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MMP-2 AND MMP-9 PROTEIN DETERMINATION IN CERVICAL CANCER SAMPLES

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ABSTRACT

Cancer is characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis. Metastasis is defined as the stage in which cancer cells are transported through the blood stream or lymphatic system, where they continue to proliferate and give rise to secondary tumours'. Cervical cancer is a malignancy of the cervix. About 80% to 90% of cervical cancer and squamous cell carcinomas, which are composed of cells that resemble the flat, thin cells called squamous cells that cover the surface of the endocervix. The remaining 10% to 20% of cervical cancer are adenocarcinomas. Matrix metalloproteinases (MMPs) secreted by cervical and ovarian cancer, especially MMP-2 and MMP-9, play crucial roles in tumor invasion and metastasis. The protein concentrations were found to be 1.84 and 2.48 for normal sample and diseased sample respectively. The molecular weight of MMP-2 and MMP-9 of cancer subjects were determined as 62 KDa and 78 KDa approximately and in normal subjects as 52 KDa and 72 KDa by using SDS-PAGE.

Key Words: Cancer, Cervix, Matrix Metalloproteinases, SDS-PAGE, Zymograph.

INTRODUCTION

Cancer is characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis. Metastasis is defined as the stage in which cancer cells are transported through the blood stream or lymphatic system, where they continue to proliferate and give rise to secondary tumors. Cervical cancer is a malignancy of the cervix. Cervix is the lower part of the uterus (womb). The cervix connects the body of the uterus to the vagina (birth canal). The part of the cervix closest to the body of the uterus is called the endocervix. The part next of the vagina is ectocervix. Most cervical cancers start where these two parts meet. Worldwide, Cervical cancer is the second most common cancer of women. It may present with the vaginal bleeding but symptoms may be absent until the cancer is in its advanced stages. Cervical cancer tends to occur in midlife. Half of women diagnosed with this cancer are between the ages of 35 and 55.

A critical event in tumor cell invasion is degradation of the extracellular matrix (ECM), a complex network of extracellular macromolecules such as collagen, proteoglycans, fibronectin, laminin and many other glycoproteins that acts as a barrier to the spread of cancer cells to distal sites by restricting tumor growth and invasion [1, 2, 3].

MMPs are initially expressed as inactive pro-enzymes that require proteolytic processing to

release the active enzyme [4]. Over twenty different MMPs act on a broad spectrum of substrates, including collagen type I, II, III, IV and stromyelin and are divided into subgroups based on their structure and substrate specificity [5]. These subgroups include collagenases, stromelysins and stromelysin-like, matrilysins, gelatinases and membrane-type MMPs (MT-MMPs) [6]. Among the many MMPs that have been identified, gelatinases, especially MMP-2 (gelatinase A) and MMP-9 (gelatinase B), are thought to a play a key role in degradation of type IV collagen and gelatin, the two main components of ECM.

MMP-2 (72 kDa) and MMP-9 (92 kDa) are secreted in their latent zymogenic form and cleaved by other MMPs or proteases to yield the activated forms of 68, 58 and 54 kDa for MMP-2 and 94 kDa for MMP-9. Many human tumors have been reported to be associated with increased expression of MMP-2 and MMP-9 [7, 8, 9].

MATERIALS AND METHODS

Sample collection:
Blood samples of cervical cancer and normal subjects were obtained from KIDWAI cancer research center, Bangalore. All the patients were of the same age group ranging from 40-45 years. The Blood sample of patients and healthy volunteers were collected, according to ethical standards, with informed consent of normal subjects and patients respectively. Blood was preserved at room temperature for a period of 30 minutes and then was centrifuged at 10,000 rpm for 10 minutes. Serum was transferred from the centrifuged tubes in clean eppendorf tubes and was stored at 4°C.

Estimation of total protein:
Total protein estimation for the serum samples was carried out by Eosin Y method. 10 µl of sample was made up to 0.1 ml with 0.15M NaCl. 0.1ml of 0.6% citric acid solution and 1 ml of 0.012% Eosin Y dye solution was added to the above mixture subsequently. The contents were mixed well by vigorous vortexing and were incubated at room temperature for 10 min. Eosin Y reacts with proteins under acidic conditions and form an intense pink protein-eosin Y complex which can be analysed by spectrophotometer at a wavelength of 535-545 nm.

