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VEGF and MMP-9 Gene Expression Caused by Treatment with Helicobacter Pylori Neutrophil-activating Recombinant Protein in a Breast Cancer Model

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ABSTRACT

BACKGROUND AND OBJECTIVE: Breast cancer is a major cause of death in women, worldwide. Cytotoxic drugs may lead to various adverse side-effects in patients. Helicobacter pylori neutrophil-activating protein (HP-NAP) is one of the most important proteins, produced by helicobacter pylori. The purpose of this study was to investigate the expression of VEGF and MMP-9 genes, caused by HP-NAP treatment in a breast cancer model.

METHODS: In this experimental study, 18 female BALB/c mice, aged 6-8 weeks (19-23 g), were used. The mice were allocated to three groups: test group (treated with recombinant HP-NAP), positive control group (treated with an anti-cancer drug called trastuzumab), and negative control group (receiving phosphate-buffered saline). Expression and purification of HP-NAP were performed using nickel-resin affinity chromatography (Qiagen). The mice with breast tumors were treated with HP-NAP. MMP-9 and VEGF gene expression was measured using real-time polymerase chain reaction (PCR).

FINDINGS: Protein expression was confirmed, using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The analysis showed the expression of a 20,000 dalton protein. Moreover, MMP-9 and VEGF gene expression decreased more significantly in the test group (due to treatment with HP-NAP), compared to the negative control group. The reduced expression in the test group was almost a quarter of the expression in the control group ($p<0.01$).

CONCLUSION: The results showed that HP-NAP can reduce the expression of genes involved in metastasis in a mouse model of breast cancer. Therefore, this protein can be exploited for future therapeutic strategies in cancer treatment.

KEY WORDS: Neutrophil-Activating Factor, Breast Cancer, Metastasis, Immunotherapy.

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Introduction

Helicobacter pylori neutrophil-activating protein (HP-NAP) is one of the most important proteins, produced by helicobacter pylori. During bacterial growth, some HP-NAP molecules are released from the bacterium and some remain attached to the outer membrane. A cell-bound HP-NAP can mediate the binding between the bacterium and carbohydrates on the surface of the host cell (1).

This 150-kDa protein is coded by NAP gene and is present in all H. pylori strains; however, its expression varies in different strains. Moreover, this protein is widely conserved among isolates. HP-NAP, as the agonist of TLR2, has immunomodulatory properties and can induce the expression of IL12 and IL23 by human monocytes and neutrophils. This protein has the potential to modify immune responses and change the phenotype from Th1 to Th2 by the production of interferon gamma and tumor necrosis factor alpha, which triggers the start of an effective immune reaction against infection or cancer (2).

Breast cancer is the most common malignancy among women in Iran, accounting for 18.9% of cancer cases in this gender. Despite the significant improvements in the diagnosis and treatment of breast cancer, mortality as a result of metastatic breast cancer in women still remains a medical challenge (3). Cancer progression can jeopardize the patient's survival and lead to failure in treatment strategies. For the invasion of cancer cells to the surrounding tissue, these cells must first penetrate into the basement membrane and destroy the extracellular matrix barrier. At this level, protease enzymes such as metalloproteinases (MMP) by digesting the extracellular matrix and its compounds facilitate the invasion of cancer cells to other tissues and play a major role in tumorigenesis, metastasis, and cancer development (4).

So far, it has been well established that tumor cells produce higher levels of protease, compared to normal cells. Moreover, the positive relationship between cell invasion and protease level has been demonstrated in experimental tumor models and human clinical studies (3, 4). Therefore, it is believed that these enzymes play an important role in tumor development. Previous studies have shown that MMP expression in tumor cells is influenced by growth factors and cytokines; therefore, its expression is different from that of normal tissues (5). Angiogenesis is the growth of blood vessels in and around tumors. This process leads to the discharge of waste products from the tumor and delivers oxygen and nutrients to the tissue. Angiogenesis is activated due to the secretion of angiogenic factors such as vascular endothelial growth factor (VEGF) from cancer cells to the surrounding normal cells. Cancers that are able to express VEGF are also able to grow and metastasize (6, 7). If a compound is able to reduce the production of proteins involved in neovascularization and metastasis of cancer cells, it can be used to prevent the progression of this incurable disease. Since metastatic and angiogenic genes play a significant role in cancer cell invasion to various parts of the body, we aimed to investigate the expression of VEFG and MMP-9 genes, caused by HP-NAP treatment in a breast cancer model.

