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Herpesvirus entry mediator as a potential biomarker in breast cancer compared with conventional cytotoxic T-lymphocyte-associated antigen 4

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Abstract. Breast cancer (BC) is the most common cancer in women worldwide, with 2.3 million cases recorded in 2020. Despite improvements in cancer treatment, patients with BC still succumb to the disease, due to regional and distant metastases when diagnosed at later stages. Several immune checkpoint inhibitors have been approved for BC treatment, based on their expression and role in maintaining immunosurveillance against tumors. The present study aimed to evaluate the expression of 12 immune checkpoints in patients with BC, and assess their role as diagnostic and therapeutic markers. Expression levels were measured using reverse transcription-quantitative polymerase chain reaction. Among the 12 immune markers, herpesvirus entry mediator (HVEM) was found to be significantly upregulated in patients with malignant BC compared to non-malignant controls, with a relative fold change (FC) of 1.46 and P=0.012. A similar finding was observed for cytotoxic T-lymphocyte-associated antigen 4 (CTLA4; FC=1.47 and P=0.035). In addition, receiver operating characteristic curve analysis revealed that HVEM expression allowed significant differentiation between groups, with an area under the curve of 0.74 (P=0.013). Upregulation in both HVEM and CTLA4 was revealed to be significantly associated with the human epidermal growth factor receptor-2 (HER2)-enriched phenotype (FC=3.53, P=0.009 and FC=5.98, P=0.002, respectively), while only HVEM was significantly associated with the triple-negative phenotype (FC=2.07, P=0.016). Furthermore, HVEM was significantly higher in patients with grade III tumors (FC=1.88, P=0.025) and negative vascular invasion (FC=1.67, P=0.046) compared with non-malignant controls. Serum protein levels were assessed by multiplex immunoassay, and a significant increase in HVEM was detected in patients with malignant BC compared with that in non-malignant controls (P=0.035). These data indicated that HVEM may serve as a potential biomarker and target for immunotherapy, especially for certain types of BC.

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Introduction

Breast cancer (BC) is the most common malignancy among women, with 2.3 million new cases and 685,000 cancer-related deaths reported globally in 2020 (1). According to the World Health Organization (WHO), BC is the second most prevalent cancer diagnosed in Saudi Arabia, with 14.2% new cases in 2020. Although the 5-year survival rate has improved over the years owing to developments in cancer treatment, a significant number of patients with BC still succumb to this disease, due to regional and distant metastases when diagnosed at later stages. A burden is associated with BC owing to its heterogeneity,

resulting in increased incidence and low survival rates (2). Breast tissues contain heterogeneous groups of carcinoma cells that exhibit tumorlike characteristics. Breast carcinomas appear as elongated spindle cells in the presence or absence of elongated or ovoid nuclei in pale cytoplasm. An extensive loss of epithelial morphology and a gain in the mesenchymal spindle cell profile occur due to biological plasticity processes, such as epithelial-mesenchymal transition. Therefore, different types of breast carcinomas can appear, such as epithelial, mesenchymal, or biphasic carcinomas, which contain both cell types (3).

The tumor microenvironment (TME) is highly immunosuppressive and can constrain antitumor immune responses and promote tumor progression. It has recently been reported that molecules such as macroH2A1, a histone variant with a large C-terminal portion, are associated with bone metastasis in BC. MacroH2A1 expression is higher in metastatic BC than in nonmetastatic BC (4). The release of soluble molecules can shape the bone microenvironment and promote tumor progression. Various molecules in a soluble secreted form, but also membrane-bound, contribute to the suppression of antitumor immunity. Membrane-bound molecules are shed from the cell surface and secreted into the TME (5). Targeting of these molecules has shown clinical benefits in cancer immunotherapy.

Immune checkpoint inhibitors with a role in maintaining immunosurveillance against tumors, including two antibodies against cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and five antibodies against programmed death 1 (PD-1) and its ligands PDL-1 and PDL-2, have been used in several clinical trials on BC treatment (6). These immune checkpoint inhibitors are effective in patients with CTLA4-positive and PD-1-positive BC, where differential expression levels are associated with the effectiveness of related blockades as tumor suppressor agents. Hence, only a small proportion of patients with BC benefit from these cancer immunotherapies (7,8).

In addition, BC is considered as a weakly immunogenic cancer (7). Along with CTLA4, PD-1, PDL-1 and PDL-2, B- and T-lymphocyte attenuator (BTLA) has immune inhibitory effects. BTLA is induced in Th1 cells after activation of T cells but is not expressed by naive T cells. The interaction of BTLA with its ligand attenuates the production of interleukin-2 (IL-2), thus affecting T-cell proliferation and suggesting that it acts as a third immune checkpoint (9). BTLA binds to the herpesvirus entry mediator (HVEM), a tumor necrosis factor (TNF) receptor. Cytotoxic T-lymphocyte 8 (CD8-T)-cell suppression has been shown to be mediated by aberrant expression of BTLA and HVEM (7), which has been linked to leukemia (9) and B-cell malignancy (10). HVEM was first detected in hematopoietic cells but later was also observed in cancer cells such as BC (11), colorectal carcinoma (12), hepatocellular carcinoma (13), and renal carcinoma (14). A negative correlation between HVEM expression and survival rate was reported in human esophageal squamous cell carcinoma, in which high levels of HVEM were associated with lower SR (15). HVEM$^+$ melanomas suppressed the proliferation of BTLA$^+$ tumor-specific CD8$^+$ T cells and inhibited the production of interferon-γ (IFN-γ). This is due to the phosphorylation of immunoreceptor tyrosine-based inhibition motifs (ITIMs) and Src homology 2 (SH2) domain-containing phosphatase 1 (SHP-1)/SHP-2, resulting in the reduction of both T-cell proliferation and cytokine production (9). In addition, HVEM expression in melanoma samples has been correlated with an aggressive gene signature, such as MITF (16). These results suggest that the interaction between HVEM and BTLA contributes to tumor evasion from immunosurveillance. Indeed, antagonizing both BTLA and PD-1 has been shown to restore NY-ESO-1-specific CD8$^+$ T-cell proliferation and cytokine production in melanoma compared with the use of anti-PD-1 alone (17). However, other studies have shown that HVEM overexpression in pancreatic and bladder cancers improves the survical rate (18,19). In addition, in melanoma treatment, the transfer of CD8$^+$ BTLA$^+$ tumor-infiltrating lymphocytes (TILs) in adoptive cell therapy was revealed to be associated with improved clinical results (20). Owing to the contrasting results of these studies, further HVEM investigations are required.