Gelatin Zymography:
8 % SDS-Polyacrylamide gel was prepared containing gelatin at a concentration of 1mg/ml without boiling or reduction. Sample at concentration of 120µg total protein was mixed with zymography sample buffer, kept at room temperature for 30 minutes and were loaded to the wells. Electrophoresis was carried out at 50 V until the sample reached the separating gel and then the voltage was increased to 100 V until the dye reached the bottom of the gel. After electrophoresis the gel was washed twice with 0.25 % of Triton X-100 for a period of 20 minutes each. Then the zymogram was transferred into digestion buffer and was incubated for 18 hours at 37 °C. After incubation, the gel was rinsed briefly with distilled water followed by staining with 0.25 % Coomassie Brilliant Blue (CBB) for 1 hours. Then, the gel was destained with 7% acetic acid solution for several hours till the bands are clear and sharp. Area of gelatinase activity appeared as clear area over the blue background, indicating gelatin digestion.

RESULTS

Estimation of total protein:
Concentration of protein present in the normal and cancer-affected blood serum was estimated by Eosine Y method. Plotting the standard graph performed estimation and the concentration were found to be 1.84 and 2.48 for normal sample and diseased sample respectively (Figure 1).

Zymography of MMP-2 and MMP-9 in normal subjects:
Gelatin zymography for the purified serum proteins of normal samples were performed and the zymogram was documented. The concentrations of the MMPs were analyzed based on the thickness/intensity of the zymogram produced. A graph is generated based on the intensity of MMP-2 and MMP-9 to

analyze the difference in expression of MMPs in various samples (Figure 2 and 3).

Figure 1: Total protein estimation from the blood samples

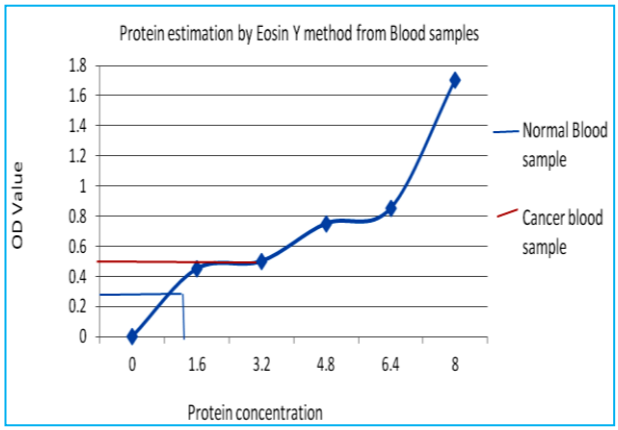


Figure 2: Zymogram of MMP-2 and MMP-9 activity in Normal subjects.

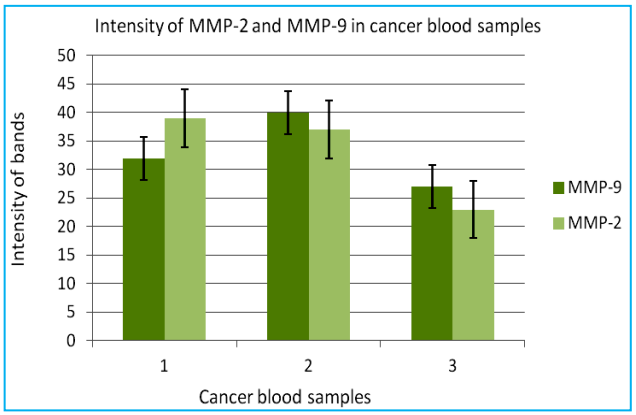
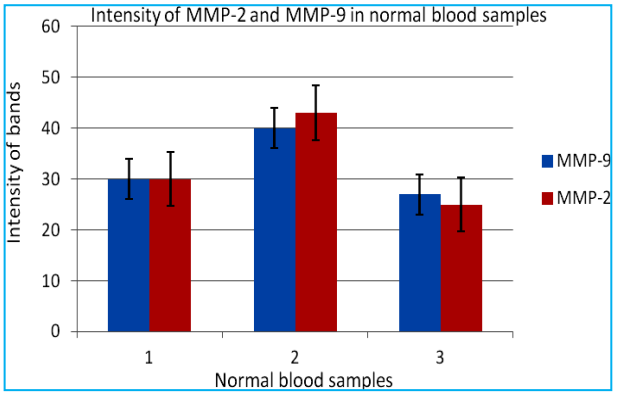


