. Tissue microarrays (TMAs) allow the simultaneous analysis of many tumours using small-diameter cores sampled from larger blocks of tissue. Hence present study was taken up to validate TMA technology.

**Aims and Objectives:** To analyse and compare the immunohistochemical (IHC) expression of EGFR in OSCC using TMA technology and in whole tissue sections.

**Materials and Methods:** Study included 34 cases of OSCC. Three tissue cores, each 1 mm in diameter were placed into a recipient paraffin block using a precision microarray instrument finally containing 102 spots. EGFR expression was analysed. Agreement between whole sections and TMA scores was analysed using Cohen's weighted Kappa.

**Results:** EGFR expression was seen in 61.8% of whole section cases. In TMA out of 102 cores 75.50 % of the disks were confirmed to represent an adequate amount of tumor tissue. In TMA 48.5% cases showed EGFR expression. The EGFR expression of whole.

**Conclusion:** Some OSCC express high EGFR and this expression may be an independent Wfactor of certain clinico-pathological variables. TMA may be used as an adjunct with conventional method of evaluation of OSCC especially in larger sample sized studies keeping in mind its limitations.

**Keywords:** Epidermal growth factor receptor, oral squamous cell carcinoma, tissue microarray

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## INTRODUCTION

Oral cancer is becoming a pressing problem in the world and the WHO predicts a continuing worldwide increase in

the number of patients with oral cancer.<sup>[1]</sup> Lip and oral cavity cancer was the most common incident cancer in males in

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India in 2016.<sup>[2]</sup> Oral carcinogenesis involves numerous genetic events that alter normal functions of oncogenes and tumor suppressor genes.<sup>[3]</sup> Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that constitutes one of four members of the erbB family of tyrosine kinase receptors.<sup>[4]</sup> Aberrant expression of proto-oncogene EGFRs believed to contribute to cancer development.<sup>[3]</sup>

Even though immunohistochemistry (IHC) is an established and widely used technique quality control is one of the major issue.<sup>[5]</sup> Tissue microarray (TMA) is a purely mechanical technique, which involves taking tissue cores from multiple donor blocks with precise insertion (arraying) into an empty “recipient” block.<sup>[6]</sup> TMA allows for rapid analysis of the large number of tissues under standardized laboratory and evaluation conditions.<sup>[7]</sup> TMA technique is not constrained by application as slides can be probed using any assay protocol developed for whole tissue sections in a nondestructive manner such as histology, IHC and fluorescence *in situ* hybridization.<sup>[5]</sup> A potential disadvantage is that the donor cores may not be representative for the whole tumor, particularly in case of heterogeneous tumors.<sup>[7]</sup>

The present study was taken up to validate TMA in oral squamous cell carcinoma (OSCC) by analyzing EGFR expression.<sup>[8,9]</sup>

## MATERIALS AND METHODS

Thirty-four cases of OSCC were included in the study. Thirty-two were graded as well differentiated squamous cell carcinoma (SCC) and two two cases were graded as moderately differentiated SCC. No patients had distant metastasis at the time of diagnosis. In overall samples, 76.47% ( $n = 26$ ) of cases were T2 (tumor size < 2 cm), 11.76% ( $n = 4$ ) cases were T1 (tumor size 2–4 cm), 8.82% ( $n = 3$ ) of cases were T3 (tumor size > 4) and 2.94% ( $n = 1$ ) case were T4. Histologically confirmed metastatic lymph nodes were seen in 47.06% ( $n = 16$  cases) of cases and 52.94% ( $n = 18$ ) cases did not show lymph node metastasis. Among cases with metastatic lymph nodes 26.47% ( $n = 9$ ) were N2, 20.59% ( $n = 7$ ) were N1. In overall cases, 50% cases ( $n = 17$ ) presented with advanced stage tumors (Stage III and IV) and 50% ( $n = 17$ ) cases with early tumor stage (Stage I and II).

### Tissue microarray construction

Representative tumoral areas of interest avoiding areas of necrosis and keratin pools were selected on hematoxylin and eosin slide of each case. Three tissue cores, each one

mm in diameter, were placed into a single paraffin block using a precision microarray instrument (Beecher Manual Tissue microarrayer) finally containing 102 spots (Figure 1). The section was studied to confirm the presence of tumor (Figure 2).