Furthermore, three inhibitory receptors in dysfunctional T cells in cancer have been found to maintain tumor tolerance including lymphocyte activating gene 3 (LAG-3), T-cell immunoreceptor with immunoglobulin (Ig) and ITIM domains (TIGIT), and T-cell immunoglobulin and mucin-3 (TIM-3) (21). Indoleamine 2,3-dioxygenase (IDO) is a modulatory enzyme that interferes with T-cell survival (10) and has been detected in BC, especially in the triple-negative subtype, resulting in cancer escaping antitumor immunity (21). Unlike previously described molecules, the glucocorticoid-induced tumor necrosis factor (TNF) receptor-related (GITR) gene provides co-stimulatory signals to CD4$^+$ and CD8$^+$ T cells through the activation of the NF-κB pathway, resulting in the production of inflammatory cytokines. GITR agonists induce effector antitumor cells and overcome self-tolerance (22).

In the present study polymerase chain reaction (PCR) was used to assess the expression levels of 12 genes with immune-inhibitory effects. The clinicopathological characteristics of patients with BC were then linked to genes that showed substantially different values. Furthermore, the serum levels of the differentially expressed genes were determined and linked to clinicopathological features. The present study may lead to enhanced understanding of the expression status of immune inhibitory-related genes, which may be used as biomarkers for BC prognosis.

Materials and methods

Study subjects. Ethical approval (approval no. HA-02-J-008) was granted by the Biomedical Ethics Research Committee of King Abdulaziz University Hospital (KAUH), Jeddah, Saudi Arabia. Patients with recurrent BC who had begun treatment were excluded from the study, and only those diagnosed with BC for the first time were included. A total of 32 age-matched female patients, 16 with malignant BC and 16 non-malignant control subjects (mean age ± SEM, 47.03±1.141 and 46.56±2.1, respectively), were recruited between October 2016 and September 2017 from the Department of Radiology, Mammogram Section, KAUH. The baseline characteristics of the participants, obtained from the completed questionnaires, are presented in Table I. The participants were provided with information concerning the study and requested to sign a consent form. Clinicopathological data were acquired from

Table I. Baseline characteristics of studied subjects.

	Total			Non-malignant BC			Malignant BC		
Parameters	Mean ± SEM	Median	IQR	Mean ± SEM	Median	IQR	Mean ± SEM	Median	IQR
Number of patients, n (%)	32 (100)			16 (50.0)			16 (50.0)		
Age, years	47.03±1.41	48.00	12.25	47.5±1.92	49.00	11.75	46.56±2.13	45.00	10.50
BMI, kg/m^2	29.56±0.96	28.85	6.17	30.4±1.40	29.80	8.50	28.71±1.31	28.10	5.87
Waist circumference, cm	89.09±3.12	92.00	19.00	93.0±3.53	85.50	30.00	85.19±5.08	93.00	19.00
Hip circumference, cm	103.8±3.50	106.00	16.8	107.2±3.57	105.00	14.50	100.4±6.03	106.00	18.55
W/H ratio	0.86±0.02	0.865	0.105	0.87±0.03	0.870	0.085	0.85±0.02	0.865	0.15
Age of first menstruation, years	13.41±0.31	13.00	3.00	13.19±0.41	13.00	2.75	13.63±0.47	13.50	3.00
Age since menopause, years	50.91±1.37	50.00	9.00	51.80±2.11	52.00	7.50	50.17±1.92	50.00	10.25
Age of first pregnancy, years	21.89±0.80	21.00	6.00	20.92±1.19	20.00	7.25	22.67±1.06	22.00	6.00

Data are presented as the mean ± SEM, median. BC, breast cancer; SEM, standard error of the mean; IQR, interquartile range; n, number of samples; BMI, body mass index; W/H, waist/hip.

the Department of Laboratory Medicine and Pathology, KAUH (23).

Collection and storage of blood samples. Peripheral blood samples were collected in two different tubes. First, 5 ml of whole blood was collected in PAXgene™ Blood RNA Tubes (BRT; Qiagen, Inc.) according to the manufacturer's instructions. The samples were stored at -80˚C until RNA extraction. Second, serum was separated from the DB Vacutainer® SSTTM tube by centrifugation at 2,000 x g for 20 min at room temperature. The serum was then aliquoted and stored at -80˚C for multiplex immunoassays.

RNA extraction. Total RNA was extracted from the samples using the PAXgene™ blood RNA kit (Qiagen, Inc.) following the manufacturer's instructions. In brief, the BRT was centrifuged for 10 min at 4,800 x g and the pellet was washed twice with RNase-free water before adding 40 μl of proteinase K (PK). The lysate was then directly pipetted into a PAX gene shredder spin column. DNase I was added directly to the spin column membrane, incubated for 15 min, and centrifuged at 16,000 x g for 1 min. The elution step was repeated twice, and the quality and quantity of the extracted RNA were confirmed using a DeNovix DS-11 spectrophotometer (DeNovix, Inc.) and gel electrophoresis on 1.2% agarose gel. The isolated RNA was aliquoted and stored at -80˚C until further analysis. All steps of this protocol were carried out at room temperature.

