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RESEARCH ARTICLE

Activated Salivary MMP-2 - A Potential Breast Cancer Marker

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Abstract: It has been reported that Matrixmetalloproteinase-2 (MMP-2) is involved in the pathogenesis of cancer. The over expression of MMP-2 is associated with the progression of malignancy of several types of carcinoma. Human saliva is a biological fluid with several advantages for non-invasive diagnosis and prognosis of diseases. The aim of this study was to detect MMPs expression and activity in biological fluids (saliva, urine *etc*.) derived from breast cancer patients. Here, our results showed that the activity of MMP-2 was higher at the time before the surgery than after the saliva collected from the same patients. Therefore, we suggested that the highly active form of MMP-2 presented in saliva could be used as a novel potential biomarker for non-invasive diagnosis of breast cancer.

Keywords: Saliva, MMP-2, non invasive, breast cancer.

INTRODUCTION

The matrix metalloproteinases (MMPs), is a family of proteases involved in extracellular matrix remodeling, whose activity has been implicated in a number of important normal and pathologic processes [1 - 5]. Growth of tumor, progression, and metastasis as well as angiogenesis are associated with these events. As a result, these proteases are important therapeutic and diagnostic targets for detection of human cancers [6 - 10]. Successful targeting of this enzyme as diagnostic and prognostic predictors of human cancer has very interesting potential. Matrix metalloproteinase-2 (MMP-2, 72 Kd gelatinase A), a member of zinc dependent endopeptidases, degrades matrix proteins like type IV collagens in basement membranes. The expression of MMP-2 is strongly associated with the progression of malignancy of several types of carcinoma. Presence of active MMP-2 is related to breast cancer development [11 - 15]. The role of MMP in cancer biology especially in migration of malignant tumor cells (metastasis) is well documented [16 - 25].

Human saliva is a biological fluid of varying diagnostic potential with several advantages for disease diagnosis and prognosis [26]. Salivary markers have been reported for oral cancer detection [27]. A review on salivary proteomics and genomics has been reported [28]. In chronic periodontitis the role of MMP-2 and 9 has been observed [29]. It has also been reported that salivary protein factors are elevated in breast cancer patients [26]. Gelatinolytic activity of gingival crevicular fluid has also been observed. While saliva is a source of easily accessible bodily fluid, little efforts have been done to study its potential in cancer diagnosis. In this present project we aimed to develop a non-invasive method to detect MMPs in saliva or urine which may be used for diagnosis and prognosis.

Here we report that MMP-2 is present in a highly active form in saliva of breast cancer patients collected before surgery, however, 7 days after surgery the activity of the MMP-2 decreases as shown in comparative zymogram, indicating potential of salivary MMP-2 as breast cancer marker.

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MATERIALS AND METHODS

Patients

Saliva (500 ul) and urine (10 ml) were collected from 36 female breast cancer patients at different stages of the disease (I-IV) before and 7 days after surgery. Saliva from 20 female patients with benign breast tumor was also collected before and 7 days after surgery from R G Kar Medical College, Kolkata following the ethical guidelines. The samples were collected in autoclaved tubes, centrifuged at 5000 rpm for 15 mins at 4°C and clean supernatants were stored (adding protease inhibitors cocktail) at - 20°C until use. Saliva of female breast cancer patients (100 cases) at pre and post surgical conditions (stages I-IV) were also collected, randomly. Saliva of 25 normal (Indian standard, without any disease) female volunteers at ages between 25-50 years were also collected.

Reagents

MMP-2 monoclonal antibody was purchased from Santa Cruz, USA. Protease inhibitor cocktail and NBT/BCIP were purchased from Roche, Germany. All other chemicals like Gelatin, acrylamide, bis-acrylamide, SDS, TRIS *etc* were purchased from Sigma, USA.

