The Effect of Recombinant HopH Protein of *Helicobacter pylori* on the VEGF Expression in Metastatic Breast Cancer Model

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Abstract- Breast cancer imposes a considerable amount of cancer-related mortality and morbidity among women worldwide. Many efforts are in progress to reduce the disease burden and amongst the bacterial-based products received considerable attention as potential anti-cancer drugs. In the present study, the effect of recombinant pro-inflammatory outer membrane protein (HopH) of *Helicobacter pylori* on the angiogenic factor and tumor development in metastatic breast cancer model was evaluated. The HopH gene was cloned into Pet28a vector, induced by IPTG and expressed and purified by Ni-NTA affinity chromatography. The expressed protein was confirmed by SDS-page. The breast cancer tumor induction was performed using Breast cancer cell line (4T1). The mice were divided into different groups and underwent treatment by recombinant HopH and Herceptin, subsequently. The treatment effectiveness on tumor size was followed, and the expression level of vascular endothelial growth factor was evaluation by real time PCR. The SDS-PAGE analysis confirmed the expression of HopH protein with an approximate 34KD weight. Based on our results, the expression level of VEGF was significantly reduced in HopH-treated mice group comparing to the control and Herceptin group. Our results have shown that the recombinant HopH protein can effectively reduce VEGF expression in breast cancer tumor which was associated with reduction of tumor size. The HopH protein can be considered as a potential anti-cancer agent for future cancer therapeutic studies.

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Introduction

Breast cancer is the most frequent malignancy among women worldwide; it has constituted the 18.9% of cancer incidence which has been reported in females. As the most cause of cancer-related mortality affecting the woman, the improvement in diagnosis and therapeutic strategies were not favorable enough to efficiently cure and detect the disease especially at the metastatic stage (1). One of the essential parameters for determining patient's survival is tumor progression which can efficiently affect treatment strategies. One of the critical steps for cancer cell growth and proliferation is angiogenesis which facilitates the transformation of in situ proliferated cells to invasive carcinoma. The process of angiogenesis is mediated by several factors and molecular pathways however vascular endothelial growth factor (VEGF) has been acknowledged as one of the key mediators of angiogenesis. The VEGF gene is located with the cytogenetic location 6p21 in the human genome which is organized in 8 exons. The first Exon is responsible for signaling of secretory section of protein, and the coding region of the protein is coded by the rest of exons. The VEGF protein is secreted by many cells regulating cell physiological functions and development. In addition, overexpression and function of VEGF help cancer cells as a signal protein to stimulate vasculogenesis and angiogenesis. It is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate (2). VEGF's normal function is to accelerate new blood vessels formation during embryonic development, wound healing and hematopoiesis. Several in vitro and in-vivo studies demonstrated that over-expression of VEGF enable cancer cells to growth and spread (3). Therapeutic strategies based on reducing those proteins that are

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involved in cancer progression and invasion are considered as promising molecular targets to prevent cancer progression. In accordance, bacterial-based products like-wise bacterial proteins and toxins received considerable attention for regulating cancer cell growth. Outer inflammatory protein A of *Helicobacter pylori* (HopH) is one of the bacterial outer membrane proteins that is involved in inflammatory processes (4). *Helicobacter pylori* is a gastric pathogen inducing mucosal inflammation leading to chronic gastric associated disease. This protein is one of the virulence factors of *Helicobacter pylori* with high antigenic feature which results in elevation of serum IL-8 (5,6). It has been shown that HopH is involved in the pathogenesis of *Helicobacter pylori* and the adverse effect of this bacteria on human health (9). Based on recent evidence, HopH is involved in gastric carcinogenesis through re-organizing cell cytoskeleton mediating focal adhesion kinase (FAK) and ERK pathway (10) Accordingly, manipulation of such proteins will open up new insights into cancer therapeutic strategies. To aim this, the current study is designed to evaluate the effusiveness of microbial products in controlling cancer pathogenesis. More specify, the expression pattern of VEGF following treatment by HopH (*Helicobacter* recombinant protein) in metastatic breast cancer model was surveyed in this study.

Materials and Methods

Cloning and transformation

The HopH gene was amplified using PCR and the PCR product as well as the Pet28a vector were subjected to digestion by two enzymes *BamHI* and *XhoI* (Fermentas Co., Lithuania. Interpolation was performed at 4° C using the enzyme T4 DNA Ligase (Fermentas Co., Lithuania) and was transformed into cloning host DH5α cells.