Complementary DNA (cDNA) synthesis. Using a QuantiTect Reverse Transcription kit (Qiagen, Inc.), 400 ng of extracted RNA was reverse-transcribed according to the manufacturer's instructions. The cDNA produced was maintained at -20˚C until analysis.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was used to evaluate the expression levels of 12 genes, selected based on their previously reported association with cancer and their involvement in impairing antitumor immunity, in malignant BC and non-malignant controls (Table II) (24-35). Primers targeting these genes were designed using the Primer3web tool (version 4.1.0; https://bioinfo.ut.ee/primer3/) and were evaluated using the In-Silico PCR tool of the University of California, Santa Cruz Genome Browser for Human Gene Assembly GRCh38 (http://genome.ucsc.edu/index.html). Relative gene expression levels were adjusted using the internal reference housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used are listed in Table III. Samples were processed in duplicate in 96-well plates using Bio-Rad IQ SYBR Green mix and a CFX Connect™ Real-Time PCR device (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. RT-qPCR was conducted using a single initial cycle of 30 sec at 95˚C, followed by 40 amplification cycles of 15 sec at 98˚C, and 30 sec at 60˚C. The amplified products were verified at the end of each cycle and their purity was determined by analyzing the melting curves. Relative expression was quantified using the comparative Cq method ($2^{-\Delta\Delta Cq}$) (36) and the REST 2009 software version 2.0.13 (37).

Multiplex ELISA. The MILLIPLEX® Human Immuno-Oncology Checkpoint Protein premixed immunoassay (cat. no. HCKP1-11KPX17; Merck Millipore, Inc.) was used to determine serum levels of HVEM and CTL4. The assay kit included all of the reagents required for the analysis. Serum samples were thawed to room temperature before analysis and no other pretreatment was required. Fluorescent color-coded magnetic beads (Merck Millipore, Inc.) were read after the assay was completed using a Luminex MAGPIX™ reader, according to the manufacturer's instructions. For validation, 25 μl of sample was added to the polystyrene beads on a microtiter plate and incubated overnight at 4˚C. After adding the serum samples and standards, incubating, and washing the plate, the detection antibody and streptavidin-phycoerythrin solution were set up to react with the beads independently before being washed again. Individual bead types were determined, and the fluorescence signal of the immunoassay sandwich was quantified using a MAGPIX™ analyzer (Luminex Corporation, Inc.), which

Table II. Selected genes associated with BC.

Target gene	Full name	Biological function on immune cells	Expression status in literature
BTLA	B- and T-lymphocyte attenuator	Inhibitory receptor, expressed by T and B lymphocytes and dendritic cells, able to suppress lymphocyte activation	Highly expressed in B cell malignancy (24,25) and gastric cancer and gene polymorphisms in BC (26)
GITR	Glucocorticoid-induced TNF receptor related gene	Co-stimulatory protein for T cells, highly expressed in regulatory T cells and lower in Th and CTLs	Overexpressed in regulatory T cells in peripheral blood mononuclear cells of patients with BC (27)
GITRL	GITR ligand	Associated with worse relapse-free survival	Expressed in platelets of BC patients (28)
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	Inhibitory receptor suppresses antitumor immunity through binding to B7 molecules	Overexpression detected in BC (8,29)
HVEM	Herpesvirus entry mediator	HVEM expression decreases lymphocyte infiltration, perforin, and IFN-γ, suggesting its suppressor effects	Expression detected in BC tissues (30)
LAG3	Lymphocyte-activation gene 3	Inhibitory receptor suppresses antitumor immunity	Upregulation of LAG-3 observed in BC (31)
PD1	Programmed cell death protein 1	Inhibitory receptor suppresses antitumor immunity	Upregulated in BC (32)
PDL-1	Programmed death ligand 1	Inhibitory receptor suppresses antitumor immunity	Upregulated in BC (32)
PDL-2	Programmed death ligand 2	Inhibitory ligand suppresses antitumor immunity	Increased expression in BC (33)
TIM3	T-cell immunoglobulin and mucin-domain containing 3	Inhibitory receptor suppresses anti-tumor immunity	Upregulated in BC (34)
TIGIT	T cell immunoreceptor with Ig and ITIM domains	Inhibitory receptor suppresses anti-tumor immunity	Upregulated in BC (5)
IDO1	Indoleamine 2,3-dioxygenase 1	Suppresses immune surveillance by catalyzing tryptophan to kynurenine, resulting in lack of essential amino acids for immune cells	Increased in BC (35)

BC, breast cancer; IFN-γ, interferon-γ.

was correlated with a set of standards (standard curve) measured using MAGPIX (38,39).

Statistical analysis. GraphPad Prism version 8.0.1 was used for statistical analysis of the collected data (GraphPad Software, Inc.). Significant changes in gene expression between malignant BC and non-malignant controls were detected using an unpaired two-tailed t-test. In addition, a two-tailed P-value was utilized to examine the distribution of clinicopathological features in malignant BC patients, using one way ANOVA (two-tailed and Kruskal-Wallis tests) for certain parameters as the comparison between three groups were applied. The results are displayed as the mean ± standard error of the mean (SEM). The median and interquartile range (IQR) were calculated using Excel. Using the easyROC web tool (ver.1.3.1; https://www.biosoft.hacettepe.edu.tr/easyROC/), receiver operating characteristic (ROC) curves were created to examine the sensitivity and specificity of HVEM as a possible biomarker compared to CTLA4 using their expression values ($2^{-\Delta\Delta Cq}$) in malignant BC and non-malignant controls. $P \leq 0.05$ was considered to indicate a statistically significant difference. The G-power software to calculate post-hoc power was used to verify the small sample size.