### Immunohistochemistry staining for epidermal growth factor receptor

IHC staining on sections from each representative paraffin-embedded tissue block was carried out using the polymer labeling technique. Five-micron section was mounted on super frost slides. Sections were dewaxed, washed and antigen retrieval carried out in PT Link Module with 1 mM ethylenediaminetetraacetic acid solution (pH 9) for 20 min. Endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol at room temperature for 10 min. The slide was washed with phosphate-buffered saline (PBS) briefly and incubated with primary antibody EGFR (HPA018530, Atlas Antibodies, dilution 1:100) for 60 min. The section was washed with PBS and incubated with the polymer (Envision FLEX, Dako) for 30 min. Diaminobenzidine was used as the chromogen in hydrogen peroxide for 10 min. Sections were then counterstained with hematoxylin

### Evaluation of staining

Immunostaining was evaluated by two experienced oral pathologists. Antigen expression for EGFR was defined as the presence of specific staining on the surface membrane of tumor cells. To quantify EGFR expression, a total immunostaining score was calculated using the product of a proportion score and an intensity score. The proportion score described the estimated fraction of positive stained tumor cells (0 = none; 1 = <10%; 2 = 10%–50%; 3 = 50%–80%; 4 = >80%). The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong (Figure 3, 4). The total score ranged from 0 to 12. Tumors were arbitrarily categorized into samples with high EGFR, low EGFR and no EGFR expression using a cut off value: 0 = 0, 1–3 = low and 4–12 = high [Figures 3 and 4].<sup>[9]</sup>

### Statistical analysis

Clinical and pathologic parameters were analyzed by IBM SPSS statistics for windows version 22.0 Armonk, NY. Association between various groups was calculated using Chi-Square test. Significance was set at <0.05. Agreement between whole sections and TMA scores was analyzed using Cohen’s weighted Kappa (agreement was considered poor if  $\kappa < 0.2$ , moderate if  $0.2 < \kappa < 0.4$ , reasonable if  $0.4 < \kappa < 0.6$ , substantial if  $0.6 < \kappa < 0.8$  and if  $0.8 < \kappa < 1$  agreement

**Table 1: Association between clinicopathological variables and epidermal growth factor receptor expression**

Factors	No EGFR (%)	Low EGFR (%)	High EGFR (%)	Total (%)	X <sup>2</sup>	P
Gender						
Male	9 (39.13)	8 (34.78)	6 (26.09)	23 (67.65)	0.4118	0.8139
Female	4 (36.36)	3 (27.27)	4 (36.36)	11 (32.35)		
Age groups						
Below 45	5 (27.78)	8 (44.44)	5 (27.78)	18 (52.94)	2.8573	0.2397
Above 45	8 (50.00)	3 (18.75)	5 (31.25)	16 (47.06)		
Site						
Buccal mucosa	6 (66)	1 (11)	2 (22)	9 (26.47)	4.667	0.097
GBS	4 (25)	6 (37)	6 (37)	16 (47.05)	0.500	0.779
Tongue	2 (33.2)	3 (50)	1 (17.7)	6 (17.64)	1.000	0.607
Others	1 (33.2)	2 (66.4)	0 (0)	3 (8.82)	0.333	0.564
Tumour size						
T1 and T2	13 (43.33)	8 (26.67)	9 (30.00)	30 (88.24)	5.2377	0.0729
T3 and T4	0	3 (75.00)	1 (25.00)	4 (11.76)		
Histological grading						
WDSCC	12 (37.50)	11 (34.38)	9 (28.13)	32 (94.12)	1.0707	0.5855
MDSCC	1 (50.00)	0	1 (50.00)	2 (5.88)		
Lymph node status						
N0	9 (50.00)	5 (27.78)	4 (22.22)	18 (52.94)	2.3043	0.3160
N1, N2	4 (25.00)	6 (37.50)	6 (37.50)	16 (47.06)		
Stages						
Stage I-II	9 (52.94)	5 (29.41)	3 (17.65)	17 (50.00)	3.6140	0.1642
Stage III-IV	4 (23.53)	6 (35.29)	7 (41.18)	17 (50.00)		
Total	13 (38.24)	11 (32.35)	10 (29.41)	34 (100.00)		

EGFR: Epidermal growth factor receptor, GBS: Gingivobuccal sulcus, WDSCC: Well-differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma ( $P < 0.05$ )

