Molecular Quantification and Detection of Interleukin-2 by PCR and ISH in Oral Squamous Cell Carcinoma in Relation to Metastasis

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Abstract:

Background: Cytokines play an important role in oral cancer having both cytotoxic and growth stimulating effects on neoplastic epithelial cells. Cytokines may also be involved in tumor metastasis. They are mainly expressed in the connective tissue; however, some neoplastic epithelial cells in some tumors express cytokines.

Material and methods: A total of 24 oral squamous cell carcinoma (OSCC) cases were obtained as archival blocks from the Department of Oral Pathology, Faculty of Oral and Dental Medicine, Cairo University. Twelve cases were non-metastatic and 12 cases showed metastasis to regional lymph nodes. Five µm thick sections from each paraffin block were prepared for real time (RT)-polymerase chain reaction (PCR). Only sections rich in the epithelial element (by examination of hematoxylin and eosin sections) were chosen for RT-PCR. Three to 4 µm thick sections mounted on tissue adhesive glass slides (OptiPlus) were used for in situ hybridization (ISH).

Results: RT-PCR showed that interleukin-2 (IL-2) gene expression was down regulated in neoplastic epithelial cells in all cases of OSCC compared to the control group (C group). The metastatic group (M group) showed a significant lower level of IL-2 gene expression than the non-metastatic group (NM group). Molecular detection of IL-2 RNA by ISH revealed its expression in some neoplastic epithelial cells of the NM group. However, in the M group, expression of IL-2 RNA was seen mainly in few inflammatory cells surrounding the cell nests.

Conclusion: Down regulation of IL-2 gene expression in neoplastic oral epithelial cells is associated with oral carcinogenesis and seems to be associated with the increased metastatic potential in OSCC.

Keywords: Oral squamous cell carcinoma, interleukin-2, metastasis, PCR, ISH.

Introduction

Oral cancer is a serious disease (Mangalath et al., 2014). Its incidence and mortality rate have increased over the past decades. Despite sophisticated surgical, chemo and radio therapeutic modalities, it is characterized by poor prognosis and low survival rates (Noguti et al., 2012). Moreover, it has a liability to metastasize to cervical lymph nodes (Okura, 2009). Metastasis of oral cancer is a complex process involving regulation of cell motility, invasion, proliferation and evasion through the lymphatic system or blood vessels (Noguti et al., 2012).

Cytokines play a role in carcinogenesis. They stimulate growth of tumor cells through promotion of angiogenesis (Wiseman et al., 1988), modification of matrix proteins (Turner et al., 2010) or expression of adhesion molecules (Chen et al., 2001). On the other hand, cytokines are cytotoxic to tumor cells,

inhibiting the progression of the tumor or causing necrosis (Riddell, 2015). Thus, cytokines have not only a cytostatic action, but also have a pathogenic role in tumor development being further implicated in invasion and metastasis (Nakano et al., 1999).

Determining which cells express cytokines in tumor tissue is confusing. Neoplastic epithelial cells in oral carcinoma (Reichert et al., 1998 and Nakano et al., 1999) and ovarian carcinoma (Watson et al., 1990 and Naylor et al., 1993) contain cytokines. Whereas those in other neoplasms, such as breast cancer (Pusztai et al., 1994**),** renal cell carcinoma (Waase et al., 1992) and gastric carcinoma (Ohno et al., 1994) do not express cytokines.

Interleukin-2 (IL-2) is a 15.5 kDa cytokine secreted predominantly by Ag-stimulated $CD4^+$ T cells, but it can also be produced by $CD8^+$ T cells, natural killer cells and activated dendritic cells. IL-2 is the main factor responsible for the maintenance of $CD4^+$ regulatory T cells and plays a role in their differentiation into a variety of subsets with different T cell functions (Rosenberg, 2011).