METHODS

Substrate Gel Electrophoresis

Equal amount (100 ug) of proteins from saliva and urine (determined by Lowry's method) run in 8% SDS-PAGE impregnated with 0.1% Gelatin. The gel was run at 15 mA using Tris/Glycine/SDS buffer (pH 8.3). The gel was washed in 2.5% Triton-X for 15 mins and then incubated in buffer A (NaCl 0.2M, CaCl2 4.5 mM, Tris 50mM, pH 7.4) overnight at 37°C. The gel was stained with Coommassie Brilliant Blue to develop the zymogram [16].

Immunoprecipitation

100 ug of salivary proteins (saliva collected before surgery) of breast cancer patient was immunoprecipitated with MMP-2 monoclonal antibody and Protein G-Agarose. A zymogram was developed with the immunoprecipitates.

ELISA

To assay salivary MMP-2, TIMP-2, VEGF 25 ug of salivary proteins were used to develop ELISA using their respective monoclonal antibodies followed by 2^{nd} antibody coupled to horseradish peroxidase (HRP). O.D was taken at 450 nM [16].

Immunoblot Development

100 ug of salivary proteins were run on 8% SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and immunoblots were developed using monoclonal antibody against MMP-2 (Santa Cruz, USA) followed by alkaline phosphatase coupled 2^{nd} antibody. The color was developed using NBT/BCIP [16].

Statistical Analysis

Statistical Analysis was performed with help of Epi Info (TM) 3.5.3. EPI INFO is a trademark of the Centers for Disease Control and Prevention (CDC). Descriptive statistical analysis was performed to calculate the means with corresponding standard deviations (s.d.). Test of proportion was used to find the Standard Normal Deviate (Z) to compare the difference proportions. t-test was used to compare the means. $p < 0.05$ was taken to be statistically significant.

RESULTS

Expression and Activity of MMP-2 in Saliva & Urine of Breast Cancer Patients

Fig. (**1A**) shows comparative zymogram of salivary MMPs of breast cancer patients (before and after surgery of the same patient) at different stages of the disease (I-IV). The results show higher activity of MMP-2 (increasing stage wise) in saliva of breast cancer patients collected before surgery (72 kD pro MMP-2 when activated broken down to smaller fragments). The activity of MMP-2 is at background level in post-operative saliva of the same breast cancer

patients. Fig. (**1B**) shows that urine samples (collected before and after surgery) of the same breast cancer patients (of Fig. **1A**) express very weak background of MMP activity (for example saliva of one breast cancer patient at stage IV (before surgery) is showing MMP activity from 140 Kd to 25 Kd in comparison with the same patient's urine showing background activity). Fig. (**1C**) shows the salivary MMP-2 activity of the breast cancer patients randomly collected before and after surgery. The comparative zymogram shows higher MMP-2 activity (from stages I-IV) as compared to background activity in saliva collected after surgery. Fig. (**1D**) shows benign breast tumors, comparative zymogram (saliva collected before and after surgery) shows background activity at MMP-2 region (pro or activated) and Fig. (**1E**) shows background MMP activity in case of normal female volunteers.

