

Original Research Article

A Comparative Study Of Immunohistochemical Expression Of MMP-9 and Its Inhibitor TIMP-1 In Adenoid Cystic Carcinoma and Polymorphous Low Grade Adenocarcinoma Of The Salivary Glands

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Abstract

The Aims of current study were to assess the immunohistochemical expression of TIMP-1 and MMP-9 in the salivary glands adenoid cystic carcinoma and polymorphous low grade adenocarcinoma and immunoexpression matching of these proteins with the outcomes of clinicopathology.

Twenty five blocks of salivary polymorphous low grade adenocarcinoma and another twenty five of salivary adenoid cystic carcinoma attained from the oral pathology department archives of College of Dentistry in Baghdad University and the hospital of Al-Shaheed Ghazi were retrospectively involved in our study which are inserted in archival formalin fixed paraffin. We gained and immunostained sections of four micrometer using monoclonal antibody against MMP-9 and TIMP-1. The detection of immunoexpression was by the existence of stain which are brown in color seen in the cytoplasm of cells of tumor. The quantity of cells that expressed the stain was related with the clinicopathological documents of the patients.

MMP-9 expression was found positive in 21 cases of PLGA and 22 cases of ACC restricted in tumor cells. TIMP-1 expression was found positive in 20 cases of PLGA and 19 cases of ACC limited in tumor cells.

The relation was seen non-significant [P=0.357] in concerning MMP-9 appearance in both types of tumor and [P=0.937] was seen about TIMP-1 expression in two types of tumor. The relation was seen non-significant in examples of gender [p= 0.117], sites [p= 0.991] and stage [p= 0.853] of PLGA and ACC was seen concerning MMP-9 and TIMP-1.

Non-Significant relation was seen with grade [p= 0.951] of ACC was detected regarding TIMP-1 and MMP-9. An anxious equilibrium was seen between TIMP-1 and MMP-9 in malignant salivary gland neoplasm. Imbalanced of MMP-9/TIMP-1 expressions might offer the tumor cells a double growth benefit because uninhibited TIMP-1; deposition of ECM is joint with increase MMP-9; degradation of ECM. A multistep process created due to this communication which is capable to excite and possibly will show a portion in the genesis of salivary gland tumor; may chain forces to normalize invasive events related to these neoplasms.

Key Words: Polymorphous low grade adenocarcinoma, Adenoid cystic carcinoma, immunohistochemistry, MMP-9 and TIMP-1.

دراسة مقارنة للظهور الكيميائي النسيجي المناعي لأنزيم الماتركس ميتالوبروتينيز 9 والمثبط 1 للماتركس
ميتالوبروتينيز 9 للسرطان الغدي المتعدد الأشكال ذو الدرجة الواطئة والسرطان الكيسي الغدي للغدد اللعابية

الخلاصة

تقييم الظهور الكيميائي النسيجي المناعي لأنزيم الماتركس ميتالوبروتينيز 9 والمثبط 1 للماتركس ميتالوبروتينيز 9 للسرطان الغدي المتعدد الأشكال ذو الدرجة الواطئة والسرطان الكيسي الغدي للغدد اللعابية، ومقارنة درجة ظهور هذه العوامل مع الخصائص السريرية والمرضية.

تضمنت الدراسة 25 عينة لأشخاص مصابين بالسرطان الغدي المتعدد الأشكال ذو الدرجة الواطئة و 25 عينة لأشخاص مصابين بالسرطان الكيسي الغدي جمعت هذه العينات من ارشيف قسم امراض الفم والوجه والفكين / كلية طب الاسنان / جامعة بغداد ومختبر الامراض العامة /

مستشفى الشهيد غازي الحريري للجراحات التخصصية ، وباستخدام قوالب شمعيه حاوية على النسيج المحفوظ في الفورمالين واجري لها الفحص النسيجي للتأكد من التشخيص بعد تطعيمها الى شرائح دقيقة ويسمك 4 ما يكو متر . بعدها تم اجراء الفحوصات المناعية النسيجية الكيميائية للما تركس ميتالوبروتينيز 9 والمثبط 1 للما تركس ميتالوبروتينيز 9 على شرائح نسيجية بنفس السمك المذكور سابقا مع اجراء اختبار السيطرة السالبة والموجبة ثم تقييم النتائج الى بعضها البعض والى الخصائص السريرية والمرضية ايضا.

