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ORIGINAL ARTICLE

Influence of *Helicobacter pylori*-derived outer membrane vesicles (OMVs) on Snail/β-Catenin cascade and metastasis-related proteins in 4T1 breast cancer cells

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ABSTRACT

Background and Objectives: This study investigates the impact of *Helicobacter pylori (H. pylori)*-derived outer membrane vesicles (OMVs) on the regulation of Snail/β-Catenin cascade and the production of metastasis-related proteins, such as E-cadherin and Vimentin, in the 4T1 cell line.

Materials and Methods: OMVs were purified from *H. pylori* (ATCC 700392) cultures and applied to 4T1 cells at concentrations of 1, 5, and 10 µg/mL, with untreated cells serving as controls. The MTT assay was employed to quantify cell viability. Expression profiles of *+vimentin*, *Snail*, *α-SMA*, and *β-catenin* genes were evaluated by qRT-PCR, while protein expression of E-cadherin and Vimentin was analyzed via immunohistochemistry. Data were analyzed using appropriate statistical methods with SPSS and GraphPad Prism software.

Results: The MTT assay showed that 1 µg/mL OMVs were safe for normal cells. At this concentration, the expression of *vimentin*, *Snail*, *α-SMA*, and *β-catenin* genes significantly increased in the treatment group ($P \leq 0.05$). Additionally, Vimentin protein decreased, and E-cadherin protein increased ($P \leq 0.05$).

Conclusion: *H. pylori*-derived OMVs activate the Snail/β-Catenin cascade, influencing inflammatory responses and metastasis-related proteins, ultimately reducing migration in advanced cancer stages by modulating Vimentin and E-cadherin expression.

Keywords: Breast neoplasms; *Helicobacter pylori*; Outer membrane vesicles; Metastasis; β-catenin; Transcription factors

INTRODUCTION

Breast cancer constitutes a major global health challenge, remaining one of the principal causes of cancer-associated global death rates (1, 2). With more than 2.3 million new diagnoses annually, breast cancer represents the most commonly occurring cancer type most frequently diagnosed in women around the

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world (3). The broader and more far-reaching impact of this disease highlights the critical need for more effective control strategies and continued research to confront its complex challenges (4).

Recent advances in cancer immunotherapy have focused on enhancing anti-tumor immune responses (5). One key signaling axis that has gained significant attention in this regard is the Snail/β-Catenin cascade, a central player in cancer biology. The transcription factor Snail is a key regulator in initiation of epithelial–mesenchymal transition (EMT), a cellular reprogramming process that facilitates metastatic dissemination of tumor cells (6). EMT is characterized by a shift in gene expression patterns, marked by reduced expression of epithelial indicators like E-cadherin and increased levels of mesenchymal markers like Vimentin and the transcriptional regulator Snail (7). By repressing E-cadherin, Snail facilitates tumor cell invasion and metastasis (6). Furthermore, the Wnt/β-catenin signaling pathway activation often triggered by DNA damage—has been implicated in promoting loss of genomic stability and facilitating the conversion of normal stem cells into tumor-initiating cells. Notably, Snail can amplify this pathway, establishing a positive feedback loop that perpetuates oncogenic signaling (8).

Another critical protein in EMT is α-smooth muscle actin (α-SMA), which increases the contractile capacity and motility of cancer cells, facilitating their dissemination to distant tissues. Elevated α-SMA expression has been correlated with higher invasive behavior and metastatic capability in breast cancer patients (9).

Bacterial-derived outer membrane vesicles (OMVs) have emerged as potential anticancer agents, primarily given their powerful capacity to influence immunological functions (10, 11). OMVs are nano-sized, spherical structures released by gram-negative bacteria during active growth, carrying diverse bioactive molecules including enzymes, lipids, nucleic acids, toxins and membrane-associated proteins (12). These vesicles can stimulate innate immune cells, polarize macrophages and induce pyroptosis, thereby enhancing anti-tumor immunity (13).