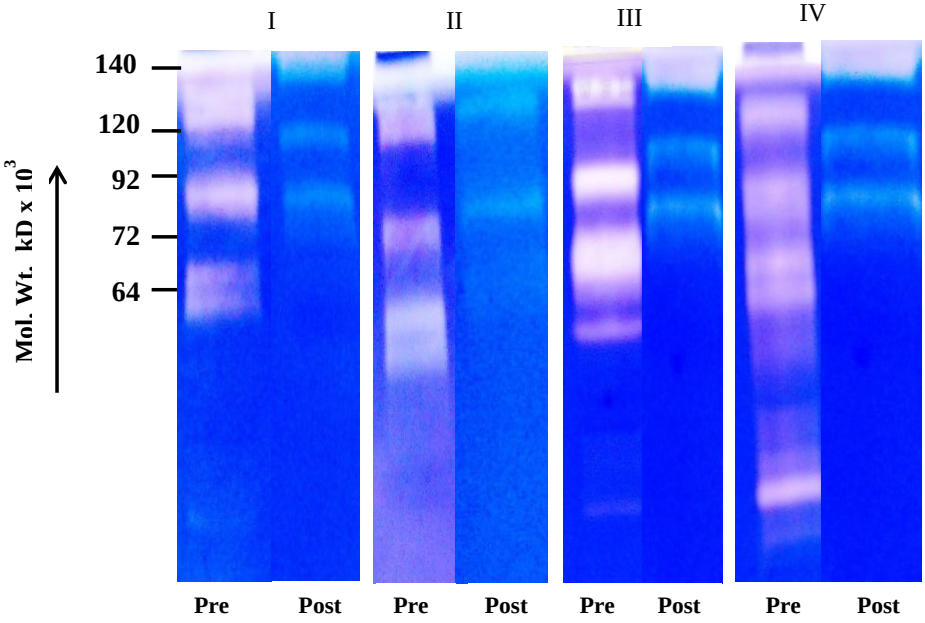

Fig. (1A). before and after surgery of stages I-IV.

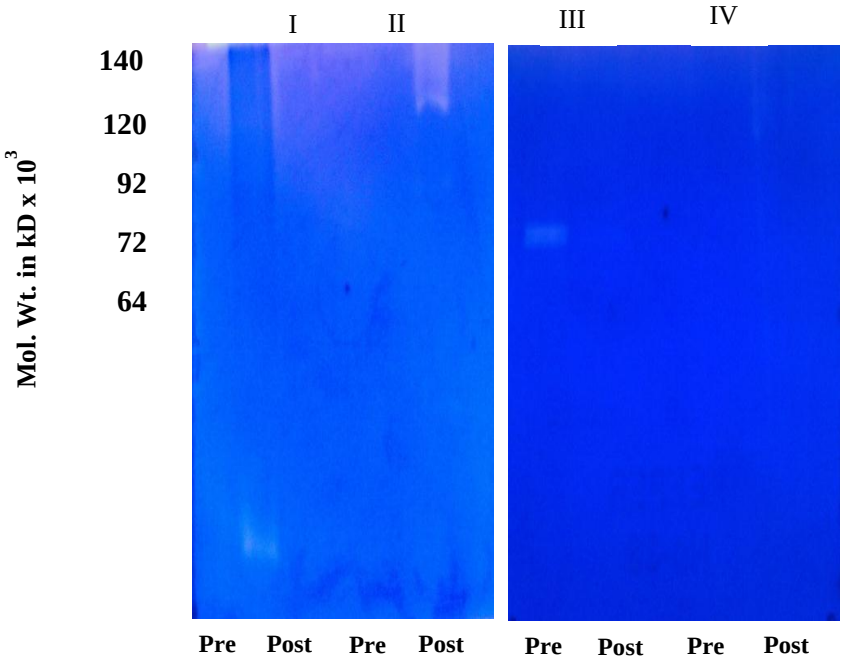

Fig. (1B). Urinary MMP-2 profile of breast cancer Patients (same) before and after surgery of stages I-IV.