اظهرت النتائج ان الظهور الكيميائي النسيجي المناعي للما تركس ميتالوبروتينيز 9 كان موجبا في 21 عينة للسرطان الغدي المتعدد الاشكال ذو الدرجة الواطنة و 22 عينة للسرطان الكيسي الغدي كما اظهرت النتائج ان الظهور الكيميائي النسيجي المناعي للمثبط 1 للما تركس ميتالوبروتينيز للسرطان الكيسي الغدي. لوحظ عدم وجود علاقة معنوية بين الماتركس ميتالوبروتينيز 9 والمثبط 1 للما تركس ميتالوبروتينيز 9 في السرطان الغدي المتعدد الاشكال ذو الدرجة الواطنة والسرطان الكيسي الغدي. مع جنس المريض ،الموقع التشريحي و نظام تحديد الانتشار لوحظ عدم وجود علاقة معنوية بين الماتركس ميتالوبروتينيز 9 والمثبط 1 للما تركس ميتالوبروتينيز 9 مع درجة التمايز في السرطان الكيسي الغدي. أظهرت الدراسة وجود درجة من عدم التوازن بين الكيميائية الماتركس ميتالوبروتينيز 9 والمثبط 1 للما تركس ميتالوبروتينيز 9 في سرطانات الغدد اللعابية ، ان عدم التوازن هذا اعطى للخلايا السرطانية قدرة النمو مضاعفة بسبب عدم السيطرة على المثبط 1 للما تركس ميتالوبروتينيز 9 وزيادة الماتركس ميتالوبروتينيز 9. هذا التداخل والتفاعل يحفز عمليات بخطوات متعددة تساعد على حدوث سرطان الغدد اللعابية وتساهم في تنظيم عمليات الهجوم والنزعة الخبيثة لهذه السرطانات.

الكلمات المفتاحية: السرطان الكيسي الغدي، السرطان الغدي المتعدد الاشكال ذو الدرجة الواطنة، الظهور الكيميائي النسيجي المناعي، TIMP-1، MMP-9 .

Introduction

Cytological evenness, associate infiltrative growth pattern, morphological variety and little metastatic potential are very significant characterizing features of polymorphous low grade adenocarcinoma [PLGA] that could be a malignant neoplasm of salivary gland of epithelial origin [1,2].

The morphologic nonuniformity is answerable for the diagnostic trouble and confusion with adenoid cystic carcinoma. It is central and insistent to differentiate it from tumors like adenoid cystic carcinoma [ACC] which has myoepithelial differentiation so we have got a diagnostic challenge [3,4].

An association between clinical progression and histological characteristic has been documented; the aggressive clinical manners is currently joined with papillary cystic pattern.

Adenoid cystic carcinoma [ACC] is a tumor of each the major and minor salivary glands. [ACC] ascend from malignant alteration of the intercalated duct reverse cell soil it's malignant neoplasm of changed myoepithel island ductal cells that form three important characteristic growth patterns that include cribriform, tubular, and solid and contains perineural invasion tendency. ACC is branded by regionally invasive growth and shows great propensity for resident recurrence and distant metastasis [5,6].

Among many MMP families; [MMP-9] is recognized to possess acute roles in neoplasm metastasis and invasion concluded their capability to destroy varied element of extracellular matrix and liberating many cytokines and triggering completely different growth factors. [MMP-9] might show a very important role in angiogenesis and neovascularization.

TIMP-1 is an accomplished of inhibiting the actions of all best-known matrix metalloprotenases and intrinsically plays a significant role to keep the equilibrium seen in numerous physiological processes between extracellular matrix deposition and degradation and inhibits neoplasm

growth, metastases and invasion, and this has been related to their matrix metalloproteinase repressing activity [7,8].

The equilibrium between [MMP-9] and [TIMP-1] which represent their tissue inhibitors is concerned within the histogenesis of normal salivary glands also as within the mechanism of neoplasm invasion and metastases [9,10].

