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Investigation of the effects of antimicrobial peptides and vitamin D in breast cancer

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Abstract

Breast cancer is the most common cancer in women. In recent years, there have been studies on the effectiveness of cathelicidins, one of the antimicrobial peptides, especially in breast cancer. Cathelicidin expression is regulated by a rather complex mechanism. Under normal conditions, vitamin D induces the release of cathelicidin from human neutrophils via Neutrophil Elastase (NE). Elafin, on the other hand, inhibits cathelicidin as a Neutrophil Elastase inhibitor. However, the effect of these molecules on tumor progression is unclear. In our study, we aimed to investigate the effects of vitamin D and Elafin on cathelicidin in breast cancer patients. Cathelicidin, Elafin, and Vitamin D Receptor levels in the pre and post-operative blood samples of control patients and patients diagnosed with breast cancer were analyzed by western blot method. According to our findings, it was observed that cathelicidin levels were especially high and elafin levels were low in the preoperative group. Since an inflammatory environment occurs in the presence of a tumor, there is an increase in vitamin D receptors and elafin in the postoperative group. It was thought that the low Elafin in the preoperative group caused vitamin D to secrete more Cathelicidin and increased the progression of the tumor, but the formation of cathelicidin was inhibited due to the increase in Elafin levels in the postoperative period. Thus, it was concluded that cathelicidin, which has an effect on the development of breast cancer, can be controlled by Elafin and Vitamin D receptors.

Keywords: Breast cancer, cathelicidin, elafin, vitamin D receptor

Introduction

Antimicrobial peptides, also known as host defense peptides (AMP), constitute an component of innate immunity, serving as natural antibiotics encoded by genes. These peptides are synthesized by diverse cell types and tissues. These proteins are small peptides containing 12-50 amino acids with broad-spectrum microbiocidal activity. (MW≤5 kDa) [1,2].

Antimicrobial peptides were first identified in blood, leukocytes and lymphatic tissues in the late 19th century. In later studies, it was shown to kill bacteria, viruses, fungi, and even altered and cancerous cells. Additionally, AMPs can initiate or stop apoptosis depending on the biological situation [3]. Major antimicrobial peptides; cathelicidins, defensins, histatins, granulizins, lactoferrin and hepcidins. Cathelicidins are cationic peptides containing a structurally variable antimicrobial region at the C-terminal end. While there are many cathelicidin genes in mammals such as pigs and cattle, there is only one gene (CAMP) in humans. Antimicrobial protein 18 (hCAP18), the precursor protein of cathelicidin in humans, is produced by many cells in the skin (keratinocytes, mast cells, neutrophils etc.). Neutrophil proteases cleave hCAP18 to LL-37, an extracellularly active antimicrobial peptide. The active peptide begins with two leucine amino acids and contains 37 amino acids, hence the designation LL-37, and is the only known human cathelicidin peptide [3,4].

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Production of cathelicidins is very low under normal conditions. However, various microbial factors, infection, inflammation and injury cause increased cathelicidin production. Research has demonstrated that this peptide plays a significant role in cancer. However, it is regulated by a complex mechanism and varies according to cell types. LL-37 causes tumorigenic effects in breast, ovarian, pancreatic, lung, prostate cancers as well as malignant melanoma and cutaneous squamous cell carcinoma. In contrast, LL-37 shows anticancer effects in colon cancer, gastric cancer, hematological malignancy, and oral squamous cell carcinoma [4,5].

Cathelicidin is released from human neutrophils by Neutrophil Elastase (NE), and NE is inhibited by elafin. However, it is not clear whether elafin can suppress tumor progression via elastase [6-8]. It has been reported that another molecule that regulates cathelicidin expression is vitamin D. Vitamin D induces cathelicidin expression in normal conditions but reduces it in the presence of inflammation [9,10]. There is no study in the literature that comparatively evaluates these markers together in breast cancer.

According to this information, our aim was to investigate the effects of vitamin D and elafin on cathelicidin in breast cancer patients. We also compared the levels of these molecules with the levels after the operation.

