Expression of Endothelial Protein C Receptor in Prostate Cancer

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Honors Thesis

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Abstract

Prostate cancer is the second leading cause of cancer deaths in men in the United States (Siegel 2019). This honors thesis quantified the expression of Endothelial Protein C Receptor (EPCR) in the serum of patients with varying stages of prostate cancer and determined its expression in the prostate cancer cell line, PC3. EPCR and its ligand, protein C, play an important role in the anticoagulation cascade that prevents thrombosis from occurring at the sight of a vascular injury (Esmon 1992). Previous research regarding tumor metastasis and the coagulation cascade in ovarian cancer has shown that there is also an association between the expression of EPCR and the metastasis of cancer cells, as cells that expressed EPCR were more prone to survive, divide, and eventually become metastatic (Ducros et al. 2012). The expression of EPCR on breast tumor cells promotes their metastasis by activating the cytoprotective mechanisms of Activated Protein C (APC) and allowing them to invade the circulatory system and avoid immune detection by the host organism (Griffin 2012). Researchers have investigated the use of EPCR concentration in patient serum samples and in ovarian cancer cell lines as a potential biomarker for ovarian cancer. These researchers found that there was a correlation between the stage of ovarian cancer and the concentration of EPCR found in the patient serum (Ducros et al. 2012).

Since previous research has shown that EPCR is expressed in breast cancer and ovarian cancer cells, this study investigated if EPCR is also present in prostate cancer cells. The expression of EPCR in serum samples may be used to confirm the diagnosis of prostate cancer and may also be used to determine the stage of prostate cancer. EPCR expression

in the prostate cancer cell line, PC3, and the concentration of EPCR in serum from patients with prostate cancer were determined. An immunoprecipitation and western blot was performed on PC3 cell media and cell lysates to determine if EPCR is expressed by prostate cancer cells. Bands appeared in all lanes of the western blot at 49 kDa indicating EPCR was expressed in the media and lysates of PC-3 cells. An ELISA was performed to quantify the concentration of EPCR in prostate cancer patient serum samples. There was a significant differences between the means of EPCR concentrations in patients with varying Gleason Scores of prostate cancer ($p<0.001$), with patients with prostate cancer having elevated concentrations of EPCR compared to controls.

We conclude that EPCR is expressed by prostate tumor cells and that patients with prostate cancer Gleason grades 4-8 have statistically elevated concentrations of serum EPCR when compared to the levels of EPCR expression in control serum samples. This indicates that the expression of EPCR in serum of patients can be used as a biomarker for prostate cancer. The concentration of EPCR in serum samples can indicate the severity or score of the prostate cancer the patient has.

Introduction

The Coagulation Process and the Role of Thrombin

It is common for blood vessels to be damaged during an injury and for this damage to lead to bleeding at the site of the wound. In order to maintain hemostasis at the site of the vascular injury, both coagulation and anticoagulation cascades are utilized at

appropriate times to ensure the cessation of bleeding from the blood vessel and the prevention of clot formation within the blood vessel known as thrombosis. The coagulation process begins when a platelet plug is formed at the site of the vascular injury. Following the formation of a platelet plug, the procoagulant enzyme, thrombin, is eventually generated from its precursor prothrombin. Thrombin then converts fibrinogen to fibrin. Fibrin adds stability to the platelet plug and forms what is known as a secondary plug at the location of the vascular damage (Palta 2014). This process is responsible for the cessation of bleeding at the site of the vascular injury.

Activated Protein C and Endothelial Protein C Receptors

During the coagulation process, the levels of the plasma protein, prothrombin, are nearly 100 times greater than the amount of thrombin that is needed for clot formation. This excess prothrombin leads to the overproduction of thrombin and causes massive clotting, known as thrombosis, which is detrimental to the organism (Esmon 1989). This excess thrombin binds to the endothelial cell surface protein, thrombomodulin (TM), which with the coreceptor, EPCR, activates the anticoagulant enzyme protein C to activated protein C (APC) (Griffin 2012). The process by which Protein C is activated is shown in Figure 1 below.

To regulate the procoagulant effect of thrombin, the anticoagulant enzyme, APC, and its cofactor protein S then inhibit the two cofactors, V_a and $VIII_{a,}$ that are needed for the formation of thrombin (Figure 1). When bound to APC, Protein S causes a

conformational change that positions the active site of APC closer to the cell membrane so that it is in the optimal position to inhibit the V_a and $VIII_a$ cofactors of prothrombin (Griffin 2012).

Endothelial Protein C Receptor (EPCR) enhances the rate of activation of APC by thrombin bound to TM (Ruf 2014). As indicated by their name, EPCR's are integral membrane proteins found on endothelial cells lining the blood vessels (Fukudome 1994). Once Protein C binds to EPCR, it is oriented in a manner that better exposes it to the thrombin-TM complex for activation (Figure 1). Once activated, Protein C inhibits the procoagulant effect of the thrombin molecules that normally promote the formation of fibrin as described above. Protein C inhibits this to avoid thrombosis within the vessel (Griffin 2012).

