DETECTION OF MMP3 BY IN SITU – HYBRIDIZATION TECHNIQUE IN ORAL SQUAMOUS CELL CARCINOMA

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Abstract

Background: MMP3 is a member of zinc-dependent proteinases family that is associated with extracellular matrix degradation in physiological and pathological conditions such as tumor metastasis. Hence, they form part of the invasive machinery of cancer cells.

Objectives: The objective of this study is to evaluate MMP3 signals (by in situ- hybridization technique in the cancer cells and the surrounding stroma, whether it is at an early or deep invasive site) in oral squamous cell carcinoma, and correlate such expression with histopathological grading.

Materials and Methods: MMP3 signals were identified by ISH technique in paraffin blocks of 40 oral squamous cell carcinoma and in 10 normal oral mucosa. Chi- square test and Pearson's correlation were applied for analysis. $P < 0.05$ was considered statistically significant.

Results: In normal oral mucosa, the stromal cells displayed MMP3 signals while surface epithelia did not. In oral squamous cell carcinoma, 18 cases (45%) did not reveal any signals (negative cases). Positive signals expression (whether in cancerous or stromal cells) were seen in 22 OSCC (55%), with the following localization; in cancerous cells only (4.5%) , in stromal cells only (18.1%) and in both of them (77.2%).

Both early and deep invasive lesions illustrated great combined cancerous and stromal signals (52.6% and 33.3% respectively). Regarding signals localization and their distribution with respect to the depth of the lesion, significant relation was noted with histopathological grading. Early invasive lesions had more superficial stromal signals of 31.5%, while deep invasive lesions revealed great combined stromal signals of 33.3% .**Conclusion:** MMP3 signals were recognized in more than half of the sample of 55%, and

are more frequently seen with combined cancer and stromal localization in early invasive lesion which is significantly related to histopathological grading.

Keywords: Oral squamous cell carcinoma, MMP3 signals, stromal cells

Abbreviations:
OSCC: Oral quamous cell carcinoma, MMP3: matrix metalloproteinase 3, ECM: extracellular matrix, EMT: epithelial-mesenchymal transition, ISH: in situ hybridization, WDSCC: well differentiated squamous cell carcinoma, MDSCC: moderate differentiated squamous cell carcinoma, PDSCC: poor differentiated squamous cell carcinoma

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common types of human cancer. It is associated with severe disease and treatment-related morbidity, and has a 5-year survival rate of 50%. Recent molecular studies have advanced the understanding of the disease and provided a rationale to develop novel strategies for early detection, classification, prevention, and treatment (Kudo et al., 2006; Mascolo et al.,2012). Despite considerable advances in surgical techniques and the addition of adjuvant treatment modalities, its overall prognosis has not improved (Humayun and Ram Prasad, 2011; de Aguiar Júnior et al., 2012). Invasive and metastatic process, is characterized by increased cell motility and invasiveness, and it has been hypothesized that these processes may be associated with an epithelial-mesenchymal transition (EMT) (Thiery and Sleeman, 2006 and Choi and Meyers, 2008).

EMT is a process in which epithelial cells change from a polarized epithelial phenotype to a fibroblast-like mesenchymal phenotype, leading to the dissolution of epithelial integrity, increased migration, local invasion and ultimately metastasis. Loss of epithelial cell polarity and acquisition of motility require loss of cell-cell adhesion, reorganization of the cytoskeleton and the redistribution of organelles, including alterations in the gene expression profiles of cancer cells (Krisanaprakornkit and Iamaroon, 2012).

MMP3, is responsible for degradation of collagen type IV. It also plays an important role in the activation of proMMP-1 into the active form of MMP-1 in malignant tissues (Brinckerhoff et al., 2000). Its expression is low in normal tissues but it is altered during tumor formation, where remodeling of the extracellular matrix is required (Chaudhary et al., 2010).

