5-2022

The influence of MMP14 on angiogenesis in chemotherapy-treated tumors

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The influence of MMP14 on angiogenesis in chemotherapy-treated tumors

An undergraduate honors thesis

submitted to

the Department of Biomedical Engineering

College of Engineering

University of Arkansas

Fayetteville, AR

April 2022

By

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Abstract

Colorectal cancer is the third most common cancer in the world, and it is the fourth most common cause of cancer related death (1). There have been many significant advancements regarding the treatment of cancer which aim to shrink the size of tumors in patients. However, there is still more that needs to be understood about the many different factors that play a role in colorectal cancer development.

Angiogenesis is the process of forming new blood vessels from existing ones and it requires breaking down and remodeling of the extracellular matrix (ECM) in order to allow endothelial cells to migrate and invade into the surrounding tissue so that new blood vessels can successfully sprout. Among the many signals that initiate and nurture angiogenesis, vascular endothelial growth factor (VEGF) is the one of interest.

Matrix metalloproteinases (MMPs) are endopeptidases that break peptide bonds of non-terminal amino acids. Among all the classes of MMPs, it has been found that membrane-type MMPs, such as MMP14, play a role in the process of angiogenesis. MMPs are known to enhance angiogenesis by releasing ECM bound angiogenic growth factors such as VEGF (4).

The most widely used chemical carcinogen used to induce colon tumors in mice is azoxymethane (AOM). 5-fluorouracil (5-FU) is one of the anti-cancer drugs used to treat colorectal cancer (6). The AOM mouse model will be used to study the effects of colorectal cancer and the effects of 5-FU treatment.

The goal of this research was to understand how 5-FU treatment of AOM mice affect the gene expression of VEGF and HIF-1 and the quantification of MMP14, a membrane-type MMP, in colon tumors. Since it is known that VEGF and HIF-1 play a significant role in angiogenesis, it is important to investigate the role that MMPs play in angiogenesis as well.

Introduction

Background

Colorectal cancer is the third most common cancer in the world, and it is the fourth most common cause of cancer related death (1). There have been many significant advancements regarding the treatment of cancer, one of them being neoadjuvant chemotherapy (NAC) through fluoropyrimidine-based anti-cancer drugs. NAC is typically administrated clinically before surgical resection, such as tumor biopsies, with the aim of shrinking tumors in patients (7). Fluoropyrimidine-based anti-cancer drugs have been widely used in the treatment of neoplastic diseases such as colorectal, gastric, and breast cancers (6).

Angiogenesis is the process of forming new blood vessels from existing ones and it requires breaking down and remodeling of the extracellular matrix (ECM) in order to allow endothelial cells to migrate and invade into the surrounding tissue so that new blood vessels can successfully sprout. Vascular endothelial growth factor (VEGFs) are proangiogenic growth factors which are secreted by inflammatory cells, pericytes, keratinocytes, or tumor cells (4). It has been found that there is a synergistic relationship between therapeutic doses of 5-FU and the inhibition of VEGF-induced proliferation, migration, and survival (5). To further understand this relationship, the factors that affect angiogenesis must be further researched.

Matrix metalloproteinases (MMPs) are endopeptidases that break peptide bonds of non-terminal amino acids. MMPs are predominantly produced by activated macrophages and are secreted as proenzymes, which are inactivate precursors of enzymes, and require extracellular activation through proteolytic cleavage (3). Among all the multiple classes of MMPs, it has been found that membrane-type MMPs, such as MMP14, play a role in the process of angiogenesis. MMP14 is known to enhance angiogenesis by releasing ECM bound angiogenic growth factors

such as VEGF. They can also inhibit angiogenesis by generating angiogenic inhibitors like bioactive cleaved forms of collagen which bind to endothelial cell surface integrins causing the inhibition of proliferation and migration (4).