APC also performs many other cytoprotective activities on cells through interactions with EPCR. The interaction between APC and EPCR has been linked with anti-apoptotic, anti-inflammatory, and cell barrier protection responses in cells to protect them from many harmful agents (Griffin 2012). After the APC is activated, it remains active until it is inactivated by Protein C Inhibitor (PCI) or α_1 anti-trypsin. However, Protein C is inactivated very slowly with a half-life of 15 minutes (Esmon 1989).

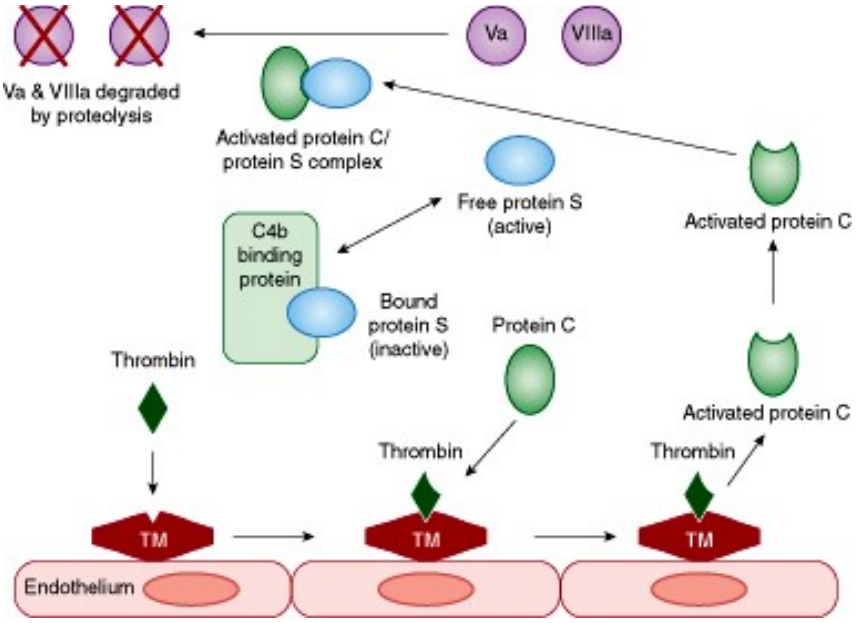

Figure 1: Protein C Pathway The binding of thrombin to the transmembrane protein thrombomodulin and the binding of Protein C to EPCR causes the activation of Protein C to proceed at an enhanced rate. Once activated, APC, along with its cofactor Protein S, inhibits the cofactors Va and VIIIa of prothrombin that are necessary to form more thrombin. (*https://basicmedicalkey.com/bleeding-and-thrombotic-disorders/*)

Tumor Growth and Metastasis

Cancer can develop in any cell that has mutations that result in uncontrolled cell division. This may produce tumors that can either be malignant or benign. Benign tumors do not spread into neighboring tissue or other parts of the body. Malignant tumors are capable of spreading to nearby tissue, lymph nodes, and other parts of the organism.

Metastasis is the process by which cancerous tumor cells spread to multiple locations within an organism from an initial tumor growth site. For a tumor to become malignant and metastasize, several conditions must be met. Tumor cells first break free from their intercellular connections with other tumor cells and interact with the

extracellular matrix (ECM) of neighboring tissues. Tumor cells secrete enzymes that degrade the ECM. Following this degradation, the tumor cells are able to propel themselves through the ECM and become invasive. As the tumor continues to grow and invade surrounding tissue, it may eventually invade the vascular network of the host and enter the circulatory or lymphatic systems where they may make their way to other locations in the organism. To metastasize, the tumor cells must also avoid the host's immune cells in the vascular network. To invade a secondary tissue, they must also adhere to the endothelial cells of the vasculature before attempting to invade tissue at a secondary location (Abbas 2017).

Tumors require oxygen and nutrients to continue growing, therefore, they cannot continue growing past 1-2mm without their own vascular network. Angiogenesis is triggered in growing tumors by an increase in angiogenesis promoters and a decrease in angiogenesis inhibitors. Tumors undergoing angiogenesis form their own vascular networks that stem from preexisting capillaries. Access to these new vessels allows the tumor cells to enter the bloodstream and metastasize throughout the organism (Abbas 2017).

Correlation between EPCR and Tumor Cell Metastasis

There has been recent evidence proving that the coagulation system plays a role in tumor cell metastasis and cancer progression. Earliest research found that when anticoagulants were used to treat cancer patients, the anticoagulants limited the growth and

metastasis of the tumor cells. This treatment method was developed from studies that revealed that even minute amounts of thrombin enhance the rate of cancer metastasis (Sluis 2010). This sparked an interest in the association of cancer progression and the APC anticoagulation pathway.

