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Impact of *Helicobacter Pylori*-Derived Outer Membrane Vesicles on Inflammation, Immune Responses, and Tumor Cell Migration in Breast Cancer Through the Snail/B-Catenin Pathway

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Abstract

Background: Breast cancer remains a significant global health concern, with challenges in treating advanced stages necessitating the exploration of novel therapeutic approaches. Bacterial outer membrane vesicles (OMVs) have shown promise in cancer immunotherapy by targeting cancer cells and modulating immune responses. This study investigated the effects of Helicobacter pylori-derived OMVs on the activation of the Snail/β-Catenin gene cascade in regulating inflammation and cell migration in a mouse model of breast cancer.

Methods: The OMVs were extracted from the culture of *H. pylori* strain 26695 (ATCC 700392) using ultracentrifugation. In the mouse model, the vesicles were injected intraperitoneally into Balb/c mice with breast tumors. Tumor growth was assessed through histological examination of tumor samples. IgA and IgG antibodies were measured using ELISA. The expression of E-cadherin and vimentin proteins was evaluated by immunohistochemistry, and real-time PCR was used for vimentin, Snail, α-SMA, and β-catenin in serum samples from the different groups.

Results: The OMV treatment led to a significant increase in the expression of α-SMA, β-catenin, Snail, and vimentin genes, indicating a potential induction of epithelial-mesenchymal transition and enhanced cancer cell growth. Additionally, a decrease in vimentin expression and an increase in E-cadherin expression were observed, suggesting inhibition of cell migration. The study also revealed alterations in systemic IgA and IgG antibody levels, indicating potential immunomodulatory effects of OMVs.

Conclusions: These findings highlight the therapeutic potential of OMVs derived from *H. pylori* in breast cancer treatment by targeting gene cascades involved in cancer progression and modulating immune responses.

Keywords: Breast cancer, Helicobacter pylori, Inflammation, Membrane vesicles, Neoplasm Metastasis.

Introduction

Breast cancer is a leading cause of cancer-related deaths globally, with over 2.3 million new cases reported each year (1, 2). It is the most common type of cancer. While rare in men, the impact of breast cancer on male patients, should not be underestimated (3). Certain subtypes of breast cancer are aggressive and have a high risk of spreading, emphasizing the urgent need for improved treatment options in the clinic (4, 5). Research is ongoing to better understand and address the far-reaching effects of breast cancer, with

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a focus on developing more effective control strategies (6, 7).

The potential of bacterial outer membrane vesicles (OMVs) as a therapeutic intervention for cancer treatment is promising, as they are potent stimulators of the immune system (8, 9). The OMVs are small structures released by bacteria during the their rapid growth phase (10). They contain a variety of components, including enzymes, phospholipids, nucleic acids, toxins, and proteins from different parts of the cell (10). Recently, the use of OMVs derived from *Helicobacter pylori* has shown great potential as a new and effective approach for developing vaccines and immunotherapies (11). However, further studies are needed to understand the mechanisms by which bacterial vesicles activate immune system cascades in breast tumor cells, such as the Snail/β-Catenin pathway, which plays a crucial role in cancer progression (12).

Epithelial-mesenchymal transition (EMT), a prerequisite for metastasis, involves the downregulation of epithelial markers like E-cadherin and the upregulation of mesenchymal markers such as vimentin, Twist, and Snail (13). The transcription factor Snail, which inhibits E-cadherin expression, plays a significant role in promoting EMT and tumor invasion (14). The Wnt pathway can be activated in response to DNA damage, leading to genomic instability and the transformation of non-tumorous stem cells into tumorigenic cells, such as glioblastoma cells. Activation of Snail can stimulate the Wnt/β-Catenin pathway, which, in turn, activates Snail through positive feedback (15).

By elucidating these mechanisms, we may uncover new strategies for enhancing anticancer immune responses and improving outcomes for cancer patients. Therefore, building on past studies that highlight the role of OMVs in cancer control and the importance of breast cancer research, this study aims to investigate the effect of OMVs derived from *H. pylori* on the Snail/β-Catenin cascade pathway in a mouse model of breast cancer.