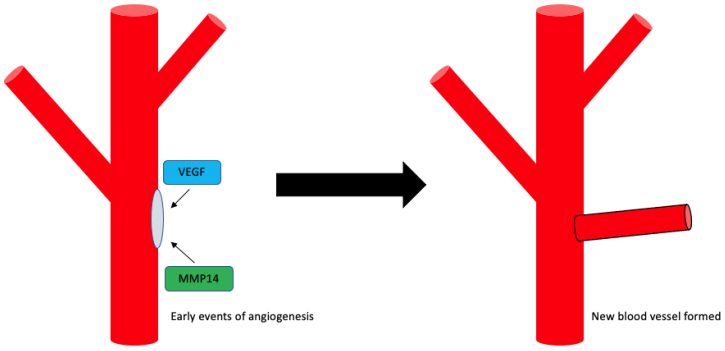

Figure 1*: Sprouting of angiogenesis. Shows the early events of angiogenesis and how MMP14 enhances angiogenesis through the release of angiogenic growth factors such as VEGF (4)***.**

Figure 7 *later shows how angiogenesis can be measured.*

Carcinogen-induced colorectal cancer in mice can comparatively simulate the phases of initiation and progression of colon tumors that occur in humans. In addition, carcinogen-induced colorectal cancer mice models are highly reproducible, and their pathogenesis can summarize that of human colorectal cancer (7). One of the most widely used chemical carcinogen used to induce colon lesions is azoxymethane (AOM). This model is used to study anti-tumor drugs and different dosing strategies for NAC. 5-fluorouracil (5-FU) is a fluoropyrimidine-based anti-cancer drug used to treat colorectal cancer and it is the chemotherapy used in this study (2). The standard NAC regimen is based on the maximum- tolerated dose (MTD) approach where treatment is cycled such that the patient will alternate being on and off treatment every other week (8). This approach has an unintended side effect of allowing tumors to recover in the week while being off treatment (9).

Research Question

How does 5-FU treatment of AOM mice in the MTD group affect the gene expression of VEGF and HIF-1 and the quantification of MMP14 in colon tumors?

Hypothesis

If colon tumors from MTD group mice express VEGF and HIF-1, then an increase in MMP14 quantification in tumors would be expected.

Project Aims

Aim 1 – The goal of this research was to understand how MMP14, a membrane-type MMP, plays a role in angiogenesis and how it is affected by 5-FU treatment. It is known that MMP14 significantly contributes to angiogenesis regulation by cleaving ECM molecules in addition to being a key effector in the production of pro-angiogenic factors (4). The purpose of this study was to specifically understand how the MTD group of 5-FU treatment affects the expression of VEGF and hypoxia-inducible factor 1 (HIF-1), as well as the quantification of MMP14 concentration. It is known that VEGF is a pro-angiogenic factor and HIF-1 induces hypoxia which promotes the formation of blood vessels.

Aim 2 – Another goal of this study was to understand how 5-FU affects colon tumor characteristics such as size, color, and vascularity. In the AOM model, it has been observed that the animal model induces colon tumors of various sizes in mice colon. The purpose of this study was to investigate how the MTD group plays a role in this. Some tumors shrink significantly, change color, and change in amount of vascularity in between treatment weeks. It was hypothesized that if a tumor has high vascularity, then it would be expected that tumor would express VEGF and have high MMP14 protein concentration.

Materials and Methods

AOM mouse model – The process for preparing AOM mice for tumor biopsies is a 25-week process split into three phases: AOM phase, rest phase, and colonoscopy phase (visualized in **Figure #**). In the AOM phase, AOM injections were administered to mice over the period of 6 weeks to induce tumors. Then, mice rested for another 14 weeks to let tumors develops. Finally, mice were split into groups of 4 based on similar tumor development. Weekly colonoscopies were performed, and images and videos of tumor biopsy extractions were taken. Biopsies of approximately 660 µg were taken on a weekly basis and placed in a sterile 1.5 mL microcentrifuge tube (022600001, Eppendorf) and stored in a -80°C freezer for future experimentation.