MMP3 is synthesized primarily by fibroblasts and to a lesser extent by activated macrophages and keratinocytes adjacent to the sites of injury (Vairaktaris et al., 2007). Conventional TNM staging obviously does not

provide all the needed information. Further analysis of the gene expression profiles underlying the pathologic covariates of what had been seen under the microscope, would greatly aid in directing more appropriate treatment modalities such as the need for neck dissection, postoperative radiation chemotherapy or even targeted therapy (Kao et al., 2009).

Materials and Methods

A total of 40 archival paraffin blocks previously diagnosed as OSCC were collected from Baghdad and Sulaimani histopathological centers. Ten blocks of mucocel containing normal oral mucosa were obtained from the Oral Pathology Department at Sulaimani University. However, the study was approved by the ethical committee of the college.

Two serial 4µm sections were cut and mounted on positively charged slides, one section stained with hematoxylin and eosin to confirm histopathological grading and the other section was stained with c DNA probe by ISH technique . Sections were deparaffinized and rehydrated, then antigen retrieval were done by boiling the sections in 95ºC for 10 minutes using (Citrate buffer, pH 6) and incubated with diluted 1X proteinase K solution at 37 ºC for 30 min in order to achieve effective deproteinization. The prepared c DNA probe/hybridization solution from eppendorf tube in crushed ice was placed on the tissue sections in a humid chamber. Then a cover slip was placed carefully over each slide to avoid trapping any air bubbles. Slides were kept in an oven at 70°C for 10 min to denature the secondary structure of RNA. The slides were incubated at 37°C overnight to allow hybridization of the probe with the target nucleic acid. The slides were soaked in 1X prewarmed detergent wash at 37°C until the cover slips fell off. RNase A was placed on sections at 37°C for 30 min. The slides were washed with protein block (prewarmed) at 37°C for 5 min. 1-2 drops of ready- to- use conjugate red were placed on the tissue sections and kept in a humid chamber at 37°C for 45 min. Slides were rinsed in detergent wash buffer for 5 min. 1-2 drops of ready- to -use substrate (brown) was placed on the tissue sections, and incubated at 37°C for about 25 min, or until blue colored color development is complete. Slides were counter-stained using nuclear fast red stain for 4-5 min according to the manufactural instructions (Maximbio USA). Positive control was normal connective tissue (Tsukifuji et al., 1999), while negative control was performed by omitting the probe and using the hybridization solution only according to manufactural instruction. Proper use of the hybridization detection system was judged when an intense blue–black color at the nucleus of hybridized probe in positive control was developed.

SPSS statistical software was used to estimate Chi-square and Pearson's correlation coefficient. Thus, probabilities of less than 0.05 were accepted as significant.

Results

In normal oral mucosa, the stromal cells displayed high signals specific for MMP3, appeared as nuclear blue granules, while epithelial cells showed no signaling (Figure 1-A and B).

In OSCC, positive signals (whether in cancerous or stromal cells) were observed in 22 cases (55%) (19WDSCC, 2 MDSCC and 1 PDSCC) (Figure 1-C and D) (Table 1). On the other hand, 18 cases (45%) were negative (Figure 1- E) (Table 1).

Positive signals were either located in cancerous cells alone or associated with stromal cells or only in stromal cells. The majority of OSCC had combined cancer and stromal signals (77.2%), followed by signals in stromal cells alone (18.1%) and only one case (4.5%) showed negative stromal cells signals.

Concerning the histopathological differentiation, results showed that 78.9% of WDSCC expressed combined signals in cancerous and stromal cells. While the two positive cases of MDSCC had simultaneous percentage (50%) in showing positive cancer cells signalling with or without signals in stromal cells. But out of 3 PDSCC, only one case was MMP3 positive and revealed combined cancer and stromal signals. Statistical analysis showed significant relation with histopathological grading (X^2= 3.90, P= 0. 048) (Table 1).