Materials and Methods

OMVs isolation

The isolation and confirmation of *H. pylori*-derived OMVs were described in our previous study. Briefly, the *H. pylori* strain 26695 (ATCC 700392) was cultured in Brucella broth (Qlab, Canada) supplemented with fetal bovine serum (FBS) (GIBCO, USA) and incubated for 72 hours until reaching the mid-logarithmic phase. The culture supernatant was harvested when the optical density (OD) at 600 nm ranged from 1.0 to 1.4. OMVs were subsequently isolated through centrifugation and filtration processes. The extracted OMVs were stored at -80 °C. It is important to note that ultra-centrifugation should be performed without using the brake to avoid disrupting the vesicles.

Animal groups

In this study, six male BALB/c mice, approximately 6 to 8 weeks old, were purchased from the Pasteur Institute of Iran. The mice had unrestricted access to food and water and were housed in a light-controlled environment with a 12-hour light-dark cycle, a temperature maintained at 22 ± 3 °C, and humidity levels of 50 ± 10% in the animal facility of the Faculty of Basic Sciences at Islamic Azad University, Shahrekord branch. All experiments were conducted one week after the mice had acclimatized to their new environment.

The experimental procedures adhered to the guidelines for the care and use of laboratory animals, and the ethical principles approved by the Faculty of Basic Sciences at Islamic Azad University, Shahrekord branch, were strictly followed. Efforts were made to minimize animal sacrifice, surgical interventions, stress, and suffering, and the minimum number of mice necessary for the experiments was utilized. The mice were randomly divided into two groups: a control group and a group receiving the selected dose of extracellular vesicles. Each group consisted of three mice.

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Breast cancer model

BALB/c mice were divided into two groups and anesthetized with an intraperitoneal injection of ketamine-xylazine (Alfasan, Netherlands) (3:5 ratio). To establish a breast cancer model, 40,000 tumor cells in 100 microliters were injected subcutaneously into the backs of the mice. Additionally, the second group received intraperitoneal injections of 100 microliters of extracellular vesicles extracted from *H. pylori* at a selected dose three times a week for two weeks. Tumor-bearing mice in the treatment group were administered extracted OMVs at a concentration of 1 µg/ml directly into the tumors. To evaluate the effectiveness of the treatment, blood samples were collected from the mice's hearts, and serum was separated. The mice were then euthanized using CO2. Tumor growth progression and tumor histology were compared between the treatment and control groups.

H&E Staining

Mice were anesthetized with a combination of ketamine (50 mg/kg) and xylazine (5 mg/kg) 24 hours after the final injection dose. Tumor tissue was carefully excised and immediately fixed in 10% formalin. The fixed tissues were dehydrated by gradually placing them in increasing concentrations of alcohol (50%, 70%, 90%, and absolute alcohol). This step replaces water in the tissue with alcohol, which enhances the penetration of subsequent embedding materials, such as paraffin. After dehydration, the tissues were clarified using xylene and then impregnated with melted paraffin. The samples were embedded in molds filled with molten paraffin and allowed to solidify. Once solidified, the paraffin-embedded samples were sectioned into thin slices (5 to 10 microns) using a microtome. The sections were mounted on albumin-coated slides, and further clarification and dehydration steps were performed. For routine histological examination, the sections were stained with hematoxylin and eosin (H&E), to study any morphological changes under an optical microscope (Olympus, Japan).

RNA extraction and cDNA synthesis

Total RNA was isolated from tumor tissues of the mouse model of breast cancer treated with a dose of 1 µg/ml OMVs using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA). The RNA concentration and quality were assessed using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies). Complementary DNA (cDNA) was synthesized from the isolated RNA using the ThermoScript RT kit (Life Technologies, Rockville, MD), following the manufacturer's guidelines.

Gene expression in breast cancer model mice treated with OMVs

To evaluate differences in gene expression of the Snail/β-Catenin cascade genes (Snail, β-Catenin, α-SMA, and Vimentin), quantitative real-time PCR (qRT-PCR) was performed using the Takara SYBR Green I kit (Takara, Dalian, China) on the Roche LightCycler 2.0 System. The qRT-PCR reaction was conducted with 200 ng of total RNA in a 20 µL reaction volume. The amplification protocol included an initial incubation at 95 °C for 5 minutes, followed by 40 cycles of amplification with denaturation at 95 °C for 15 seconds, annealing at 60 °C for 20 seconds, and extension at 72 °C for 30 seconds. A final extension was performed at 30 °C for 20 minutes. The reaction mixture consisted of 1 µL of cDNA, 10 µL of SYBR Master Mix (Addbio, Korea), 7 µL of water, and 1 µL of each primer. Primer sequences used in the experiment are detailed in Table 1. Data analysis involved normalization to the GAPDH housekeeping gene and calculation of ΔCT values. Fold changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method, with ΔCT values from the control group serving as the reference. Each reaction was performed in triplicate, and control samples were included.

