The Detection of Transcription Factor Nuclear Factor Kappa-B in Oral Squamous Cell Carcinoma by in Situ Hybridization.

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ABSTRACT:

BACKGROUND:

Nuclear factor – kappa B is an important transcription factor that plays a pivotal role in cell biology and the control of apoptosis. The vast majority of studies focused on the regulatory roles of this factor on apoptosis suggest that it is acting on the upstream pathways of apoptosis, either negatively or positively. It has got an important role in the development of oral squamous cell carcinomas.

OBJECTIVE:

To detect mRNA of nuclear factor- kappa B in oral squamous cell carcinoma by in situ hybridization compared to its presence in foci of dysplasia in premalignant lesions and to link it to tumor grade and degree of dysplasia.

MATERIALS AND METHODS:

Forty two cases, including 30 cases of oral squamous cell carcinoma and 12 cases of oral premalignant lesions containing foci of dysplasia were included in this study. Sections on positively charged slides were made from their paraffin blocks and were used for the detection of nuclear factor-kappa B mRNA using in-situ hybridization technique.

RESULTS:

Nuclear factor-kappa B mRNA was detected in 10(83.33%) cases of oral dysplasia and 24(80%) cases of oral squamous cell carcinoma. A significant orrelations was found between the marker and the degree of dysplasia, but it was not significant regarding tumor grade.

CONCLUSION:

The highly significant increased intensity of nuclear factor kappa B m RNA may indicate a role in increasing the degree of dysplasia in oral squamous epithelium. In keeping with the malignant phenotype, the functions of other genes are needed besides nuclear factor –kappa B.

KEYWORDS: oral squamous cell carcinoma, oral dysplasia and nuclear factor-kappa B.

INTRODUCTION:

Basic cancer research has produced remarkable advances in our understanding of cancer biology and cancer genetics. Among the most important of these advances is the realization that apoptosis and the genes that control it have a profound effect on the malignant phenotype and it is now well documented that most cytotoxic anticancer agents induce apoptosis, raising the intriguing possibility that defects in apoptotic programs contribute to treatment failure.[1]

Because the same mutations that suppress apoptosis during tumor development also reduce treatment sensitivity, apoptosis provides a conceptual framework to link cancer genetics with cancer therapy. It is clear that multiple genes are probably involved in apoptosis regulation in a tumor cell population.[1, 2]

Nuclear factor – kappa B (NFκB) is an important transcription factor that plays a pivotal role in cell biology and the control of apoptosis.[3] The vast majority of studies focused on the regulatory effect of NFκB on apoptosis suggest that NFκB is acting on the upstream pathways of apoptosis, either negatively or positively.[4] NFκB has got an important role in the development of oral squamous cell carcinomas (OSCC).[5, 6, 7] It also represents a new target for treating this cancer.[8]

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In Iraq, oral squamous cell carcinoma accounts for about 91.5 % of all oral cancers. Its incidence reaches 4.5 % of all cancer cases.[9]

The aim of the study was to detect the mRNA of NF-κB by in situ hybridization in oral squamous cell carcinoma compared to its presence in foci of dysplasia present in pre- malignant lesions and to correlate between the two pathologies and this marker regarding the tumor grade and the degree of dysplasia.

MATERIALS AND METHODS:

Thirty biopsies, histologically diagnosed as oral squamous cell carcinoma and 12 biopsies of oral premalignant lesions containing squamous dysplasia were randomly retrieved from the archives of Oral Pathology Department, College of Dentistry/University of Baghdad through the period from January 2000 to March 2004. The malignant lesions included 19 cases of well differentiated carcinoma and 11 cases of poorly differentiated carcinomas. The premalignant lesions included leukoplakia with mild dysplasia in 7 cases, moderate dysplasia in 3 cases and sever dysplasia in 2 cases.

Five histologically normal gingival biopsy blocks from patients who have already undergone extraction of wisdom teeth were used as a control group. Two cases of ovarian carcinoma already positive for NFκB mRNA ISH served as positive control for NFκB. Negative controls were used in each batch whereby thr hybridization reaction was conducted without a probe.

Biotinylated Long DNA probe for Human NFκB as well as in-situ DNA hybridization/detection system were purchased from Maximbiotech (USA). The kit contains a biotinylated house keeping human DNA gene probe as a positive control as well.