Results

Evaluation of selected gene expression in malignant BC and non-malignant control samples. The expression levels of the selected immune checkpoint protein genes (BTLA, GITR, GITRL, CTLA4, HVEM, LAG3, PD1, PDL-1, PDL-2, TIM3, TIGIT, and IDO1) were evaluated. Among these 12 genes, CTLA4 and HVEM showed significantly different

Table III. PCR primer sequences of target genes.

Gene symbol	Gene name	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
BTLA	B- and T-lymphocyte attenuator	NM_001085357	GTCATACCGCTGTTCTGCAA	CTGCTTGCCATTTCGTCCTT
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	NM_005214	ATCCCTGTCTTCTGCAAAGC	TACTCACACACAAAGCTGGC
GITRL	Ligand for receptor TNFRSF18/AITR/GITR	NM_005092	GATCATCCTGGAAGCTGTGG	CTCCTTAGCAGTCTCTAATTGGA
GITR	Glucocorticoid-induced TNFR-related protein	NM_004195	TGTCCAGCCTGAATTCCACT	AGCCAAAACTGAATTTCCCCT
HVEM	Herpesvirus entry mediator	NM_003820	ACCTACATTGCCCACCTCAA	CGTTCTCTGTCCTGGAGCA
LAG3	Lymphocyte-activation gene 3	NM_002286	CCAGGCCTCGATGACTGC	TCAGGGCGGCTGAAGGAG
PD-L1	Programmed death ligand 1	NM_014143	CCTGAGGAAAACCATACAGCTG	TGGCTCCCAGAATTACCAAGT
PD-L2	Programmed death ligand 2	NM_025239	ATCATCTATGGGGTCGCCTG	GCTCTACCTCATCTGTTTCTGG
PD1	Programmed cell death protein 1	NM_005018	CTCTGTGGGGCCATCTCC	TCTGCCCTTCTCTCTGTCAC
TIM3	T-cell immunoglobulin and mucin-domain containing 3	NM_032782.5	CAGACACTGGGGAGCCTC	TTGCTCCAGAGTCCCGTAAG
TIGIT	T cell immunoreceptor with Ig and ITIM domains	NM_173799	GGGACGTACACTGGGAGAAT	CACCACGATGACTGCTGTG
IDO1	Indoleamine 2,3-dioxygenase 1	NM_002164	TTCGTGATGGAGACTGCAGT	CAAAGTGTCCCGTTCTTGCA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	NM_002046	TCACCAGGGCTGCTTTTAAC	GATGATCTTGAGGCTGTTGTCA

expression levels between malignant BC and control subjects, with fold changes (FC) of 1.47 (P=0.036) and 1.46 (P=0.012), respectively (Table IV and Fig. 1). ROC curves were created using gene expression values in patients with malignant BC and non-malignant controls to evaluate the sensitivity and specificity of HVEM as a potential BC biomarker. ROC curve analysis revealed that HVEM expression allows significant differentiation between patients with malignant BC and controls, with an area under the curve (AUC) equal to 0.74 (P=0.013). The same was observed for CTLA4, with an AUC equal to 0.69 (P=0.063). Therefore, at the genetic level, HVEM may act as a potential biomarker for malignant BC (Fig. 2 and Table SI).

Association between gene expression levels of CTLA4 and HVEM with clinicopathological characteristics of patients with BC. The clinicopathological characteristics of the patients under investigation, including hormone receptor phenotype, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), lymph node involvement, tumor size, BC histotype, vascular invasion, and margin invasion, are presented in Table SII. Significant associations between CTLA4 and HVEM expression levels and some of these clinicopathological parameters were detected among patients with BC (P≤0.05; Fig. 3). Association with hormone receptor phenotype in Fig. 3A, revealed significantly higher expression of CTLA4 (FC=5.98, P=0.002) and HVEM (FC=3.53, P=0.009) in the HER2-enriched phenotype of patients with malignant BC compared with the non-malignant control reference baseline levels; whereas a significantly high expression of HVEM only was observed in the triple-negative phenotype (FC=2.07, P=0.016) and no significant differences were found in the expression of CTLA4 and HVEM in the luminal-like phenotype. Furthermore, significantly high expression levels of both CTLA4 and HVEM were detected in negative ER/PR (FC=1.94, P=0.030 and FC=1.79, P=0.012, respectively; Fig. 3B and C); while, a significantly high expression of HVEM only was detected in HER2$^-$ (FC=2.07, P=0.016; Fig. 3D). Significantly different levels of CTLA4 and HVEM were also associated with negative lymph node involvement (FC=1.94, P=0.018 and FC=1.69, P=0.028, respectively; Fig. 3E). Furthermore, HVEM was significantly higher in patients with grade III tumors compared with non-malignant controls (FC=1.89, P=0.025), in contrast to CTLA4 that exhibited no significant differences in tumor grades (Fig. 3F). Moreover, no significant differences were found in the expression of CTLA4 and HVEM with regard to tumor size or BC histotype (Fig. 3G and H). A significant difference of HVEM expression levels was detected in negative vascular invasion of patients with malignant BC and non-malignant controls (FC=1.68; P=0.046; Fig. 3I); as well as, of both CTLA4 and HVEM in negative margin invasion (FC=1.73, P=0.046 and FC=1.68, P=0.036, respectively; Fig. 3J).