Figure 3: Intensity of MMP-2 and MMP-9 in normal blood samples.



Zymography of MMP-2 and MMP-9 in cervical cancer subjects:

Gelatin zymogram for the serum protein of cervical cancer was developed as described above and the concentration or levels of expression of MMPs were analyzed based on the graph plotted. The zymogram showed a high intensity band, which suggests the increased level of MMP in cancer samples (Figure 4 and 5).

Figure 4: Zymogram of MMP-2 and MMP-9 activity in cancer subject blood subjects.

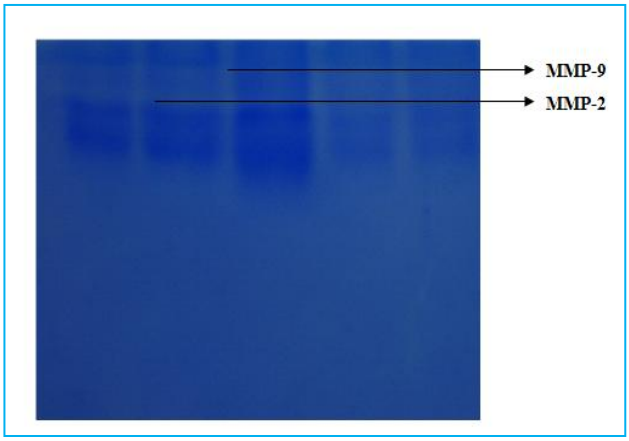
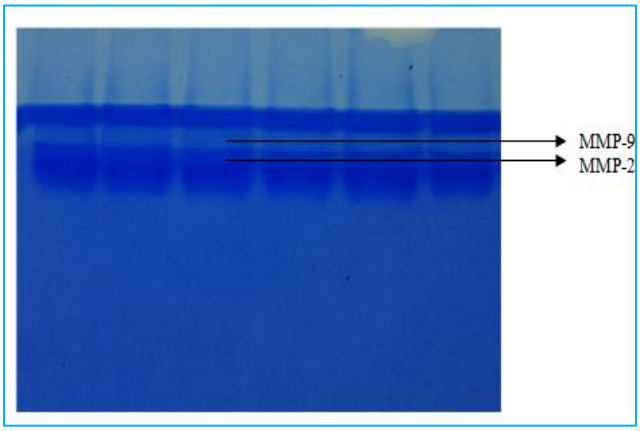


Figure 5: Intensity of MMP-2 and MMP-9 in cancer blood samples.



Comparison of expression levels of MMPs in normal and cervical cancer subjects:

The comparison of expression levels of MMPs was studied by comparing the intensity of bands formed on gelatin zymogram in the previous experiments. The comparison study supported the earlier studies, as the expression of both the MMPs were 2-3 times higher in cancerous

sample than normal. this shows the role of MMPs in cancer metastasis. For better understanding of the expression levels, the zymogram of normal and affected subjects were performed together and analyzed (Figure 6 and 7).

Figure 6: Zymogram of MMP activity in Normal subjects and Cervical Cancer patients (Lane 1, 2, 3: MMP-2 and MMP-9 activity in Normal subjects Lane 4, 5, 6, 7: MMP-2 and MMP-9 activity in cervical cancer patients)