Furthermore, the sections were assessed according to the depth of the lesion into 19 early invasive lesions and 21 samples with deep invasive lesions and MMP3 signals were revaluated accordingly. High combined signals were detected in early invasive lesions (52.6% versus 33.3%), stromal signals alone were nearly equally found in early and deep invasive lesions (10.5% versus 9.5 %). Finally, single case with cancer signals only was related to deep invasive lesion. WDSCC revealed more combined signals in their early invasive lesions. One of the two MDSCC tends to show combined signals in its early invasive lesion while the other case had only cancer signals in its deep invasive lesion. Finally, the single PDSCC case had combined signals expression in its early invasive lesion. The above differences were statistically significant (X^2=5.67, P = 0.017) (Table 2).

Special concern was paid for positive stromal signals and were reevaluated in respect to its extent around the cancer islands whether in surface or deep parts of both early and deep invasive lesions. It was found that the stroma around OSCC had great chance to show superficial signals in early invasive lesions of 31.5%, in contrast to the deep invasive lesions

which had great expression of combined superficial and deep signals of 33.3%. Statistical analysis did not show significant difference in MMP3 stromal signals distribution in relation to histopathological grading , although each of single positive MDSCC and PDSCC cases revealed the combined (superficial and deep) stromal signals in their early invasive lesions (Table 3).

Discussion

The lack of unique molecular markers and the diversified phenotype/genotype of OSCC needs more powerful tools to demonstrate its gene expression profiles and link them to the clinical behavior (Kao et al,2009).

To the best of author knowledge, this is the first study that considered MMP3 signaling expression using ISH technique in OSCC and provided the reference data for future identification, validation and comparison, as one could identify abnormal mRNA expression and interfere with such expression in order to correct the defect before the translation process.

MMPs are a family of zinc-dependent endopeptidases, that are up-regulated in almost every type of human cancer. Their expressions are mediated by cancer cells or by surrounding stroma cells and their over-expression is often related to poor prognosis (Chaudhary et al, 2010; Singh et al, 2011).

In this study, the total positive MMP3 signals were recognized in more than half of the sample (55%). Previous researchers demonstrated over-expression of MMP3 in OSCC by IHC ranging between 42.6–88.5% (Kusukawa et al 1995; Kurahara et al,1999; Ylipalosaari, 2005) . On the other hand, by using cDNA microarray analysis, immunocytochemistry and PCR Nagata et al. (2003), Erdem et al. (2007) and Liu et al. (2007) respectively showed that MMP-3 expression levels were higher in OSCC tissues compared with normal oral mucosa. Furthermore, Ye et al, 2008 in their study in tongue SCC using microarray method reported MMP3 gene up regulation (8.43 fold). Therefore, MMP3 expression might be analyzed at many levels (gene transcription or protein) and considered as one of several interesting candidate genes associated with OSCC.

Despite the relatively small sample size, several interesting conclusions might be drawn based on the results of this study. Regarding signal localization, significant increase in MMP3 signals were observed with combined cancer and stromal cells localization (77.27%). This would probably modify the believe that MMP3 is secreted by the mesenchymal cells surrounding the cancerous cells (Chiu et al,2008) and indicated that in epithelial cancers, most of the up regulated MMPs were expressed by carcinoma cells beside their adjacent supporting stroma (Newman and

Rosenthal , 2010). Such an expression was not always predictable since (18.1%) of our cases did not reveal carcinomatous signals and on the opposite side, only one case of 4.5% did not show stromal signals. This might demarcate that stromal mensenchymal cells had probably greater role than epithelial cancer islands in OSCC.

Tsukifuji et al. (1999) in their study of skin carcinoma, also reported that the majority of MMP3 signals were of combined cancer and stromal expression of 72.2% with greater stromal signals of 27.2% than that reported in this study; thus they were no cases without stromal signals .

Lesions with early invasion revealed higher positive MMP3 signals in both cancer and stroma compared to the advanced deep invasive lesion. Thus, in order to facilitate their invasion, cells in superficial carcinomatous islands, dissolve the ECM that restrains them and start spreading to the surrounding tissue, they up regulated their MMPs in both tumor cells and associated stromal cells. In addition, WDSCC revealed slightly more combined (cancer and stroma) signals in their early invasive lesions, indicating that even at an early stage of tumor growth prior to the deep invasion, a dual action of tumor and stromal cells were clearly observed. The step of carcinomatous cells invasion into the surrounding stroma as a late event in the sequence of metastasis process (Choi and Meyers, 2008), might occur at an unpredictable time and site within the heterogeneous carcinomatous growth.

