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Association between Matrix Metalloproteinase2, Membrane Type 1-Matrix Metalloproteinase and Hepatocyte Growth Factor in Breast Cancer Patients

MOHAMED A. MOHAMED, Ph.D. and GEHAN A. ABD EL-WAHAB, Ph.D.*

Biochemistry & Molecular Biology Unit Department of Cancer Biology, Cell Chemistry Unit, Department of Clinical Pathology, National Cancer Institute, Cairo University.*

ABSTRACT

Membrane type-1 matrix metalloproteinase (MT1-MMP) is essential for breast cancer invasion and metastasis. Many studies have shown that MT1-MMP has an important role in MMP-2 activation in cell membranes but only few reports about its clinical value are valid. In this study, we investigated the relationship between MT1-MMP protein expression and matrix metalloproteinase type 2 (MMP2) activity as well as hepatocyte growth factor (HGF) serum level in human breast cancer. For this purpose 34 human breast cancer tissues, also blood samples from the patients and 15 healthy controls were collected and sera were separated and analyzed for HGF determination. MT1-MMP protein expression was detected by Western Blot, MMP2 by zymographic analysis and HGF was measured by ELIZA. The results revealed that 79.4% of breast cancer tissue exhibited positive MT1-MMP expression, while it was 26.4% in the normal surrounding tissues. (p-value <0.001). There was a significant association between MT1-MMP expression and MMP-2 activity (p-value=0.007) in the tumor tissue. Also a significant association was detected between MT1-MMP expression and lymph node involvement (p-value=0.014). Neither grade nor tumor size showed significant correlation with MT1-MMP expression. MMP2 activity displayed a highly significant difference (p-value=<0.001) between the tumor tissues and their normal correspondence, while no significant association was detected between MMP2 activity and any of clinicopathological features. The serum levels of HGF were significantly increased in group of breast cancer patients as compared to the control group (p-value: 0.001). The best cut-off value for HGF was ≥430pg/ml with 91.2% and 73.3% sensitivity and specificity respectively. There was a highly significant association between serum level of HGF and MMP-2 activity in cancer breast tumor but the association between its serum level and MT1-MMP protein expression didn't reach a significant level. There was no significant correlation between serum level of HGF and any of clinicopathological features.

In conclusion: MT1-MMP can be used as a predictor for the ability of breast cancer invasion and metastasis and the association between it and MMP2 in the tumor tissue confirms that MT1-MMP is the tumor-specific activator of proMMP-2. The significant correlation between MMP2 and HGF level may reflect the regulation role of HGF on transcription of MMPs genes.

Key Words: *Breast cancer – MMPs – HGF.*

INTRODUCTION

Matrix metalloproteinase (MMP)-2 in breast cancer, is a protease produced essentially by stromal cells. In vitro studies have clearly demonstrated that it degrades molecules that are abundant in the extracellular matrix (ECM) **[1]**. MMP-2 is also one of the major targets of recently developed synthetic MMP inhibitors **[2]**. The recently literature demonstrates that the mechanism of action of MMP-2 is complex and that other molecules modulate its activity **[3]**.

MMP-2 is secreted in an inactive pro-enzymatic form and, unlike other MMPs, its activity is modulated by tissue inhibitor of metalloproteinase (TIMP)-2 and the membrane type-1 MMP (MMP-14) **[4]**.

Membrane type matrix metalloproteinases (MT-MMPs) localized to the invasive front of highly motile cancer cells **[5]** were shown to be directly involved in matrix breakdown **[6]**. So far, six members of the MT-MMP subfamily have been identified and partially characterized **[7]**. MT1-, MT2-, and MT3-MMP strongly contribute to tumor cell invasion **[8]**. MT1-MMP, the first member of a more recently established group of MMPs containing a membrane-spanning sequence, has been shown to have an important role in MMP-2 activation in cell membranes implicated in tissue-remodeling

events that range from tumor invasion and angiogenesis to growth and development **[9]**.

Current evidence indicates that MT1-MMP regulates matrix turnover by means of its ability to degrade matrix-associated molecules either directly or via the activation of downstream MMPs. As rate of activation of MMP-2 in tumor tissue is well-controlled to the expression levels of MTI-MMP and to the tumor spread, **[10]** thus MT1-MMP is believed to be the in vivo proMMP2 activator during cancer cell invasion. Animal studies showed that carcinoma cell lines transfected with MT1-MMPproduced higher levels of active MMP-2 and developed more lung metastases compared to parent tumor cells **[11]**.