EPCR expression on vascular endothelial cells prevents the adhesion and migration of tumor cells within the vessels, which suggests that the anticoagulation cascade prevents tumor cell growth and metastasis (Sluis 2010). However, EPCR expression is found to be upregulated on breast cancer cells. This expression of EPCR on cancer cells allows them to activate protein C and its inflammatory and anti-apoptotic effects. These signals transduced by ECPR-bound APC in cancer cells has been shown to promote cancer metastasis and angiogenesis (Sluis 2010).

Other research focused on the growth of breast cancer in mammary glands. This research performed by Ruf and Schaffner (2014) found that there was a direct correlation between tumor cells that expressed EPCR and breast cancer metastasis. As stated previously, APC alone has no prometastatic effects on tumor cells. In fact, this research found that Activated Protein C and EPCR expressed in vascular endothelial cells reduce the metastatic effects of the thrombin produced by the interaction with tumor cells and epithelial cells. This research focused on the expression of EPCR in cancer stem cells. They found that the most aggressive breast cancer cell lines could be identified by their expression of EPCR. The cancer cells that expressed EPCR were more capable of growth and survival. This research also suggested that these cancer cells may use the expression

of EPCR to trigger the host's innate immune system to regulate the differentiation and retention of stem cells, allowing for the overproduction of tumor cells (Ruf 2014).

Ducros et al. (2012), who investigated the expression of EPCR in ovarian cancer, indicated that EPCR could be used as a possible biomarker for the onset of ovarian, breast, and lung cancer.These researchers found that the concentration of EPCR in the plasma samples of patients with ovarian cancer was significantly higher than in the plasma samples obtained from healthy patients (Ducros et al. 2012). The expression of EPCR in plasma was also found to be related to cell survival, invasion, and immune down regulation. This suggested that elevated levels of EPCR in plasma indicates the onset of ovarian cancer and can, therefore, be used as a potential biomarker (Ducros et al. 2012). While this research focused primarily on ovarian cancer, our study investigates the potential use of EPCR expression as a biomarker for prostate cancer.

Prostate Cancer

Prostate cancer is the second leading cause of death by cancer in men (Seigel 2019). To determine if EPCR is expressed in prostate cancer cells, cells from the prostate cancer cell line PC3 were cultured in the lab. The cultured prostate cancer cells were then used for an immunoprecipitation, SDS-PAGE, and Western blot analysis to determine if the EPCR protein was present. Plasma samples collected from Carolinas Medical Center (Atrium Health Medical Facility) were used to perform ELISAs and quantify and compare the concentration of EPCR in patients with varying stages/grades of prostate cancer to control

serum (no prostate cancer). Due to the fact that there was a correlation between the expression of EPCR and the invasion and metastasis of breast cancer cells, it was predicted that EPCR would be found in both the PC3 cells and the serum samples of prostate cancer patients and that patients with prostate cancer would have elevated serum concentrations compared to controls.

Materials and Methods

Immunoprecipitation

The PC3 cell line was used to determine if EPCR is expressed in prostate cancer cells. PC3 cells (ATCC) were cultured in DMEM media supplemented with L-glutamine, sodium pyruvate, and 10% Fetal Bovine Serum (Sigma Aldrich). PC3 cells were maintained in T25 cell culture flasks with 5ml of media at 37ºC with 5% CO_2. When cells were 80% confluent, media was replaced with serum free media for 12 hours. EPCR was then immunoprecipitated from 1ml of cell media and cell lysates from these cells. For the immunoprecipitation process, immunoprecipitation buffer (0.1 M Tris – HCl, 25 mM EDTA, 5 mM benzamidine, 5 mg/ml bovine serum albumin, 2.5% Triton X-100, 2.5% deoxycholate) was then added to the 1mL of media and cell lysis buffer (150 mM sodium chloride, 1.0% NP-40 (Triton X-100 can be substituted for NP-40), 50 mM Tris pH 8.0) was added to the cells remaining in the flask. Following 5 minutes of incubation with the cell lysis buffer, the cells were transferred to a tube and centrifuged to remove debris. 1mL

of the supernatant was transferred to a clean tube to which the immunoprecipitation buffer was then added.

Sixty µl of α-mouse IgG-agarose (Sigma) was added to both the precipitated media and cell lysate for 20 minutes. This was then zip spun and the supernatant was removed. 2µl of the EPCR-MAb (Sigma) was then added to the supernatant and it was allowed to shake overnight at room temperature. 60 µl of α-mouse-IgG-agarose was then added for one hour at 4ºC followed by centrifugation. The beads were then washed with 300µl TBS and 40µl of non-reduced SDS-PAGE sample buffer was added for 10 minutes at 90ºC. Finally, following centrifugation, the supernatant was removed and a 10% SDS-PAGE and Western was performed.