IL-2 up regulation was illustrated in head and neck squamous cell carcinoma cell lines and tumor tissue and this expression correlated to increased proliferation and to the histological grade (Reichert et al., 1998). In contrast, another interleukin (IL-6) was down regulated in undifferentiated thyroid carcinoma cells (Basolo et al., 1998)

As regards the relation between cytokines and metastasis, IL-6 was up regulated in epithelial cells of oral squamous cell carcinoma (OSCC), but wasn’t associated with lymph node metastasis (Nakano et al., 1999). Moreover, IL-2 has been used in treatment of metastatic renal cell carcinoma (McDermott and Atkins, 2006 and Moore, 2007) and metastatic melanoma (Dutcher, 2002).

Reviewing the dental literature, little information is available on IL-2 gene levels in relation to metastasis in OSCC. Hence, this study was designed to investigate IL-2 gene expression in oral carcinoma cells in metastatic and non metastatic cases and correlate this expression with the metastatic potential of oral carcinoma.

Material and methods

Tissue specimens

A total of 24 OSCC cases were obtained as archival blocks from the Department of Oral Pathology, Faculty of Oral and Dental Medicine, Cairo University. Cases were divided into two groups: non-metastatic (NM) and metastatic (M) groups. Twelve cases were NM and 12 cases showed metastasis to regional lymph nodes. Twelve normal mucosa cases obtained as archival blocks from the normal mucosa adjacent to the neoplastic tissue were used as the control group (C group).

Quantitative real time (RT)-polymerase chain reaction (PCR) for IL-2

Five µm thick sections from each paraffin block were prepared for RT-PCR. Only sections rich in the epithelial element (by examination of hematoxylin and eosin sections) were chosen for RT-PCR. Total RNA was extracted from paraffin sections using the standard method and the RNeasy mini kit (Qiagene, Germany), followed by DNase I treatment. For reverse transcription, RT kit (Qiagene) with cyber green master mix was used. The following oligonucleotide primers were used for amplification of the IL-2 gene:

IL-2 sense5’-GTCACAAACAGTGCACCTAC-3’

IL-2 anti-sense 5’CCCTGGGTCTTAAGTGAAAG-3’

The PCR amplification was done for IL-2 in each sample using RT- PCR. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used to check the removal of all contaminating genomic DNA. RT-PCR values for NM and M groups were measured as fold change of the C group. RT-PCR was performed in the Biochemistry Department, Faculty of Medicine, Cairo University. (Nakano et al., 1999)

In situ hybridization (ISH) for IL-2

Three to 4 µm thick sections mounted on tissue adhesive glass slides (OptiPlus) were used for ISH. This work was carried out using Digene tissue hybridization kit which is based on the presence of nucleic acid in cells or tissues. In brief, nucleic acid was visualized by hybridization of labelled DNA primer with biotin to target RNA in human OSCC tissues.

The sections were deparaffinized and digested to expose the fixed targeted RNA, which was simultaneously changed to cDNA by reverse transcription. Hybridization was detected using alkaline phosphatase conjugated with streptavidin which binds specifically to the biotinylated primer. Dephosphorylation of the substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP) by alkaline phosphatase in the presence of nitrobluetetrazolium (NBT) resulted in the deposition of a purplish blue precipitate at sites of hybridization to IL-2. Finally, the sections were counterstained with nuclear fast red allowing for morphological examination along with visualization of target RNA. ISH was performed in the Biochemistry Department, Faculty of Medicine, Cairo University. (Nakano et al., 1999)

Statistical analysis

Data for the NM and M groups was expressed as means of fold change of the C group ± standard deviations. Data collected was statistically analysed (Statistical software package R version 2.15.2). One way analysis of variance (ANOVA) test was used to compare the C, NM and M groups followed by Tukey test for pair-wise comparisons between each two groups.

Results

Clinical findings

NM cases showed an age range from 40-55 years (mean age 46.8 years); 10 cases (83.3%) were males and 2 cases (16.7%) were females. M cases showed an age range from 37-65 years (mean age 51 years); 4 cases (33.3%) were males and 8 cases (66.7%) were females.