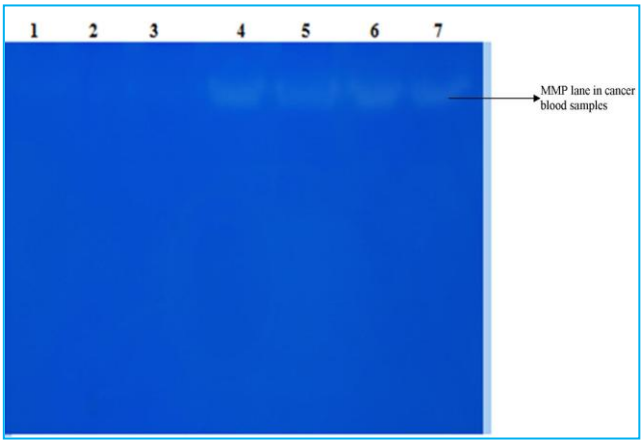
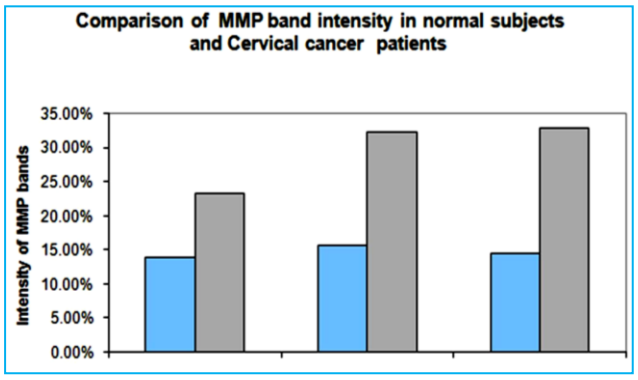
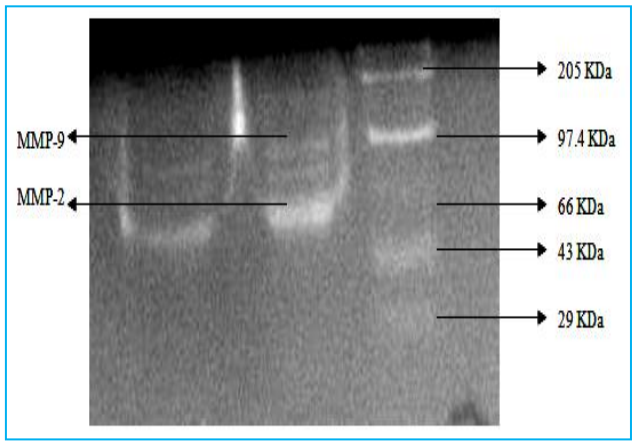


Figure 7: Intensity of MMP-2 and MMP-9 in normal blood and cervical cancer samples.



Determination molecular weight of MMPs:
The molecular weight of MMP-2 and MMP-9 of cancer subjects were determined as 62 KDa and 78 KDa approximately and in normal subjects as 52 KDa and 72 KDa by using SDS-PAGE .The sample was electrophoresed along with the standard marker (Figure 8).

Figure 8: Zymogram of MMP-9 and MMP-2 Activity In normal Subjects and Cervical cancer patients (Lane 1: MMP-2 and MMP-9 activity in Normal subjects. Lane 2: MMP-2 and MMP-9 activity in cervical cancer patients. Lane 3: Protein ladder).



DISCUSSION

In various experimental and clinical studies a correlation between increased MMPs and tumor progression and metastasis [10, 11, 12, 13, 14, 15] have been reported. Thus, knowledge of MMP regulation is of great importance for developing therapeutic strategies. MMP expression is regulated at both pre and post-transcriptional levels. A number of extracellular factors, including cytokines, growth factors, cell contact with ECM, and inducers and inhibitors, have been implicated in the regulation of MMP expression in different types of tumor cells [16, 17].

The expression of MMP-2 has shown to increase with the increasing grade of CIN and further in invasive of cancer [18, 19, 20, 21]. Inhibition of MMP activity has demonstrated a direct effect in reducing tumor cells invasion and angiogenesis [22, 23, 24]. The MMP inhibitor GM 6001 was shown to block SMC migration [25]. The synthetic MMP inhibitor BB94 (Batimastat) inhibits gelatinases A and B with IC50 values of 4 and 10 nmol/L, respectively [26] and is able to reduce intimal thickening after arterial injury by decreasing both SMC migration and proliferation [27].

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