Among gram-negative bacteria, *Helicobacter pylori* is known to secrete OMVs containing several virulence factors, most notably CagA, which can phosphorylate and activate β-catenin. CagA also binds to E-cadherin, promoting β-catenin accumulation in the cytoplasm and nucleus and altering epithelial cell behavior (14, 15). Moreover, *H. pylori* infection has been associated with increased expression of mesenchymal-associated genes/proteins, including Vimentin and α-SMA, downregulation of E-cadherin, and enhanced anti-apoptotic signaling—molecular alterations that collectively promote malignant transformation, tumor progression and fibrotic tissue remodeling (16).

Given these dual roles, OMVs appear to function as a double-edged sword, with their impact on cancer progression depending largely on the bacterial source. Therefore, this study aims to investigate the potential role of *H. pylori* and its OMVs in either promoting or suppressing tumor development in breast cancer—a malignancy located outside the gastrointestinal tract, considering their established immunomodulatory and inflaammatory effects. This study complements and supports another study conducted by our team on a in vivo breast cancer model using mice (PMID: 39995644) (17).

MATERIALS AND METHODS

OMVs isolation and characterization. The *H. pylori* 26695 strain (ATCC 700392) was cultured at 37°C under defined microaerophilic conditions (10% CO_2, 85% N_2, 5% O_2) using Brucella broth (Qlab, Canada) enriched with 10% fetal bovine serum (FBS; GIBCO, USA). After 72 hrs of incubation, the culture reached the mid-log phase with an OD600 nm of 1.0–1.4, reflecting an approximate value of 6×10^6 CFU/ml based on viable counts from horse blood agar. To isolate and purify OMVs, the culture was centrifuged at 4°C for 20 minutes at 12000 ×g. The supernatant was filtered through a 0.45 µm membrane filter and subjected to ultra-centrifugation at 200000 ×g for 3 hours at 4°C. The resulting OMV pellets were resuspended in 1 ml sterile PBS, followed by an additional filtration step using a 0.22 µm before storage at -80°C. It is crucial to avoid using the brake during ultra-centrifugation. OMV characterization was performed using scanning electron microscopy (SEM) (Hitachi, S4169 Japon) and dynamic light scattering (DLS) (Malvern Instruments, UK) (17).

Cell culture and OMV inoculation. The 4T1 mouse breast cancer cell line and MCF10A cell line which is a non-cancerous model of human breast ep-

ithelial cells were procured from the Pasteur Institute of Iran for experimental use. 4T1 cells were cultured in DMEM (Dulbecco's modified Eagle medium; Gibco) containing 2 g/L sodium bicarbonate, 2 mM L-Glutamine, 100 U/mL penicillin-G, 100 µg/mL streptomycin, and 10% heat-inactivated FBS (Gibco, Paisley, UK) in T25 flasks (Sorfa, Zhejiang, China). The cultures were incubated under standard physiological conditions (37°C, 5% CO_2, humidified atmosphere) until reaching a confluency level of approximately 60-70%. They were then seeded into six-well plates and infected with OMVs at doses of 1, 5, and 10 µg/mL in triplicate, while uninfected cells served as controls. The infected cells were maintained in supplemented DMEM (1% FBS) for 24 and 48 hours. MCF10A cells were cultured in DMEM/F-12 media supplemented with 5% horse serum, 10 µg/ml insulin and 0.5 µg/ml hydrocortisone. To prepare the growth medium, appropriate additives were premixed and sterile-filtered through a 0.2 µm filter before being added to the DMEM/F12 medium bottle. For passaging, cells are mixed with 4.0-5.0 ml of medium, and 1.0 ml of the cell suspension was plated into 10 cm culture dishes containing 10 ml of MCF-10A growth medium, resulting in a 1:5 to 1:6 dilution. Additionally, the ensured MCF-10A cells were maintained with epidermal growth factor (20 ng/ml) (18, 19). We evaluated the cell viability by using the Trypan Blue exclusion method (Sigma-Aldrich, Germany), following the manufacturer's protocol (20).