Methods

Bacteria and culture medium: In this experimental study, Escherichia coli (E. coli) BL21 strain was used as a host for the expression of cloned napA gene in pet28a expression vector. Luria-Bertani medium (LB, 1 µg/ml), containing kanamycin (50 mg/ml), was used to amplify strains carrying the plasmid pET-28a. Induction and expression of napA recombinant proteinIn this study, napA gene sequences (manufactured by Genscript Company, USA) were used. The cloned gene was transferred to pet28a vector. To express the recombinant napA, E. coli BL21 was used as the expression host. For the induction of napA, the bacterium was cultured overnight in the presence of 50 µg/ml kanamycin. The induction process was performed at a high volume after the optical density reached about 0.8-1 (550 nm wavelength).

For the expression of the recombinant protein, isopropyl-beta-thiogalactopyranoside (IPTG) solution with a final concentration of 5.1 mM/ml was used. Incubation was performed in a shaking incubator at 37 °C for 24 hours (8). Purification and expression of napA recombinant protein Considering the designed primer and the histidine-tagged vector, nickel-resin affinity chromatography was used for protein purification. To prepare the cell lysate, the cultured E. coli sediment was suspended in 100 ml of IBW buffer and washed using a sonicator during ten cycles (40 sec) at 4 °C (90% speed). The resulting suspension was centrifuged at 4 °C for 20 min at 12000 rpm. The final supernatant was collected and passed through a 0.45 µ filter. After passing the protein supernatant through the filter, the column was rinsed with a washing buffer until the absorbance at 280 nm reached zero. Then, the second elution buffer was added to the column and the output was stored in a

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separate container. Eventually, the third elution buffer was added and the final output was separately collected. After collecting the proteins from the column, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to verify the purity of protein samples. To remove imidazole from the protein solution, buffer dialysis in the presence of phosphate-buffered saline (PBS) was performed (9).

Evaluation of the recombinant protein by SDS-PAGE: To investigate the expression of the recombinant protein, SDS-PAGE (polyacrylamide gel with a concentration of 5.12%) was performed at a voltage of 100 in the presence of a pre-stained protein marker (pre-stained ladder SM0607, Fermentas Company) and a 200 kDa protein ladder. Finally, the polyacrylamide gel was stained with Coomassie G-250 dye, and the band was investigated in the presence of a protein marker, which indicated the recombinant protein (8). Cell cultureThe metastatic breast cancer cell line (4T1) was purchased from Pasteur Institute of Iran. The cells were grown in a Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum and 1% penicillin-streptomycin antibiotic under incubation conditions at 37 °C (5% CO2). After 3 days, the cells were passaged using trypsin 0.25% and ethylenediaminetetraacetic acid 0.02% (EDTA). All cell culture materials were purchased from Gibco Company (USA) (10). The mouse model and tumor maintenance conditions in miceIn total, 18 female BALB/c mice, aged 6-8 weeks (19-23 g), were purchased from Pasteur Institute and allocated to three groups (6 mice per group).

Induction of breast cancer: The injection site was disinfected with cotton and alcohol. On day zero, 4T1 cancer cells were subcutaneously injected in all the mice. The injections were performed near the bottom left mammary gland. On the fifteenth day of injection (manifestation of the tumor), recombinant proteins were intraperitoneally injected in mice in the test group at a concentration of 50 µg/ml for two weeks at specific intervals. The positive control group received an anti-cancer drug (trastuzumab) under similar conditions and PBS was injected for the negative control group.