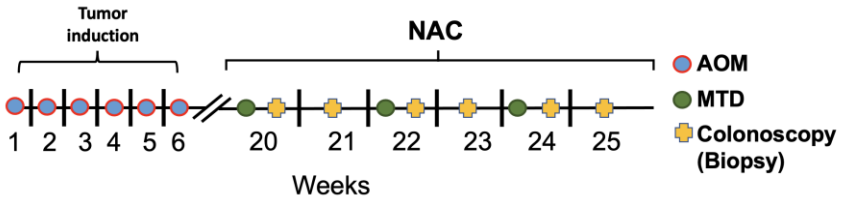

***Figure 2**: Diagram illustrating the timeline of the AOM mouse model (10).*

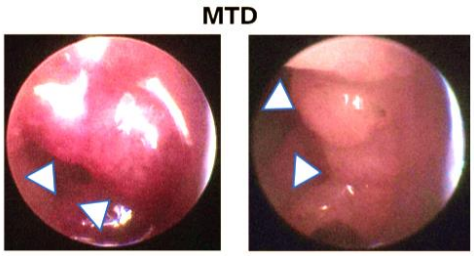

***Figure 3**: Image from endoscopy of mouse colon. Representative endoscopic image of tumor size from mouse in MTD group (10).*

Enzyme-linked Immunosorbent Assay (ELISA) – ELISA is a plate-based assay technique used to quantify soluble substances such as peptides and proteins. For this study, an ELISA MMP14 kit (MBS2516088, MyBioSource) was used to quantify MMP14 concentration in tumor biopsy samples. Biopsies were collected from mice colon tumors during the colonoscopy

phase of the AOM mouse model. Biopsies were placed inside an empty microcentrifuge tube and stored in a -80°C freezer until it was time to prepare the samples for the ELISA. For sample preparation, biopsies were minced and homogenized in 20 µL of fresh lysis buffer over ice using a sonicator (Q55 Sonicator, Qiagen). The homogenates were then centrifuged for 1 minute at 10,000×g. The samples were then diluted 64-fold (seen in **Figure #**) to make sure that optical density reading would fall within the typical calibration curve of MMP14, Mouse ELISA. Concentration of MMP14 in mouse colon tumors was expected to be between 0.1-0.4 µg/mL (11).

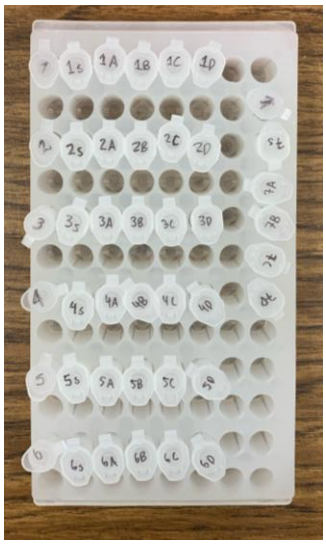

Figure 4*: Sample preparation tubes and sample dilutions prepared for ELISA.*

For reagent preparation, standard dilutions were prepared using Standard Diluent, included in ELISA kit as shown in **Figure 5** below. 7 points of standard dilutions were made at 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL, and 0 pg/mL to serve as the blank. Detection Reagent A and B were prepared by using Detection Reagent A and B from the kit and diluting them to the working concentration 100-fold with Assay Diluent A and B, respectively. 300 mL of Wash Solution was then prepared by diluting 10 mL of Wash Solution with 290 mL of distilled water.