Evaluation of CTLA4 and HVEM serum levels in patients with malignant BC compared with non-malignant controls associated with clinicopathological characteristics of patients. A positive association was observed between the expression levels of CTLA4 and HVEM in patients with malignant BC. To assess whether the expression of these genes could

Table IV. Relative change in expression of selected genes in patients with malignant breast cancer compared with non-malignant control.

Gene name	Relative expression fold change	SEM	Median	IQR	P-value
BTLA	0.853	0.161	1.156	0.962	0.636
GITR	1.573	0.178	2.145	1.269	0.062
GITRL	1.861	0.396	1.450	3.990	0.147
CTLA4	1.473	0.143	1.657	1.232	0.036[a]
HVEM	1.459	0.087	1.543	0.708	0.012[a]
LAG3	1.372	0.164	1.668	1.353	0.107
PD1	1.490	0.177	1.643	1.430	0.083
PDL-1	1.616	0.259	1.845	1.098	0.076
PDL-2	1.449	0.297	1.456	1.826	0.193
TIM3	1.345	0.158	1.194	0.797	0.122
TIGIT	1.292	0.130	1.402	1.645	0.170
IDO1	1.249	0.264	1.539	0.995	0.384

[a]Significant at P≤0.05. SEM, standard error of the mean; IQR, interquartile range.

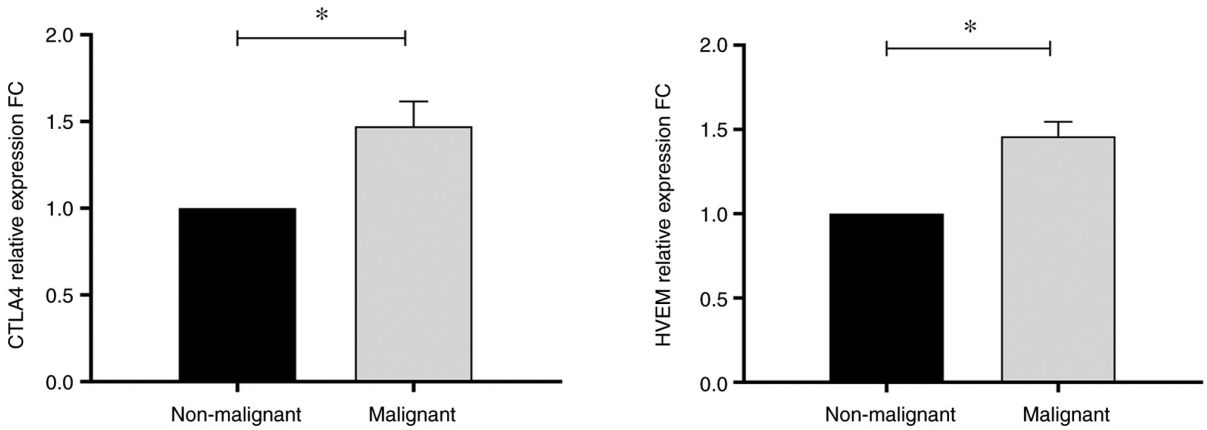

Figure 1. Relative expression fold change of CTLA4 and HVEM in patients with malignant BC compared with non-malignant controls. Gene expression of CTLA4 and HVEM was determined by reverse transcription-quantitative polymerase chain reaction, and GAPDH expression was normalized. Error bars represent the standard error of the mean. *P≤0.05. CTLA4, T-lymphocyte-associated antigen 4; HVEM, herpesvirus entry mediator; FC, fold change.

induce the production of related proteins, the serum levels were measured using a multiplex immunoassay. In healthy subjects, CTLA4 serum levels were measured at an average of 10 pg/ml, whereas HVEM exhibited much higher levels of ~3,000 pg/ml. The results in Fig. 4 revealed that patients with malignant BC exhibited a significant increase in the serum levels of HVEM compared with non-malignant control subjects, whereas CTLA4 levels remain unaltered. In addition, the increase in HVEM serum levels was not associated with any specific clinicopathological characteristic in the patients with malignant BC (Table V).

Discussion

Although cancer immunotherapy is still emerging, it is considered a promising cancer treatment (6). Clinical trials of several immune checkpoint inhibitors have shown positive disease outcomes in different types of cancer, such as melanoma, lung, kidney, and bladder cancers, as well as Hodgkin's lymphoma (5). However, immune checkpoint inhibitors are considered controversial in BC, and only a minority of patients with BC have benefited from them (40). Combining immunotherapy with chemotherapy results in improved overall survival but causes severe adverse effects (41). Chemoresistant patients with PDL-1-positive metastatic BC treated with PD-1 blockade pembrolizumab exhibited an 18% objective response rate (ORR) compared with the 15% ORR when using a combination of PD-1 and CTLA4 blockade (40). Numerous trials have investigated combinations of other targeted immune checkpoints (42). Therefore, there is still a need to investigate additional immune checkpoint molecules that may play potential roles in BC diagnosis and treatment. Numerous studies have focused mainly on PD-1 and CTLA4, but there are a few other promising immune checkpoints, such as TIGIT, GAT-3, BTLA, and HVEM (43), which require further investigation.

In the present study, the gene expression of 12 selected immune checkpoint molecules, namely BTLA, GITR, GITRL,

Table V. Distribution of HVEM serum levels associated with the clinicopathologic data of the patients.