Induction and expression of recombinant proteins HopH

The *E. coli* BL21 stain was applied as a host for expressing recombinant protein HopH. The transformed bacteria were grown and screened on solid LB medium containing the antibiotics kanamycin (Mast UK companies), and the isolated colonies were cultured in 3 ml of liquid LB medium containing 50 mg ml kanamycin and incubated in rotary shaker incubator with rotation (200 rpm) at 37° C overnight and have been used for subculture in 15 ml of LB liquid medium with the mentioned condition. When the turbidity of the culture at 600 wavelengths (nm) reached to 0.6-1 mM, the recombinant plasmids were induced with 1 µM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fermentas Co., Lithuania). The measurement of Protein expression levels was performed at different times intervals using 12.5 % polyacrylamide gel by SDS-PAGE at the voltages of 100 V, in the presence of a molecular weight marker (Fermentas, and Ladder 200 kDa. Eventually, the polyacrylamide gel was stained with Kumasy G-250 color (Fermentas Co., Lithuania) and the resulting protein bands were evaluated (6,8,11). The Nickel reluctant resin (Ni-NTA affinity chromatography) (BioVision CO, California) was used for protein purification due to the embedded His-tag in the vector construct (Following collection of protein fractions, the SDS-PAGE electrophoresis was applied to confirmed the protein identity. The dialysis buffer PBS (Merk Co., UK) was used in order to remove the imidazole from the protein solution. The final concentrations of protein were determined by Bradford method (12,13).

Cell line and culture condition

The metastatic breast cancer cell line (4T1) was purchased from the national cell bank of Pasteur Institute, Tehran, Iran. The cells were cultured in Gibco® high glucose Dulbecco's Modified Eagle Medium (Life Technologies, Carlsbad, CA, USA), respectively. The medium was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies). All cells were grown at 37° C in a humidified atmosphere of 5% carbon dioxide.

Animals model

Animal studies have been conducted according to relevant national and international guidelines of the Weatherall report, and Institutional Animal Care and Use Committee (IACUC) Pasteur Institute, Tehran, Iran. Animals were housed in pens exceeding the stipulated size requirements. All inbred female BALB/c mice (6-8 weeks old with 19-23 gr weight, purchased from Iran Pasteur Institute) were maintained in large group houses under 12-hour dark and light cycles and were given access to food and water ad libitum. Female BALB/c mice were divided into 3 groups including the control group, HopH treated group and, Trastuzumab (Herceptin®) treated group which contained 6 mice in each group.

Tumorgenicity

4T1 cells were trypsinized and resuspended in 10-fold excess culture medium. After centrifugation, cells were re-suspended in PBS, and 1×10^6 cells were used for injection (0.1 ml, s.c) using a 21-gauge needle in the right flank of BALB/c mice under Ketamine and Xylazine (10 mg/kg, i.p) anesthesia. As mentioned above, 3 different groups namely rHopH, Trastuzumab, PBS Were determined. The tumors were observed two weeks following injection. On the fifteenth day of injection (tumor visualization) mice in treated groups were received intraperitoneally injection 50 µgr/ml of recombinant protein during a two-weeks with specified time intervals. Trastuzumab (the anticancer drug) was used in same conditions as a control group.

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was extracted from tumor tissues with Trizol (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's instructions. The quantity and quality of the isolated RNA were determined by Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, Delaware, USA) and agarose gel electrophoresis (1% agarose; Gibco/BRL), respectively. The amount of the 260/280 OD ratio of all samples was between 1.8 and 2.2, indicating their high purity. cDNA was synthesized using a RevertAid Reverse transcriptase cDNA Synthesis Kit (Fermentas, Germany) according to the manufacturer's protocol and stored at -20° C until use. Primers were designed onto two adjacent exons with a very long intron in the middle by AlleleID 6.0 (http://www.premierbiosoft.com). All of the selected primers sequences were further analyzed with the Oligo software (http://www.cambio.co.uk/index.php). GAPDH was used as internal housekeeping control gene to normalize the mRNA expression levels. Real-time PCR was performed with a light cycler instrument (Applied Biosystems 7500, USA) using 5x HOT FIREPol® EvaGreen® HRM Mix (ROX) (Solis BioDyne Inc.). In a total volume of 10 µl, 2 µl eva Green master mix, 1 µl of cDNA samples, 0.5 µl of forward and reverse primers (10 pmol), and 7.5 µl of nuclease-free water (Qiagen, Hilden, Germany) were added into each capillary tube. The PCR condition included an initial denaturation of 1 min at 95˚C followed by 40 cycles at 95˚C for 15 s, at 62˚c for 30 s and at 72˚C for 30 s. The specificity of the PCR products was assessed by verifying a single peak in melting curve analysis. For complementary length verification, PCR products were visualized on 1.5% agarose gel stained with ethidium bromide. No template controls were included in each run. Primer efficiencies were calculated by LinregePCR 12.17.1.0 and fold change in relative expression of each target mRNA calculated on the basis of comparative Ct (2-ΔΔCt) method (7). The sequences of primers are listed in (Table 1).

