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RESEARCH ARTICLE

The Ratio of MMP-2 to TIMP-2 as Probable Prognostic Biomarkers in Indian Cancer Patients

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ABSTRACT: Cancer is an uncontrollable growth of abnormal cells that leads to tumor formation, invading neighboring tissues by migrating to distant organs to form secondary tumors through metastasis. Metastasis also involves the degradation of the Extracellular Matrix (ECM), which maintains the integrity of the organs. ECM degradation occurs during pathophysiological conditions by matrix metalloproteinases (MMPs). MMPs remodel the components of the ECM by their proteolytic activity. MMPs have a role in normal biological processes, like embryogenesis, morphogenesis, tissue remodeling, etc., and pathological processes like atheroma, arthritis, cancer, etc. MMPs are regulated at the transcription level by activating endogenous inhibitors-tissue inhibitors of metalloproteinases (TIMPs) that control the local activities of MMPs in tissues. Variations in MMPs and TIMPs expression contribute to the development and metastasis of cancers. Therefore, MMPs and TIMPs have become important therapeutic and diagnostic cancer targets. We studied MMP-2, MMP-9, TIMP-1, and TIMP-2 plasma levels in cancer patients and healthy controls. MMPs and TIMPs levels were analyzed by ELISA (Immunoassay). We hypothesized that the ratio of MMP-2 to TIMP-2 and MMP-9 to TIMP-1 in cancer patients could be higher compared to healthy controls, contributing to their pathophysiological implication.

KEYWORDS: Biomedical and Health Sciences; Extracellular Matrix; Tissue Remodeling; Diagnostic Biomarkers; Matrix Metalloproteinases; Tissue Inhibitors of Metalloproteinases.

Introduction

Cancer is defined as the uncontrolled and abnormal proliferation of cells. It can develop in any body part, be benign (not cancerous) or malignant (cancerous), and spread to other tissues via the lymphatic or circulatory systems.[1] Cancer can develop from any factor that can cause cells to divide uncontrollably. Cancer can be called a genetic disease or induced by environmental factors, lifestyle, etc.[2] There are numerous distinct cancer types, each with its own causes, signs, and therapies.[3] In India, Cancer is increasingly prevalent. The number of new cancer patients in 2022 is 14,61,427. It is predicted that the number of new cancer patients will increase by 12.8% before the end of 2025.[4]

Cancer-causing genetic mutations mainly affect three categories of genes found in human DNA: proto-oncogenes, tumor suppressor genes, and DNA repair genes. Proto-oncogenes are essential for normal cell growth and division. When these genes become more active than usual, they can become cancer-causing genes known as oncogenes. Alterations in the tumor suppressor gene can also cause cancer.[5] DNA repair genes are involved in the rectification of damaged DNA. Cells that have mutations in these genes are more prone to produce additional mutations. These alterations, when combined, may cause the cells to become malignant.[6] These malignant cells then spread to different body parts through metastasis by invading tumor cells into the basement membrane and migrating to other organs. Metastasis usually involves the migration of cells through the basement membrane into the lymphatics and possibly also to the blood vessels, which finally find their way into other organs. Tumor cells cross the extracellular matrix barrier to accomplish this transition.[7] The Extracellular Matrix (ECM) barrier helps prevent metastasis and maintains polarity. The extracellular matrix comprises macromolecules that support everything from local tissue growth to maintaining an entire organ.[8] These molecules are all secreted by neighboring cells. Upon being secreted, the proteins will undergo scaffolding. The viscous consistency of the matrix will be maintained by these stiff, albeit temporary, protein structures. The extracellular matrix has a role in supporting growth and wound healing.[9]

Cancer must be diagnosed in the early stages so that the patient gets the proper treatment and has a higher chance of survival.[10] Currently, cancer diagnosis is invasive and expensive, reducing the accessibility to low-income society.[11] Biomarkers are available in the blood plasma of any human.