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Table 1. Primers used in the experiment.

Gene	Sequence (5'->3')	Length (bp)	Tm (°C)
snail (NM_005985)	F-CCTGTCTGCGTGGGTTTTTG	22	60.02
	R-ACCTGGGGGTGGATTATTGC	20	60.25
β-catenin ***(***NM_001904)	F-AAGGTGATTTGATGGAGTTGGA	21	58.92
	R-AGAGAAGGAGGTGTGGTAGTG	21	58.93
α-SMA ***(***NM_001613)	F-AGGGAAGGTCCTAACAGCCC	22	59.11
	R-AGGATTCCCGTCTTAGTCCC	20	58.69
vimentin ***(***NM_003380)	F-TCCGCACATTCGAGCAAAGA	22	58.93
	R-TGATTCAAGTCTCAGCGGGC	21	60.55
gapdh ***(***NM_002046)	F-CTTTGGTATCGTGGAAGGAC	20	58.96
	R-GCAGGGATGATGTTCTGG	21	59.25

Immunohistochemistry analysis (ICC)

E-cadherin and vimentin proteins were measured by immunohistochemistry in tumor tissues from breast cancer model mice treated with a dose of 1 μg/ml of OMVs derived from *H. pylori*. For this procedure, the slides were first boiled in 1X TBS (tris-buffered saline) solution and then washed three times with PBS (phosphate-buffered saline) (Sigma–P4417), with each wash lasting 5 minutes. Cell membranes were permeabilized using Triton X-100 (Sigma-T8787), followed by another wash with PBS. To block nonspecific binding, the slides were incubated with 10% goat serum. The primary antibody was applied at a **dilution of 1:100 in PBS, and the slides were incubated at** 4 °C for 24 hours. After multiple washes with PBS, the secondary antibody was applied at a dilution of 1:150 and incubated in the dark at 37 °C for 90 minutes. The slides were then washed again, stained with DAPI (4',6-diamidino-2-phenylindole) (D9542 - Sigma), and mounted with a glycerol/PBS solution. Fluorescent imaging was used to visualize and confirm the presence of E-cadherin and vimentin markers.

Humoral immune response assessment

Humoral immune responses were evaluated using an optimized enzyme-linked immunosorbent assay (ELISA) with serum samples, as previously described (16). Briefly, 96-well microplates were coated with *H. pylori* antigen and incubated at 4 °C overnight. Following this, the wells were washed with PBST (PBS containing 0.05% Tween 20) and blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich, USA) in PBS for 2 hours. After blocking, diluted serum samples (ranging from 1:100 to 1:51,200) were added in duplicate to the wells and incubated for 2 hours. The plates were then washed with PBST, and HRP-conjugated goat anti-mouse IgG and anti-mouse IgA antibodies (Sigma-Aldrich, USA) were applied as secondary antibodies and incubated for 2 hours. Following another wash with PBST, 3,3′,5,5′-Tetramethylbenzidine (TMB) (Thermo Fisher Scientific, USA) substrate was added to the wells. The reaction was stopped with a stop solution, and the optical density was measured at 450 nm using an ELISA plate reader (Epoch2, BioTek Instruments, USA).

Statistical analysis

Mucosal samples were collected by sacrificing six mice from each group two weeks after the final vaccination (on day 45). The lungs were excised, homogenized, and then centrifuged. The supernatants obtained were collected and stored at -20 °C for

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subsequent analysis. Statistical analysis was performed using SPSS software version 16.0 and GraphPad Prism 8.0.2. The mean values and standard deviations were reported. For comparisons between two normally distributed groups, Tukey's HSD post-hoc test was used. Differences between multiple groups were assessed using one-way ANOVA, with statistical significance set at $P<0.05$ (*$P<0.05$, **$P< 0.01$, ***$P< 0.001$, ****$P< 0.0001$).