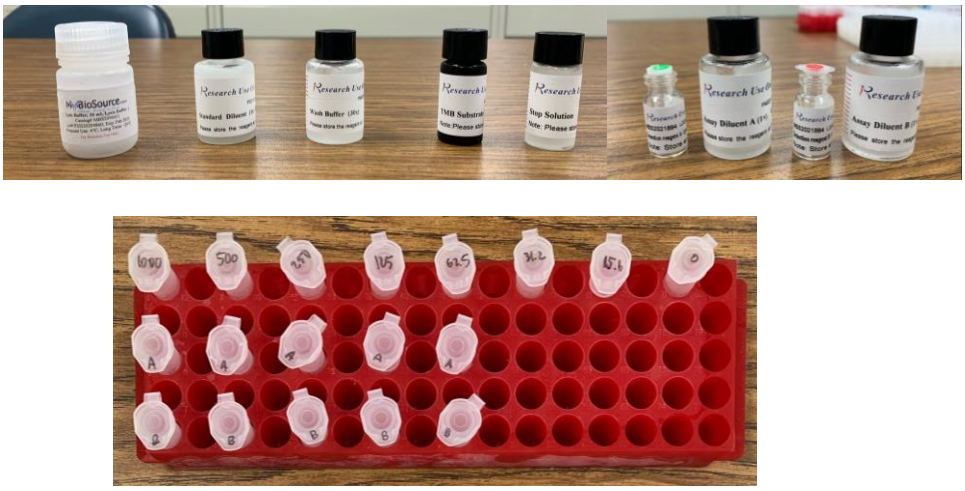

Figure 5*: Reagents included in MMP14, Mouse ELISA kit (on top) and reagent preparation from MMP14, Mouse ELISA kit (on bottom). Standard dilutions (first row of tube rack) and Detection Reagent A and B dilutions (bottom 2 rows of tube rack, respectively).*

For assay produre, 100 µL of each of the standard dilutions, blank, and sample dilutions were added into the appropriate wells. Well plate was covered with plate sealer and incubated at 37°C for 1 hour. Liquid from each well was removed onto an absorbent paper and 100 µL of Detection Reagent A working solution was added to each well. Well plate was then covered with plate sealer and incubated at 37°C for another hour. 350 µL of Wash Solution was added to each well and allowed to sit for 1-2 minutes. All liquid was removed each well and was washed again for a total of 3 times. After all liquid was removed again, 100 µL of Detection Reagent B working solution was added to each well. Well plate was again covered with plate sealer and incubated at 37°C for 30 minutes. The previous wash step was then performed again 2 more times, for a total number of 5 washes. 90 µL of Substrate Solution was then added to each well. Substrate solution was protected from light as TMB Substrate is easily contaminated. Well plate was again covered with plate sealer and incubated at 37°C for 10-20 minutes. 50 µL of Stop Solution was added to each well and color change to yellow was observed in each well (seen in **Figure 6** below). Well

plate was immediately taken to the spectrophotometer (Synergy Neo2 Multimode Microplate Reader, BioTek) to measure absorbance at 450 nm.

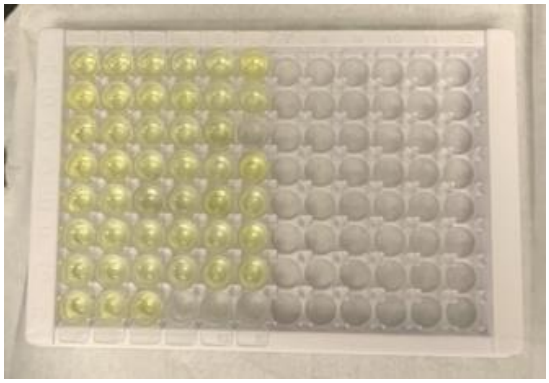

Figure 6*: Image of well plate after completion of ELISA procedure. Yellow color change in each well indicates positive reaction.*

Real Time Quantitative Polymerase Chain Reaction (qPCR) – qPCR was used to measure and quantify gene expression of VEGF and HIF-1. Multiple biopsies were taken and stored in sterile 1.5 mL microcentrifuge tube (022600001, Eppendorf) and stored at -80°C. Samples were prepared as previously reported (10). Biopsies were homogenized over ice using a using a sonicator (Q55 Sonicator, Qiagen) and mRNA extraction was performed using a RNeasy kit (74004, Qiagen). The quality of the resulting extracted mRNA was determined using a spectrophotometer (NanoDrop One, ThermoFisher). Reverse transcription to cNDA was performed using a cDNA Synthesis kit (11754050, ThermoFisher). cDNA concentration for all samples was normalized at 2.5 ng/µL and DNA primers were acquired from Integrated DNA Technologies. qPCR performed using Luna Universal qPCR Master Mix (M3003, New England BioLabs) and manufacture protocol was followed to execute experiment. Samples were performed in triplicates in a CFX96 Touch Real-Time PCR Detection System (BIO-RAD) and relative mRNA gene expression was quantified using Pfaffl's method, using murine glyceraldehyde-3-

phosphate dehydrogenase (GAPHD) as the housekeeping gene. This methodology was performed as previously reported (10, 12, 13).