Invasion and metastasis involve a large number of molecules, including angiogenic factors, growth factors and their receptors, adhesion molecules, proteases, intracellular signaling molecules, and transcription factors **[12-14]**.

Hepatocyte growth factor (HGF) was suggested to play an important role in the regulation of mitogenesis, motogenesis, angiogenesis, migration and invasion for various types of cells, and acts through a specific membrane receptor encoded by c-met proto-oncogene **[15]**.

Elevated hepatocyte growth factor content in tumor tissue was reported to predict a more aggressive biology in non-small cell lung cancer patients. However, there is still limited knowledge about the role of HGF in breast cancer. Hepatocyte growth factor (HGF) was previously reported to induce expression of Ets-oncogene family transcription factor (E1AF) gene whose product in turn positively regulates transcription of MMP genes **[16]**.

This study was carried out to investigate the expression of the activated form of gelatinase A (MMP2), MT1-MMP and serum level of pre operative HGF in breast cancer patients and to evaluate the correlation between them and clinicopathological features.

MATERIAL AND METHODS

This study was performed on 34 newly diagnosed breast cancer patients, the tumors were used for Western Blot and zymography to measure expression level of MT-1MMP and MMP2 respectively. The normal surrounding corresponding of each tissue sample was taken as control. Also venous blood samples (5ml) were collected from the patients before surgery and venous blood samples (5ml) were collected from 15 apparently healthy age matched females who serve as controls for determination of serum Hepatocyte Growth Factor (HGF). The patients under went surgical excision in National Cancer Institute, Cairo university. All the patients met the following criteria: (a) having been diagnosed as having primary invasive breast cancer (b) having no clinical manifestation of infection, (c) having received no blood transfusion during the previous 3 weeks, (d) having no known liver, renal dysfunction.

The patients were 34 females with mean age of 54.8±13.6 years (range, 28-76 years). The average tumor size was 4.6±2.4cm (range, 1.5-11cm). There were 24 tumors of differentiation grade 2 while 7 tumors of grade 3 (3 patients were unclassified). There were 24 tumors with +ve lymph nodes while 10 with –ve lymph nodes.

The tumor material was fixed in 10% neutral formalin and embedded in paraffin. The diagnosis of tumors were based on a light microscopic examination of H&E stained section.

Western blot:

To detect MT1-MMP expression, a piece of each tumor (1cubic cm) was homogenized in 1% (w/v) sodium dodecyl sulfate and diluted to one fold in water before measuring protein content against bovine serum albumin using Bradford srandard, (1979). Samples were diluted with SDS-PAGE sample buffer in the presence of mercaptoethanol, heated at 100C in boiling water bath for 5min. and allowed to cool to room temperature. Each aliquot containing 100µg was separated on 10% SDS-PAGE at 30mA and transferred to nitrocellulose membranes (Bio-RAD, USA) in transfer buffer at 10V constant for 30min (Semi-dry Bio-Rad unit, USA). The membranes were blocked for two hours at room temperature in 2.5% non fat milk powder in PBS. The membranes were then incubated overnight with monoclonal antibody specific for MT1-MMP (Santa Cruz Biotechnology, Inc.). The bands were visualized by using a horseradish-peroxidase conjugated goat antiserum against mouse IgG (Sigma Chemical CO., St. Louis, Mo), hydrogen peroxide (BDH) and diaminobenzedine (Sigma Chemical CO.,

St. Louis, Mo). The bands were evaluated (area under the peak of each sample) using Dual-Wavelength flying spot scanning Densitometer P, N206 (Shiamadzu. CO, Japan). Value of normal tissue has been taken as 100% for each sample.

Zymography analysis:

The proenzyme and activated forms of MMP2 were detected by zymography using SDS-polyacylamide gels copolymerized with 1mg/ml gelatin to detect gelatinolytic activities **[17]**.