Assessment of the cell viability. To examine the toxicity of *H. pylori* OMVs on MCF10A cell viability, an MTT assay was performed. Cells (1.5×10^5 per well) were seeded into 96-well plates and treated with either 300 nM OMVs or phosphate-buffered saline (PBS) as a control for 24 hours. Following the treatment period, 20 µL of MTT solution (5 mg/mL; Sigma–Aldrich) was dispensed into each well to initiate the colorimetric viability assay, followed by a 150-minute incubation at 37°C to allow for formazan formation. The culture medium was then carefully removed, and 150 µL of solubilization buffer composed of 0.1% NP-40 and 4 mM HCl in isopropanol was added to dissolve the crystals. The absorbance of each well was recorded at 570 nm using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) to quantify formazan production. All assays were conducted in triplicate to ensure the consistency and the reproducibility (20).

Quantitative real-time PCR (qRT-PCR). To evaluate changes in gene expression after treatment with *H. pylori*-derived OMVs, RNA isolation from 4T1 cells was performed with TRIzol reagent (Invitrogen, Grand Island, NY) following the supplier's protocol. RNA concentration and purity were assessed spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, RNA samples were reverse transcribed into cDNA employing the ThermoScript Reverse Transcription System (Life Technologies, Rockville, MD) as directed by the supplied protocol.

Gene expression analysis of Snail/β-Catenin pathway components (*Snail*, *β-catenin*, *α-SMA*, and *vimentin*) was conducted via quantitative real-time PCR. Amplification was conducted using SYBR Green I Master Mix (Takara, Dalian, China) on a Roche LightCycler 2.0 system. Each 20 µL reaction mixture contained 1 µL of cDNA template, 10 µL of SYBR Green master mix, 7 µL of nuclease-free water, and 1 µL each of forward and reverse primers (10 µM). The thermal cycling protocol comprised an initial denaturation step at 95°C for 5 minutes, followed by 40 amplification cycles alternating between 95°C for 15 seconds (denaturation) and 60°C for 20 seconds (combined annealing/extension), with a final elongation at 72°C for 30 seconds and subsequent temperature equilibration at 30°C for 20 mins.

Gene expression levels were normalized to *GAPDH* as an endogenous reference control. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method for quantitative comparison across samples, with ΔCt values calculated for each sample and compared to the average ΔCt of the control group. Technical triplicates were run for all qRT-PCR assays to guarantee reliable and consistent results. The primer sets used, along with their amplicon lengths are detailed in Table 1.

Immunohistochemistry analysis (IHC). IHC testing was conducted to assess the immunoreactivity of 4T1 breast cancer cells treated with *H. pylori*-OMVs, with a specific focus on assessing the expression levels of E-cadherin and Vimentin proteins. For experimental procedures, cell suspensions were carefully dispensed into 24-well culture plates and incubated for 16-24 hrs under standard physiological conditions (37°C, 5% CO_2) to ensure proper cellular attachment prior to subsequent treatments. Subsequently, cells were exposed to *H. pylori*-derived OMVs at concen-

Table 1. Oligonucleotide sequences and PCR product lengths for target genes in the Snail/β-Catenin axis

Gene	Sequence (5'->3')	Length of PCR products	Ref
Snail	F-CCTGTCTGCGTGGGTTTTTG	198	(17)
	R-ACCTGGGGGTGGATTATTGC		
β-catenin	F-AAGGTGATTTGATGGAGTTGGA	127	(17)
	R-AGAGAAGGAGGTGTGGTAGTG		
α-SMA	F-AGGGAAGGTCCTAACAGCCC	156	(17)
	R-AGGATTCCCGTCTTAGTCCC		
vimentin	F-TCCGCACATTCGAGCAAAGA	163	(17)
	R-TGATTCAAGTCTCAGCGGGC		
GAPDH	F-CTTTGGTATCGTGGAAGGAC	126	(17)
	R-GCAGGGATGATGTTCTGG		