Results

OMVs isolation

Cell-free OMVs from the *H. pylori* strain 26695 were purified using ultracentrifugation (Beckman, Germany). The phenotypic characteristics of the OMVs were analyzed by dynamic light scattering (DLS) (Horiba, SZ100, Japan) and scanning electron microscopy (SEM) (TESCAN, MIRA III, Czech Republic). These analyses revealed that the OMVs were spherical with a bilayer membrane, with diameters ranging from 50 to 450 nm. The purification process effectively removed contaminants from other bacteria, ensuring that the OMVs were suitable for subsequent experiments. Detailed results regarding the isolation and confirmation of OMVs are reported in our previous study.

Gene expression in breast cancer model mice treated with OMVs

The expression levels of genes in the Snail/β-Catenin cascade—specifically α-SMA, β-Catenin, Snail, and Vimentin—were evaluated in a breast cancer mouse model treated with *H. pylori* OMVs (1 μg/ml) using qRT-PCR. The GAPDH gene was used as the housekeeping gene for normalization. The results showed that the expression levels of α-SMA, β-Catenin, Snail, and Vimentin were significantly higher in the OMV-treated group compared to the control group ($P< 0.05$) (Fig. 1).

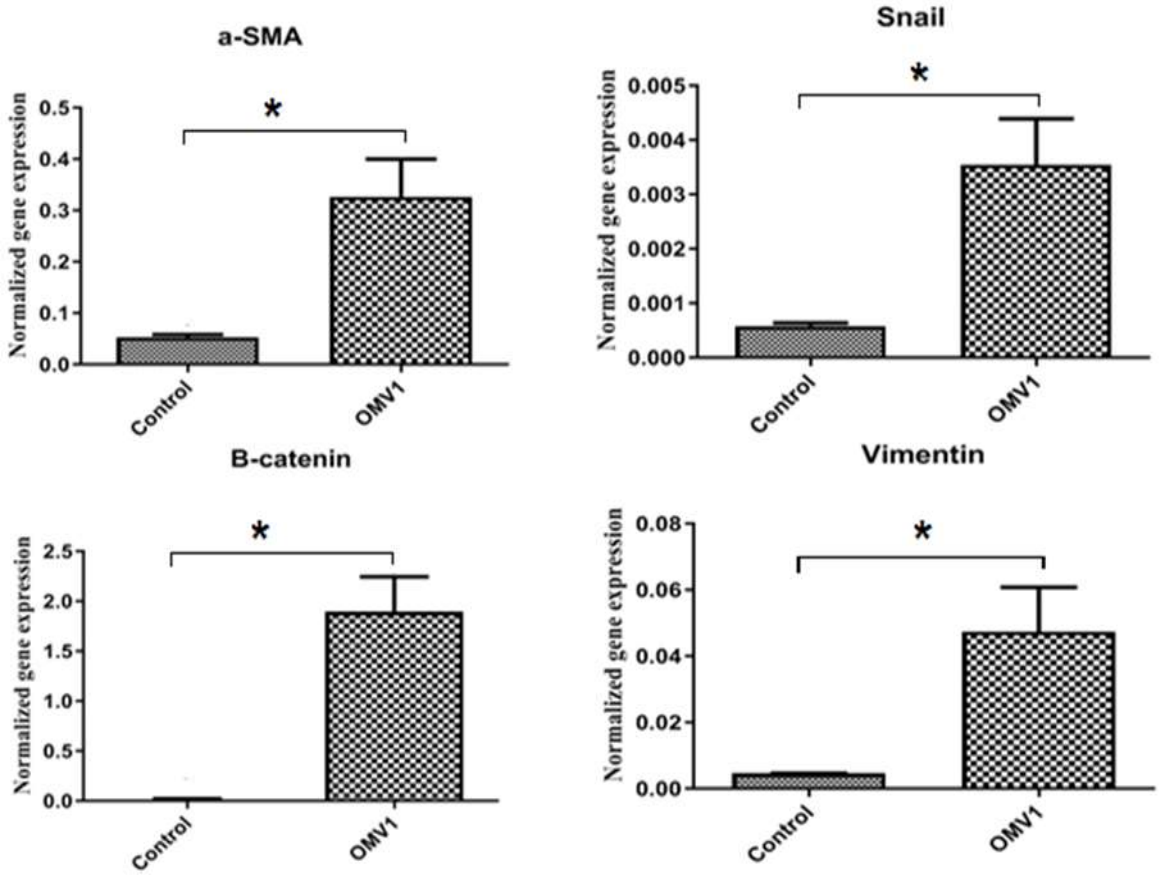

Fig. 1. The relative level of expression of α-SMA, β-Catenin, Snail, and Vimentin genes in the treated mice of the control group and the group receiving a dose of 1 μg/ml of OMVs. *$P< 0.05$.