Fifty µg from surgical specimen was homogenized in sample buffer. Samples were separated by electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS and 1mg/ml gelatin as substrate. After electrophoresis, Gels were washed at room temperature in renaturation buffer containing 2.5% Triton-X 100 for 10 minutes and for 20 minutes in water. Thereafter the gels were incubated for 18 hours at 37°C in Tris-based buffer. Gels were stained with a solution containing 30% methonal, 10% glycial acetic acid containing 0.5% Coomassie brilliant blue R250. Destaining was done in 10% methanol and 10% acetic acid, and gelatinolytic activity was detected as clear bands; 68kDa and 62kDa and analysed by densitometric scanning using a computer-assisted analysis. The band of active form was classified as negative (same as control) and positive (10% more than the control). Unstained areas corresponded to zones of MMP proteolytic activities.

Determination of HGF by quantitative sandwich enzyme, immunoassay (ELISA) technique:

Using a kit from R and D systems, Minneapolis MN, USA. The samples and standard were added to micro wells precoated with the captured antibody, after washing any unbound substances, an-enzyme linked polyclonal antibody for HGF was added. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of HGF. The intensity of the color was measured **[18]**.

Statistical analysis:

Statistical package for social sciences (SPSS) version 9 was used. Quantitative variables were summarized using mean and SD. Qualitative data were summarized using frequencies and percentage. The relation between quantitative variables was tested by Spearman correlation. Chi-square test was used to test the association between the different qualitative variables. *t*-test and Mann-Whitney U were used to compare numerical data between groups. Cut-off value was calculated. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated using ROC curve.

RESULTS

Detection of MTI MMP expression in tumor tissue and corresponding surrounding normal tissue by Western blot:

Table (1), showed that MT1-MMP expression was positive in 79.4% in breast tumor tissue compared to 26.4% in normal corresponding. The difference was highly significant (*p*:value=<0.001) Fig. (1).

Table (1): Comparison between MT1-MMP expression in tumor tissue and normal surrounding tissue of breast cancer patients.

MT1-MMP Expression	Normal tissue		Tumor tissue		*p* value
	No.	%	No.	%	
Negative	25	(73.6)	7	(20.6)	**<0.001
Positive	9	(26.4)	27	(79.4)	

***p*-Value is highly significant (<0.001).

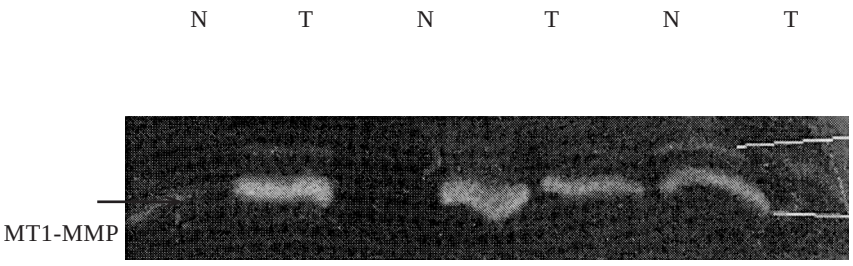

Fig. (1): Showed western blot analysis of MT1-MMP expression in breast cancer patients.
N: Normal surrounding tissue T: Tumor tissue from the same patients.

Table (2) showed the association between MT1-MMP expression and lymph nodes status in cancer breast patients. There was a significant association between MT1-MMP expression in tumor tissue and the presence of positive lymph nodes (*p*value=0.014). However there was no significant association between MTI-MMP expression and tumor grade or tumors size.

Detection of MMP2 expression in tumor tissue and corresponding surrounding normal tissue by zymography:

61.8% of tumor cases showed high activity of MMP2 while 38.2% showed the same activity of control correspondence (*p*value:0.001) Fig. (2).

There was strong association between MMP2 activity and MT1-MMP Expression in tumor tissue of breast cancer patients (*p*-value: 0.007).

The association between MMP2 activity with other clinical and pathological parameters were evaluated. As shown in Table (4), there was no relationship between the expression of MMP2 and lymph node status, tumor grade or tumor size.

Determination of HGF serum level by (ELISA) technique:

A highly significant increase of serum HGF levels was found in breast cancer patients when compared to controls (*p*:value=<0.001).

The cut-off value of HGF was ≥430pg/ml. It detects breast cancer patients with sensitivity 91.2% and exclude normal control with specificity of 73.3%, the positive predictive value was 88.6% and the negative predictive value 78.6%.