This study showed that in early invasive lesions, the stroma revealed great signals at superficial part, while in the deep invasive lesions, great stromal signals distribution were seen at both superficial and deep part within the lesion. The two positive cases in each of MDSCC and PDSCC tend to show combined stromal signals in their early invasive lesion. This supports the concept of stromal role in the tumor microenvironment for establishing the field of cancerization, as it is generally accepted that tumor arises from genetic changes within normal epithelium. However, the possibility of genetic alteration within the stroma has also been recently identified in HNSCC (Newman and Rosenthal, 2010).

Conclusion

MMP-3 signals were observed in nearly half of the sample 55% of OSCC. Significantly, great signaling were combined with cancer and stromal distribution, specifically at the early stage of invasion of OSCC. Furthermore, stroma play a major role in tumor microenvironment.

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Table-1: The distribution and localization of MMP3 signals in total OSCC in relation to the histopathological grading

Total No	Negative cases		Positive cases		Cancer cells 18				Stroma 21		
					Without stroma		With stroma		Without cancer cells		
40	No	%	No	%	No	%	No	%	No	%	
	18	45	22	55	1	4.54	17	77.27	4	18.18	
Histopathology	WDSCC(10)		19	65.51	0	0	15	78.94	4	21.05	S*
	MDSCC(6)		2	25	1	50	1	50	0	0	X^2=3.90
	PDSCC (2)		1	33.33	0	0	1	100	0	0	P=0.048

Table-2: The distribution of 22 OSCC with MMP3 positive signals in total sample and different histopathological grading in relation to invasion.

Positive cases	Total	Positive cancer cells and positiv stroma		Negative cancer cells and positive stroma		Positive cancer cells and negative stroma		Negative cancer cells and negative Stroma	
		No.	%	No.	%	No	%	No	%
22	Early invasive(19)	10	52.63	2	10.52	0	0	7	36.84
	Deep invasive (21)	7	33.33	2	9.52	1	4.76	11	52.38
WDSCC(19)	Early invasive	8	42.10	2	10.52	0		X^2=5.67 P = 0.017	
	Deep invasive	7	36.84	2	10.52	0			
MDSCC(2)	Early invasive	1	50	0	0	0			
	Deep invasive	0	0	0	0	1	50		
PDSCC(1)	Early invasive	1	100	0	0	0			
	Deep invasive	0	0	0	0	0			

Table-3: The distribution of 21* MMP3 positive stromal signals in relation to their depth in both early and deep invasive lesions and in different histopathological grading

	Sample	Expression of stromal cells					
		Surface		Deep		Both	
		No.	%	No.	%	No.	%
Total positive (21) cases	Early invasive lesion (19)	6	31.57	2	10.52	4	21.05
	Deep invasive lesion (21)	1	4.76	1	4.76	7	33.33
WDSCC(19)	Early invasive	6	31.57	2	10.52	2	10.52
	Deep invasive	1	5.26	1	5.26	7	36.84
MDSCC(1) #	Early invasive	0	0	0	0	1	100
	Deep invasive	0	0	0	0	0	0
PDSCC(1)	Early invasive	0	0	0	0	1	100
	Deep invasive	0	0	0	0	0	0