trations of 1, 5, and 10 μg/mL and incubated for an additional 48 hrs in a humidified incubator with 5% CO_2 at 37°C. After treatment, the cells were fixed using 4% (w/v) paraformaldehyde for 20 minutes, followed by three consecutive PBS washes at 5-minute intervals (PBS; Sigma-P4417). Permeabilization was achieved by incubating the cells with 0.3% (v/v) Triton X-100 for 30 minutes, after which they were rinsed again with PBS. To block non-specific binding, 10% goat serum was added for 45 minutes. Next, primary antibodies (Biorbyt, orb156677 for E-cadherin and orb214735 for Vimentin) were diluted 1:100 in PBS and applied to the samples for 24 hours at 2-8°C. After four PBS washes, the cells were incubated at 37°C for 1.5 hours with secondary antibodies (Biorbyt, orb688924) diluted at a ratio of 1:150. After further washes, DAPI (Sigma-D9542) was added for 20 minutes in the dark, followed by a final PBS wash. Fluorescent images were captured with the aid of an Olympus microscope (17).

Statistical analysis. Quantitative data are presented as mean values accompanied by standard deviation (SD), where relevant. Statistical comparisons between two normally distributed datasets were performed using Tukey's Honestly Significant Difference (HSD) post hoc test. For comparisons across more than two groups, one-way analysis of variance (ANOVA) was applied. Statistical significance was defined at $P < 0.05$, with the following notations: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. All statistical analyses were conducted using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 8.0.2 (GraphPad Software, La Jolla, CA, USA).

RESULTS

***H. pylori*-derived OMVs characterization.** To assess phenotypic characteristics, DLS and SEM analyses were conducted on the isolated cell-free OMVs. The OMVs were predominantly round, characterized by a bilayer membrane, and ranged in size from 50 to 450 nm in diameter (Fig. 1). The purification process effectively ensured that the OMVs could be utilized in subsequent experiments without contamination from other bacterial species.

Cell viability assay. Cell viability assays and direct microscopic examination showed that the rate of decrease in cell viability of normal MCF10A cells at a concentration of 1μg/mL (OMV1) had no meaningful variation between treatment and control conditions after 24 and 48 hrs of exposure ($P > 0.05$). Therefore, this concentration is not lethal to normal cells and if its inhibitory effect on the 4T1 tumor model cells is confirmed, it could be a safe candidate for treating tumor masses in breast cancer model mice (Fig. 2).

Snail/β-Catenin cascade genes expression in 4T1 cells. As illustrated in Fig. 3, qRT-PCR analysis revealed that exposure to H. pylori-derived OMVs at 1 μg/mL was associated with a considerable rise in the expression levels of *β-catenin, α-SMA, Snail,* and *vimentin* comparing to the untreated controls ($P < 0.05$). In contrast, exposure to elevated non-significant alterations in gene expression were observed following treatment with OMVs at either 5 μg/mL or 10 μg/mL concentrations ($p > 0.05$).