There was a highly significant increase in the serum levels of HGF in patients with positive MMP2 activity than those with negative MMP2 activity (*p*value:0.001). Meanwhile, there was also increase in its levels in patients with positive MTI-MMP expression compared to those with negative MT1-MMPbut the difference did not reach to significant level.

The serum level of HGFdid not correlated with age of the patients (*r*=–.139, *p*value:0.44) or with tumor size (*r*=0.146, *p*:value=0.41) and tumor grade (*r*=0.084, *p*:value=0.65). Also, there was no statistically significant difference in its level between cases with positive lymph nodes and those with negative lymph nodes (mean ± SD, 553.13±105.96pg/ml and 521.1± 97.06pg/ml respectively) with *p*value=0.42.

Table (2): Association between MT1-MMP expression and lymph nodes status, tumor size and tumor grade in cancer breast patients.

Clinical parameters	MT1-MMP expression		*p* value
	–ve No. (%)	+ve No. (%)	
Total patients no.	7	27	
Lymph nodes:			
(–)	5 (71.4)	5 (18.5)	*0.014
(+)	2 (28.6)	22 (81.5)	
Tumor size:			
≤2cm	2 (28.6)	5 (18.5)	0.59
>2cm	5 (71.4)	22 (81.5)	
Tmor grade:			
II	5 (71.4)	18 (66.7)	0.18
I11	0 (0)	8 (29.6)	

*p-value <0.05 significant (2 tailed).

Table (3): Showed the association between the MT1-MMP expression and the MMP2 activity in tumor tissue.

MTI -MMP Expression	MMP2 activity		*p* value
	–ve (no=13)	+ve (no=21)	
Negative (no=7)	6 (85.7%)	1 (14.3%)	*0.007
Positive (no=27)	7 (25.9%)	20 (74.1%)	

*p-value <0.05 is significant (2-tailed).

Table (4): Showed the association between MMP2 activity and lymph nodes status, tumor size and tumor grade.

Clinical parameters	MMP-2		*p* value
	–ve no (%)	+ve no (%)	
Total patients no.	13	21	
Lymph nodes:			
(–)	5 (38.5)	5 (23.8)	0.36
(+)	8 (61.5)	16 (76.2)	
Tumor size:			
≤2cm	4 (30.8)	3 (14.3)	0.25
>2cm	9 (69.2)	18 (85.7)	
Tmor grade:			
I	10 (83.3)	14 (73.7)	0.68
II	2 (16.7)	5 (26.3)	
III			

*p-value <0.05 is significant. (2-tailed).

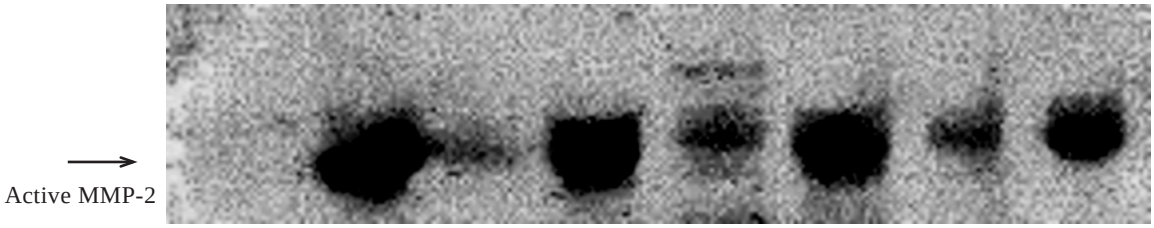

Fig. (2): Showed zymographic analysis of MMP2 in breast cancer patients.

Table (5): Serum levels of HGF in healthy controls and breast cancer patients.

Parameter	Healthy control (no=15)	Patients (no=34)	*p* value
HGF (pg/ml) Mean ± SD	409.67± 104.89	543.71± 103.03	**<0.001

***p* value <0.001 highly significant (2-tailed).

Table (6): Comparison between Serum HGF levels in patients and MT1-MMP and MMP2 expression.