Nestin-1 quantification – End of study colons were stored in 10% neutral-buffered formalin and later professionally embedded in paraffin and slides were prepared by IHC World. Samples were sectioned in the sagittal plane at a thickness of 5 µm and then stained with Nestin-1. Slides were imaged under an upright Nikon widefield microscope using a 10x objective and a gain of 1. Tumor images were then processed in ImageJ to visually separate the image into 3 different categories: tumor tissue, colon tissue, and slide background. This is seen in **Figure 7** below.

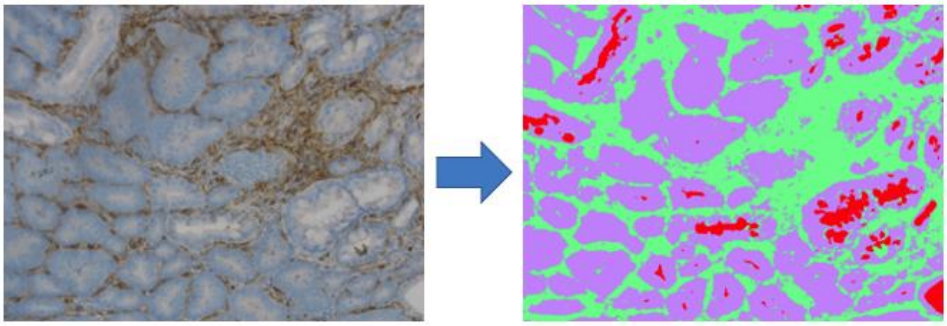

Figure 7*: Nestin-1 stained tumor slide (40x) from end of study colon (on left). Image produced in ImageJ to segment the 3 different categories of the tumor slide (on right). This method will be used to measure angiogenesis from Nestin-1 stained tumor slides.*

Data analysis – All biopsy samples were collected from weeks 5 and 6 of colonoscopy phase to study the effects of MTD treatment regimen in near end-of-study colon tissue. MMP14 concentration was plotted against relative gene expression of VEGF and HIF-1 and Nestin-1 microvessel density (MVD). Resulting correlation plots were analyzed to determine line of best fit equation, coefficient of determination (R^2), and Pearson's correlation coefficient (r^2) for each respective plot. Data analysis was performed using Prism and MATLAB.

Results and Discussion

ELISA – Absorbance for each of the samples (n=7) was measured at 450nm using a spectrophotometer (Synergy Neo2 Multimode Microplate Reader, BioTek). Results were further analyzed to calculate the optical density by calculating the negative log of each absorbance reading. Apparent concentration was calculated for each sample using the typical calibration curve for MMP14, Mouse ELISA as seen in **Figure 8** below. Actual concentration of MMP14 in each biopsy sample was calculated by multiplying the apparent concentration by a factor of 64 to account for the 64-fold dilution in each sample.

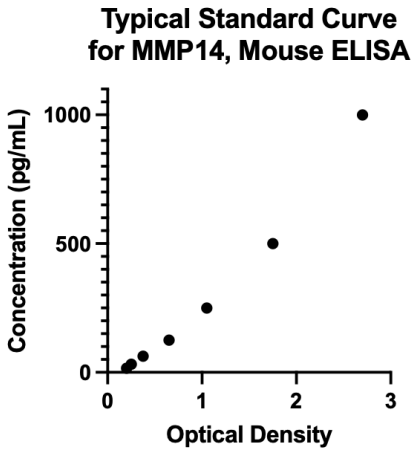

Figure 8*: Typical Standard Curve for MMP14, Mouse ELISA. Gathered from MMP14, Mouse ELISA kit manual.*

qPCR – Data was collected and further analyzed to quantify relative gene expression of both VEGF and HIF-1. Results were calculated as the norm ratio using GAPHD as the housekeeping gene, as previously discussed. This number represented the relative gene expression of each respective gene tested.