**Table 2: Epidermal growth factor receptor expression in tissue microarray Core 1, Core 2 and Core 3**

Whole sections	Number of cases (%)		
	Core 1	Core 2	Core 3
No EGFR	26 (78.78)	15 (51.72)	16 (59.23)
Low EGFR	4 (12.12)	12 (41.37)	10 (37.04)
High EGFR	3 (9.09)	2 (6.89)	1 (3.70)
Total	33 (100.00)	29 (100.00)	27 (100.00)
X <sup>2</sup> , $P < 0.05$	30.729, 0.001*	9.586, 0.008*	12.667, 0.002*

\*  $P$  value set to be  $< 0.05$ , EGFR: Epidermal growth factor receptor

was considered good.

## RESULTS

### Clinical features

Mean age of patients was 46.8 years (23–65 years range). Male to female ratio was 2:1. Majority of lesions (47.06%) occurred in gingivobuccal sulcus ( $n = 16$ ).

### Epidermal growth factor receptor expression in whole tissue sections

EGFR expression was seen in 61.8% ( $n = 21$ ) of cases, 38.2% ( $n = 13$ ) cases did not show EGFR expression, wherein low EGFR expression was seen in 32.35% ( $n = 11$ ) cases while high EGFR expression was seen in 29.41% ( $n = 10$ ) of cases. The difference between the groups was statistically nonsignificant (Chi-square = 0.4120,  $P = 0.8122$ ). EGFR expression was not significantly associated with any clinical and pathological variables [Table 1].

### “EGFR expression in TMA and comparison of EGFR expression between whole tissue sections and single core TMA (Core 1 or Core 2 or Core 3).”

Among 102 cores 75.50% of the disks were confirmed to represent an adequate amount of tumor tissue. While 12.74% of tissue cores were lost completely during processing and 11.76% of tissue cores were devoid of tumor cells. Correlation between whole tissue sections and single core TMA values [Table 2] showed a weighted kappa value of 0.4000 for core 1 correlation ( $P = 0.0005$ ), 0.3495 core 2 correlation ( $P = 0.0085$ ), and 0.1456 for core 3 correlation ( $P = 0.1367$ ).

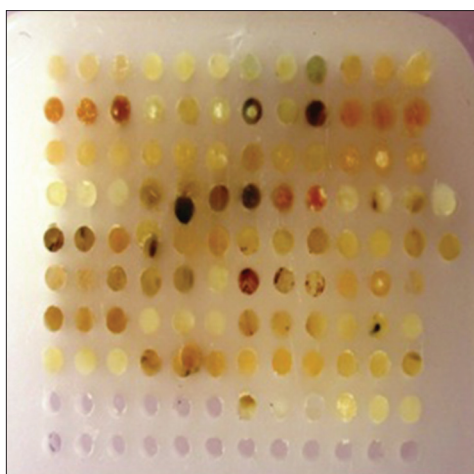
Comparison of EGFR expression between whole tissue sections and maximum score of TMA

For TMA, the highest score of the three core discs was taken. Thus, 51.5% ( $n = 17$ ) of cases showed no EGFR expression and 48.5% ( $n = 16$ ) cases showed EGFR expression (36.36% cases,  $n = 12$  showed low EGFR expression and 12.12%,  $n = 4$  high EGFR expression).

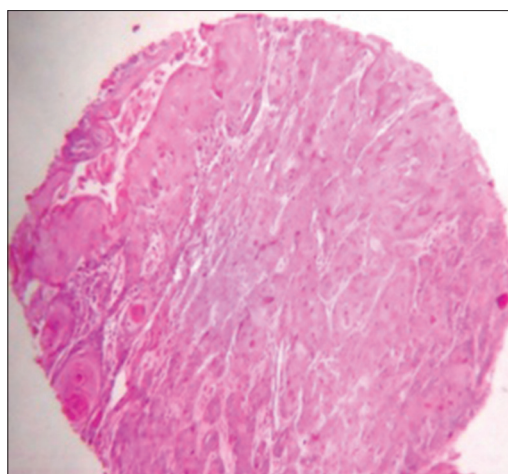
The co-relation between whole tissue sections and TMA (triple core) showed a reasonable agreement with a weighted kappa value of 0.4931,  $P = 0.0008$ .