SDS-PAGE and Western Blot

A vertical polyacrylamide gel was used to separate the EPCR based on molecular weight. The gel was loaded with 20 µl samples of both cell media and cell lysate along with a rainbow molecular weight ladder. The gel was run at 15 mA for 15' and then at 30-35 mA until the bands reached the bottom of the gel. Following SDS-PAGE, a Western Blot was used to visualize the protein bands. The gel was transferred onto nitrocellulose at 25 V for 1 hour. The gel was then removed and Coomassie stained to detect any remaining protein. The nitrocellulose was used for western blot probing and detection. The nitrocellulose was blocked with 3% milk in 1xTBS buffer for 30 minutes. It was then rinsed with TBS and the 1 µg primary antibody EPCR-MAb in 1% milk was added and allowed

incubate at 4°C overnight. The nitrocellulose was then rinsed three times with TBS for five minutes. The secondary antibody α-mouse IgG-agarose was then added and allowed to rock for one to three hours. This was then rinsed with TBS twice for five minutes each time and was left in distilled water. Enhanced chemiluminescent (ECL) detection was performed to detect the protein present.

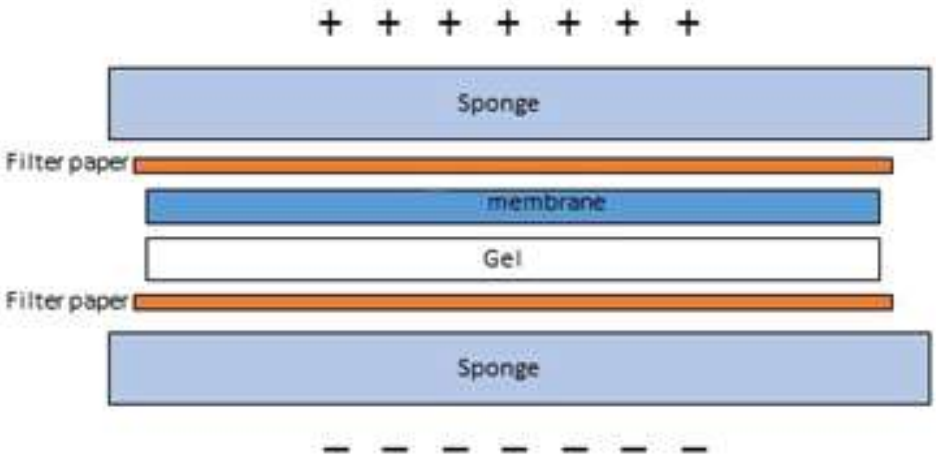

Figure 2: Arrangement Order for Western Blot Transfer To transfer the protein from the gel to the nitrocellulose membrane, they were placed in a cassette with four sponges and four pieces of filter paper. They were arranged in the following order: two sponges, two pieces of filter paper, the gel, the nitrocellulose membrane, two pieces of filter paper, and two sponges. (*https://www.clinisciences.com/it/read/western-blot-516/western-blot-protocol-1727.html*)

ELISA

Serum samples were collected from patients with various grades of prostate cancer from Carolinas Medical Center, Charlotte, NC using an IRB approved protocol (Drs. Glasscock and Teigland). These samples were then de-identified by Dr. Glasscock to

maintain patient privacy. These samples were stored in liquid nitrogen and at -20ºC up until 24 hours before the assay occurred. 24 hours before assaying the samples, the samples were stored at 2-8ºC. Serum samples were then centrifuged and diluted 1:2 before use. A Human EPCR PicoKine ELISA Kit (Boster) was then used to perform the assay. The serum samples were activated using solution A (1N HCl) and B (1.2N NaOH/ 0.5M HEPES). An EPCR standard was reconstituted to 316 pg/ml as directed by the instructions provided in the ELISA kit. The biotinylated anti-human EPCR antibody working dilution was then prepared by adding 10mL of antibody diluent buffer to the whole anti-human EPCR antibody. The avidin-biotin-peroxidases complex working solution was also prepared by adding 10mL of ABC dilution buffer to 130µL of the avidin-biotin-peroxidases complex.

A plate coated with EPCR-Mab from mouse, was then prepared. 100µl of the standard curve was added to the plate in triplicate (0 ng/ml –800 ng/ml). 100µl of each activated serum sample and the control female serum sample were then added in triplicate. The plate was covered and placed in a 37ºC with 5% CO_2 incubator for 30 minutes. The contents were then removed from the plate and 100µl of the biotinylated anti-human EPCR antibody working solution was added to each well. This was allowed to incubate for 60 minutes at 37ºC, 5% CO_2. Following incubation, the plate was washed three times with 0.01M PBS. 100µl of ABC working solution was then added to each well and the plate was once again allowed to incubate for 30 minutes at 37ºC, 5% CO_2. Following incubation, the plate was washed five times with 0.01M PBS. 90µL of the TMB color developing agent was then added to each well and the plate was left in the dark at 37ºC for 15-20 minutes

until a color change from blue to yellow was observed as shown in Figure 3. The absorbance at 450nm was then read and the data was analyzed to determine the mean concentration +/- standard deviation of EPCR for each patient. Three ELISAs were performed.