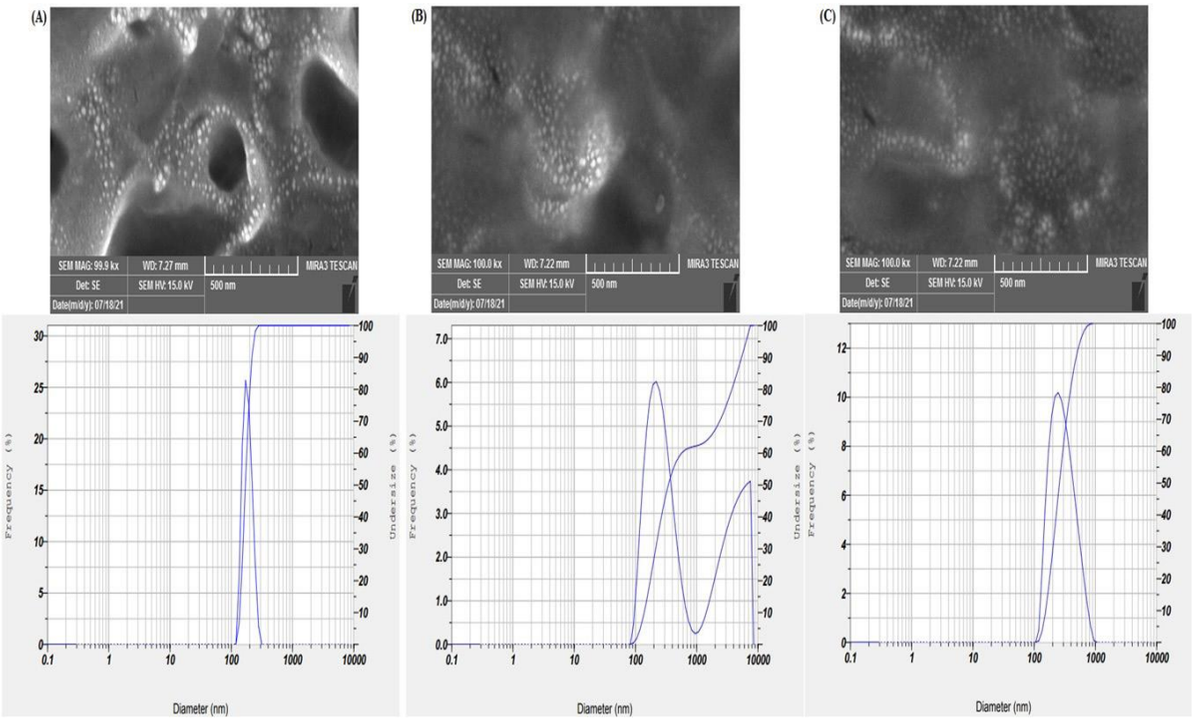

Fig. 1. The SEM and DLS characterization of OMVs derived from *H. pylori* at different concentrations, A. 1 µg/mL (OMV1), B. 5 µg/mL (OMV2), and C. 10 µg/mL (OMV3). SEM analysis demonstrated the presence of spherical, bilaminate vesicular structures with heterogeneous size distribution, measuring approximately 50-200 nm in diameter. DLS measurements identified nanoscale OMVs within an approximate size distribution of 50 to 450 nm, exhibiting a major peak between 150 and 200 nm.

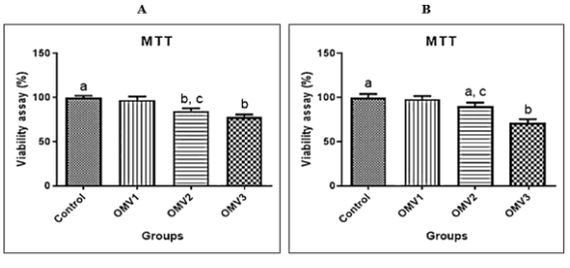

Fig. 2. OMVs effects on MCF10A cell viability. A. After 24 hrs, B. After 48 hours. OMV 1, OMV2, and OMV3 show concentrations of 1, 5, and 10 µg/mL, respectively. All quantitative data are shown as mean ± SEM. Significant differences are shown by unlike letters (a, b, and c).

OMVs increased the protein expression of Vimentin and E-cadherin in 4T1 cells. Quantitative immunocytochemistry showed markedly enhanced E-cadherin levels in treated samples compared to control conditions ($P < 0.05$), with the most pronounced differences observed between the second and third doses of OMVs (Fig. 4A). Furthermore, quantitative analysis demonstrated dose-dependent reductions in Vimentin expression following OMV treatment at all tested concentrations (OMV1: 1 µg/mL, OMV2: 5 µg/mL, OMV3: 10 µg/mL) relative to the control group ($P < 0.05$), with a more substantial reduction detected at the 5 and 10 µg/mL doses (Fig. 4B). The safe concentration of 1 µg/mL, determined in the MTT assay, demonstrated sufficient efficacy in reducing Vimentin protein expression and increasing E-cadherin expression.