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Immunocytochemistry for Vimentin and E-Cadherin Proteins

Immunocytochemistry was utilized to assess the levels of Vimentin and E-cadherin proteins, which are critical markers of EMT activated through the Snail/β-Catenin pathway. The protein expression patterns for Vimentin and E-cadherin generally corroborated the gene expression findings obtained from qRT-PCR. The expression of E-cadherin and Vimentin proteins was evaluated in serum samples from breast cancer model mice treated with *H. pylori* OMVs (1 µg/ml). Notably, Vimentin protein expression significantly decreased following OMV treatment compared to the control group ($P< 0.001$). This reduction in Vimentin expression further supports the role of OMVs in inhibiting cell migration (Fig. 2).

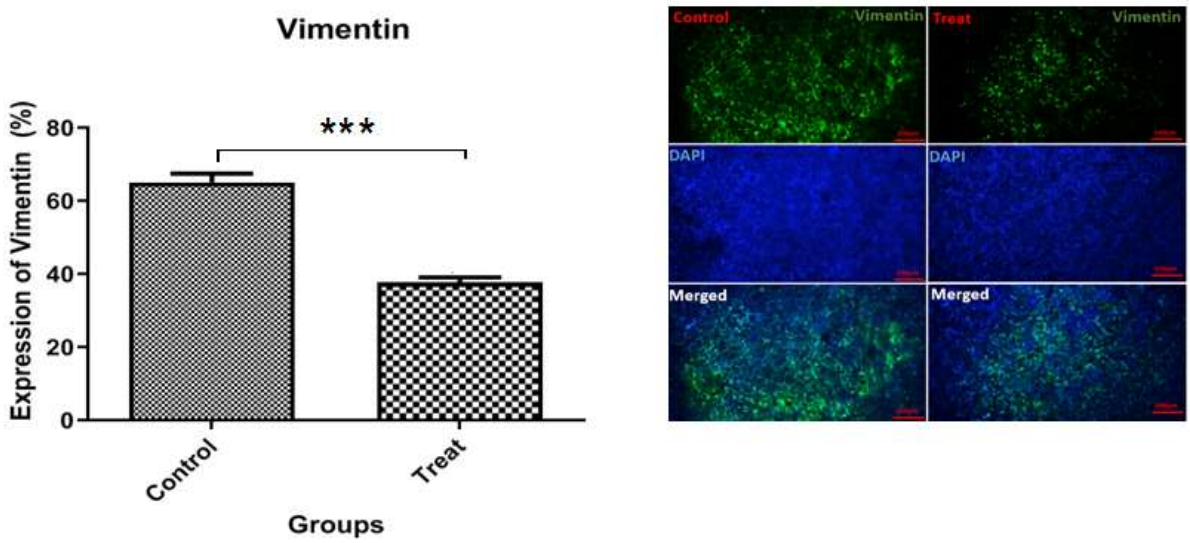

Fig. 2. Expression of Vimentin protein in breast cancer model mice treated with *H. pylori* OMVs (1 µg/ml). Data are presented as means ± SEM from three independent experiments. Statistical significance was determined using one-way ANOVA, with ***$P < 0.001$ indicating significant differences. Scale bar: 20 µm; magnification: 200X.

On the other hand, E-cadherin protein expression in breast cancer model mice increased significantly following treatment with *H. pylori* OMVs compared to the control group ($P< 0.05$) (Fig. 3). This notable increase in E-cadherin expression suggests that these vesicles play a role in inhibiting cell migration.