RT-PCR results

There was a significant difference ($p<0.0001$) in mean RT-PCR values among the C, NM and M groups (1±0, 0.803±0.176 and 0.605±0.131, respectively). Pair-wise comparisons between each two groups showed that the NM and the M groups showed significant less ($P<0.01$) mean RT-PCR values compared to the C group. Moreover, the M group showed significant less ($P<0.01$) values compared to the NM group (table1 and fig. 1).

Table (1): Mean RT-PCR values for IL-2 gene in the C, NM and M groups.

Groups / Parameter	C group	NM group	M group	P-value (ANOVA)
Mean±SD	1^{a}±0	0.803^{b}±0.176	0.605^{c}±0.131	P<0.0001
P-value (Tukey test)	P<0.01			

Level of significance set at P<0.05

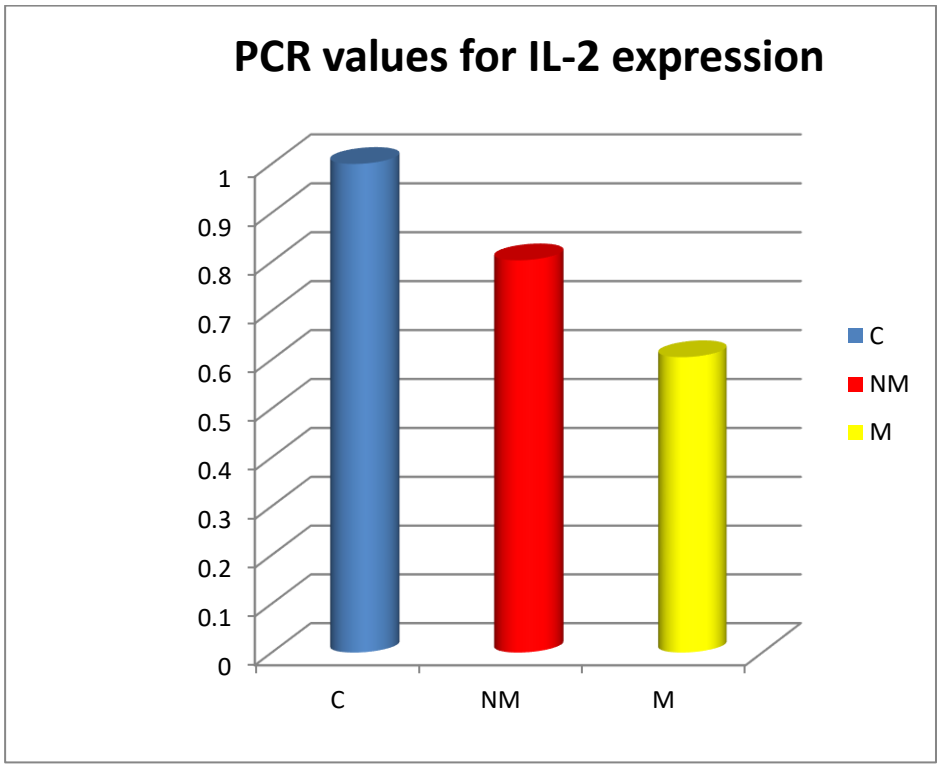

Fig.(1): Bar chart showing mean RT-PCR values for IL-2 gene among the studied groups.

In situ hybridization (ISH) results

In the NM group, IL-2 RNA was detected as a bluish purplish precipitate in the cytoplasm of some malignant epithelial cells (fig. 2A). However, in the M group, IL-2 RNA was mainly detected in some inflammatory cells surrounding the cell nests of OSCC (fig. 2B).