Material and Methods

This study was supported by Manisa Celal Bayar University Scientific Research Projects Coordination Unit with project number 2022/009 and an ethics committee report was received received on 24.11.2021 (Decision no: 20.478.486/1030). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Working group; It consists of 40 cases (20 preoperative and 20 postoperative) and 20 control groups. The case group (n=40) was selected from patients who applied to the General Surgery clinic of the University of Health Sciences Dr. Abdurrahman Yurtaslan Oncology Hospital and were diagnosed with breast cancer after a fine needle aspiration biopsy and decided to undergo surgery. Those who were over 18 years of age and did not have any other pathology or chronic disease were included in the study. While blood was taken from the case group for routine control purposes before the surgery and on the 30th day after the surgery, approximately 2 cc blood was taken for the study. The control group (n=20) consisted of healthy individuals without any health problems in the same age group as the patient group. People who met these criteria and wanted to participate in the study signed a consent form. The serum of the blood samples was separated and stored at (-80°).

After the proteins in the control and study group serum samples were isolated, Cathelicidin, Elafin and VDR protein values were were analyzed by Western Blot technique. Proteins with known molecular weights (Beta Actin; 40 kD, Cathelicidin; 19 kD, Elafin; 14kD, VDR; 41 kD) were run on the gel and then transferred to the nitrocellulose membrane and displayed on the membrane. For the isolation of proteins, 1 mL of 1x PBS was added to the serum and was centrifuged at 2500 rpm for 3 min. After the PBS was removed, 1 ml ProtinEx Total Protein Extraction Solution and 10 µL protease inhibitor coctail were added to the pellet and pipetted. It was kept in ice for 10 minutes. 15 min at 16000 rpm at +4°C. was centrifuged. The supernatant was taken into a clean tube. Protein determination was made using the Abp biosciences bradford protein assay kit. 5x PAGESTA Reducing sds-page buffer (751-001) was added to the protein sample to be placed according to the Bradford calculation (Figure 1). Distilled water was added to make the total volume 20 µl. The prepared mixture was heated at 95 °C for 10 minutes. It was incubated and then placed on ice. It was run on 4-12% bis-tris gradient gel (Invitrogen, nupage 4-12% bis-tris gel) in an Xcell Surelock vertical gel system tank (Invitrogen) for 35 minutes (200 volt, 135 amper). Proteins were transferred to nitrocellulose membrane within 7 minutes (Iblot transfer stack nitrocellulose kit, (IB401001)). Membranes were blocked by incubating for 1 hour at room temperature in the blocking solution prepared by adding 5% BSA (bovine serum albumin) into TBS-T solution containing 0.1% Tween-20. After washing the membranes with PBSX3, they were incubated with primary antibodies Cathelicidin, Elafin, Vitamin D Receptor (DF7741, DF14564, AF6159 Affinity biotech, respectively) overnight at +4°C with shaking. After washing, it was incubated with Secondary antibody (goat-anti-rabbit IgG (h+1), hrp conjugate) (Affinity Biotech, S0001) for 1 hour at room temperature. After the washing process, the membranes were placed in ECL solution (Nzy Supreme Ecl HRP Substrate, nzytech, MB19301) and placed in the imaging device (chemidoc-IT2, UVP).

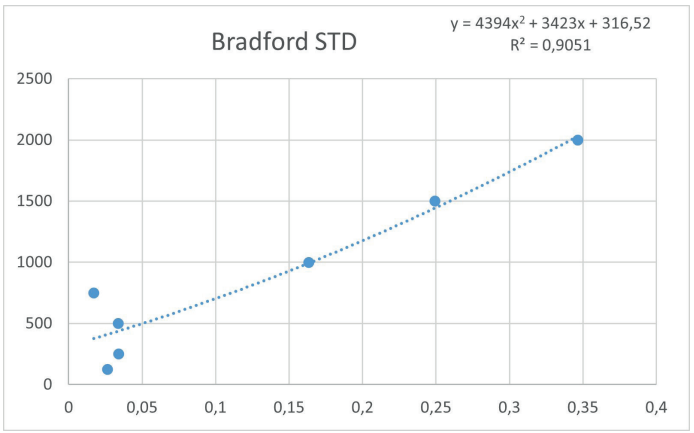

Figure 1. Standard graph and quadratic formula used in the quantitative determination of proteins by the Bradford method

SPSS 23.0 (SPSS Inc.; Chicago, IL, USA) package program was used to evaluate the data. Data analysis focused on mean±SD values.