Using biomarkers, the diagnosis and prognosis of cancer become much simpler. The variations in the concentration of specific biomarkers can indicate the presence or progress of a specific disease.[12]

Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases TIMPs are biomarkers.[13] MMPs are a class of zinc-dependent extracellular matrix (ECM) remodeling endopeptidases. They can degrade the ECM.[14] TIMPs are inhibitors of MMPs. They bind with their complementary MMPs and inhibit their activity.[15] In cancer, biomarkers such as Matrix Metalloproteinase 2 (MMP2), Matrix Metalloproteinase 9 (MMP9), Tissue inhibitors of matrix metalloproteinase

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1 (TIMP1), and Tissue inhibitors of matrix metalloproteinase 2 (TIMP2) can help determine the prognosis of a patient and determine if treatment was helpful and determine the future course of treatment.[16]

MMPs and TIMPs vary in concentration in the blood of cancer patients and healthy controls. The presence of cancer cells usually causes this variation. MMPs are generally expressed in higher concentrations if cancer cells are in the body. This is because MMPs promote the spread of cancer.[17] Some TIMPs are expressed in lower concentrations in cancer because the lack of TIMPs mainly leads to metastasis, hence their cancer promotion.[18] We have selected these biomarkers specifically as the samples of blood used in this experiment were collected from a cancer hospital that treats head and neck cancer patients as the majority. A literature survey revealed that MMP2 and MMP9 are active in head and neck cancers.[19]

Our study investigated the correlation of plasma MMP2: TIMP2 and MMP9: TIMP1 in cancer patients compared to healthy controls. The rationale of this work was to explore whether these correlations can offer a prognostic value to assess the stage of disease in these subjects. Our results in Indian subjects demonstrated that MMP9 was higher in the blood plasma of cancer patients than in healthy controls, and MMP2 was lower in cancer patients than in healthy controls. TIMP1 and TIMP2 investigated in our research were higher in the blood plasma of healthy controls than in cancer patients. Our research aims to find the ratio between the complementary MMPs and TIMPs for cancer patients and healthy controls. This ratio can then be used to determine the patients' prognosis for cancer.

Methods

Study Population:

Eight head and neck cancer patients, irrespective of their gender, age, type, and stage of cancer, and seven healthy controls were selected for our study. The cancer patient population was selected under the framework of the research regulations of the Central Ethics Committee (CEC) of Health Care Global (HCG) Hospitals in Bangalore, India. All the healthy controls and the patients whose blood samples were used for the study gave informed consent before their enrollment in the investigation.

Collection of Blood:

4 ml blood was collected from cancer patients and healthy controls by venous puncture using the standard blood bank procedure in EDTA vacutainers (32). All blood samples were screened for infectious diseases before use in experiments.

Collection of Plasma from Peripheral Blood:

The peripheral blood was centrifuged at 1500 rpm for ten minutes to separate the plasma from the blood. Plasma was collected and stored at -20°C for 4-5 days until further evaluation of the cytokines.

Cytokine detection by ELISA:

The presence of cytokines was determined quantitatively using ELISA, and the concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2 were evaluated according to the manufacturer's instructions (Elabscience). Capture antibody pre-coated 96-well microtiter plates were used for the assay.

The procedure for sandwich ELISA is summarized as follows: 100 µL of standards, samples, and blank were added to the precoated wells and then incubated at 37°C for 90 minutes. The liquid was removed, and 100 µL of biotinylated detection antibody was added to all the wells and incubated at 37°C for 1 hour. The plate was washed three times with wash buffer, and 100 µL of horseradish peroxidase conjugate solution was added to the wells and incubated at 37°C for 30 minutes. The plate was again washed five times, and 90 µL of the (transparent) substrate was added and kept at 37°C for 15-30 minutes. Lastly, 50 µL of stop solution was added to all of the wells, and the optical density values of the samples were determined at 450nm using an ELISA plate reader (Lisaquant).

The concentration of each cytokine in ng/mL was estimated by plotting standard and blank values in MS Excel and fitting them with the equation y = mx + c. We used the sample diluent as a negative control, the standards as a positive control, and the O.D values of the blank wells were subtracted from the experimental samples. The Plasma samples were diluted 100-fold in MMP-2 and MMP-9, and 50-fold dilutions were included for TIMP-1 and TIMP-2.

Statistical analysis:

Statistical analyses were performed using an Excel Spreadsheet. Cancer patients (n=8). Healthy controls (n=7). Data values were expressed as mean values mean ± SD. Statistical significance was analyzed by ANOVA single factor. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered statistically significant (n=2, where n=2 means that all the samples were run in duplicates).

Results and Discussion

Several studies have demonstrated different MMPs and TIMPs as biomarkers for cancer prognosis.[20] Not many studies have been done on the Indian population of head and neck cancer patients. We wanted to know if there was a correlation between the concentration of MMPs and TIMPs in both cohorts and if it could be used as a prognostic marker. Therefore, we undertook a study to evaluate the concentrations of MMPs and TIMPs in cancer patients' and healthy controls' plasma samples. Eight Cancer patients' and seven healthy controls' plasma samples were chosen for the study. The concentrations of Matrix metalloproteinase-2 and 9 and Tissue inhibitors of Matrix metalloproteinase-1 and 2 present in these plasma samples were quantitatively analyzed using sandwich Enzyme-Linked Immunosorbent Assay (ELISA).