Slide preparation for in-situ hybridization included their baking at 60 C for overnight, deparaffinization and rehydration at room temperature (RT) (25˚C) by dipping slides in xylene, serial dilution of ethanol and de-ionized water. For ISH, after heating at 98˚ C in citric buffer, tissue sections were deproteinized using proteinase-k enzyme solution (1X concentration) for 10 minutes at RT. Slides were dehydrated at RT by sequential dipping in ethanol (70%, 95% and 100%) each for 1 minute and dried by incubation at 37˚C for 5 minutes. NFκB DNA probe was diluted to 8%, denatured at 95˚C for 5 minutes and ice-quenched immediately. One drop (10 µl) of the probe was placed on the tissue section, covered by a cover slip and heated in an oven at 70˚ C for 10 minutes. Hybridization was carried out in a humidity chamber at 37˚C for 3 hours, followed by soaking in 1X detergent wash, addition of RNase- A and washing with 3X protein block at 37˚C for 3 minutes. The hybridized probe was detected by streptavidin– alkaline phosphatase (streptavidin-AP) conjugate. Upon addition of the substrate solution which is 5-brom-4 chloro-3 indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT), an intense blue signal appeared at the specific site (probe hybridization to target mRNA). Nuclear fast red was used as a counter stain. Slides were dehydrated and mounted. Using the light microscope, scoring was done at X400 to determine positive hybridization signal. Scoring was conducted independently by two pathologists. Positive cells were counted out of 100 nucleated cells in10 different high power fields. The mean percentage of positive cells was determined assigning cases to one of the four following score categories: [10] Score 1: 1-25%, Score 2: 26-50%, Score 3: 51-75% and Score 4: 76-100%. The ISH signaling intensity was assessed using a scale of negative, low, moderate and high intensities of signaling.[10]

Statistical analysis was performed using the SPSS 16 for windows evaluation version and Microsoft office 2003. Data were summarized using standard descriptive statistics. Associations between categorical variables were assessed via cross tabulation and chi-square. Spearman rank correlation coefficient was used to express relative relation between any two ordinal variables. In all statistical analyses, a P value < 0.05 and <0.01 were considered significant and highly significant respectively.

Photographs were taken by a digital camera at X1000.

RESULTS:

An intense blue hybridization signal was detected at the site of the specific mRNA sequence in different cells. Both nuclear and cytoplasmic signals were recorded. Hybridization signals appeared as dots representing mRNAs copies. Nuclear fast red aided in the background visualization since it has tendency to stain nuclei with red color. Blue signals

which were associated with red staining represented nuclear signals. Those which were only blue represented cytoplasmic ones.

Positive NFκB mRNAs hybridization signals were observed in 10 cases (83.33%) of leukoplakia with dysplasia. Five cases (41.66 %) were with mild dysplasia, 3 cases (25%) were with moderate dysplasia and two (16.67%) were with severe dysplasia. Frequency distribution of NFκB positive percentage scores

revealed that score 2 was the predominant one whereby it was detected in 7 cases (58.33%). Regarding the NFκB signaling intensity, 6 cases (50%) revealed moderate intensity followed by low intensity in 3 cases (25%) and only one case (8.33%) revealed high intensity (Tables-1 & 2) (Figure-1A & B).

The results revealed a non-significant correlation between the degree of dysplasia and NFκB percentage score (P>0.05). A highly significant positive correlation with NFκB signaling intensity (P<0.01) was observed (Tables- 1 & 2).

Positive NFκB mRNA hybridization signals were observed in 24 cases (80%) of OSCC. From these, 15 (50%) cases were well differentiated and 9 cases (30%) were poorly differentiated squamous cell carcinomas. The frequency distribution of NFκB percentage scores revealed equal frequencies of scores 2&3, 9 cases (30%) of each. Concerning signaling intensity of NFκB, 10 cases (33.33%) revealed high intensity followed by moderate intensity in 9 cases (30%) (Table- 3 & 4) & (Figure-1C, D, E & F). A non-significant correlation was observed between OSSC and both of percentage scores and signaling intensity (p>0.05) (Tables-3 & 4).

The difference between well differentiated and poorly differentiated OSCC showing positive NFκB mRNA ISH signals was statistically not significant (p> 0.05) (Table-5).

The difference between oral dysplasia and OSCC showing positive NFκB mRNA ISH signals was statistically not significant (p>0.05) (Table-6).

DISCUSSION:

NFκB is an important molecular switch for development of head and neck SCC.[11] It modulates expression of programs of genes functionally linked to proliferation, apoptosis and angiogenesis either directly or indirectly .[12]

On observing the results obtained in table 5, a higher number of well differentiated carcinomas were positive for NFκB signals, however, the differences between them and the poorly differentiated ones was not significant (p > 0.05). The difference is most likely attributed to the number of specimens included in both samples. Results could reflect possible transfer from dysplasia to carcinoma in comparing the number of positive cancer cases with that of dysplastic cases in table-6, in spite of being non-significant. A better view to the subject is the highly significant positive correlation (p <0.01) observed between NFκB intensity and the degree of dysplasia. A low intensity is associated with mild dysplasia. Higher intensities reflect an increase in the copy number of mRNA of NFκB which seems to be important in increasing the degree of dysplasia until it reaches carcinoma. Passing into higher tumor grades involves other pathways in addition to NFκB.[5] In another word, NFκB effect is no more predominating and this is reflected by the irregular distribution of both scores and intensities in between well and poorly differentiated SCC as it is seen in tables 3 & 4.