DISCUSSION

Breast cancer continues to be a leading contributor to global morbidity and mortality, and there is increasing evidence of the role of microbial factors, including *H. pylori*, in cancer progression beyond the gastrointestinal tract. Despite *H. pylori* being widely recognized for its contribution to gastric carcinogenesis, recent studies have implicated its OMVs in modulating host signaling pathways relevant to cancer development and metastasis (14, 21).

This investigation examined how OMVs derived

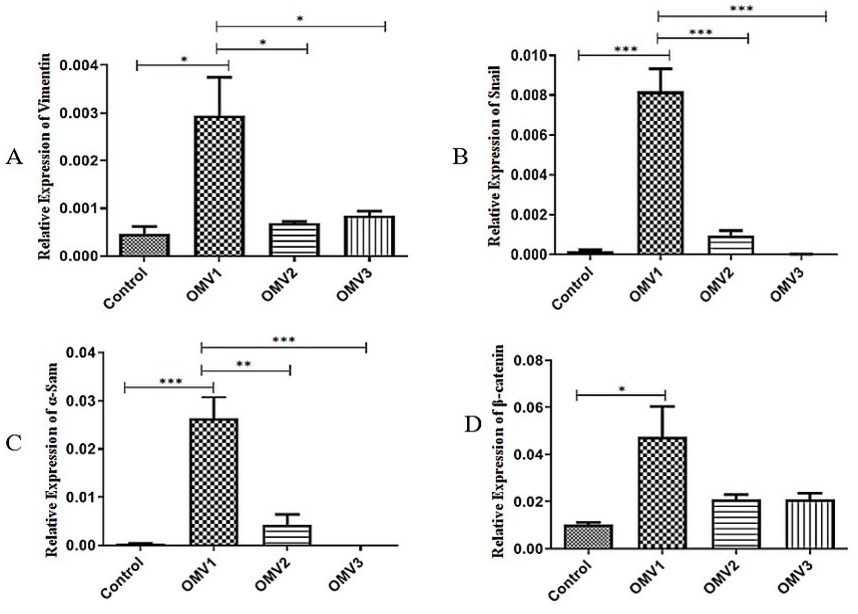

Fig. 3. Transcriptional profiles of Snail/β-Catenin pathway-related genes were evaluated in 4T1 cells following treatment with varying doses of *H. pylori*-derived OMVs. A, B, C, and D depict the expression of *vimentin*, *Snail*, *α-SMA*, and *β-catenin*, respectively. The OMVs were administered at three concentration levels: OMV1 (1 μg/mL), OMV2 (5 μg/mL), and OMV3 (10 μg/mL). All data are expressed as the mean ± standard error of the mean (SEM), derived from three biologically independent experiments. We evaluated the statistical differences among groups using one-way analysis of variance (ANOVA), followed by post hoc comparisons. The asterisk notation system denoted significance levels as: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

from *H. pylori* influence the expression of key genes and proteins involved in EMT in 4T1 breast cancer cells. EMT is a fundamental cellular process in which epithelial cells experience a transition, characterized by the loss of polarity and intercellular adhesion, accompanied by the acquisition of mesenchymal traits that promote enhanced motility and invasiveness (22). Our findings provide compelling evidence that low concentrations of OMVs (1 μg/mL) can induce the EMT phenotype in breast cancer cells using modulating the Snail/β-Catenin signaling cascade.

The physicochemical characterization of the extracted vesicles confirms their nanoparticulate nature, with a uniform size distribution predominantly ranging between 150 and 200 nm. This consistency underscores the structural integrity and homogeneity of the OMVs derived from *H. pylori*, in alignment with previous reports on bacterial extracellular vesicles (23).

To evaluate the biosafety profile of these vesicles, cytotoxic effects were quantified through the MTT colorimetric assay. Treatment with OMVs at a concentration of 1 μg/mL did not significantly affect the viability of MCF10A normal epithelial cells following 24- and 48-hour exposures, suggesting minimal cytotoxicity at this dose. These findings indicate that OMVs, at this concentration, may be considered as biocompatible and potentially safe for therapeutic application. Nevertheless, prior studies have demonstrated dose- and time-dependent OMV-induced cytotoxicity at higher concentrations, such as 15 μg/mL (24). Additionally, extended incubation periods may amplify cytotoxic responses, although no significant differences were noted between 24- and 48-hour exposures in the current study.