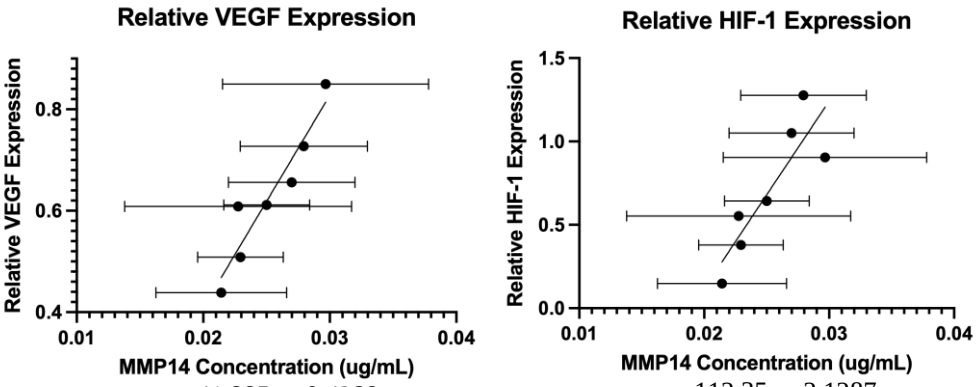

Figure 9*: Correlation plots of MMP14 concentration from ELISA plotted against relative VEGF and HIF-1 gene expression, respectively. Line of best fit equation, coefficient of determination (R^2), and Pearson's correlation coefficient (r^2) displayed below each respective plot.*

Nestin-1 quantification – Samples were fixed in formalin and embedded in paraffin blocks and stained for Nestin-1 to visualize vascular areas. ImageJ image segmentation was used to separate the three distinct sections on the tumor slide and then MATLAB analysis was performed to quantify MVD.

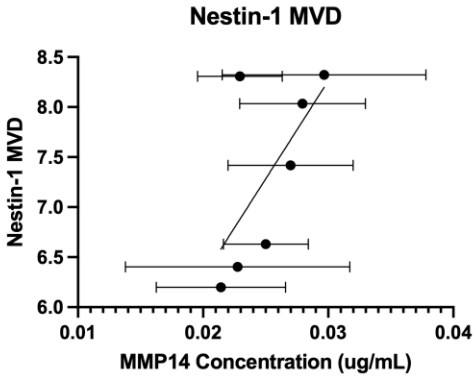

Figure 10*: Correlation plot of MMP14 concentration from ELISA plotted against Nestin-1 MVD. Line of best fit equation Line of best fit equation, coefficient of determination (R^2), and Pearson's correlation coefficient (r^2) displayed below the plot.*

Conclusion and Future Directions

Correlation analysis showed positive correlation between MMP14 concentration and VEGF gene expression, HIF-1 gene expression, and Nestin-1 MVD, although with a lower value in the latter. Sample size was a limitation in this study as the number of samples available for ELISA were limited, so it would be ideal to be able to collect more biopsies for a larger ELISA sample size. It would also be favorable to perform longitudinal biopsies on the exact same tumor to quantify MMP14 concentration as well as gene expression of VEGF and HIF-1. This would provide more accurate results for the relationship of MMP14 and gene expression of VEGF and HIF-1 in each specific colon tumor, instead of being generalized over a group from the study. Additionally, biopsies could be collected over the course of the whole study to assess changes in MMP14 concentration and gene expression over time, instead of examining only end of study colon tumors. Lastly, it may be beneficial to research the role of MMP14 in different chemotherapy treatment regimen groups, such as low dose chemotherapy (metronomic group), as compared to only the MTD group.

Acknowledgements

I would like to thank Dr. Timothy Muldoon for his support and guidance for this project. I would also like to thank Dr. Ariel Mundo for allowing me to work on his mouse study and helping me with data analysis and interpretation.

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