		HVEM serum level				
Parameters	Categories	Mean, pg/ml	SEM	Median	IQR	P-value
Hormone receptor phenotype	Luminal	4470	664.7	4025	2074	0.6499
	HER2-enriched	6171	0.000	6171	0	
	Triple negative	5107	913.7	4628	3354	
ER status	ER^-	5320	739.0	5507	3155	0.0959
	ER^+	3899	393.5	3872	2019	
PR status	PR^-	5320	739.0	5507	3155	0.0959
	PR^+	3899	393.5	3872	2019	
HER2 status	$HER2^-$	5107	913.7	4628	3354	0.3226
	$HER2^+$	4183	443.6	4025	2074	
Lymph node involvement	Negative	4701	795.8	4450	3548	0.9273
	Positive	4843	664.5	4843	1329	
Size of tumor, cm	≤2	4354	750.3	3748	2019	0.0873
	>2	6580	910.7	6171	3678	
Tumor grade	I	4178	0.000	4178	0	0.08998
	II	4703	875.3	4445	3095	
	III	4994	688.8	4690	2968	
Histotype	DCIS	5103	525.8	5142	2527	-
	LCIS	No samples				
Vascular invasion	Negative	4622	668.4	3963	2499	0.2513
	Positive	6171	0.000	6171	0	
Margin	Negative	5026	684.7	4843	2997	-
	Positive	No samples				

Data were collected by averaging the protein concentration in each subgroup of clinicopathological parameters. HVEM, herpesvirus entry mediator; SEM, standard error of the mean; IQR, interquartile range HER2, human epidermal growth factor receptor-2; ER, estrogen receptor; PR, progesterone receptor.

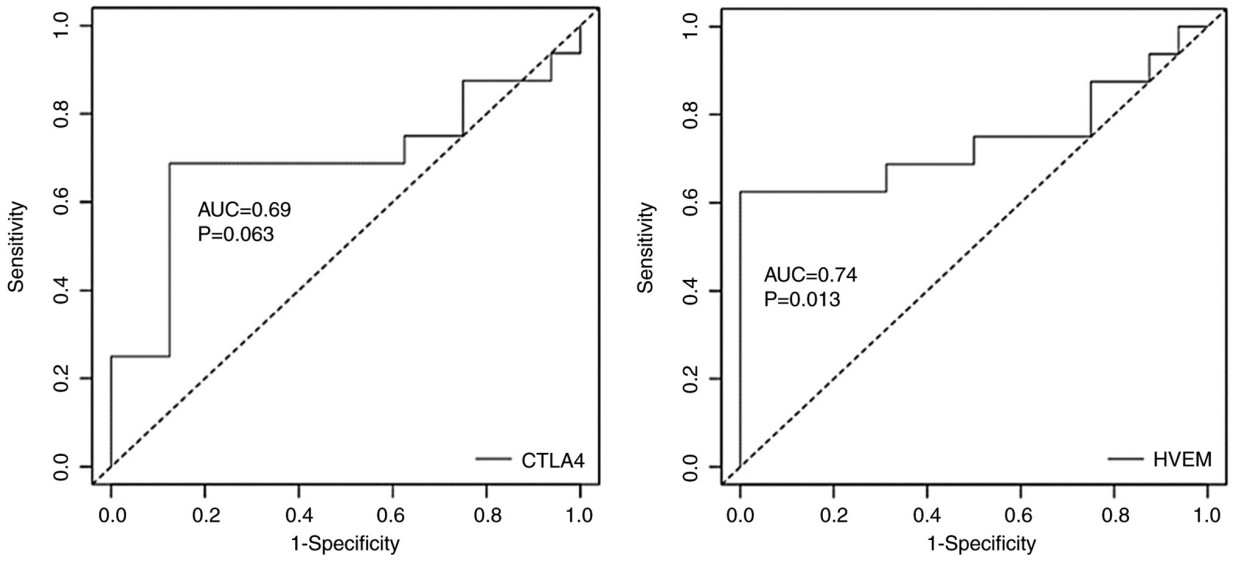

Figure 2. Receiver operating characteristic curve for T-lymphocyte-associated antigen 4 and HVEM gene expression, indicating that HVEM is a potential biomarker in patients with BC. CTLA4, T-lymphocyte-associated antigen 4; HVEM, herpesvirus entry mediator; AUC, area under the curve.

CTLA4, HVEM, LAG3, PD1, PDL-1, PDL-2, TIM3, TIGIT, and IDO1, was measured in the blood samples of patients with BC to identify a systematic BC biomarker. The gene expression patterns in BC were distinct; among the 12 selected genes,