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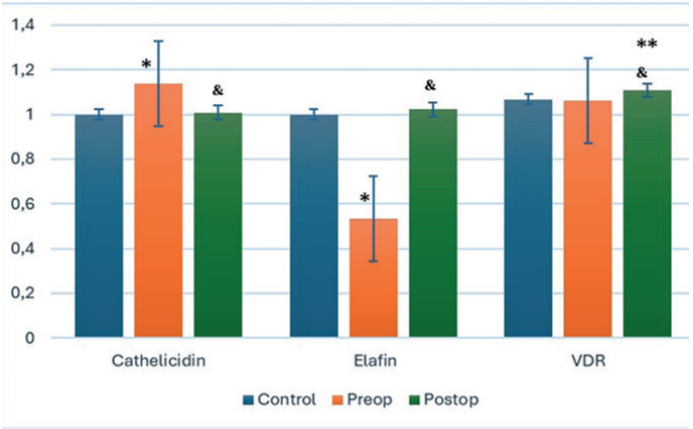
Results

Beta Actin, Cathelicidin, Elafin, VDR protein Western Blot results are given in (Graph 1, Figure 2). As a result of the analysis, we observed that Cathelicidin was statistically significant increase in the Preop group compared to the control group (p=0.001). A statistically significant decrease was observed in the postoperative group compared to the preoperative group (p=0.05). While Elafin expressions were statistically significantly lower in the preop group compared to the control group (p=0.001), There is a significant increase in the postop group compared to the preop group(p=0.00). While VDR protein level is no statistically significant difference between the preop and control groups, There is a significant increase in the postop group compared to the control and preop groups(p=0.02, p=0.014).



Graph 1. Graphical representation of protein values; *p≤0.001 preoperative compared to control, **p≤0.05 postoperative compared to control, p≤0.05 according to preop and postop