Table 1. List of the primers for the VEGF gene and for the housekeeping gene, GAPDH, which were used in real-time PCR

Gene	Designed oligonuclotide	Amplicon length
VEGF	F 5′ AGGCTGCTGTAACGATGAAG3′ R 5′ GTGCTGGCTTTGGTGAGG3′	197
GAPDH	F 5′ CCCACTCCTCCACCTTTGAC3′ R 5′ CATACCAGGAAATGAGCTTGACAA3′	270

Abbreviations: F, forward primer, R, reverse primer, GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VEGF, vascular endothelial growth factor

Histopathology examination

Hematoxylin and Eosin (H and E) was done on test and control group tumor tissue.

Results

Expression and purification of HopH recombinant protein in the host E. coli BL21

The result of SDS-PAGE was shown that the HopH recombinant protein was expressed by the 34kDa weight as is indicated by Marker band area. The HopH protein expression was started to appear following three hours, and it was reached to the maximum amount of its expression after four hours. No significant differences were observed while time was passed especially after fifth hours, seventh hours, and nightly induction. The

surveys were revealed that the highest amount of protein was produced in the fourth hour as a form of water-insoluble solid particles (inclusion bodies) in sedimentary phase. According to measurements by the Bradford method (with Standard BSA) and spectrophotometry, the extraction of sedimentary protein was measured approximately 270 micrograms from 1 milliliter of bacteria culture (Figure 1).

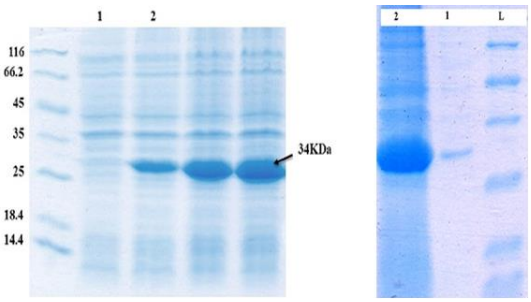

Figure 1. Evaluation of protein expression HopH in 12% SDS-PAGE gel: well L: Weight Markers with Low-range (14.4±97.4 kDa), well 1: Non-Induced HopH, well 2: induced HopH by 0.1mM IPTG in the fourth hour. B. purified protein HopH. well L: Weight Markers with Low-range (14.4±97.4 kDa), well 1: purified protein HopH, well 2: induced HopH

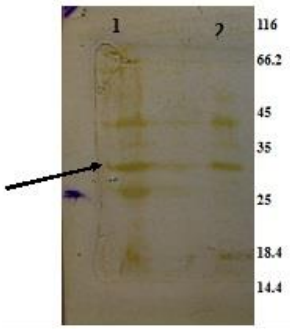

Figure 2. Western blot analysis

Analysis of gene expression by real-time PCR

In order to evaluate the effectiveness of recombinant HopH protein in the outcome and tumor development of breast cancer, inbred female BALB/c mice were injected by 4T1 cells and following tumor visualization, the mice were divided into 3 groups that were treated by indicated amount of HopH recombinant protein, Herceptin and PBS for further analysis. After adequate time interval, the related breast tumors were removed, and the RNA was extracted as mentioned above and subjected to evaluate the expression level of VEGF using Real-Time PCR. Our results have shown that the expression level of VEGF gene had significant reduction in treated groups compared to the control groups (Figure 3).

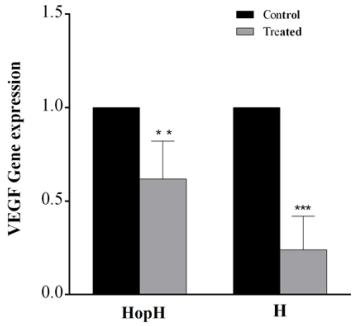

Figure 3. The Effect of HopH recombinant protein and Herceptin (Trastuzumab) on suppressing VEGF gene expression

Notes: Data expressed as mean ± standard deviation; **P*<0.05; ****P*<0.001 compared to control

Histopathology examination

Hematoxylin and Eosin (H and E) was shown on test and control group tumor tissue that the HopH protein induced (cell death) apoptosis (Figure 4).