At the molecular level, treatment with 1 μg/mL of *H. pylori* OMVs led to a significant upregulation in the expression of key genes within the Snail/β-Catenin signaling axis, including *Snail*, *β-catenin*, *vimentin*, and *α-SMA*. Interestingly, this effect was not observed at higher concentrations (5 and 10 μg/mL), suggesting a possible threshold effect or regulatory saturation. The observed gene expression profile is indicative of the early activation of EMT pathways, which are closely linked to tumor progression and metastatic potential. These results are consistent

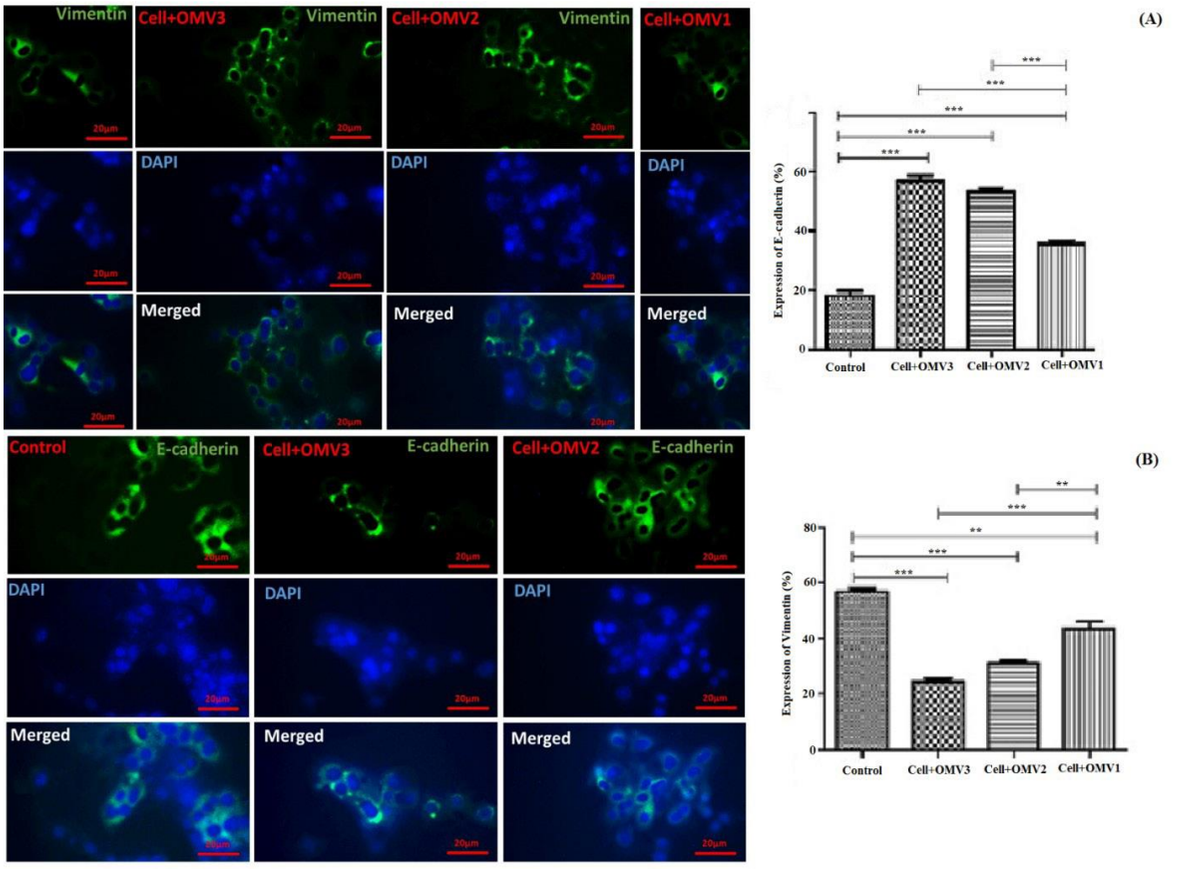

Fig. 4. A. Analysis of Vimentin protein levels in 4T1 cells following exposure to *H. pylori*-derived OMVs. OMV1, OMV2, and OMV3 represent treatments at 1, 5, and 10 µg/mL, respectively. Experimental outcomes are represented as mean ± SEM, calculated from three distinct and independently conducted replicates to ensure data reliability. Statistical evaluation was conducted by means of one-way ANOVA (**P < 0.001). Scale bar: 20 µm; magnification: 200×. B. Evaluation of E-cadherin expression in 4T1 cells treated with *H. pylori*-derived OMVs at concentrations of 1, 5, and 10 µg/mL (corresponding to OMV1, OMV2, and OMV3). Data represent mean ± SEM. One-way ANOVA revealed statistically significant differences (*P < 0.01, **P < 0.001). Scale bar: 20 µm; magnification: 200×.

with findings from previous investigations, such as those involving *H. pylori*-derived vesicles in hepatic stellate cells (LX-2), which demonstrated similar EMT-related gene expression patterns (25).

Protein-level analyses further support these findings, revealing a dose-dependent downregulation of Vimentin at the protein level and a corresponding upregulation of E-cadherin protein expression following OMV treatment. Given that Vimentin is a mesenchymal marker associated with enhanced motility and metastatic behavior, while E-cadherin is an epithelial marker critical for maintaining cell-cell adhesion, these opposing trends suggest an anti-migratory influence of OMVs on cancer cells. This modulation of EMT markers has been previously reported across both in vitro and in vivo experimental systems (26-30). Notably, animal studies have confirmed that *H. pylori* vesicles can upregulate E-cadherin and suppress Vimentin expression, further supporting their potential role in impeding metastasis (31, 32).