## DISCUSSION

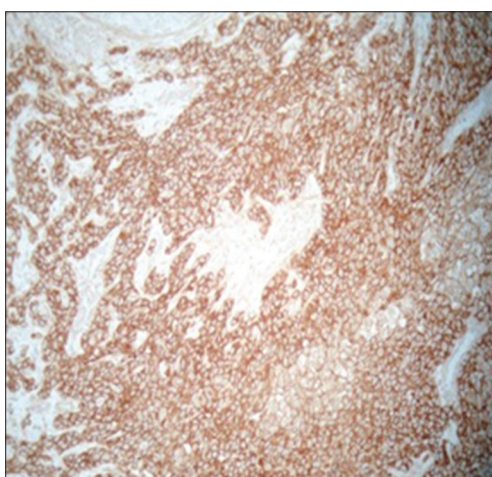
OSCC is an aggressive malignant cancer, with high mortality and morbidity, which commonly occurs in male middle-aged and older individuals.<sup>[10]</sup> Several studies have



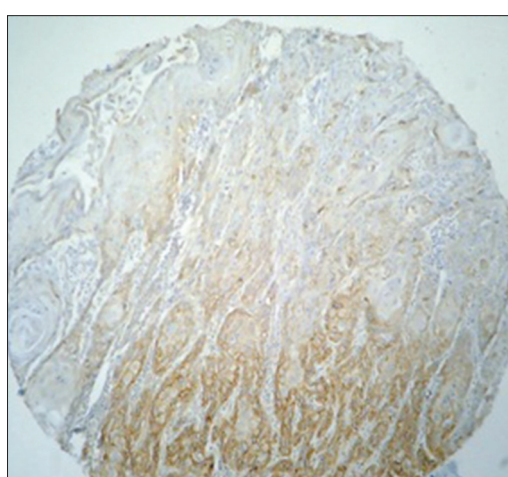
**Figure 1:** Tissue microarray paraffin block



**Figure 2:** Tissue microarray, H and E



**Figure 3:** Whole section showing epidermal growth factor receptor intense expression x10



**Figure 4:** Tissue microarray showing intense epidermal growth factor receptor expression

shown that EGFR overexpression is an independent prognostic marker that correlates with increased tumor size, decreased radiation sensitivity and increased risk of recurrence.<sup>[11]</sup>

TMA technology has been developed to address the limitations of conventional histopathological techniques and to facilitate genome-scale molecular pathology studies linking novel genes with clinical end points.<sup>[6]</sup>

In the present study, EGFR expression was seen in 61.8% of cases while the remaining 38.2% cases showed no EGFR expression whole sections. Wide variation in the expression of EGFR has been reported ranging from 15% to 97%.<sup>[12,13]</sup> Some studies have analyzed EGFR expression based only on the staining intensity in >10% of cells contrary to the present study where the product of intensity and extent was used. In the study of Verma *et al.*

25% of cases showed strong, 60% showed intermediate, 13% showed weak EGFR expression and 2.1% showed no expression (based on product and intensity score).<sup>[14]</sup>

In view of varying reports on the EGFR expression in OSCC, there is a need for a standardized procedure for immunohistochemical evaluation of EGFR expression. In the present study, EGFR expression was analyzed in relation to various clinicopathological parameters such as age, gender, tumor size, lymph node metastasis, degree of differentiation and staging which did not show significant association. Similar findings were observed in the study of Diniz-Freitas *et al.* which may suggest that EGFR is an independent factor and not influenced by any of the clinical and pathological statuses of patients with oral cancer<sup>[12]</sup> and in another study significant correlation was seen between EGFR expression and advanced T stage of the primary tumor, an advanced pathological stage and a high incidence

of neck metastasis.<sup>[15]</sup> Some studies evaluated EGFR expression and its association with overall survival rate and obtained varied results, however in the present study, survival rate could not be assessed due to incomplete information available during follow up.

Christensen *et al.* reported that the extent of EGFR staining reaction on OSCCs varies inversely with cellular differentiation.<sup>[16]</sup> Thus the decreased expression of EGFR in the present study may be attributed to the majority of cases being graded as well differentiated.