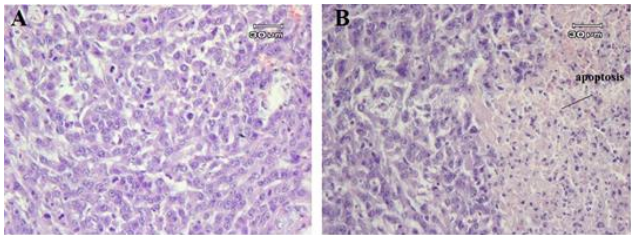

Figure 4. Hematoxylin and Eosin exanimation.A. normal tissue. B. apoptosis tissue

Discussion

Breast cancer imposes a considerable amount of cancer-related mortality and morbidity among women worldwide. Several therapeutic strategies are applied to reduce the disease burden however amongst, surgery still stands as the main line of breast cancer treatment. The common neoplasm treatment is based on reducing tumor size however the survival rate and the risk of recurrence are not attenuated following the existing treatments (14). Moreover, Current therapies are based on the hypothesis that tumor is composed of homogeneous population thereby, the rapid proliferation of cells are mainly targeted (15,16). However, it has been recently revealed that tumor is composed of morphologically heterogeneous population of cells which may affect the tumor responsiveness against treatments (15,17). In fact, the cancerous tissue is confined subpopulations which contains cells with the special properties of tumor metastasis, recurrence and resistant to common treatments (18,19). In accordance with this diversity, finding a general therapeutic strategy for definite cure is far to be achieved. In the study carried out by Teymournejad *et al*., at 2012, optimization of cloned genes in *E. coli* was assessed using bioinformatics techniques. Based on their results, the cloned gene was obtained by engineered primers (20). In the current study, outer inflammatory recombinant protein has been built from *Helicobacter pylori*. The highest expression level of this protein was observed in the presence of IPTG at the fourth hour after induction. The expression of the recombinant protein was confirmed by SDS-PAGE, and the results of western blot test clearly showed the presence of recombinant protein due to the specificity of related antibodies. These results further confirmed the correct production of bacterial recombinant protein with the approximate size of 34 kDa. It has been exhibited that HopH inhibits dendritic cells and induce down-regulation of CD40 and CD86 expression level (21). However, to the best of our knowledge, the impact of this protein on cancer progression and pathogenesis has not been elucidated yet. In an investigation by Soleimani *et al*., the trimethyl chitosan nanoparticles carrying recombinant protein Hp-NapA were used as a candidate for the treatment of metastatic breast cancer tumor model (22). However, information on the possible effect of HopH protein on the Breast cancer development was lacking. Recent evidence has emerged in favor of proposing HopH as a candidate for cancer treatment. Vascular endothelial growth factor (VEGF) is a well-described mediator of tumor angiogenesis. Tumor cells are exposed to the higher oxidative stress compared to normal cells. Multiple lines of evidence have shown that the high potential of VEGF expression is dependent on the intracellular oxidation-reduction (ORP) status (23,24). Our data demonstrated that the outer membrane of *Helicobacter pylori* recombinant protein reduced the expression of VEGF gene in an animal model of breast cancer in comparison with the control group. Moreover, the reduction of VEGF expression level was observed following Herceptin treatment in breast cancer mice model. As it has been indicated the expression level of VEGF was reduced more in rHopH-treated group comparing to Herceptin-treated group. Based on this data, it is suggested that rHopH can be more effective to attenuate antigenic factors comparing to control group. The same effect was detected in tumor morphology of mice since the tumor size was reduced in rHopH-treated mice group comparing to the control group (25). Eventually, it has been proven that the large set of outer membrane proteins of *Helicobacter pylori* facilitates bacterial colonization, infection, and persistence (26). Additionally, HopH is suggested to be involved in H. *pylori* adherence to gastric cells, and it is proposed that HopH is involved in the gastric cell cytoskeleton reorganization induced by *H. pylori* infection (27).

Interestingly, oral HopH-DNA vaccines exhibit efficient protection against bacterial infection which emphasis on the importance of this protein in bacterial function (28). In the study of Francesco *et al*., the relevance of angiogenesis and hypoxia-driven angiogenesis which was associated with VEGF gene was evaluated by studying on HIF-1α/GPER signaling pathway (29). In regard to cancer preventive compounds, Tsuboi *et al*., have shown that ginger effective part can reduce VEGF expression level in gastric cancer cell (AGS) (30). Taken together, the cytotoxic effect of HopH has been proposed and our results have revealed that HopH recombinant protein can reduced tumor size and attenuate VEGF expression level. Therefore, it can be considered as potential anti-cancer drug Due to the aggressive nature of cancer, as well as the complex mechanisms involved in the progression of cancer, traditional approaches like surgery, chemotherapy, and radiation are inefficient in many cases. Increasing amount of side effects besides the low specificity and probability of recurrence are considered as main limitations of these methods. Therefore, many efforts are devoted to substitute them with more effective and specific with lower side effect strategies.

In the line of this fact, our results have unraveled the possible preventive effect of recombinant HopH protein in breast cancer pathogenesis and usage of this protein as a candidate for Pharmaceutical Supplements in breast cancer therapy might lead to promising results. However, more comprehensive studies are required in this regards.

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