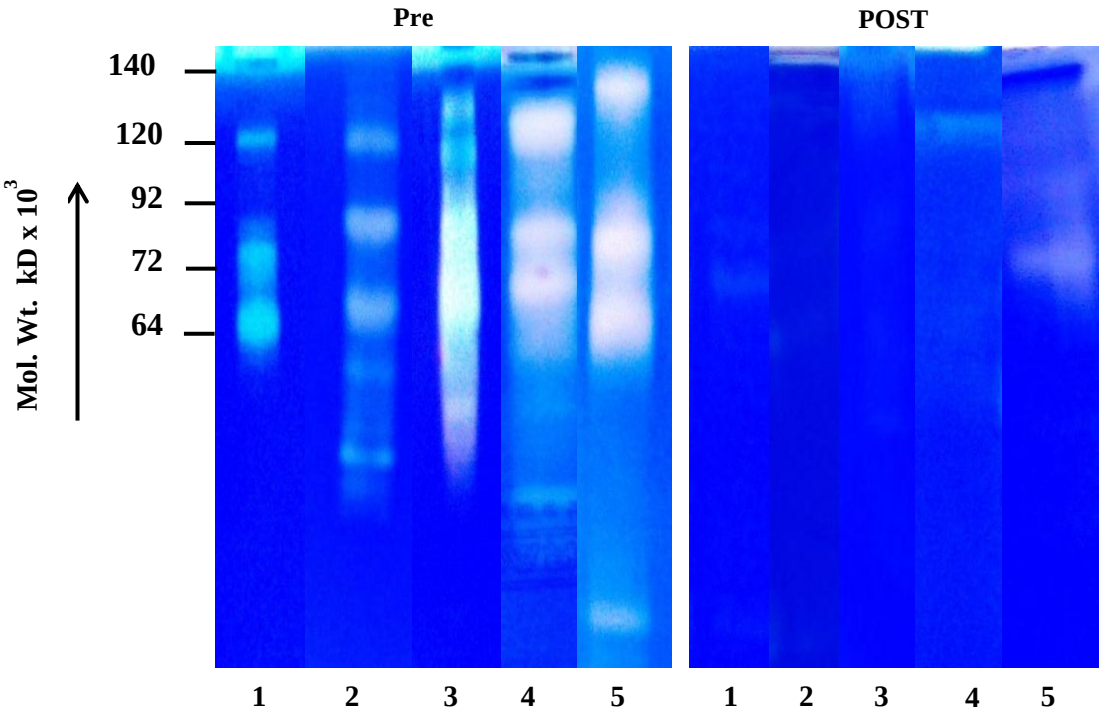

Fig. (1C). Salivary MMP-2 profile of breast cancer patients (random) before(pre stagewise) and after(post) surgery.

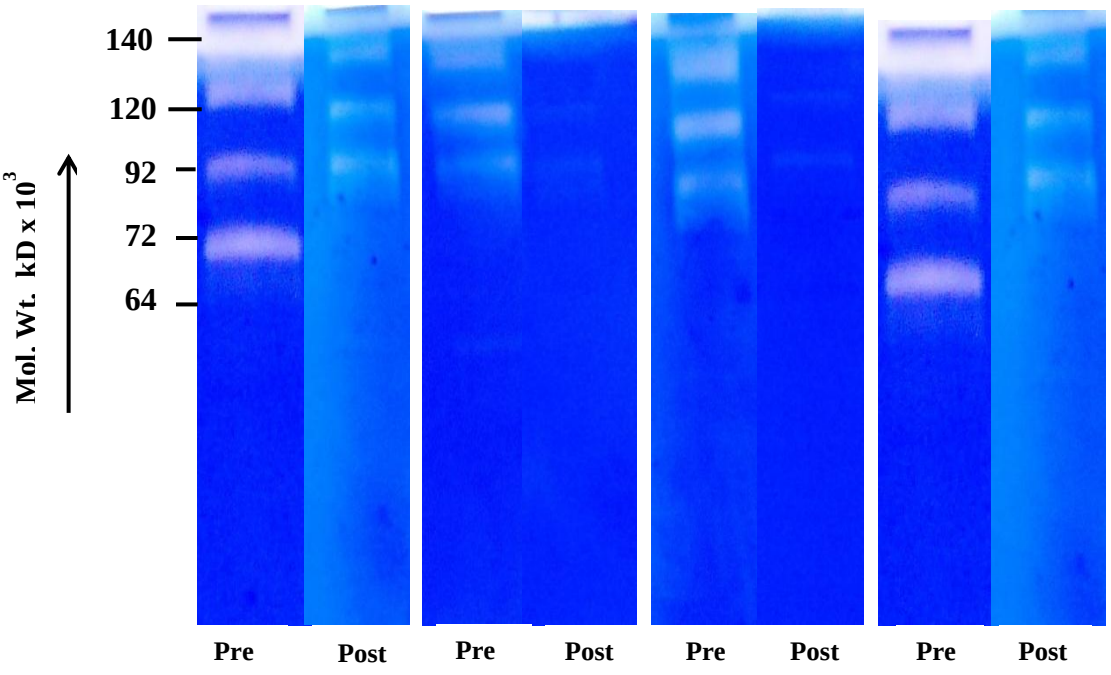

Fig. (1D). Salivary MMP-2 profile of breast tumor (benign) patients before and after surgery.