Inspection of changes in gene expression using real-time polymerase chain reaction (PCR) technique: RNA extraction: First, the tumor tissue was completely crushed in a sterile mortar in the presence of liquid nitrogen and transferred to RNase-free microtubes. One ml of triazole was added for each microtube of the crushed tumor tissue and gently pipetted several times until the tissue was resolved in triazole. Then, 200 ml of chloroform (per mL of trizole) was added to the uniform solution containing cells and triazole, and the microtube was vortexed for 15 seconds. The microtube was placed at room temperature for 5 minutes and centrifuged at 4 °C for 15 minutes (12000 rpm). After centrifugation, three major phases were formed. The upper phase contained RNA and the lower phases consisted of DNA, protein, cell components, and trizole. The upper phase was gently separated and transferred to RNase-free microtubes. The same volume of isopropanol was added to the solution from the previous step. The obtained mixture was placed at a temperature of -20 °C for 30 min. The microtube was centrifuged at 1200 g for 15 min at 4 °C. The upper phase was separated and one ml of 75% ethanol was added to the sediment inside the tube. The supernatant was removed and the microtube was placed at room temperature so that the ethanol present in the sediment would evaporate. Then, the concentration and purity of RNA were measured by a spectrophotometer.

Quality and quantity assessment of the extracted RNA: After RNA extraction, its quantity and quality were evaluated using ultraviolet-spectrophotometry (absorbance at 260 and 280 nm) and agarose gel electrophoresis (for detecting the presence of 18S and 28S ribosomal RNA bands). Identification of RNA concentration via spectrophotometry is a quantitative method; therefore, the concentration and purity of RNA sample can be evaluated using light absorbance at wavelengths of 260 and 280 nm. Overall, OD260/OD280 ratio indicates the purity of the extracted RNA. If this ratio is lower than 1:8, there is a possibility of contamination with protein and aromatic compounds such as phenol. For quality evaluation of RNA, 1 g of the sample was analyzed using horizontal electrophoresis on 1% agarose gel. Also, 18S and 28S rRNA bands, positioned at 800 and 1500 bp DNA bands, were considered as quality indicators of the extracted RNA.

cDNA synthesis from the extracted RNAs: cDNA synthesis was performed by a reverse transcriptase. For cDNA synthesis, PrimeScript ™ RT reagent kit (Takara Bio, Japan) was used. All the procedures were performed using RNase-free solutions. Also, for cDNA synthesis with the same concentration, for each sample, a fragment, containing 1000 ng RNA, was taken, considering the concentration of RNA; by adding water and Master Mix, the final volume was increased to 10 µl (table 1). Primer design In order to amplify each gene

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fragment, a pair of exclusive primers was selected for each gene, including forward and reverse primers. The primers were designed using Oligo, Allele ID, and PerlPrimer software (table 2).

Real-time PCR reaction: Real-time PCR refers to the continuous collection of fluorescent signals during polymerase chain reactions. Quantitative real-time PCR refers to the conversion of these fluorescent signals to numerical values, which are applied for each sample. The method in this study included using SYBR Green I fluorescent dye. Real-time PCR reactions were operated in the Applied Biosystems 7500 device. SYBR® Premix Ex Taq TM II (Perfect Real time, Takara Co., Japan) was used to study gene expression (table 3).

Statistical analysis: For data analysis, one-way analysis of variance (ANOVA) and Student's t-test were performed, using SPSS software. $p<0.05$ was considered statistically significant

Table 1. Preparation of Master Mix for a cDNA synthesis reaction

Reagent	Amount	Final concentration
5X PrimeScript™ Buffer	2 lμ	1X
PrimeScript™ RT Enzyme Mix I	0.5 μl	-
Oligo dT Primer (50μM)	0.5 μl	25 pmol
Random 6 mers (100μM)	0.5 μl	50 pmol
total RNA	500 ng	-
RNase-free dH2O	Variable	-
Total	10 μl	-

Table 2. The sequence and length of the fragment produced by real-time PCR primers