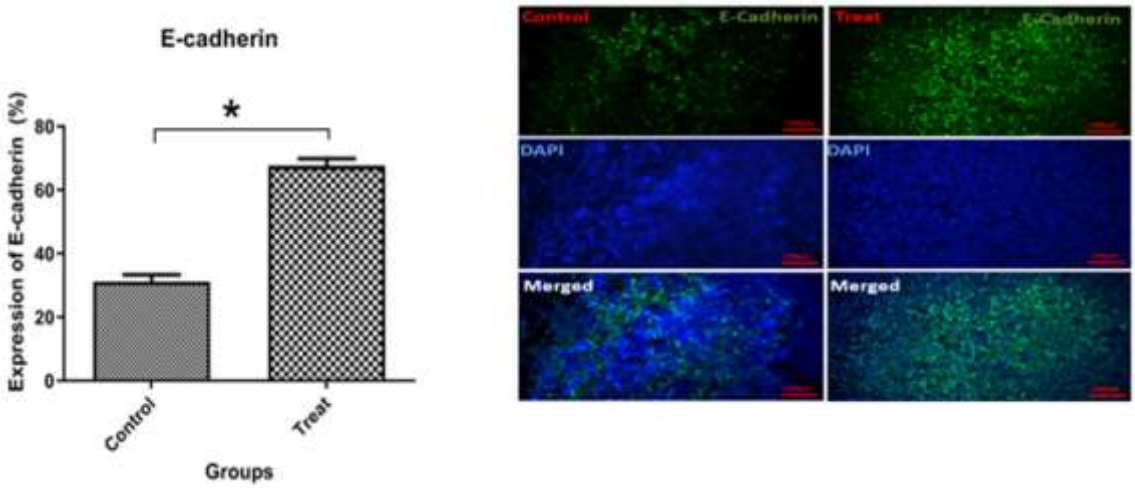

Fig. 3. Expression of E-cadherin Protein in Breast Cancer Model Mice Treated with *H. pylori* OMVs (1 µg/ml). Data are presented as means ± SEM from three independent experiments. Statistical significance was evaluated using one-way ANOVA, with * $P<0.05$ indicating significant differences. Scale bar: 20 µm; magnification: 200X.

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Humoral immune response assessment

The levels of IgA and IgG antibodies in breast cancer model mice treated with *H. pylori* OMVs were evaluated using the ELISA method (Fig. 4). Treatment with the first dose of OMVs resulted in a significant increase in IgA antibody levels compared to the control group (P<0.05), indicating that the OMVs enhance mucosal immunity. In contrast, the level of IgG antibodies significantly decreased after treatment with the first dose of OMVs compared to the control group (P< 0.05). This decrease suggests that the OMVs may suppress humoral immunity.