Results of the present study are not very much in agreement with a report on colorectal cancer showing an increased immunohistochemical expression of NFκB in transition from normal mucosa to adenoma and in transition from adenoma with low grade dysplasia to adenocarcinoma, which in turn, suggested the role of NFκB in promoting the tumor through inhibition of apoptosis. That was by finding a statistical correlation between increased levels of both NFκB and Bcl_2.[13]

In another study on gastric carcinoma, NFκB was proved to play an important role in hindering an intracellular apoptotic process followed by accelerating cancer invasion and metastasis.[14] In correlation with the present study, a significant increase in the expression of P65, a subunit of NFκB was demonstrated in head and neck squamous cell carcinoma and that the expression was mainly confined to the nucleus which means an active form and that such an activation prevented cell death (apoptosis).[15] It was proved that inhibition of NFκB activity potentiates apoptosis by blocking TNF-induced NFκB activation and

sensitized oral SCC cells to TNF killing.[16] The already mentioned studies dealt with the expression of the protein product rather than the mRNA. On the contrary, the present study deals with mRNA as a reflection of gene activity which when high shows a higher intensity.

Table 1: Frequency distribution of NFκB ISH percentage scores of 12 cases showing dysplasia

NFκB positivity Scores					
Oral Dysplasia	0 – No (%)	1 + No (%)	2 ++ No (%)	3 +++ No (%)	Total No (%)
Mild Moderate Severe	2 (16.67) 0 0	0 0 1 (8.33)	4 (33.33) 2 (16.67) 1 (8.33)	1 (8.33) 1 (8.33) 0	7 (58.33) 3 (25.00) 2 (16.67)
Total	2 (16.67)	1 (8.33)	7 (58.33)	2 (16.67)	12 (100)

Spearman correlation = 0.066
P = 0.838 NS

Table 2: Frequency distribution of NFκB ISH signaling intensity in 12 cases of dysplasia

NFκB Intensity					
Oral Dysplasia	Negative – No (%)	Weak + No (%)	Moderate ++ No (%)	Strong +++ No (%)	Total No (%)
Mild Moderate Severe	2 (16.67) 0 0	3 (25.00) 0 0	2 (16.67) 3 (25.00) 1 (8.33)	0 0 1 (8.33)	7 (58.33) 3 (25.00) 2 (16.67)
Total	2 (16.67)	3 (25.00)	6 (50.00)	1 (8.33)	12 (100)

Spearman correlation = 0.733
P = 0.007 S

Table 3: Frequency distribution of NFκB ISH percentage scores in 30 cases of oral squamous cell carcinoma.

NFκB positivity scores					
OSCC	0 No (%)	1 No (%)	2 No (%)	3 No (%)	Total No (%)
Well Poor	4 (13.33) 2 (6.67)	5 (16.67) 1 (3.33)	6 (20.00) 3 (10.00)	4 (13.33) 5 (16.67)	19 (63.33) 11 (36.67)
Total	6 (20.00)	6 (20.00)	9 (30.00)	9 (30.00)	30 (100)

Spearman correlation=0.224
p= 0.235 NS

Table 4: Frequency distribution of NFκB ISH signaling intensity in 30 cases of oral squamous cell carcinoma.

NFκB Intensity					
OSCC	Negative – No (%)	Weak + No (%)	Moderate ++ No (%)	Strong +++ No (%)	Total No (%)
Well Poor	4 (13.33) 2 (6.67)	4 (13.33) 1 (3.33)	4 (13.33) 5 (16.67)	7 (23.33) 3 (10.00)	19 (63.33) 11 (36.67)
Total	6 (20.00)	5 (16.67)	9 (30.00)	10 (33.33)	30 (100)

Spearman correlation = 0.097
P = 0.931 NS

Table 5: The difference between well and poorly differentiated OSCC regarding positive ISH signals for NFKB mRNA:

OSCC	NFκB		Total
	Positive	Negative-	
Well	15	4	19
Poor	9	2	11
Total	24	6	30

$X^2 = 0.0359$ P > 0.05 NS

Table 6: The difference between oral dysplasia and OSCC regarding positive ISH signals for NFκB mRNA

Pathology	NFκB		Total
	Positive	Negative-	
OSCC	24	6	30
Dysplasia	10	2	12
Total	34	8	42