Gene name	Sequence (3-5)	Length of PCR product
MMP9	F 5'TTCCAGTACCAAGACAAAGCC3' R 5'CACGGTTGAAGCAAAGAAGG3'	176
VEGFA	F 5'AGGCTGCTGTAACGATGAAG3' R 5'GTGCTGGCTTTGGTGAGG3'	197
GAPDH	F5'CCCACTCCTCCACCTTTGAC3' R5'CATACCAGGAAATGAGCTTGACAA3'	139

Table 3. Real-time PCR method

PCR steps	Temperature	Duration	Number of cycles
Pre-denaturation	95	15 s	1
Denaturation	95	5 s	403
Annealing	63		
Extension	72	34 s	

Results

NapA induction and expression in E. coli BL21 strain: To evaluate the protein after induction, E.coli cells were lysated and the soluble proteins were studied. The protein was separated from cell solution on polyacrylamide gel in the presence of detergent SDS and was studied after being stained with Colloidal Coomassie G-250 dye. Since napA protein was synthesized, a protein with a molecular weight of 20 kDa was produced (fig 1).

Expression and purification of recombinant HP-NAP: In this study, the expression of recombinant HP-NAP was confirmed by SDS-PAGE. The results of SDS-PAGE analysis indicated the expression of a protein with a molecular weight of 20 kDa, which was consistent with the predicted molecular weight of about 20,477 daltons (fig1). Protein expression started after three hours and reached the maximum value after 5 hours. No significant difference was observed between

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the fifth hour, seventh hour, and overnight induction. The results showed that recombinant proteins were mostly in form of insoluble inclusion bodies in the sediment phase. The sediment phase of this protein was confirmed by purifying the liquid phase of cell lysates (via native method), using urea-free buffer. The electrophoretic evaluation of the recombinant protein revealed the notable purity of this protein (fig 1). According to measurements by Bradford method (with BSA standard) and spectrophotometry, protein sediment in one liter of bacterial culture was approximately 400 mg. The integrity of the extracted RNA was evaluated by the electrophoresis of RNA samples on agarose gel (fig 2).

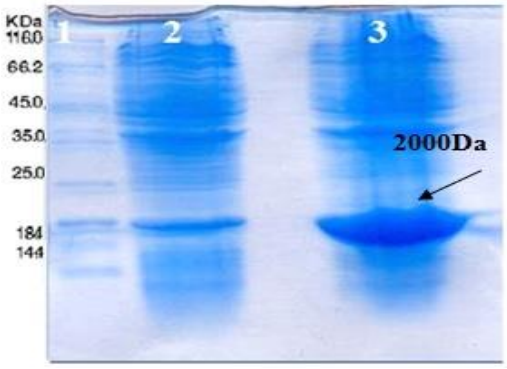


Figure 1. Evaluation of napA protein expression on SDS-PAGE gel (12%); well 1: the marker with low weight, well 2: non-induced napA, well 3: napA induced by mM 0.1 IPTG

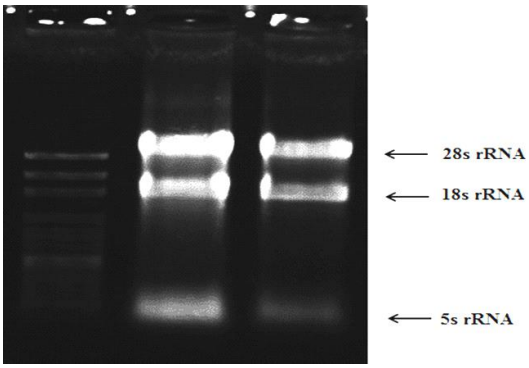


Figure 2. Electrophoresis of the extracted RNA samples on 1% agarose gel

Evaluation of MMP-9 and VEGF gene expression using real-time PCR: The results of quantitative real-time PCR showed that the expression of MMP-9 in mice treated with recombinant HP-NAP decreased more significantly than the negative control group (p<0.001). MMP-9 gene expression in the positive control group also significantly reduced (p<0.001). The mRNA expression of VEGF gene significantly reduced in the test group and the positive control group, compared to the negative control group (p <0.001) (fig 3).