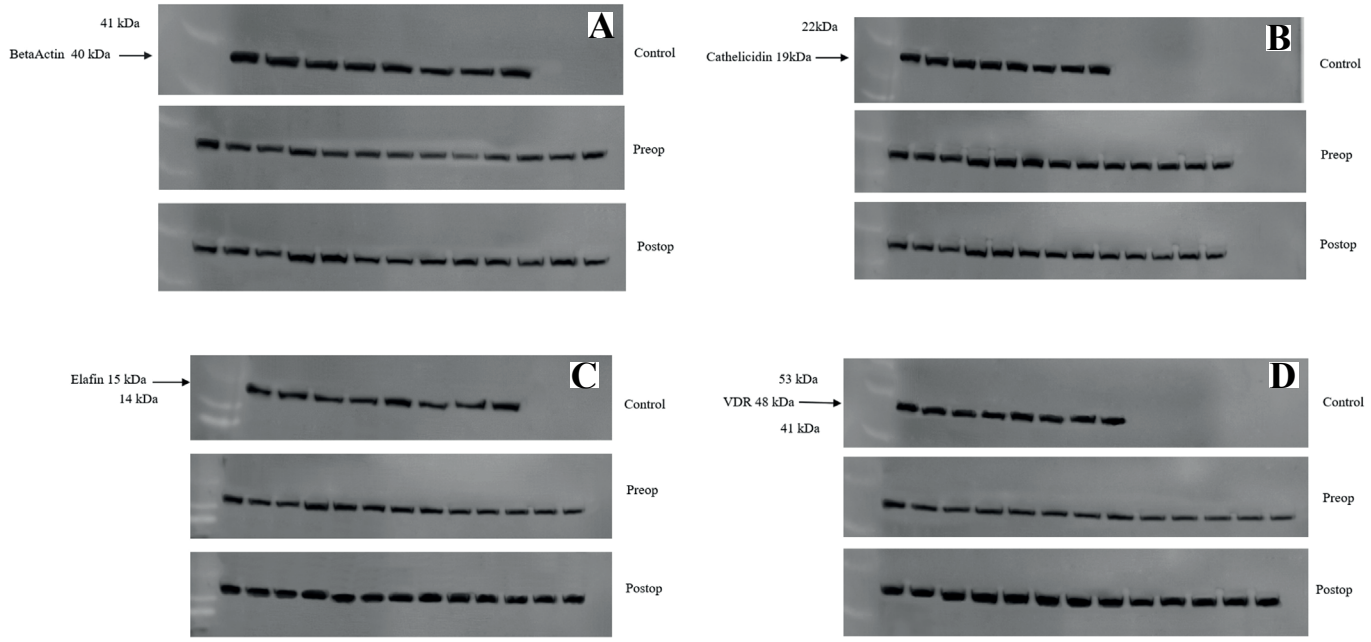


Figure 2. Western blot results of proteins in control, preop and postop groups; **A.** beta actin, **B.** cathelicidin, **C.** elafin, **D.** VDR

Discussion

Breast cancer is the most common type of cancer in women, affects more than 2 million women every year, and its incidence is increasing in almost every region around the world [11,12]. Genetic, hormonal and immune factors are of great importance in the development of breast cancer [13,14]. In recent years, there have been studies showing that cathelicidins, which are antimicrobial peptides, are particularly effective in cancer [15-17]. Recent reports suggest that Cathelisidins has an autocrine role in tumor cell proliferation, survival, and metastasis. However, it is not clear how LL-37 affects these processes and existing information is insufficient [18]. Cathelicidins exhibit conflicting roles in cancer, promoting or inhibiting tumor progression depending on the context. Under normal conditions, cathelicidin is involved in the tumor surveillance system with NK cells. However, uncontrolled expression contributes to cancer development [15]. A large body of evidence has shown that CAMP promotes tumor growth and invasion through angiogenesis initiation and recruitment of immune cells (e.g. monocytes, neutrophils, dendritic cells, mesenchymal stromal cells), and also promotes wound healing ability and angiogenesis [19]. The sensitivity of LL-37 varies among different cancer types. For example, in colon cancer, glioblastoma, hematological malignancy, gastric cancer, and oral squamous cell carcinoma, LL-37 can suppress proliferation and induce autophagy and apoptotic cell death. However, it may promote proliferation, migration, and tumor formation in other types of cancer, such as

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lung cancer, breast cancer, ovarian cancer, melanoma, prostate cancer, liver cancer, and skin squamous cell carcinoma [20,21]. The effect of cathelicidin hCAP18/LL-37 in hepatocellular carcinoma (HCC) metastasis remains unclear. In a study, LL-37 expression increases endothelial-mesenchymal transition (EMT), migration and invasion in HCC cells. LL- 37 plays a role in HCC metastases and has been confirmed to act as an important factor to weaken the inhibitory activity of 1.25(OH) 2 D3 on HCC metastasis [22]. In a study by Zhang et al., they showed that LL-37 inhibits the growth of pancreatic cancer both in vitro and in vivo and that it can do this by suppressing autophagy [23]. Consistent with its tumor suppressor role, cathelicidin has been shown to induce apoptosis in colon cancer cells via a caspase-independent pathway [24]. LL-37 also plays a role in inhibiting the proliferation of gastric cancer cells by arresting the cell cycle [25]. Cathelicidin expression in breast cancer cells has been shown to be induced by calcitriol [26]. In ovarian cancer, LL-37 caused proliferation of cancer cells and an increase in angiogenesis through cytokine and angiogenic factors [27]. It affects cytotoxicity with NK cells in breast cancer and increases metastasis [25]. Based on the above-mentioned observations, LL-37 has pro-tumorigenic or anti-cancer effects but the responsible mechanisms have not been fully elucidated. However, it has been suggested that human cathelicidin expression is strongly dependent on its ability to interact with the membrane receptor, which varies in different cancer cells, and therefore its effects may be tissue specific or dependent on other factors [28]. According to our findings, cathelicidin levels exhibited a statistically increase in the preop group compared to the control group, A statistically significant decrease was observed in the postoperative group compared to the preoperative group. Recent studies show that cathelicidin expression appears to increase with activation of the vitamin D receptor (VDR). It is activated by VDR receptor binding to the Vitamin D Response Element (VDRE) in the promoter region of the cathelicidin gene [10]. VDR mediates the action of vitamin D3 by controlling the expression of hormone sensitive genes. In addition, it has been observed that the immune response is impaired in cases of decreased VDR or vitamin D3 deficiency [29].