MMP-2 and TIMP-2 levels in cancer patients and healthy controls:

The mean concentrations of MMP-2 were significantly ($p = 0.0106$) higher (245.31 ng/mL) than the mean concentrations of TIMP-2 (122.04 ng/mL) in cancer patients. Similarly, the mean concentrations of MMP-2 were significantly ($p = 0.035$) lower (181.38 ng/mL) than the mean concentrations of TIMP-2 (363.69 ng/mL) in healthy controls. (Figure 1). MMP2 concentrations were non-significantly ($p= 0.097$) higher in cancer patients (245.31 ng/mL) when compared to healthy controls (181.38 ng/mL). Whereas TIMP2 concentrations were significantly ($p = 0.0073$) lower in cancer patients

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(122.04 ng/mL) when compared to healthy controls (363.69 ng/mL) (Figure 1).

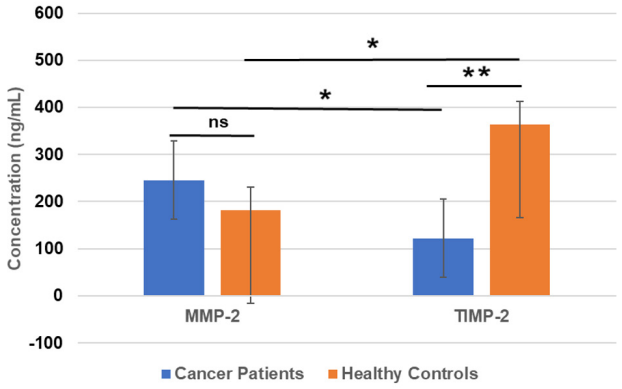

Figure 1: Mean MMP-2 and TIMP-2 concentrations in Cancer Patients' and Healthy controls' plasma samples. This is a graphic representation of MMP-2 and TIMP-2 concentrations in cancer patients (n=8) and healthy controls (n=7). Data values are represented as mean ± SD. Statistical significance was analyzed using an ANOVA single-factor test.* p0.05 and ** p0.01 are considered statistically significant. All samples were run in duplicates. Concentration is represented in ng/mL.

MMP-2 concentrations were non-significantly higher in cancer patients than in healthy controls, but their TIMP-2 concentrations, being significantly lower than those of healthy controls, could suggest hyperplasia, tumor invasion, and metastasis. MMP-2 concentrations were significantly lower than TIMP-2 concentrations in healthy controls, indicating tissue homeostasis.

Our results were supported by a study on oral cancer patients where MMP-2 concentration was higher.[21] During inflammation, hyperplasia injury, and cancer, tissues produce an increased concentration of MMP-2.[22] A study by Westermarck suggested that interaction between tumor cells and stromal cells promotes tumor invasion and metastasis.[23] Metalloproteinases are known to degrade the target matrix substrate, but their activity can be blocked by TIMPs, which are natural tissue inhibitors of metalloproteinases, through two mechanisms. In the zymogen activation stage, TIMP-2 binds to the zymogen of MMP-2 (pro-MMP-2), forming a complex, while TIMP-1 forms a specific complex with pro-MMP-9 leading to TIMP inhibiting pro-MMP activation. Later, TIMPs bind to activated MMPs, forming a 1:1 complex.[24] Under normal conditions, tissues secrete less MMP-2, and their activity is also held in check by its antagonist TIMP-2, as seen in healthy controls.

MMP-9 and TIMP-1 levels in cancer patients and healthy controls:

The mean concentrations of MMP-9 were significantly (p = 0.00000216) lower (243.65 ng/mL) than the mean concentrations of TIMP-1 (453.36 ng/mL) in cancer patients. Similarly, the mean concentrations of MMP-9 were significantly (p = 0.00103) lower (326.59 ng/mL) than the mean concentrations of TIMP-1 (520.78 ng/mL) in healthy controls (Figure 2). Also, MMP9 concentrations were significantly (p = 0.045) lower in cancer patients (243.65 ng/mL) when compared to healthy controls (326.59 ng/mL). TIMP1 concentrations were non-significantly (p = 0.074) lower in cancer patients (453.36 ng/mL) when compared to healthy controls (520.78 ng/mL) (Figure 2).