Aims of the current study were to analyze immunohistochemical appearance pattern of [TIMP-1] and [MMP-9] in [PLGA] and [ACC] and to use them as diagnostic marker for differentiation between these two tumors.

Materials and Methods

Samples

Randomly chosen fifty patients affected with malignancy of salivary glands from the pathologic specimens and file records from maxillofacial Center in the Hospital of Al-Shaheed Ghazi in Baghdad, and additionally from the archives of oral diagnosis department of Dentistry Collage in Baghdad University collected from the year 1973 to 2015.

Delivering of data which represent the demographic and clinical part was done by the operating surgeon were calm from the case sheets of patients given with the neoplasm specimens that holding patient's info regarding clinical staging of the neoplasm, site, age and sex were documented, and agreeing to the TNM classification of malignant neoplasms, the staging was administrated. All clinical and histopathologic knowledge offered were studied to reject cases demonstrating disease with secondary metastases to the salivary gland.

Control

Negative external controls conferred by 5 normal salivary gland tissues. At an equivalent time and for negative control we are neglecting of the step of primary antibody and all different reagents were added. Staining Positivity specifies an absence of specificity of the antibody, consistent with Abcam manufacturer's data sheets, gastric adenocarcinoma and liver

tissue were taken here as positive control for [MMP-9] and [TIMP-1] respectively.

Immunohistochemical Procedure

By using xylene four μm sections were deparaffinized and after that graded alcohol used for rehydrated. Hydrogen peroxide used as block in enough drops were applied to our slides, next the slides incubated in wet chamber at $37\text{ }^{\circ}\text{C}$ for ten minutes and after that in the next step the slides soaked in buffer a pair of times [for each one 5 minutes]. In tissue specimens formalin mask antigenic sites by forming protein cross-links, the slides subjected to tissue retrieving so as to reveal antigenicity. Subsequently protein used as block in adequate drops were applied to our slides and protected at 37°C for ten minutes and the buffer used for washing two times [for each one 5 minutes] ,lastly gently drained and blotted and within the next step primary antibody in dilution form was added to every slide, incubated nightlong in wet chamber at $37^{\circ}\text{ }^{\circ}\text{C}$. and within the next day, the slides were washed in buffer [for each one 4 times], lastly drained and blotted gently. In the next step adequate drops of secondary antibody which used as a reagent were applied and for 30 minutes in humid chamber at 37°C was incubated. In the next step we used the buffer four times for washing the slides [for each one 5minutes], then draining and blotting was done, now in the following step Streptavidine-HRP antibodies were applied on tissue for 30 minutes and incubated at 37°C . Now tissue prepared with [DAB] which are Diluted and kept in darkroom and incubated at $37^{\circ}\text{ }^{\circ}\text{C}$ in humid chamber for ten minutes. Then by using tap water the slides washed cautiously for 5 minutes. Then Hematoxylin used as a counter stain for bathing the slides for 1-2 minutes after that tap water was used for rinsing the slides for ten minutes . Then in the next step ethanol and xylene were used for the slides dehydration by immersing them in a jars containing these two fluids and at that time DPX in one or two drops of used as a mounting medium were used to the xylene humid sections and coated

with covers lips and let them nightlong to dry.

Our results were assessed by the existence of outcome as brown colored at the positioning of our target antigen [cytoplasm] was indicative of immunoreactive positivity. IHC positive neoplasm cells percentage per hotspot was calculated and also the percentage of mean per slide was calculated from assortment of ten high power field from the more representative area of immunostaining fields. The intensity was unnoticed as a result of its exposure to individual variation throughout checking.

Gastric adenocarcinoma was the positive control of [MMP-9] which was expressed as brown diffuse cytoplasmatic immunoreactivity additionally to stromal cells of extracellular matrix of neoplasm cells.

MMP-9 immunoreactivity was shows: [score 0] or [-ve] seen as 0% of the neoplasm cells, low [score I] or [+] seen as 1-25%, moderate [score II] or [++] 26-50%, high [score III] or [+++] 51-100% of positive cells, depending on enumeration [11].