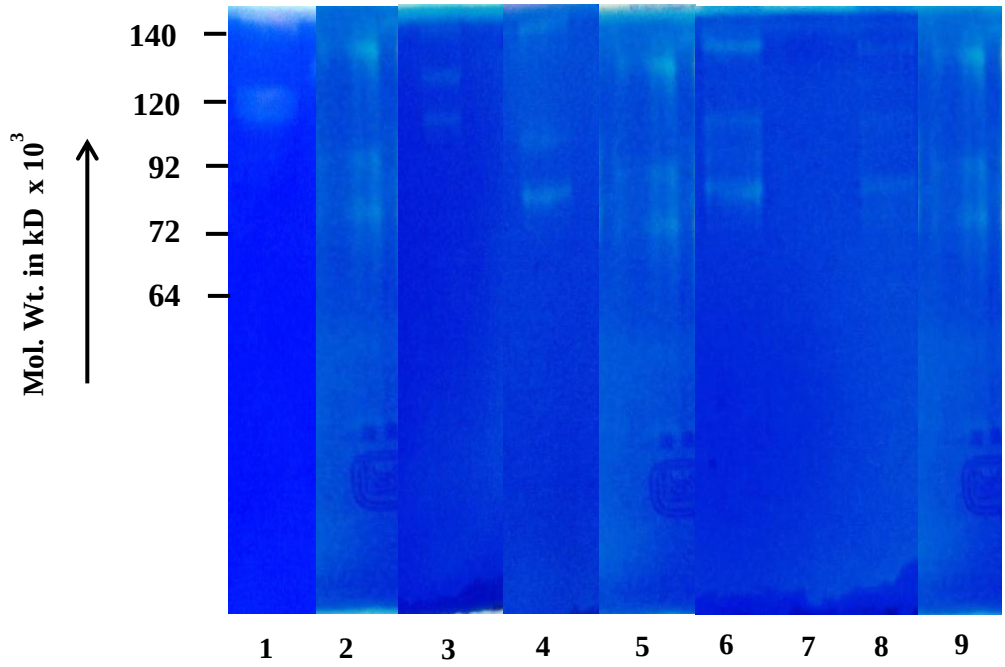

Fig. (1E). Salivary MMP-2 profile in normal femalevolunteers.

Immunoblot of Saliva Sample of Breast Cancer Patients

To identify MMP species immunoblot of salivary proteins was developed using MMP-2 monoclonal antibody which shows pro (72 kD) and activated MMP-2 (at 64 kD, 50 kD *etc*.) and 140 kD higher molecular weight form in the salivary sample of the breast cancer patients (collected before surgery) very clearly (Fig. **2**).

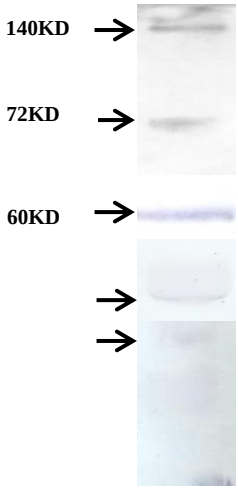

Fig. (2). Immunoblot of breast cancer patient salivary proteins with MMP-2 monoclonal antibody.

Immunoprecipitation

Fig. (**3**) shows the immunoprecipitation of salivary proteins with MMP-2 monoclonal antibody. The zymogram clearly demonstrates that most of the higher and the lower molecular bands have been precipitated by MMP-2 antibody, confirming the MMP-2 species.