MMP-9 concentrations were significantly lower than

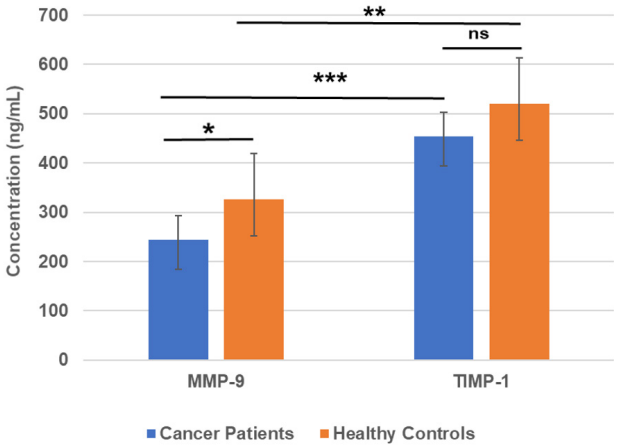

Figure 2: Mean MMP-9 and TIMP-1 concentrations in Cancer Patients' and healthy controls' plasma samples. This is a graphic representation of MMP-9 and TIMP-1 concentrations in cancer patients (n=8) and healthy controls (n=7). Data values are represented as mean ± SD. Statistical significance was analyzed using an ANOVA single-factor test.* p0.05, ** p0.01, *** p0.001 were considered statistically significant. ns = non-significant. All samples were run in duplicates. Concentration is represented in ng/mL.

TIMP-1 concentrations in both cancer patients and healthy controls, which does not indicate a diseased or healthy state.

A study by Pozzi *et al*. showed that low MMP-9 plasma levels in tumor-bearing mice decreased angiostatin levels, leading to increased tumor vascularization and growth.[25] MMP-9 is called a multifunctional modulator involved in highly complex cell-signaling cascades. MMP-9 affects cancer cells as well as other cell populations. MMP-9 can act as either a carcinoma protector or promoter depending on patient characteristics such as stage, grade, and tumor location.[26] Contradictory to our study, one study observed that the serum levels of MMP-9 in the cancer patients' group were higher than that of the control group (39.89 ± 20.54 ng/mL) with a statistically significant difference. This study, however, does not consider the complementary concentration of TIMPs. Our study does not support the findings in other studies. Hence, the results could conflict with those of others.

Comparison of the ratio of MMP-2 to TIMP-2 levels in cancer patients and healthy controls:

The ratio of concentrations of MMP-2 to TIMP-2 in cancer patients was significantly (p = 0.039) higher (2.8 or > 1), more than threefold higher when compared to that of healthy controls (0.77 or < 1) (Figure 3).

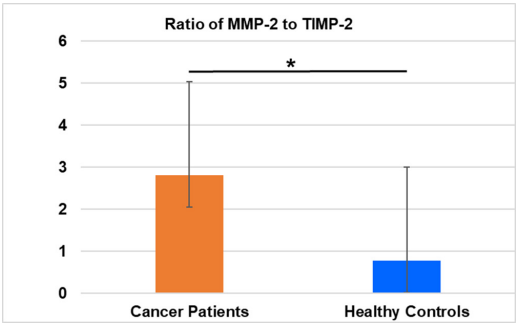

Figure 3: The ratio of mean MMP-2 to mean TIMP-2 concentration in Cancer Patients' and Healthy Controls' plasma samples. Graphical representation of the ratio of mean MMP-2 to mean TIMP-2 concentrations in cancer patients (n=8) and healthy controls (n=7). Data values are represented as mean ± SD. Statistical significance was analyzed by ANOVA single factor test *p<0.05 is considered statistically significant. All samples were run in duplicates.

The ratio of MMP-2 to TIMP-2 being significantly higher (>1) in cancer patients than the ratio in healthy controls (<1), suggests an advanced stage of their disease and a bad prognosis for the respective cancer patients.