TIMP-1 Immunoexpression was brown diffuse cytoplasmic expression in liver tissue as the positive control.

TIMP-1 immunoreactivity was shows: [score 0] or [-ve] < 5%of the neoplasm cells, low [score I] or [+] 6-25%, moderate [score II]or [++] 26-50%, high [score III]or [+++] 51-75% very high [score IV] [++++] 76%-100% of positive cells, depending on enumeration [11].

Statistical Analysis

We tend to tabulated and subjected all the immunohistochemical, histopathological and clinical relevant information to appropriate statistical analysis persecution the SPSS version 20 software system. The studied parameters were counted and given as percentage and count.

Fisher's exact test applied to check the connection concerning categories. Analysis of variance in form of ANOVA test was used to sight differences for an outstanding the markers. P value was well-thought-out

to be statistically significant if it is equal or less than 0.05.

Results

MMP-9immunoexpression

The positive control of mmp-9 was gastric adenocarcinoma expressed as brown diffuse cytoplasmic immunoreactivity additionally to cellular membrane and extracellular matrix of neoplasm cells.

MMP-9 expression in normal salivary glands seem as brown cytoplasmic immunoreactivity.

The step of primary antibody omission were used as negative control ,this is for testing the specificity of antibody utilized in our study, a scarcity of antibody specificity shows positive staining.

MMP-9 immunoreactivity was detected as staining which are brown in color localized within the cytoplasm of the PLGA and ACC tumour cells. The fascinating finding of the current study was the nuclear MMP-9 expression in some tumour cells of ACC and present in

ductal epithelial cells rather than cytoplasmic-localization.

MMP-9 expression was seen positive in twenty one PLGA cases and twenty two cases of ACC in several differential scores, the greater percentage of MMP-9 expression [score III] was seen in ten cases of PLGA [40%] whereas in ACC twelve cases [48 %] and also the greater % was situated in score II.

Non- significant relation [P= 0.357] was detected relating to MMP-9 expression in each types of tumour as in table 1 .

Non- significant relation was seen regarding MMP-9 expression with gender [P=0.117], 1 site of tumour [P= 0.991], stage [P = 0.853] in PLGA and ACC as in tables-2.

Non- significant relation was determined regarding grade [P = 0.951] in ACC as in tables 2.

Table 1: MMP-9 scores in PLGA and ACC

MMP-9 score	PLGA	ACC
Score 0	4 [16 %]	3 [12 %]
Score 1	4 [16 %]	5 [20 %]
Score 2	7 [28%]	12 [48 %]
Score 3	10 [40 %]	5 [20 %]
Total	25[100%]	25 [100%]
	NS P value= 0 .357	

Table 2: The clinicopathological finding of PLAC and ACC in relation to MMP-9 expression.

Variable		PLGA No.25		ACC No.25		P value
		MMP-9		MMP-9		
		-ve	+ve	-ve	+ve	
AGE mean±SD		57.6±15.950		42.20± 2.533		
SEX	Male	0	10	3	13	0.117 NS
	Female	4	11	0	9	
SITE	Palate	2	17	1	16	0.991 NS
	Floor of mouth Upper lip	0	2	0	3	
	Check	0	1	0	0	
	Submandibular gland	0	0	1	0	
	others	0	0	0	2	
STAGE	I	4	4	3	8	0.853 NS
	II	0	9	0	10	
	III	0	5	0	2	
	IV	0	3	0	2	
GRADE	I[tubular]	-	-	2	4	0.951 NS
	II[cribriform]	-	-	3	11	
	III[solid]	-	-	2	3	
PERINEURAL INVATION		0	0	0	2	

TIMP-1 immunoeexpression

The positive control of TIMP-1 was liver tissue expressed as brown diffuse cytoplasmic immunoreactivity.

TIMP-1 expression in normal salivary glands seem as brown cytoplasmic immunoreactivity.

Negative control presented here by using of primary antibody omission, this is for testing the specificity of antibody utilized in our study , a scarcity of antibody specificity shows positive staining.