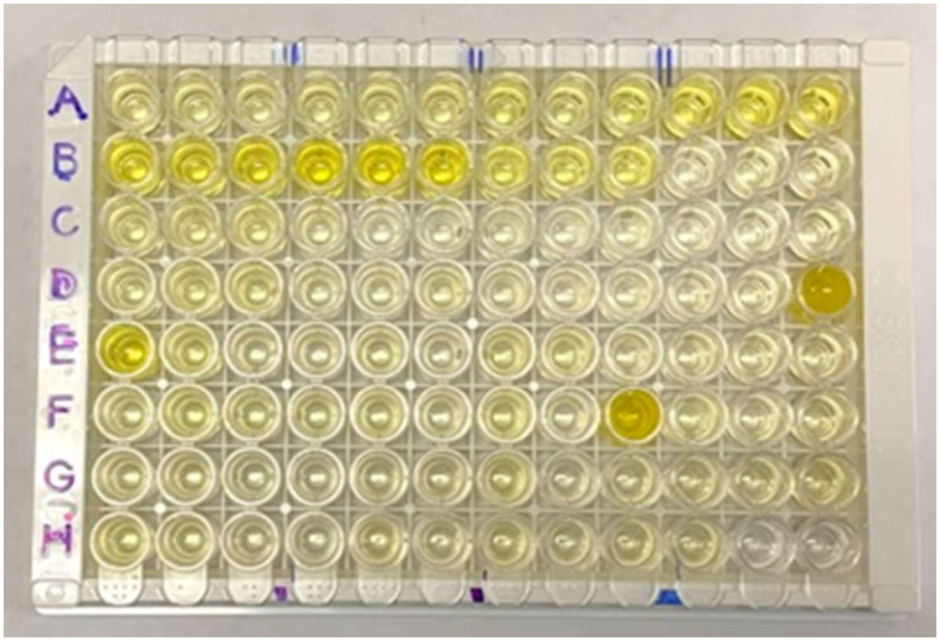

Figure 3: ELISA Plate Following TMB Color Developing Agent Color Change. Each sample and a control were plated in triplicate along with a standard curve. TMB Color Developing Agent was added and a color change was observed. Once plates turned the yellow color shown above, the OD absorbance of the plate was then ready to be read.

Results

SDS-PAGE and Western Blot

The western blot perfromed presented bands in all five lanes as shown in Figure 4 below. Lane two contained 20µg/ml purified EPCR. Both cell media and cell lystates

contained a protein that co-migrated with the postitive control with a molecular weight of 49kDa, the molecular weight of EPCR.

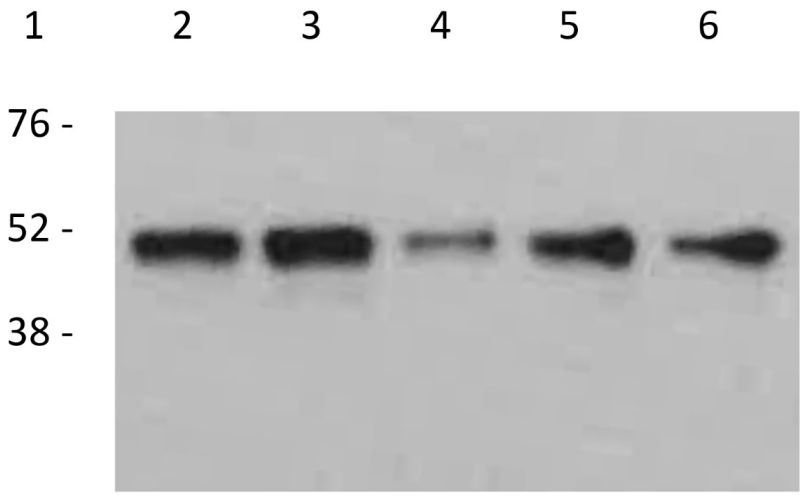

Figure 4 EPCR expression in PC3 cells. EPCR was immunoprecipitated from PC3 cell media and lysates and evaluated using Western blotting. Lane one consists of the molecular weight ladder (kDa). Lane two contains purified EPCR, 20µg/ml. Lane three contains the sample from cell media collected in November 2019. Lane four contains a sample of cell lysate also collected in November 2019. Lane five contains the sample from cell media collected in January 2020. Lane six contains a sample of cell lysate also collected in January 2020.

ELISA

Three ELISAs were done to determine the concentration of EPCR in the serum of patients with prostate cancer. A standard curve graph was constructed for each ELISA and used to determine the concentration of EPCR in each patient sample. Standard Curve 1 (Figure 5A) was used to determine the concentration of EPCR in patients 1-26 and control serum 1. Standard Curve 2 (Figure 5B) was used to determine the concentration of EPCR in patients 27-51 and control serum 2. Standard Curve 3 (Figure 5C) was used to determine

the concentration of EPCR in patients 52-76 and control serum 3. For each sample, the triplicate absorbance readings were averaged together.