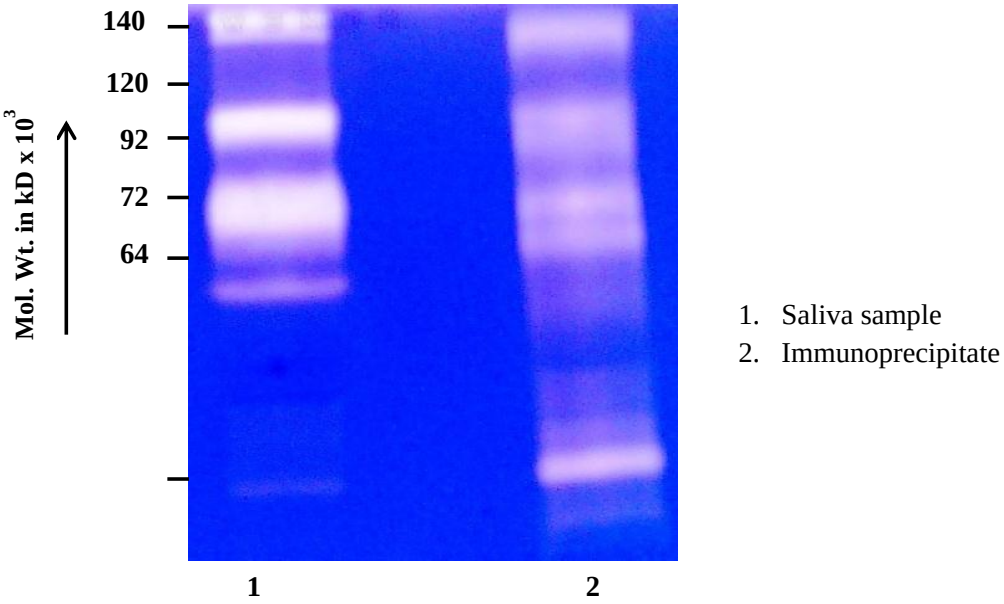

Fig. (3). Immunoprecipitation of salivary proteins with MMP- 2 monoclonal antibody.

ELISA

The quantitation of MMP-2, TIMP-2, and VEGF was performed in 10 saliva samples, collected at pre and post surgery Figs. (**4A**-**4C**). This procedure was done in triplicate. The comparative ELISA clearly shows that MMP-2 is comparatively little higher in saliva of pre surgical samples than that of post surgical samples (Fig. **4A**). TIMP-2 is appreciably higher in saliva collected after surgery (Fig. **4B**) and VEGF (Fig. **4C**) is higher in saliva collected before surgery than after surgery, $p < 0.05$.

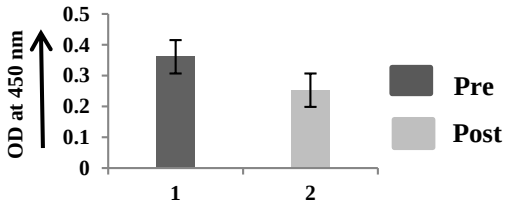

Fig. (4A). ELISA of salivary MMP-2.

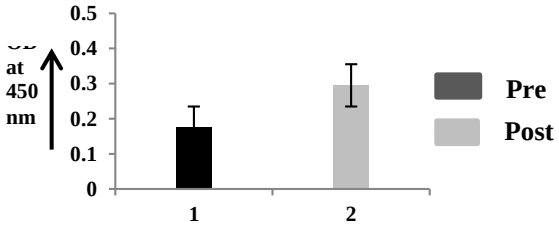

Fig. (4B). ELISA of salivary TIMP-2.

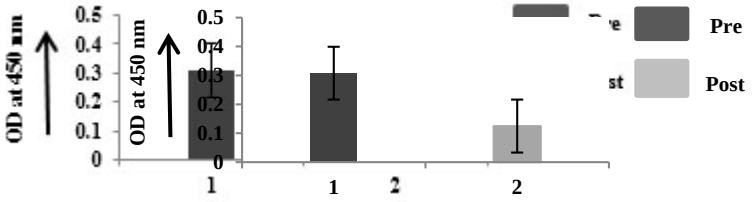

Fig. (4C). ELISA of salivary VEGF.

Clinical Characteristics of the Study Population

Table 1. Age distribution of the patients.