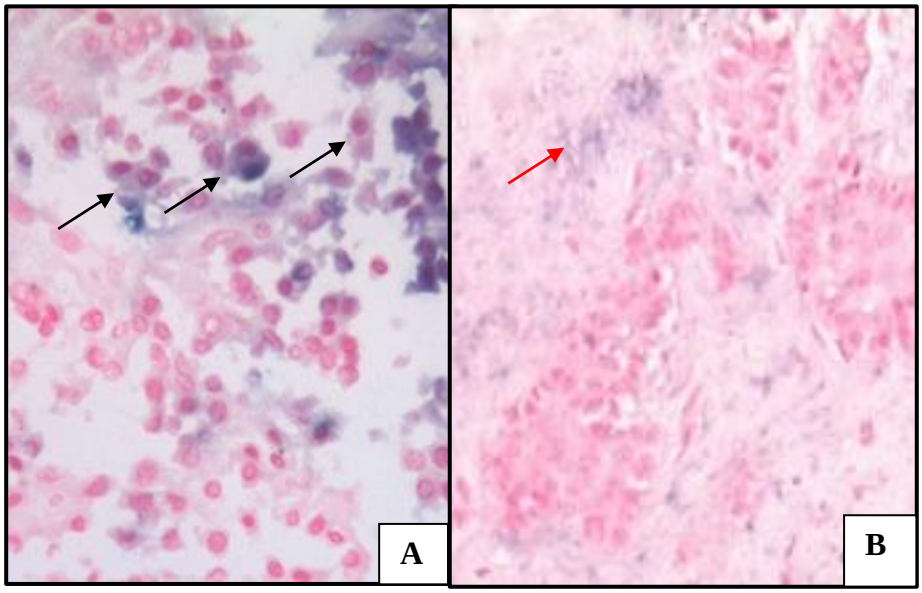

Fig. (2): Photomicrographs of IL-2 RNA expression in (A) NM group showing IL-2 expression in some malignant epithelial cells (black arrows) (ISH X 400) and (B) M group showing IL-2 expression in some inflammatory cells surrounding the cell nests (red arrow)(ISH X200).

Discussion

Since cytokine levels are useful in the determination of treatment modalities and may be predictors of prognosis (McDermott and Atkins, 2006 and Moore, 2007), information about cytokine expression in OSCC is extremely important. Therefore, this study was performed to quantify and detect IL-2 gene expression by PCR and ISH techniques in tissues obtained from OSCC

patients and to examine the significance of the observed changes in relation to metastasis.

The use of RT- PCR has many advantages over the standard PCR. RT-PCR has the ability to identify amplified fragments during the PCR process and measures the amount of the product during the exponential phase whereas standard PCR measures product during the plateau phase which do not always clearly indicate the quantity of starting material. Standard PCR requires agarose gel electrophoresis which identifies the product either by size or sequence which is time-consuming and non-automated. It is also low in specificity, since molecules of similar weights cannot be easily differentiated. RT- PCR eliminates the use of agarose gel electrophoresis (Biedermann et al., 2004).

ISH is a powerful technique for localizing specific nucleic acid targets within fixed tissues allowing spatial information about gene expression. (Segalés et al., 1999)

In the present study, epithelial rich sections were selected for RT-PCR, by examination of hematoxylin and eosin sections, to avoid any confusion in results since ILs could be expressed in neoplastic epithelial cells (Watson et al., 1990 and Reichert et al., 1998) as well as in stromal cells (Nakano et al., 1999) with variable degrees.

Results of this study demonstrated a significant down regulation of IL-2 gene expression in neoplastic epithelial cells of NM and M groups of OSCC compared to the C group. This result is in accordance with Basolo et al. (1998) who observed reduced IL-6 expression in undifferentiated thyroid carcinoma cells by PCR and Eliza techniques carried out on cultured thyroid carcinoma cells and concluded that, in thyroid pathology, down regulation of IL-6 was associated with advanced stage of tumorigenesis. In the same way,

from this study, it could be concluded that IL-2 gene down regulation in neoplastic oral epithelial cells was associated with oral carcinogenesis.