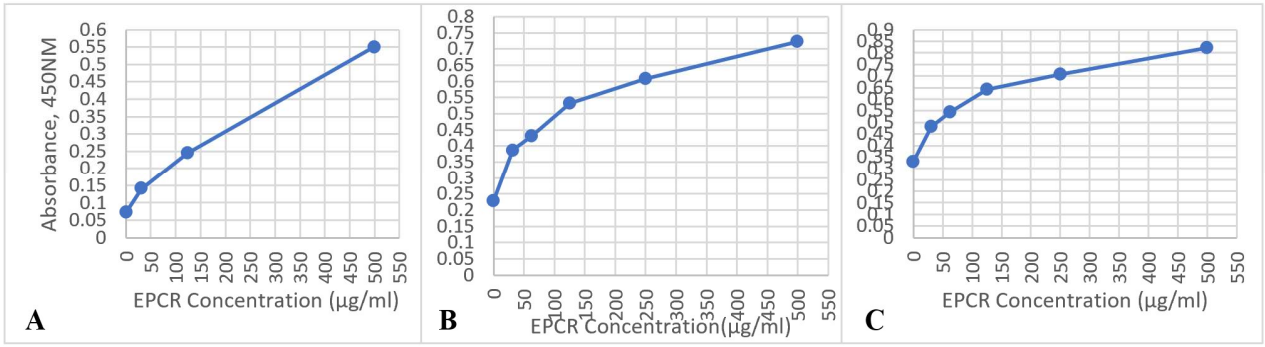

Figure 5 Standard Curves for ELISAs One, Two, and Three Standard curves were constructed for each ELISA performed and were used to determine the concentration of EPCR in each patients serum sample along with the control samples.

Each of the concentrations were then multiplied by their dilution factor to find the final concentration of EPCR in each patient's serum sample. Each patient's Gleason Score for prostate cancer was then identified. All patients had a Gleason score ranging from 5-8. Average EPCR concentration for each score was calculated for ELISAs one, two and three and are shown in Table 1 below.

	GLEASON SCORE			
	5	**6**	**7**	**8**
ELISA 1	373.69+/- 171.21	615.38+/-34.67	780.5+/-44.24	814.88+/- 84.57
ELISA 2	410+/- 118.46	659.45+/-28.07	766+/- 30.18	870+/- 0
ELISA 3	440.75+/- 198.61	600.45+/- 56.19	772.86+/- 41.16	840+/- 24.15

Table 1: Average EPCR Concentration by Gleason Score Averages of EPCR concentration (µg/ml) were calculated for patients with a prostate cancer Gleason score of 5, 6, 7, and 8. These averages were determined for each ELISA.

An ANOVA test was performed on each ELISA to determine if there was a difference in the means of EPCR concentration for each Gleason score and the control concentration. An ANOVA on ELISA 1 rejected the null hypothesis that there would be no significant difference in the means of EPCR concentrations in patients with differing Gleason scores of prostate cancer since the F calculated (23.64) was greater than the critical value (3.049) ($P<0.0001$), indicating that there is a statistical difference in the means of EPCR concentrations in patients with differing Gleason scores. A Tukey test was then performed on ELISA 1 and showed that patients with a Gleason score of 5 had a statistically lower EPCR concentration that those with scores of 6, 7 and 8 ($p<0.01$). There was also a statistical difference in EPCR concentration between patients with a score of 6 and a score of 8 ($p<0.05$). Individual patient EPCR concentrations for ELISA 1 patients 1 through 26 can be found in Appendix A below.

An ANOVA on ELISA 2 rejected the null hypothesis that there would be no significant difference in the means of EPCR concentrations in patients with differing Gleason scores of prostate cancer since the F calculated (77.08) was greater than the critical value (2.895) ($P<0.0001$), indicating that there is a statistical difference in the means of EPCR concentrations in patients with differing Gleason scores. A Tukey test was then performed on ELISA 2 and showed that patients with a Gleason score of 5 had a statistically lower EPCR concentration that those with scores of 6, 7 and 8 ($p<0.01$). There was also a statistical difference in EPCR concentration between patients with a score of 6 and a score

of 8 ($p<0.01$). Individual patient EPCR concentrations for ELISA 2 patients 27 through 51 can be found in Appendix B below.

An ANOVA on ELISA 3 also rejected the null hypothesis that there would be no significant difference in the means of EPCR concentrations in patients with differing Gleason scores of prostate cancer since the F calculated (33.19) was greater than the critical value (2.895) ($P<0.0001$), indicating that there is a statistical difference in the means of EPCR concentrations in patients with differing Gleason scores. A Tukey test was then performed on ELISA 3 and showed that patients with a Gleason score of 5 had a statistically lower EPCR concentration that those with scores of 7 and 8 ($p<0.01$). There was also a statistical difference in EPCR concentration between patients with a score of 6 and a score of 8 ($p<0.01$). Individual patient EPCR concentrations for ELISA 3 patients 52 through 76 can be found in Appendix C below.

For each ELISA performed, all concentrations of EPCR in patients with prostate cancer were significantly higher than the concentration of EPCR in the control serum ($p<0.01$). Figure 6 represents all paitents compared in each of the ELISAs. All patients with prostate cancer also had concentrations of EPCR that were greater than the 100 µg/ml basline (Figure 6).