Immunoreactivity of TIMP-1 was seen as staining which are brown color restricted within the cytoplasm of the neoplasm and stromal cells of each PLGA and ACC. TIMP-1 expression was found positive in twenty PLGA cases and nineteen ACC cases, the greater percentage of TIMP-1 expression [score I] was found

in twelve PLGA cases [48 %] and in ten ACC cases [40%].

The relation appear non-significant [P= 0.937] concerning TIMP-1 appearance in each kinds of neoplasm as in table 3.

Non- significant relation was seen regarding TIMP-1 expression with gender [P= 0.089], site of neoplasm [P=0.979] while the stage [P = 0.853] in PLGA and ACC as in tables 4.

Non- significant relation was determined regarding grade [P = 0.946] in ACC as in tables 4.

Table 3: TIMP-1 scores in PLGA and ACC.

TIMP-1 score	PLGA	ACC
Score 0	5 [20 %]	6 [24 %]
Score 1	12 [48%]	10 [40 %]
Score 2	5 [20 %]	5 [20%]
Score 3	3 [12 %]	4 [16 %]
Total	25 [100 %]	25 (100 %)
NS P value= 0.937		

Table 4: The clinicopathological finding of PLAC and ACC in relation to TIMP-1.

Variable		PLGA No.25		ACC No.25		P value
		TIMP-1		TIMP-1		
		-ve	+ve	-ve	+ve	
AGE mean + SD		57.6+ 15.950		42.20+ 2.533		
SEX	Male	0	10	5	11	0.089
	Female	5	10	1	8	NS
SITE	Palate	3	16	2	15	0.979
	Floor of mouth Upper	0	2	1	2	NS
	lip	0	1	0	0	
	Check	0	0	1	0	
	Submandibular gland	0	0	1	1	
	others	2	1	1	1	
STAGE	I	5	3	5	6	0.853
	II	0	9	1	9	NS
	III	0	5	0	2	
	IV	0	3	0	2	
GRADE	I[tubular]	-	-	2	4	0.946
	II[cribriform]	-	-	3	11	NS
	III[solid]	-	-	1	4	
PERINEURAL INVATION		0	0	0	2	

Discussion

The finding of immunohistochemical variances of [MMP-9] and [TIMP-1] between [PLGA] and [ACC] is formerly the attention of our study, essentially within the both tumors histology shared in cribriform design that has been tirelessly tried [12, 13].

Argument on this topic continues that some authors believe within the literature that immunohistochemistry doesn't require any definite diagnostic worth for recognizing [PLGA] [14 -17].

However, we have a tendency to not share this acceptance as we have a tendency to making an attempt to use immunohistochemistry to approve a diagnostic marker for histologic likeness.

The equilibrium of matrix metalloproteinases [MMPs] with their tissue inhibitors [TIMPs] is concerned within the morphogenesis of normal salivary gland further as within the invasion and metastasis of the neoplasm . [MMP-9] and [TIMP-1] role in [PLGA] and [ACC] has not been confirmed effectively.

During this study [TIMP-1]and [MMP-9] proteins appearance was matched in adenoid cystic carcinoma and polymorphous low grade adenocarcinoma, The positivity of [MMP-9] protein appearance was observed in 21cases [84%] of [PLGA] and twenty two cases [88%] of [ACC] and the positive response of [TIMP-1] protein appearance was observed in twenty cases [80%] of [PLGA] and nineteen cases [76%] of [ACC]. [MMP-9] is extremely controlled protein and this regulation includes a minimum of 3 totally diverse levels: reserve of [MMPs],transcriptional regulation and activation of hidden [MMPs] and [18]. On the extra hand,[TIMP-1]have been associated with different cellular functions, like angiogenesis, growth of cells and anti-apoptotic action [19, 20].

Our study results revealed that [MMP-9] expression raised when tissues changed from cribriform to solid and tubular pattern of growth of [ACC] indicate that [MMP-9] immunostaining will facilitate the

evaluation of the grade of histology of malignant morphological pattern of growth.

Additionally, of [ACC] 2 cases with neural invasion which are positive to [MMP-9]expression.