Age Group (in years)	Number	%
<30	1	2.8%
30-49	17	47.2%
50-69	17	47.2%
≥70	1	2.8%
Total	36	100.0%

The mean age (mean ± s.d.) of the patients was 48.52±10.90 years with range 18-70 years and the median age was 49.5 years. Proportion of patients in the age group 30 - 69 years (94.4%) was significantly higher than other age group ($Z= 12.95$; $p <0.0001$).

Table 2. Type of cancer of the patients.

Type of cancer	Number	%
Ductal	35	97.2%
Invasive Lobular (hereditary)	1	2.8%
Total	36	100.0%

Patients with ductal carcinoma (97.2% was significantly higher ($Z=13.35$; $p <0.0001$).

Table 3. Stage of cancer of the patients.

Stage of cancer	Number	%
I	4	11.1%
II	13	36.1%
III	15	41.7%
IV	4	11.1%
Total	36	100.0%

47.1% of the cases were with Stage-III followed by Stage-II (36.1%).

Table 4. Hormonal status of the patients.

Status	ER	PR	Her2 neu
Positive	5(14.7%)	4(11.8%)	2(6.1%)
Positive	15(44.1%)	14(41.2%)	7(21.2%)
Negative	14(41.2%)	16(47.0%)	24(72.7%)
Total	34*	34*	34*

*ER, PR and Her2 neu status were not available for 2 cases.

Fifty eight point eight percent (58.8%) of the cases were with ER positive and the rest 41.2% were ER negative cases. 53.0% of the cases were with PR positive and rest of the 41.2% were PR negative cases. 27.3% of the cases were with Her2 neu positive and rest of the 72.7% were Her2 neu negative cases. 14 out of 34 cases (41.1%) were TNBC (triple negative breast cancer).

DISCUSSION

In this present communication, we report the identification of activated MMP-2 (with increase in activity from early to late stage) in saliva of breast cancer patients collected before surgery. Interestingly, saliva collected after surgery (within 7 days) from the same patients, showed very weak background MMP-2 activity. The urine of the same breast cancer patients does not show any appreciable MMP activity in pre or post surgical samples. Expression of urinary MMPs has been reported by other groups in other type of cancer patients [30 - 33]. The immunoblot shows the presence of pro (72 kD), activated (64,50 kD *etc*.) MMP-2. A band at 140 kD region is also visible clearly indicating that MMP-2 may be complexed with other protein. The immune precipitation of saliva with MMP-2 antibody may strongly indicate that the salivary MMPs are mainly MMP-2 pro and activated products with higher molecular weight forms. Interestingly, out of 36 patients 35 patients are ductal carcimona only one patient is invasive lobular carcinoma but with

similar results.

Table 5. ER/PR/Her-2-Neu status and MMP-2 activity of 36 patients.