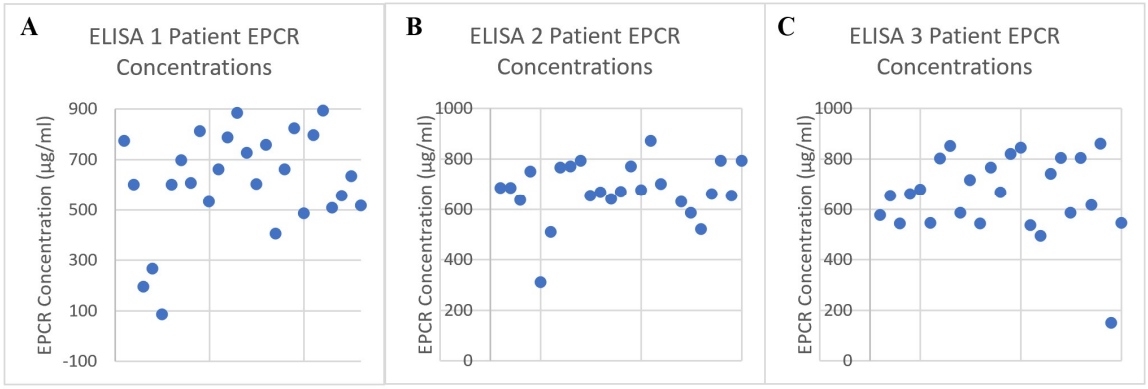

Figure 6: EPCR Concentrations for All Patients with Prostate Cancer All concentrations of EPCR for patients with prostate cancer were greater than the baseline concentration of 100µg/ml and all were significantly greater than the EPCR concentration in the control serum.

Discussion

The western blot performed with both cell lysates and cell media indicated that EPCR is expressed by prostate cancer cells. As indicated by previous research on breast cancer cell lines, the most aggressive breast cancer cell lines expressed EPCR at levels that made the cancer cells identifiable when compared with normal cells (Ruf 2014). The results presented by this western blot also correspond with those of Menschikowski et. al (2011) who found that EPCR expression is upregulated at both the mRNA and protein levels in PC-3 Cell lines. These prostate cancer cells expressing EPCR may be more capable of growth and survival as indicated by the work of Ruf and Schafer, and may utilize the hosts immune system to control the differentiation of stem cells into tumor cells (2014).

I reject the null hypothesis that there will be now statistical difference in EPCR concentrations in patients with varying prostate cancer Gleason scores. Individual ANOVAs for EPCR concentration in patients with varying Gleason scores of prostate

cancers indicated that there was a statistical difference in EPCR concentration in patients with prostate cancer ($p<0.01$). The Tukey tests performed also indicated that there were statistically significant differences in EPCR concentrations between patients with a score of 5 and those with a score of 6,7, and 8 ($p<0.01$) and a statistical difference in EPCR concentration between those with a score 6 and a score of 8 ($p<0.01$). As previous research has indicated, EPCR has potential as a biomarker for ovarian cancer as they found that the concentration of EPCR in the plasma samples of patients with ovarian cancer was significantly higher than in the plasma samples obtained from healthy patients (Ducros et al. 2012). The increase in EPCR expression may be linked to the EPCR mediated survival and anti-apoptotic signaling pathways which allows APC to act as a pro-carcinogenic agent in patients with high Gleason scores (Menschikowski et al. 2011). These results indicate that the expression of EPCR in the serum samples of patients can be used as a biomarker to indicate the onset of prostate cancer and potentially the grade of prostate cancer that the patient may possess.

These results support the hypothesis that patients with prostate cancer will have a higher level of EPCR expression in their serum than the female control serum. Those with Gleason scores of 6, 7, 8 have statistically higher levels of EPCR than those with Gleason scores of 4 and 5. This indicates that the expression of EPCR correlates with the stage of prostate cancer the patient has. This data can be used in medical facilities to determine or diagnose patients with prostate cancer.

The expression of EPCR in prostate cancer cells and in patient serum samples can be further studied to determine the average level of EPCR expression for each Gleason score of prostate cancer. This information can be used by medical facilities who wish to determine the stage of cancer the patient has based on the concentration of EPCR found in their serum.

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Works Cited

Abbas AK, Aster JC, Perkins JA, Robbins SL, Kumar V. 2017. Robbins basic pathology. 10th ed. Philadelphia: Elsevier. [cited 2020 Mar 23].

Ducros E, Mirshahi S, Azzazene D, Camilleri-Broët S, Mery E, Farsi HA, Althawadi H, Besbess S, Chidiac J, Pujade-Lauraine E, et al. 2012. Endothelial protein C receptor expressed by ovarian cancer cells as a possible biomarker of cancer onset. International Journal of Oncology 41:433–440. [cited 2020 Mar 9].