Later the most important result of current study was that [MMP-9] appearance levels were greater in myoepithelial cells of stroma compared with ductal epithelium in both tumors, it is appealing to invest that [MMP-9] is principally not created by the epithelium but the stromal myoepithelium.

Neoplasm cells could also be the supply of gelatinases in some patients and this confirmed in preceding studies [21] and stroma with neoplastic features is one of the essential parts that stimulate the conversion to invasive cancer from carcinoma in situ, within the literature it has been obviously recognized that neoplasm stroma is in a straight line associated with biological manners of tumour [22].

Our results powerfully recommend the likelihood that myoepithelial cells of stroma could also be the first source of gelatinases in [ACC] and [PLGA] and stroma maybe plays a lot of acute role than epithelium within the progression of [ACC] and [PLGA].

The amount of [MMP-9] in both the ductal epithelium and the myoepithelia of stroma of [ACC] and [PLGA] was more than in the cells of normal salivary gland.

Our result could also be due to the low proportion of normal gland tissues ductal cells, as a product of this result and former studies have demonstrated that in normal salivary gland, ductal cells specified high amounts of [MMP-9] and [TIMP-1] however acinar cells did not shows [MMP-9] and [TIMP-1] [11].

Our result showed increased [MMP-9] production by neoplastic modification of salivary gland tissues. at this time [TIMP-1]and [MMP-9] still unclear which one is more necessary in neoplasm metastasis and progression .

It's additionally valuable to note that MMP-9 appearance was not noticed [three

and four cases] of neoplasm specimens of [PLGA] and [ACC] respectively. One potential explanation of this findings is that MMP-9 could also be seen in [ACC] and [PLGA] however typically its production is unsteady and preserved at closely undetectable levels.

The rise protein expression of both [MMP-9] and its inhibitor [TIMP-1] levels delineated physiological plan to control the activity of [MMPs] and preserve a constant ratio between the 2 proteins. It documented that TIMPs antagonized [MMPs] in the gelatinolytic function during a stoichiometric manner [23], it is important to maintain this equilibrium and tissue injury by augmented proteolysis which occur due to disturbance of this balance. As matrix remodeling may be a results of the equilibrium between degradation and synthesis; it follows that down regulation of [TIMPs] will favor proteolysis. Some studies have confirmed that overexpression of [TIMP-1] resulted during a significant drop in neoplasm growth and elongated periods before the formation of tumors [24].

The current study, however, suggests the chance that myoepithelial cells could also be the first supply of gelatinases and possibly play an essential role in progression and/or development of [ACC] and [PLGA].

Protein expression of [TIMP-1] reduced within the two patterns which are cribriform and tubular . These results show that there is shut association of [TIMPs] with growth patterns of [ACC], and [TIMP-1] might play vital roles in adenoid cystic carcinoma biological character and morphogenesis . The cells ability of human salivary gland cancer for metastasis was related closely to TIMP-1 expression [25].

Salivary gland cancer cells was strictly related to altered [TIMP-1] expression. The down regulation of [TIMP-1] protein expression in tumoral cell of adenoid cystic carcinoma could be regarded as acquisition of capabilities of metastasis and recurrence by the special effects of [TIMP-1] concerning the reserve of [MMPs], anti-angiogenic activity and neoplasm growth.

[MMP-9] expression in neoplasm stromal border of [ACC] during this study indicate that stroma might show some essential role than epithelia in development of this neoplasm, it has additionally been reported that some [TIMPs]will directly have an effect on cell survival and/ or cell growth independent of their actions of [MMPs] [26, 27].

Conclusion

unbalanced between [MMP-9] and [TIMP-1] in malignant salivary gland tumors was detected. The high [MMP-9] and [TIMP-1] might justify the expected course of [ACC] and [PLGA] invasion and show that unbalanced of [MMP-9/TIMP-1] expressions would possibly provide the neoplastic cells a double growth advantage as a result of uncontrolled [TIMP]; deposition of ECM is combined with rise [MMP]; degradation of ECM. The interaction may trigger a multistep process which is able to promote and may play a role in salivary gland tumor genesis; may combine forces to regulate invasive events related to these neoplasms.

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