The reason is that most cancer patients are in the advanced stage of their disease, and the imbalance in MMP-2 and TIMP-2 concentrations could have contributed to their present state. When compared to its corresponding TIMP-2 concentrations, MMP-2 is a metalloproteinase enzyme that degrades the ECM. If its activity is not inhibited by TIMP-2, which is in lower concentration, it could have led to metastasis. Meanwhile, in the healthy control group, TIMP-2 is higher than MMP-2. The system must regulate the expression of MMPs and TIMPs to maintain homeostasis; its disruption could lead to an imbalance in homeostasis and metastasis of cancer, as seen in these patients.[27] TIMP-2, an inhibitor for MMP-2, was lower in cancer patients. Cancer tissues express MMP-2, and the enzyme is found in the peripheral blood of a cancer patient. Previous studies have found an increased concentration of MMP-2 in head and neck cancer patients. A study analyzed the MMP-2 and MMP-9 in serum and saliva of head and neck squamous cell carcinoma (HNSCC) patients. Their study showed that the salivary levels of MMP-9 in the patients' group (49.27 ± 44.5 ng/mL) were higher than the control group (44.68 ± 40.95 ng/mL). Still, this difference was not statistically significant (p = 0.736) (Table 2). In contrast, the serum levels of MMP-9 in the case group (78.77 ± 27.51 ng/mL) were substantially higher than that of the control group (39.89 ± 20.54 ng/mL) with a statistically significant difference (p < 0.001). This paper, however, does not consider the complementary concentration of TIMPs.

Table 2: Levels of MMP-2 and 9 in the Serum and Saliva of the Case and Control Group[a]

Variables	Study Groups, ng/mL		P Value
	Case	Control	
MMP-2 saliva	2.17 ± 0.62	2.21 ± 0.31	0.764
MMP-2 serum	12.48 ± 2.19	11.69 ± 2.39	0.283
MMP-9 saliva	49.27 ± 44.50	44.68 ± 40.95	0.736
MMP-9 serum	78.77 ± 27.51	39.89 ± 20.54	< 0.001

Values are expressed as mean ± SD.

Comparison of the ratio of MMP-9 to TIMP-1 levels in cancer patients and healthy controls:

The ratio of concentrations of MMP-9 to TIMP-1 (0.542 or < 1) in cancer patients was non-significantly (p = 0.204) lower than that of healthy controls (0.619 or < 1) (Figure 4).

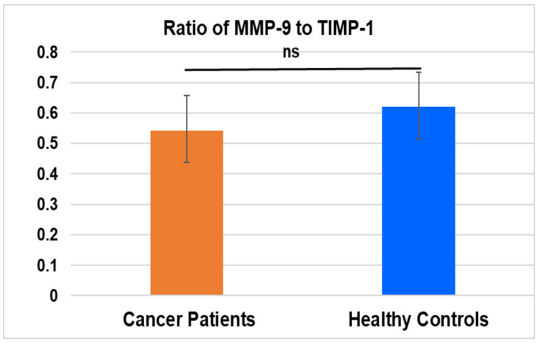

Figure 4: The ratio of mean MMP-9 to mean TIMP-1 concentration in Cancer Patients' and Healthy Controls' plasma samples. This is a graphic representation of the ratio of mean MMP-9 to mean TIMP-1 concentrations in cancer patients (n=8) and healthy controls (n=7). Data values are represented as mean ± SD. Statistical significance was analyzed using an ANOVA single-factor test. ns = non-significant. All samples were run in duplicates.

The ratio of MMP-9 to TIMP-1 being non-significantly lower in cancer patients than in healthy controls cannot be considered a prognostic indicator.

Looking at the ratio of MMP-9 TIMP-1, it is higher in healthy controls rather than in cancer patients. This means that in cancer patients, it is more likely that TIMP1 inhibits all of the MMP-9 molecules. This data does not support our hypothesis; hence, the ratio of MMP-9 to TIMP-1 cannot be considered a prognostic indicator.

It is beneficial to look at the ratios of MMP-2 to TIMP-2 and MMP-9 to TIMP-1 to obtain more useful readings.[26] Our results coincide with this study's readings when only the MMP-2 and MMP-9 concentrations were examined. In our results, the MMP-2 and MMP-9 concentrations are higher in cancer patients than in healthy controls. However, the difference between the concentrations in cancer patients and healthy controls differs. In a study analyzing MMP-2 and nine levels (tissue and plasma) in patients with colorectal cancers, a definite correlation existed between MMP-9 levels and the advancement of the disease. Although TIMP-1 is an important parameter to consider, it is not considered in this paper.[28]

Conclusion

Thus, these preliminary results showed that the ratio of MMP-2 to TIMP-2 was more than three-fold higher than that of healthy controls, and the difference was significant. Hence, the metalloproteinase 2 (MMP-2) ratio to its tissue inhibitor (TIMP-2) could be considered an index for tumor invasion, metastasis, and prognosis in head and neck cancer patients and correlate with their clinical and pathologic prognostic factors. At the same time, the difference in the Ratio of MMP-9 to TIMP-1 was not significant between the two cohorts. Since this is a preliminary study, more cancer patients

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and healthy controls must be studied to validate our results and reach a definite conclusion.

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