Furthermore, Lippitz (2013) found that IL-2 is one of the stimulatory factors required for activation of effector T cell response against cancer specific antigens. This may explain the down regulation of IL-2 in OSCC as a mechanism provided by epithelial cancer cells to escape the immune defense and provide survival of tumor cells to proliferate and metastasize.

In contrast to our results, García-Tuñón et al. (2004) immunodetected strongest IL-2 expression in the cytoplasm of epithelial cells of invasive breast carcinoma compared to in situ neoplasms and fibrocystic lesions. The authors concluded that the development of breast carcinoma is associated with an increased expression of IL-2 which seems to be associated with the malignancy of the neoplasm. The difference in the studied tissues and detection techniques between the present study and that of García-Tuñón et al. (2004) might have caused the conflicting results.

In the current study, RT-PCR values revealed a significant down regulation of IL-2 gene in the M group compared to the NM group. This is in accordance to Nakano et al. (1999) who found that elevated levels of TNF and IL-6 in OSCC by PCR, ISH, Eliza and immunohistochemistry were not related to lymph node metastasis. Therefore, it could be concluded that the down regulation of IL-2 gene, observed in this study, could be associated with the metastatic character of OSCC. The previous result could be further explained by Flieger et al. (2001) who illustrated the role of some cytokines in enhancing the expression of cell adhesion molecules in colorectal cancer, thus inhibiting cell migration and metastasis. Consequently, the observed IL-2 down regulation, in the present study, could have contributed to metastasis

through decreasing cell adhesion molecules on the surface of oral carcinoma cells.

Moreover, in accordance to our study, lower levels of plasma IL-2 were observed in metastatic head and neck carcinoma leading to tumor progression (Andersson et al., 2014). Other studies successfully used IL-2 in treatment of metastatic melanoma and renal cell carcinoma (Dutcher, 2002, McDermott and Atkins, 2006 and Moore, 2007) since it stimulates antitumor cytotoxic lymphocytes, including effector T and NK cells (Rosenberg, 2011).

ISH results of this work detected IL-2 RNA in some neoplastic oral epithelial cells of the NM group. However, in the M group, it was detected mainly in the inflammatory cells surrounding the cell nests of OSCC. Reichert et al. (1998) illustrated IL-2 expression by immunohistochemistry in some human head and neck cancer cell lines and tissue sections and showed that the strongest IL-2 expression was seen in neoplastic epithelial cells undergoing mitosis and was correlated with the histological grade. Moreover, Nakano et al. (1999) detected TNF α and IL-6 in neoplastic epithelial cells of OSCC. The same cytokines have also been shown to be expressed by epithelial cells in ovarian cancers (Watson et al., 1990 and Naylor et al., 1993). However, neoplastic epithelial cells in other neoplasms do not express cytokines such as breast cancer (Pusztai et al., 1994**),** renal cell carcinoma (Waase et al., 1992) and gastric carcinoma (Ohno et al.,1994).

Nakano et al. (1999) stated that the exact source of cytokines in tumor tissues is not well established and the authors didn't know why some tumor cells actively produce cytokines. In our study, IL-2 detected by ISH in some neoplastic epithelial cells in the NM group could result from the cellular interaction between tumor cells and stromal components (Yamamoto et al., 1991). Another study supported the previous explanation as it detected

intensified IL-6 immunoexpression in the front area of OSCC which may be ascribed to strong cellular interactions between tumor cells and stromal cells (Nakano et al., 1999).

In conclusion, the current study demonstrated that down regulation of IL-2 gene expression in oral epithelial carcinoma cells is associated with oral carcinogenesis and is closely related to increased metastatic potential of OSCC. Thus, IL-2 could be a promising molecule for anti-tumor immunotherapeutic protocols. Further studies using a larger sample size and applying various diagnostic techniques studying IL-2 expression in different grades of OSCC are recommended to elaborate the exact role of IL-2 in OSCC.

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