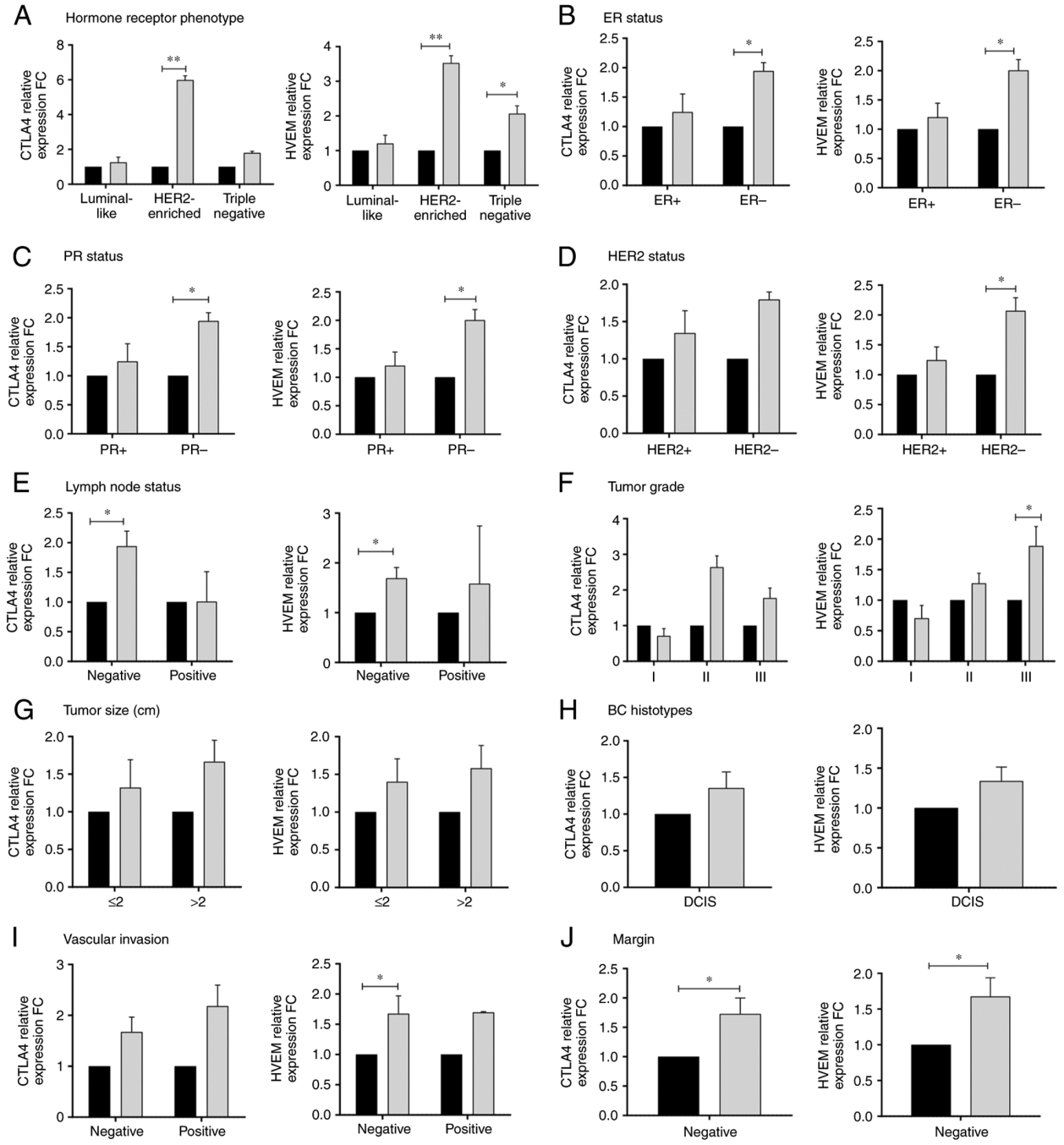

Figure 3. Relative expression fold change of T-lymphocyte-associated antigen 4 (left) and herpesvirus entry mediator (right) in association with clinicopathologic features including (A) hormone receptor phenotype, (B) estrogen receptor status, (C) progesterone status, (D) human epidermal growth factor receptor-2 status, (E) lymph node status, (F) tumor grade, (G) tumor size (cm), (H) BC histotypes, (I) vascular invasion, (J) margins, in patients with malignant BC (gray bars) compared with controls (black bars). Error bars represent the standard error of the mean; $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$. BC, breast cancer; CTLA4, T-lymphocyte-associated antigen 4; HVEM, herpesvirus entry mediator; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2.

only CTLA4 and HVEM were significantly upregulated in the blood of patients with malignant BC compared with control subjects. This result is consistent with that of a recent study by Fang *et al* on 50 immune checkpoints, which found that both CTLA4 and HVEM gene expression levels were upregulated in BC tissues (5); however, upregulation was also detected in TIGIT, PD1, IDO, and LAG3. The differences between the two studies may be due to differences in sample types. In the present study, blood samples were used to identify a systematic BC biomarker, whereas this aforementioned study (33) investigated biomarkers within the TME of BC tissues. Although this may indicate that blood signatures differ from those of tumor tissues in BC, blood samples from cancer patients differ from those of non-malignant control samples. Therefore, the blood may serve as a potential hub for systematic BC biomarkers.

CTLA4 has been approved for cancer treatment, but HVEM is still under study. It has been reported that HVEM is inducible by the TME and has a broader expression than the other immune checkpoints, such as PD-L1. The overexpression of HVEM was revealed to be directly associated with

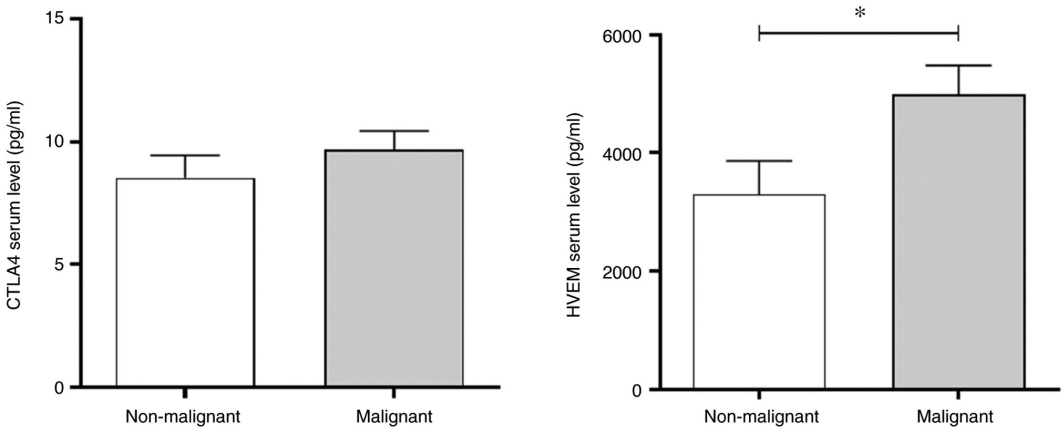

Figure 4. Serum levels of T-lymphocyte-associated antigen 4 and herpesvirus entry mediator assessed by multiplex immunoassay in patients with malignant breast cancer compared with non-malignant controls. Error bars represent the standard error of the mean; *P≤0.05. CTLA4, T-lymphocyte-associated antigen 4; HVEM, herpesvirus entry mediator.