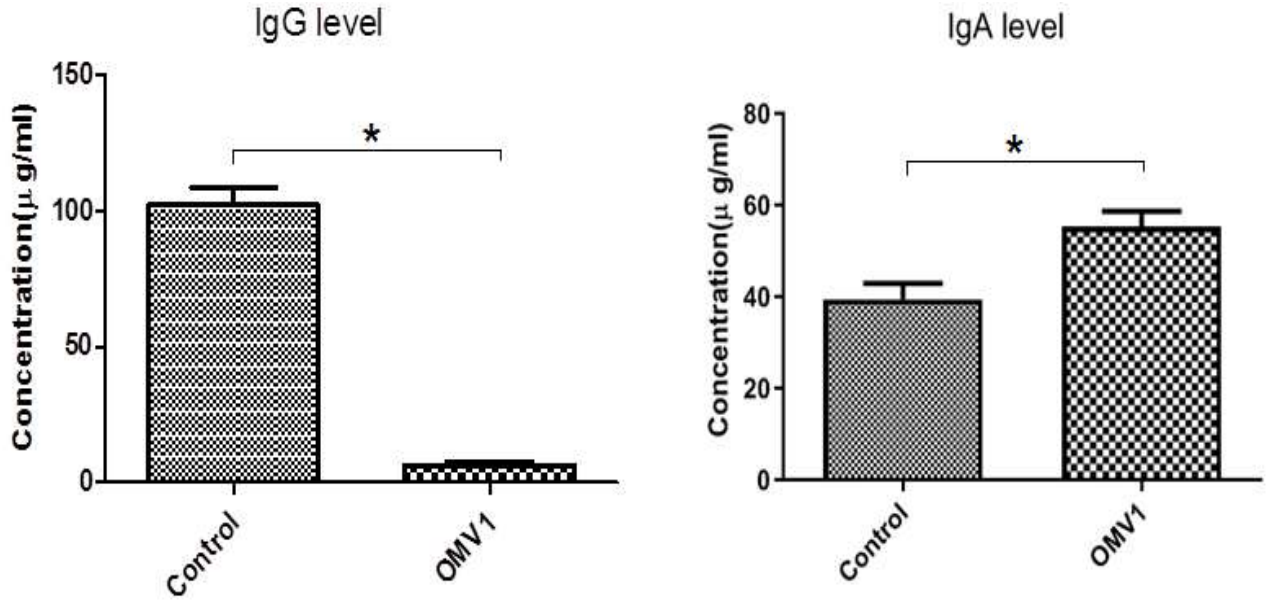

Fig. 4. Levels of IgA and IgG antibodies in breast cancer model mice treated with *H. pylori* OMVs (1 μg/ml). Data are presented as means ± SEM from three independent experiments. Statistical significance was assessed using one-way ANOVA. *: P < 0.05 indicates significant differences between the control group and the group receiving OMVs.

Discussion

This study demonstrates that bacterial outer membrane vesicles (OMVs) derived from *H. pylori* can significantly impact breast cancer treatment by modulating the Snail/β-Catenin cascade pathway, inhibiting cell migration, and altering immune responses. Specifically, our results show that treatment with H. pylori-derived OMVs led to a substantial increase in the expression levels of α-SMA, β-Catenin, Snail, and Vimentin genes in a mouse model of breast cancer. These findings indicate a possible induction of epithelial-mesenchymal transition (EMT) and enhanced cancer cell growth, consistent with previous research that has linked the Snail/β-Catenin pathway to cancer progression (17).

Our study's observation of upregulated Snail/β-Catenin cascade genes aligns with the findings of other researchers who have explored the role of these pathways in cancer development. For instance, previous studies have shown that *H. pylori* proteins in nanoparticle-based immunotherapy can modulate cytokine production and enhance anti-tumor activities, leading to reduced tumor growth in mouse models (18). Similarly, the upregulation of the Snail/β-Catenin pathway has been documented to influence inflammation and pro-inflammatory cytokine expression in gastric cancer models (19). This suggests that the OMVs from *H. pylori* may exert similar effects by affecting the molecular pathways involved in cancer progression.

In addition to the gene expression changes, we found that OMV treatment resulted in a significant decrease in Vimentin expression and an increase in E-cadherin expression. This shift indicates an inhibition of cell migration,

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which is crucial for preventing cancer metastasis. This result is supported by studies showing that the Snail transcriptional regulator promotes EMT by repressing E-cadherin expression, a process implicated in cancer invasiveness (20-22). Our findings are consistent with research indicating that *H. pylori* bacterial components can modulate these markers, thereby potentially offering therapeutic benefits in cancer treatment (23, 24).

Furthermore, our study highlights the immunomodulatory effects of *H. pylori*-derived OMVs, as evidenced by significant changes in IgA and IgG antibody levels. Increased IgA levels suggest an enhancement in mucosal immunity, which aligns with findings that IgA is a sensitive marker for gastric cancer risk in *H. pylori*-infected models (25, 26). Conversely, the decrease in IgG levels suggests a reduction in humoral immunity, which may be a result of the OMVs' effect on systemic immune responses.

Overall, our study underscores the potential of *H. pylori*-derived OMVs as a novel approach in breast cancer therapy. By modulating key gene pathways and immune responses, these vesicles offer promising avenues for enhancing cancer treatment strategies. Further research is needed to fully elucidate the mechanisms through which OMVs exert their effects and to explore their potential for broader therapeutic applications.

This study underscores the potential therapeutic benefits of bacterial OMVs derived from *H. pylori* in breast cancer treatment. Our findings indicate that these OMVs modulate the Snail/β-Catenin cascade pathway, inhibit cell migration, and enhance the immune response in a mouse model of breast cancer. The observed upregulation of Snail/β-Catenin cascade genes, along with significant changes in the expression of Vimentin and E-cadherin proteins, suggests that OMVs could play a crucial role in impeding cancer progression. Additionally, the immunomodulatory effects demonstrated by changes in IgA and IgG antibody levels point to potential new strategies for immunotherapeutic interventions in cancer prevention and treatment. Further investigation into the mechanisms by which OMVs exert their effects and their overall impact on cancer pathogenesis is essential to fully exploit their therapeutic potential.

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Ethical approval

The study was conducted in compliance with ethical standards and was approved by the Ethics Committee of Islamic Azad University, Shahrekord (IR.IAU.SHK.REC.1400.015).

Conflict of Interest

The authors declare that there are no identifiable conflicting financial interests or personal relationships that could potentially have influenced the findings presented in this paper.

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