*****One sample had negative stroma. No significant difference was found ($P < 0.05$).

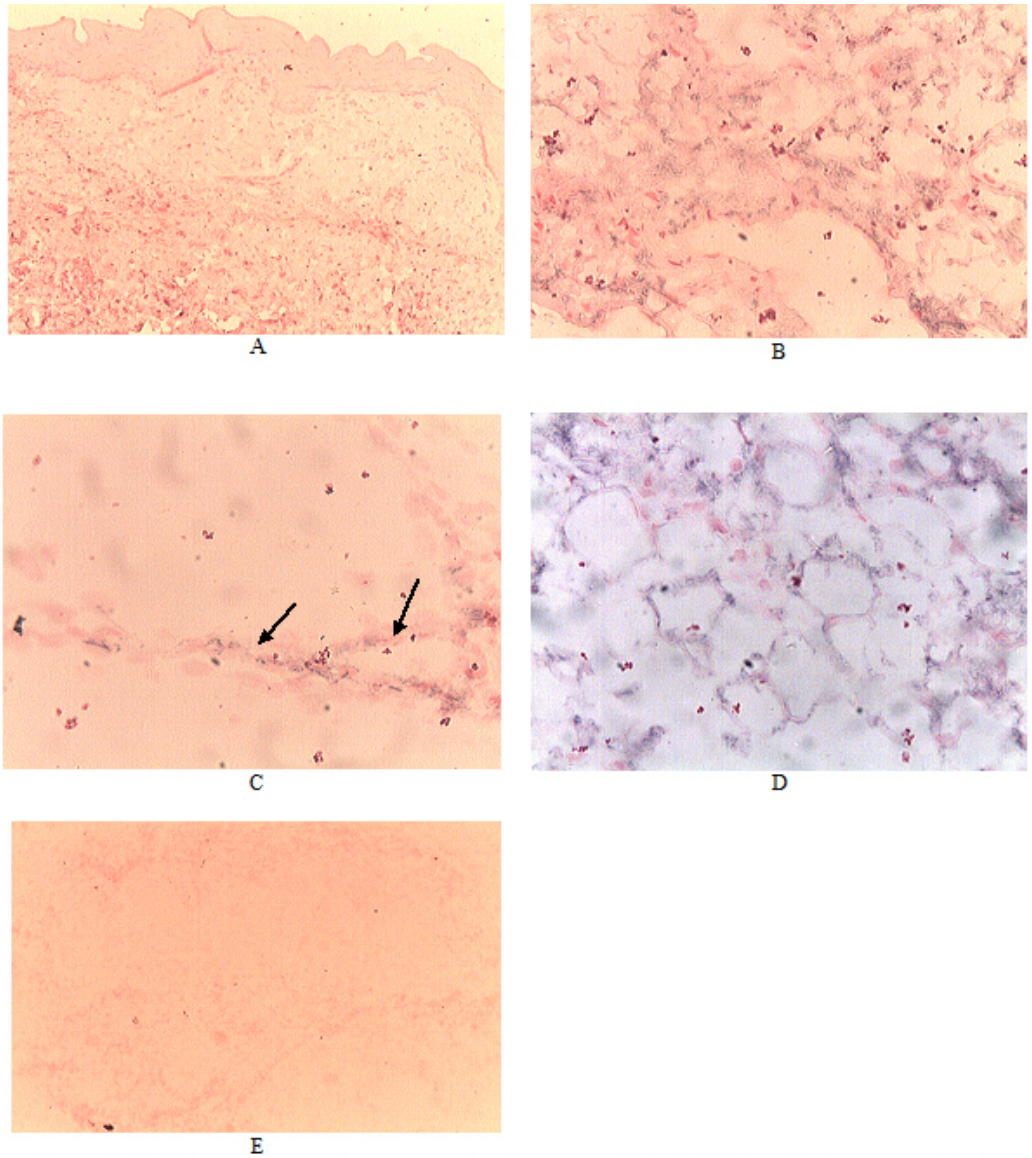

Figure-1: MMP3 signals expression in normal oral mucosa and OSCC.A-Normal oral mucosa with only positive stromal signals, epithelial surface was negative X10.B- Same picture with high magnification X40, positive signals appeared as blue granules (arrow). C. Cancer islands of WDSCC showed basically positive signals at basal cells X40. D- Stromal signals in OSCC X40. E- Negative expression of MMP3 in cancer cells X10.