Elafin, an endogenous serine protease inhibitor, is increased in normal breast epithelial cells compared to breast carcinoma cells and specifically inhibits NE. When elastase is not inhibited, metastasis of cancer cells increases. Elafin has also been shown to increase apoptosis by DNA fragmentation in tumor cells. Several studies have shown that Elafin is expressed in well-differentiated squamous cell carcinoma but is lost in poorly differentiated tumors. Elafin's control over NE activity is lost. However, it is unclear what the relationship between elafin and elastase is in cells and their implications for tumor progression [7,8]. Caruso et al. hypothesized that elafin is an important counterbalance against the mitogenic effects of NE-activity [30]. In this study, we demonstrate Elafin values demonstrated a statistically significant decrease in the preop group compared to the control group, with a subsequent increase in the postop group.

The possible anticancer effect of active vitamin D3 (1,25-(OH)2D3) has been evaluated for approximately 40 years. Research has indicated that vitamin D3 demonstrates properties that include reducing inflammation, encouraging programmed cell death, and inhibiting angiogenesis [29]. The vitamin D receptor (VDR) plays an important role in initiating these effects by modulating several genes that control cellular proliferation. Malignant cells expressing VDR are abundant. Although the binding sites of VDR to DNA and 1.25-(OH)2D3 are separate, they affect each other. Gene expression is regulated after the hormone binding VDR complex binds to DNA. The effect of the hormone depends on the VDR level in cells [31]. Vitamin D has been reported to be one of the most important regulators of Cathelicidin expression. In the literature, conflicting studies have been reported association between Vitamin D and Cathelicidin in cancer patients. Vitamin D induces cathelicidin expression in normal conditions but reduces it in the presence of inflammation [31]. There are insufficient studies in the literature comparing these markers together in breast cancer. Therefore, studies showing cathelicidin and vitamin D levels and the relationship between them in various types of cancer are needed. Lindgren et al. showed that VDR genotypes were associated with breast cancer prognosis and the association might be modified by tumor size [32] . Another studied said that the expression level of VDR in healthy tissue was significantly higher than tumoral tissue. However, there was no significant relationship between VDR and tumor grade, HER2, ER, PR, LVI, LN, disease stage, age, and tumor size [33]. In our study, we showed that there was no statistically significant difference in VDR proteins between the Preop and Control groups, but there was a significant increase in the Postop group compared to the Control and Preop groups.

Conclusion

According to the literature, the formation of Cathelicidin from vitamin D occurs by Human Neutrophil Elastase (HNE). This regulatory enzyme (HNE) is inhibited by elafin. In our study, we observed that preop cathelicidin levels were high and elafin levels were low. The fact that Elafin is low in the preoperative group causes vitamin D to activate more Cathelicidin. It has been observed that high levels of cathelicidin increase tumor progression by increasing angiogenesis in breast cancer. We observed that vitamin D receptor levels is an increase in the postoperative group in the presence of a tumor occurs inflammatory. Vitamin D causes an increase in catecilidin, but due to the increase in Elafin, it inhibits the formation of catecilidin in the postoperative period. Since Cathelicidin levels of breast Ca patients differ in the postoperative period, it was concluded that Cathelicidin may be a tumor marker and is also controlled by Elafin and VDR molecules. We suggest that more studies should be conducted on this subject with different Ca patient groups.

Conflict of Interests

The authors declare that there is no conflict of interest in the study.

Financial Disclosure

This study was supported by Manisa Celal Bayar University Scientific Research Projects Coordination Unit with project number 2022/009.

Ethical Approval

The Manisa Celal Bayar University Health Sciences Ethics Committee approved this study (Decision no: 20.478.486/1030, 24.11.2021).

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