Parameter	MMP2 activity		MT1-MMP expression	
	+ve (no=21)	–ve (no=13)	+ve (no=27)	–ve (no=7)
HGF (pg/ml) Mean ± SD	602.95± 79.48	448± 50.99	560.74± 103.2	478± 76.7
p: value	** 0.001		* 0.07	

**p*-value >0.05 not significant.
***p*-value <0.001 highly significant.

DISCUSSION

MT1-MMP is a major MMP because, it has been thought to be exclusively involved in the breakdown of the ECM components including collagens and laminin-5 **[19]**, and in the activation pathway of soluble MMPs, i.e. MMP-2 and MMP-13 **[20]**. The precise localization of MT1-MMP between cancer cells and surrounding stromal cells has been the subject of controversy. MT1-MMP was originally identified as an activator of pro MMP2 on the surface of invasive tumor cells **[9]**. MMP2 is thought to be responsible for the degradation of the basement-membranes as it degrades type IV collagen. In our study, by using western blot analysis, MT1-MMP was expressed in of breast cancer tissue of 79.4% of breast cancer patients and only in 26.4% of normal corresponding adjacent tissues (*p*:value=0.0001). Dalberg et al. **[21]** detected MT1-MMP mRNA expression in all invasive breast tumor biopsies investigated and found that it was mainly localized in the tumor cells. However, some authors stated that MT1-MMP is detectable in normal tissues, but the expression of this protease is strongly associated with aggressive, invasive malignant cells **[22]**.

MMP2 (gelatinase A) activity in this study was detected almost in all normal surrounding tissue of the breast tumors, however there was increase in the levels of its activity in breast cancer tissue in comparison with adjacent ones as 61.8% of the tumor cases showed high activity of MMP-2 while 38.2% showed the same activity of control correspondence (*p*-velue <0.01). similarly, Lee et al. **[23]** and Remacle et al. **[24]** were found by using zymography that breast cancers patients expressed higher levels of activated MMP-2 than benign lesions. The mechanism by which MMP-2 is regulated in normal tissue by malignant cells is likely to be important for the escape of neoplastic cells from tumor margins allowing invasion of adjacent normal tissues and possibly entry into the blood or lymphatic systems. However, it is difficult to imagine these mechanisms being involved in the infiltration of malignant cells at distance sites where a much more rapid regulation of MMPS is likely to be required **[25]**.

In this study a significant association between MT1-MMP expression and MMP-2 activity in tumor tissue was detected (*p*:value= 0.007). In consistence with our result Velasco et al. **[26]** and Murphy et al. **[27]** who showed that MT1-MMP may have the ability to activate pro-MMP2 through the association of an MTI-MMP with tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) extracellulary, this complex then binding pro-MMP2. The MMP2 is then brought into close proximity with a second MT-1MMP, resulting in cleavage of MMP2 prodomain. Also Cox et al. **[28]** reported that the expression of gelatinase A can frequently be

superimposed on to that of MT1-MMP. This suggests that the transcription of these two genes may be coordinated and that MT1-MMP then activates progelatinase A allowing ECM degradation. Similarly Ratnikov et al. **[29]** stated that Activation of the latent MMP-2 zymogene involves its binding to the cell surface MT1-MMP*TIMP-2 (membrane type-1 matrix metalloproteinase/tissue inhibitor of matrix metalloproteinase-2) complex with subsequent cleavage of proMMP-2 by TIMP-2-free adjacent MT1-MMP. This is followed by autolytic maturation of the activation intermediate and the release of the mature MMP-2 species from cell surfaces into the extracellular milieu.

In our study, no association between MMP2 and MT1-MMP in normal breast tissue has been found. This resultes in agreement with Naw eroke et al. **[30]**, who stated that there was no association between gelatinase A and MT1-MMP in normal lung parenchyma, so the correlation of MT1-MMP with MMP2 activation in breast cancer tumor tissue again suggests that MT1-MMP is a tumor specific activator of proMMP2.

Clinical parameters such as tumor size, grade and lymph node status have long been used to characterize breast phenotypes in relation to prognosis. In the present study, we investigated MTI-MMP and MMP-2 for their prognostic significance. We found that MT1-MMP was expressed in 91.7% of positive lymph nodes cases, while expressed in 50% only of negative lymph nodes cases. (p:value=0.014). This significant association has previously mentioned by Yao et al. **[31]** who reported that MT-1 MMP protein expression in human breast cancers had positive correlation with lymph node metastasis. Mimori et al. **[32]** also determined that the highest expression of MT1-MMP mRNA was found in breast cancer specimens showing lymph-node metastasis and/or lymph-vessel invasion.