Dunn MW, Kazer MW. 2011. Prostate Cancer Overview. Seminars in Oncology Nursing 27:241–250. [cited 2020 Feb 23].

Esmon C. 1992. The Protein C Anticoagulant Pathway. Arteriosclerosis and Thrombosis 12(2):135–145. [cited 2020 Jan 26].

Fukudome K, Esmon CT. 1994. Identification, Cloning, and Regulation of a Novel Endothelial Cell Protein C/Activated Protein C Receptor. The Journal of Biological Chemistry 269(42):26486–26491. [cited 2020 Jan 26].

Griffin JH, Zlokovic BV, Mosnier LO. 2012. Protein C anticoagulant and cytoprotective pathways. International Journal of Hematology 95(4):333–345. [cited 2020 Jan 26]. doi:10.1007/s12185-012-1059-0.

Jackson CJ, Xue M, Thompson P, Davey RA, Whitmont K, Smith S, Buisson-Legendre N, Sztynda T, Furphy LJ, Cooper A, et al. 2005. Activated protein C prevents inflammation yet stimulates angiogenesis to promote cutaneous wound healing. Wound Repair and Regeneration 13:284–294. [cited 2020 Feb 23].

Kobayashi H, Moniwa N, Gotoh J, Sugimura M, Terao T. 1994. Role of Activated Protein C in Facilitating Basement Membrane Invasion by Tumor Cells. Cancer Research 54:261–267. [cited 2020 Jan 26].

Menschikowski M, Hagelgans A, Tiebel O, Klinsmann L, Eisenhofer G, Siegert G. 2011. Expression and shedding of endothelial protein C receptor in prostate cancer cells. Cancer Cell International 11:1-10. [cited 2020 Apr 22].

Palta S, Saroa R, Palta A. 2014. Overview of the coagulation system. Indian Journal of Anaesthesia 58:515–523. [cited 2020 Feb 23].

Ruf W, Schaffner F. 2014. Role of the protein C receptor in cancer progression. Thrombosis Research 133:s85–s89. [cited 2020 Jan 27].

Sluis GLV, Büller HR, Spek CA. 2010. The role of activated protein C in cancer progression. Thrombosis Research 125:s138–s142. [cited 2020 Feb 23].

Appendix A

ELISA 1	EPCR Concentration (µg/ml)
Control1	102 +/- 4.33
P1	772.5 +/- 60.62
P2	600 +/- 85.18
P3	195 +/- 53.21

P4	267 +/- 56.29
P5	82.5 +/- 138.8
P6	600 +/- 27.04
P7	697.5 +/- 7.5
P8	606 +/- 33.82
P9	810 +/- 45.83
P10	532.5 +/- 33.82
P11	660 +/- 26.34
P12	786 +/- 73.61
P13	882 +/- 27.04
P14	727.5 +/- 37.5
P15	603 +/- 27.04
P16	757.5 +/- 30.31
P17	402 +/- 73.87
P18	660 +/- 27.04
P19	822 +/- 116.3
P20	486 +/- 43.3
P21	795 +/- 18.87
P22	892.5 +/- 30.31
P23	507 +/- 49.94
P24	558 +/- 28.39
P25	636 +/- 15.15
P26	517.5 +/- 57.28

Appendix B

ELISA 2	EPCR Concentration (µg/ml)
Control2	30 +/- 0.37
P27	685 +/- 178.49
P28	685 +/- 123.93
P29	635 +/- 151.74
P30	750 +/- 136.97
P31	310 +/- 313.74
P32	510 +/- 27.54
P33	768 +/- 92.38
P34	770 +/- 94.12
P35	790 +/- 107.16
P36	655 +/- 54.85
P37	665 +/- 98.66
P38	640 +/- 206.54
P39	670 +/- 149.75
P40	770 +/- 60
P41	675 +/- 165.40
P42	870 +/- 5.77
P43	700 +/- 124.23
P44	603.33 +/- 66.58
P45	630 +/- 161.58
P46	585 +/- 40
P47	520 +/- 49.07

P48	660 +/- 40.10
P49	790 +/- 84.12
P50	654 +/- 61.71
P51	790 +/- 86.75

Appendix C

ELISA 3	EPCR Concentration (µg/ml)
Control3	95 +/- 5
P52	575 +/- 25.98
P53	655 +/- 33.29
P54	540 +/- 11.55
P55	660 +/- 33.29
P56	680 +/- 47.70
P57	545 +/- 25.98
P58	800 +/- 92.60
P59	850 +/- 17.32
P60	585 +/- 32.79
P61	715 +/- 20
P62	540 +/- 15.28
P63	765 +/- 55.68
P64	665 +/- 44.44
P65	820 +/- 42.52
P66	845 +/- 25.98
P67	535 +/- 17.56

P68	495 +/- 53.93
P69	740 +/- 36.06
P70	805 +/- 50.08
P71	585 +/- 22.55
P72	805 +/- 73.99
P73	615 +/- 30.14
P74	860 +/- 42.52
P75	148 +/- 339.42
P76	545 +/- 23.63