$X^2 = 0.0617$ P > 0.05 NS

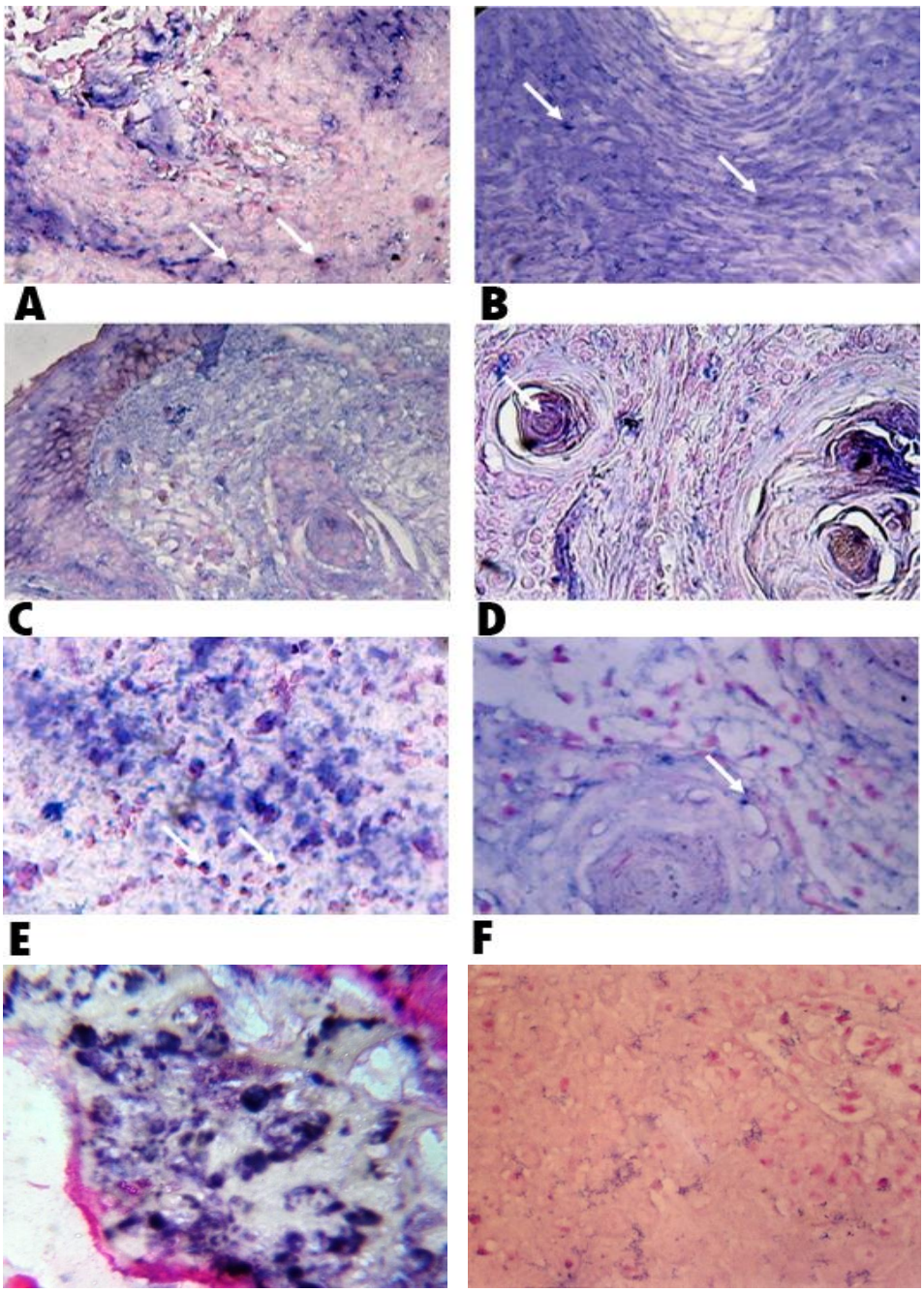

Figure-1: In situ hybridization detection of NF-κB mRNA in oral lesions (deep blue signals, white arrows x 1000): A: Sever dysplasia high intensity. B: Mild dysplasia low intensity. C: well differentiated SCC low intensity. D: Well differentiated SCC moderate intensity. E: Poorly differentiated SCC high intensity. F: Well differentiated SCC moderate intensity. G: Positive control ovarian mucinous cystadenocarcinoma high intensity. H: Negative control

CONCLUSION:

The highly significant increased intensity of NFκB m RNA may indicate a role in increasing the degree of dysplasia in oral squamous epithelium. In keeping with the malignant phenotype, other genes are needed besides NFκB.

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