Meanwhile there was only a tendency of increased MMP2 activity in positive lymph nodes cases as it expressed in 66.7% of positive lymph node cases comparing to 50% in negative lymph node ones, but the difference was not statistically significant. In agreement with our result Têtu et al. **[33]** who did not find a significant association between MMP2 activity and lymph nodes metastasis in cancer breast patients.

The association of MT1-MMP but not MMP-2 expression with lymph nodes metastasis may be explained by the fact that MT1-MMP has many substrates other than MMP-2 **[21]**. However our study demonstrated no association between MT1-MMP or MMP2 and tumor size or tumor grade. In contrary to our result He-Cheng et al. **[34]** and Yao et al. **[31]** who found that, the expression of MMP2 and MT1-MMP correlated with tumor grade and tumor size, this discrepancy could be explained by the different method of detection used.

The serum levels of hepatocyte growth factor have already been shown to be of prognostic value in other malignancy **[35]**. Semi quantitative analysis by immunohistochemical staining of tumor specimen has several drawbacks; as it is sometimes not accurate enough to evaluate the intermediate patterns of staining although it is sufficient to differentiate negative versus positive reaction, also different results could due to possibility of heterogeneity within tumor specimens. So the choice of serum for a quantitative analysis in this study could possibly avoid the abovementioned disadvantages of a semiquantitative analysis by immunohistochemical staining.

The present study showed highly statistically significant elevated levels of serum HGF in cancer breast patients when compared with controls. (p-value<0.001). Our results agree with Sheen-Chen et al. **[36]** who reported that there was a highy significant increase in preoperative serum level of HGF in cancer breast patient in comparison with control. Also HGF is found to be involved in carcinogenesis, Jeffers et al. **[38]** reported that co transfection of HGF was able to induce morphologic transformation in vitro and tumorigenicity in vivo in a nontumorigenic mouse cell line C127.

The cut-off value of HGF was ≥430pg/ml, it detected breast cancer patients with sensitivity 91.2% and exclude normal control with specificity of 73.3% so, serum HGF could be used as a non invasive marker for diagnosis of breast cancer. On the other hand the present study showed that there no significant correlation between HGF serum level in cancer breast patients and lymph nodes status, tumor grade or tumor size. In disagreement with our result, Sheen-Chen et al. **[36]** who stated that the preoperative level of serum HGF may reflect the severity of invasive breast cancer.

Furthermore the results of the present study showed highly significant increase in the serum levels of HGF in cancer breast patients with positive MMP2 activity in their tumor tissue than those with negative MMP2 expression with *p*-value<0.001. So there was a strong positive association between MMP2 and HGF. Meanwhile there was a tendency of increased serum levels of HGF in cancer breast patients in cases with positive MT1-MMP expression than those with negative MT1-MMP expression but the difference did not reach a significant level (*p*:value=0.07). Previous reports have shown that PEA3/E1AF/ETV4 gene (which encodes an Ets-related transcription factor that is expressed in the epithelial cells of the mammary gland) can up-regulate promoter activities of many genes associated with tumorigenesis. A significant fraction of those encode matrix metalloproteinases (MMP genes) **[38,16]**. Also Motoaki Hanzawa et al. **[16]**, reported that the levels of MMPs mRNAs increased in cells treated with HGF and correlated with E1AF up regulation.

Conclusion:

- MT1-MMP can be used as prognostic factor predicts the possibility of breast cancer invasion and metastasis, so the enzyme may be used a therapeutic target in breast cancer.
- The significant association between MT1-MMP protein expression and MMP2 activity in tumor tissue confirms the previous results that MT1-MMP is the tumor-specific activator of proMMP-2.
- Serum HGF may be used as diagnostic marker for breast cancer and the highly significant association between it and MMP2 activity reflects and confirms the regulatory role of HGF on MMPs genes.
- Follow-up of cases and further study with larger number of patients may be of great value to achieve more substantial conclusion and confirms the usage of the three parameters which investigated as a diagnostic or prognostic markers.

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