In addition to modulating EMT-related processes, *H. pylori*-derived components have shown promise as immunomodulatory agents in nanoparticle-based cancer therapies. Recent studies have demonstrated that these vesicles can alter cytokine expression profiles, enhance anti-tumor immune responses, and ultimately suppress tumor growth in breast cancer models (33). For example, Liu et al. reported that administration of *H. pylori* OMVs in murine models resulted in significant immunomodulation and improved anti-tumor efficacy (34).

Collectively, these results underscore the multifac-

eted biological activities of *H. pylori*-derived OMVs, including their low cytotoxicity, modulatory effects on EMT-related signaling, and capacity to enhance anti-tumor immune responses. These characteristics position OMVs as a promising candidate for further exploration in targeted breast cancer therapies.

CONCLUSION

Cancer remains a multifaceted disease with complex mechanisms underlying its development, progression and treatment. Recent researches have progressively investigated the participation of *H. pylori* and its OMVs in the induction of malignancies beyond the gastrointestinal tract. The present study offers novel insights into the bidirectional effects exerted by *H. pylori*-derived OMVs on breast cancer cells. While OMV exposure enhanced the expression of Snail and β-Catenin—markers associated with tumor progression—it also led to decreased Vimentin and increased E-cadherin expression, which are indicative of reduced metastatic potential. According to the results, although *H. pylori* OMVs may contribute to cancer cell proliferation, they appear to inhibit cell migration and metastasis. Overall, this duality underscores the complexity of OMV interactions with cancer cells and highlights their potential role in modulating tumor behavior. Further in vivo studies and broader mechanistic evaluations, including various signaling pathways and cancer models are essential to unravel the full therapeutic or pathological implications of *H. pylori*-derived vesicles in cancer biology.

Declaration of competing 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. We employed Liner's AI-powered editing features to review and refine the manuscript. Liner's capabilities in identifying and correcting grammatical errors, as well as suggesting stylistic improvements, were valuable in preparing this article for publication.

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