Sl No	Age	Type of Carcinoma	Stage	ER	PR	Her-2-Neu	MMP- 2 Activity	
							Pre	Post
1.	61	Ductal	I	-	-	-	2.0	0
2.	50	Ductal	I	+	+	_	2.0	0
3.	58	Ductal	I	+3	+3	0	2.0	0
4.	70	Ductal	I	-	-	-1	2.0	0
5.	35	Ductal	II	-	-	-	2.0	0
6.	47	Ductal	II	-	-	-	2.0	0
7.	36	Ductal	II	+	_	_	3.0	0
8.	47	Ductal	II	-	-	-	2.0	0
9.	60	Ductal	II	-	-	-	2.0	0
10.	47	Ductal	II	+	-	-	3.0	0
11.	52	Ductal	II	+3	+3	+3	3.0	0
12.	45	Ductal	IIB	+	+	_	3.0	0
13.	64	Ductal	II B	NA	NA	NA	3.0	0
14.	60	Ductal	II B	3+	3+	-1	3.0	0
15.	55	Ductal	II B	3+	3+	-1	3.0	0
16.	65	Ductal	II B	NA	NA	NA	3.0	0
17.	50	Ductal	IIB	+	+	_	3.0	0
18.	48	Ductal	III	+	+	+	4.0	0
19.	38	Ductal	III	-	-	-	4.0	0
20.	40	Ductal	III	_	-	-	4.0	0
21.	46	Ductal	III	+	+	+	4.0	0
22.	53	Ductal	III	+	+	-	3.0	0
23.	58	Ductal	III	-	-	-	4.0	0
24.	40	Ductal	III	+	+	+	4.0	0
25.	57	Ductal	III	-	-	-	4.0	0
26.	50	Ductal	III	-	-	-	3.0	0
27.	48	Ductal	III	+	+	-	4.0	0
28.	55	Ductal	III	+3	+1	+3	4.0	0
29.	50	Ductal	III	+	+	+	4.0	0
30.	36	Ductal	III A	+	+	+	3.0	0
31.	33	Ductal	III A	-	-	-	4.0	0
32.	18	Invasive Lobular(hereditary)	IIIC	_	_	_	4.0	0
33.	49	Ductal	IV	+	+	_	5.0	0
34.	56	Ductal	IV	_	_	_	5.0	0
35.	38	Ductal	IV	+	+	+	5.0	0
36.	32	Ductal	IV	+	+	+	5.0	0

ER/PR/Her-2 Neu status and comparative zymography of saliva (pre and post surgery) were done in 36 cases of same breast cancer patients at different stages. $P < 0.05$.

Comparative salivary MMP-2 activity of pre and post surgical cases of the same patients and the ER/PR/Her-2-neu status of 36 patients were listed in Table-5. The activity of MMP-2 was expressed as number of bands (lower mol. wt) the pro MMP-2(72 kD) was converted (being active) stagewise (I-IV).

VEGF has been reported to be an important regulator of MMP-2 activity [34 - 38]. The VEGF concentration of saliva of pre and post-surgical samples were measured and was found to be appreciably higher in pre surgical saliva as compared to post-surgical saliva. The TIMP-2 reported to be MMP-2 inhibitor [39 - 43] was found to be higher in post-surgical saliva as compared to pre surgical saliva. The higher concentration of TIMP-2 in saliva collected 7 days after surgery and higher VEGF concentration of saliva collected before surgery highlight the higher MMP-2 activity in saliva of breast cancer patients collected before surgery. Table **5** shows the comparative picture of salivary MMP-2 activity in before and after surgery (same patient) and ER/PR/Her-2 Neu status of 36 breast cancer female patients at different stages of the disease.

Descriptive statistical analysis was performed and reported in Tables **1**-**6**. In summary, we propose that saliva is a novel biological fluid to detect highly activated MMP-2, a very important MMP species in the development of cancer. Our observation that the expression and activity of salivary MMP-2 is appreciably high in saliva of breast cancer patients (stagewise I-IV) collected before surgery as compared to salivary MMP-2 collected 7 days after surgery of the same breast cancer patients is a novel finding. This salivary MMP-2 activity may be translated to potential breast cancer marker for diagnosis which is reproducible, cost effective and a non-invasive method for breast cancer detection.

Table 6. Comparison of pre and post-surgery salivary MMP2 activity of the patients.

MMP2 Activity	Before Surgery (n=36)	After Surgery (n=36)	t-test	p-value
Mean±s.d.	3.33±0.95	0.0±0.0	21.03	<0.0001*
Median	3.0	0.0		
Range	2 - 5	0 - 0		

* Statistically Significant.

Before surgery the mean MMP2 activity (mean ± s.d.) of the patients was 3.33±0.95 with range 2-5 and the median was 3. After surgery the MMP2 activity all the patients was found zero. t-test showed that the mean MMP2 activity before treatment was significantly higher than that of post treatment (t_{70} = 21.03; p <0.0001).

CONCLUSION

Saliva of breast cancer patients (before surgery) expresses highly active MMP-2 (stagewise I-IV) as compared to saliva collected after surgery from the same patients. This finding may have potential to establish MMP-2 activity as a marker for breast cancer using non-invasive method.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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