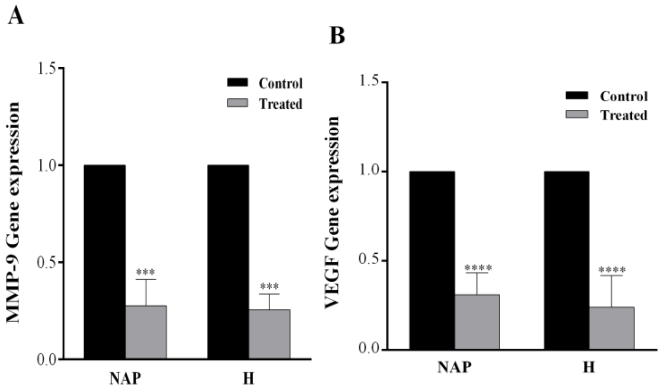


Figure 3. Reduced expression of mRNA in MMP-9 (A) and VEGF (B) after treatment with recombinant HP-NAP and herceptin

*** Compared to the negative control group (p<0.001)

**** Compared to the negative control group (p<0.00)

Discussion

HP-NAP has immunomodulatory properties and is able to induce the expression of cytokines, which are activators of human monocytes and neutrophils (11). Since this protein has the potential to change the response of the immune system, it can change the phenotype from Th2 to Th1 by producing interferon gamma and tumor alpha necrosis factor. In fact, HP-NAP can be exploited for directing the immune system, particularly dendritic cells, towards a Th1 response and enhance the stimulation of these cells. Haghighi et al. used the bioinformatic method for cloning optimization of a specific gene in E. coli. The results showed that the cloned gene (with the engineered primers) and finally the produced protein was about 20 kDa (12).

VEGF is an important factor for tumor angiogenesis (13). In the present study, the expression of MMP-9 and VEGF genes in the group treated with recombinant HP-NAP showed a significant decrease, compared to the negative control group. Also, this recombinant protein had effects similar to trastuzumab (herceptin) in inhibiting the expression of MMP-9 and VEGF genes. Tumor cells, compared to normal cells, are more exposed to oxidative stress. Studies have shown that VEGF expression is highly dependent on intracellular oxidation/reduction potential (14). Mousavi et al. studied the effects of saffron extract on VEGF-A gene

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expression in cell lines in an in vitro study. The results showed a significant reduction in the expression of VEGF-A gene due to treatment with 100, 200, 400 and 800 µg/ml of saffron extract, unlike the control group; in fact, the highest reduction in gene expression was associated with the highest concentration of saffron extract (15).

Moreover, De Ferancesco and colleagues by studying the signaling pathway of HIF-1α/GPER examined hypoxia-induced tumor angiogenesis in association with VEGF gene (16). Additionally, Tsuboi et al. evaluated the effect of a part of ginger plant on the expression of VEGF gene. The results showed that this plant causes a reduction in VEGF gene expression in gastric cancer AGS cells (17). Moreover, Boonrao et al. studied the effects of a curcumin derivative on breast cancer cells. The evaluation of the expression of MMP-3 gene matrix showed that this derivative can reduce the expression of the mentioned gene and cell migration (10). However, before the present study, no research had been conducted regarding the effects of recombinant HP-NAP protein on the expression of VEGF and MMP-9 genes in an animal model for cancer treatment.This research was conducted for the first time and suggested that HP-NAP can be effective for the prevention of metastasis and angiogenesis in metastatic breast cancer cells in a mouse model. According to the results, this protein, given its potential for reducing the expression of MMP-9 and VEGF genes (which are specific biomarkers of angiogenesis), can be effective for eliminating cancer cells if used along with anti-cancer drugs. Considering the mentioned properties, this recombinant protein can be a convenient and effective tool for immunotherapeutic applications in cancer treatment. This study can pave the way for cancer treatment considering the anti-angiogenetic aimmunomodulatory effects of HP-NAP.

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