For EGFR expression in TMA block with 102 cores, 12.74% of tissue cores were lost completely during processing and 11.76% of tissue cores were devoid of tumor cells. This may be attributed to incorrect punching of the representative areas out of the donor block. Possible causes for the absence of cores are the size, the fragility of the tumor tissue used and the aggressive nature of mechanical tissue processing applied.<sup>[7]</sup> In a study by Chen *et al.* 2003, 13% of the discs were completely lost and 13% of discs contained no or too few tumor cells.<sup>[8]</sup> In the published data, the percentage of unavailable cylinders ranges from 6% to 33%.<sup>[9]</sup> Thus arraying three core biopsies from each case reduces the number of unsuccessful discs which cannot be analyzed when all the cores of a case are lost.

For TMA EGFR expression analysis, the highest score of the three core discs was taken. Thus, 51.5% of cases showed no EGFR expression and 48.5% cases showed EGFR expression (36.36% cases showed low EGFR expression and 12.12% high EGFR expression). In the study of Bernardes *et al.* positive (moderate or intense) EGFR expression was seen in 53.8% cases of OSCC and in the study of Monteiro *et al.* EGFR expression was moderate or intense in 85.3% cases and absent or weak in 14.7% cases, which is significantly high as compared to present study but the scoring was based only on the staining intensity in >10% of cells.<sup>[9,17]</sup>

In the present study, the EGFR expression of the whole section was compared with TMA maximum score and according to Cohen weighted Kappa scoring, a reasonable agreement was observed ( $k = 0.4931$ ). A Similar result was also observed in the study of Chen *et al.*<sup>[8]</sup> However in the study of Monteiro *et al.* high concordance between the whole section and TMA maximum score was observed.<sup>[9]</sup>

Decreased EGFR expression on TMA compared to whole sections was observed in the present study. A plausible explanation for this difference is that for some antigens

there appears to be a trend for TMA-labeled sections to show slightly more or less extensive labeling than whole-tissue sections. The reason for this is not obvious but may relate to TMA sections having a lower proportion of the section containing tissue-binding sites for antibodies (due to the spaces between the cores) compared with whole-tissue sections or to different rates of oxidation of antigens after cutting sections.<sup>[6]</sup>

In the present study, the correlation between the scores for the TMA and the full sections was not as high as previously reported. Various reasons may be attributed for this such as heterogeneity of staining within tumor, the difference of scoring technique and a large proportion of intermediate staining results known to cause a larger interindividual and intraindividual variation.<sup>[9]</sup> As far as the scoring technique is concerned, some studies did dichotomize positive versus negative for EGFR expression (based only on the intensity of staining) and compared whole sections with TMA. However, in the present study, the product of intensity and extent was taken and cases were divided into three groups for comparison.

Correlation between whole tissue sections and single core TMA, showed a weighted kappa value of 0.4000 for core 1 correlation ( $P = 0.0005$ ), 0.3495 for core 2 correlation ( $P = 0.0085$ ) and 0.1456 for core 3 correlation ( $P = 0.1367$ ) which is less when the maximum score of the three was taken for correlation. The mean probability of disc predicting whole specimen scores increases with an increase in the number of discs. Hence, the number of cores used in TMA may solve heterogeneity tumor problems but the exact number of discs to be arrayed is controversial. Zhang *et al.* pointed out that a single 0.6-mm disc yielded 97% agreement in TMA and whole sections while Monteiro *et al.* in their study recommended the use of 1.5 mm dual cores and showed a strong correlation between whole sections and TMA.<sup>[9,18]</sup> In adverse Boone *et al.* recommended utilizing three biopsy cores while Gomaa *et al.* recommended using the mean results from four cores for biological studies.<sup>[6,7]</sup> However, Jourdan *et al.* reported that the addition of a fourth core did not add to the percentage of agreement.<sup>[19]</sup> Moreover, the more cores punched per case, the fewer number of different cases can be placed into the TMA reducing throughput.

Thus, TMA is a specialized technique which offers various advantages over conventional techniques by allowing rapid analysis of a large number of tissue without damaging the parent block under standardized laboratory conditions. It also reduces the number of consumables used and increases

cost-effectiveness. Potential problems with the use of TMAs include technical aspects of TMA preparation and the extent to which TMA cores are representative of a whole tumor because of tissue heterogeneity.

## CONCLUSION

Some of the OSCC express high EGFR and this expression may be an independent factor of certain clinicopathological variables. The agreement in the scoring of the whole section and the tissue arrays in the present study was reasonable. TMA may be used as an adjunct with the conventional method of evaluation of OSCC, especially in larger sample sized studies. The treatment of OSCC with anti-EGFR therapy may be a promising area.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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