aggressiveness and a poorer cancer prognosis. A previous study concluded that HVEM has a significant oncogenic role in breast carcinogenesis and suggests HVEM as a tumor-specific target (30). In addition, in melanoma, HVEM was revealed to be a negative prognostic marker with potential as a treatment target (16). Therefore, a comparative study of CTLA4 and HVEM gene expression levels associated with clinicopathological data was conducted. A significant association between HVEM expression and tumor grade (grade III) was detected, consistent with the findings of Tsang *et al* indicating that HVEM gene expression in BC tissues and blood is associated with higher tumor grades (30). In contrast to HVEM, no significant correlation was found between CTLA4 expression and tumor size or grade. Moreover, the findings of the present study showed no link between HVEM gene expression in the blood of patients with BC and tumor size. This suggests that when tumors grow, HVEM is overexpressed in BC tissue, and this expression level is maintained in cancer cells and is not secreted into the HVEM soluble form in the blood. Consistent with this previous study (30), significant differences in HVEM expression were associated with the triple-negative phenotype, which is considered the most aggressive type of BC. It was also reported that the presence of HER2 is associated with positive outcomes by increasing levels of TILs (30). In the present study, it was observed that HVEM expression, in contrast to CTLA4 expression, was significantly associated with negative HER2 status. This suggests that HVEM expression in the blood of patients with HER2-negative BC may reduce infiltration, resulting in worse outcomes by downregulating the immune response. In addition, the present study demonstrated that HVEM expression may directly be associated with tumor grade. HVEM was significantly higher in the blood samples of patients with malignant BC with tumor grade III, compared with non-malignant controls, in contrast to CTLA4 which exhibited no significant differences between patients with malignant BC and non-malignant controls in all tumor grades. Similar results have been previously observed in BC tissues, as HVEM was revealed to be associated with aggressive forms of BC with high grade (30). Effective antitumor immunity is evidenced by the production of perforin, granzyme B, and IFN-γ (44), which is reduced by HVEM in hepatocellular carcinomas (13). HVEM

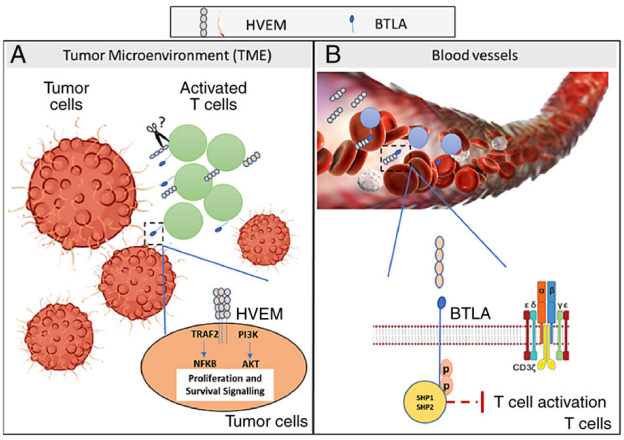

Figure 5. (A) Proposed diagram of the effects of herpesvirus entry mediator on tumor cells and T cells in the tumor microenvironment and (B) blood vessels. HVEM, herpesvirus entry mediator; BTLA, B- and T-lymphocyte attenuator; TME, tumor microenvironment; SHP1, Src homology 2 (SH2) domain-containing phosphatase 1; SHP1, Src homology 2 (SH2) domain-containing phosphatase 2.

expression in BC tissues has been demonstrated to be associated with shorter overall survival. Significantly higher HVEM expression has been reported in tissue samples from early recurrent BC than in those from later cancer recurrence (30). This suggests the involvement of HVEM shedding in tumor tissues. Moreover, the integration of gene expression and metabolites as serum proteins may provide unique insights into the pathways associated with patients with malignant BC (45). Therefore, the serum levels of HVEM were assessed in the present study. Notably, significant differences in serum HVEM levels were observed in patients with malignant BC compared with control subjects, whereas no difference in CTLA4 levels was detected. HVEM may act as an agonist that interacts with BTLA in its circulating, soluble form. BTLA is strongly expressed on naïve and effector antitumor cells, in contrast to PD-1, which is strongly expressed on activated T cells. Therefore, circulating soluble HVEM can affect naive T cells, resulting in their arrest in an inactive state and preventing them from infiltrating the TME. HVEM has also been demonstrated to promote tumor

cell progression. Furthermore, it has been shown to act as an oncogene to promote the colorectal cancer cell cycle as its silencing induces cell cycle arrest (15).

The model presented in Fig. 5 is based on the results of the present study. The TME plays a role in immune editing, resulting in the shedding of HVEM from immune cells. Activated T cells express both HVEM and its ligand BTLA. Following T-cell activation, HVEM can be shed from the surface of immune cells within the TME, which then circulate in the blood. However, HVEM in tumor cells remains bound to cells, providing them with survival signals. Ligation of circulating HVEM with BTLA on T cells in the blood provides an inhibitory signal to T cells, preventing their activation. This suggests that the TME accelerates shedding of HVEM into its soluble form (Fig. 5).

In conclusion, the present study demonstrated the upregulated expression of HVEM and CTLA4 genes in the blood of patients with malignant BC, which suggests that the upregulated HVEM gene translates into HVEM protein, which could then be secreted in a soluble form, as indicated by increased HVEM serum levels, in contrast to CTLA4, which remains bound to cells. HVEM at both genetic and protein levels may serve as a prognostic and diagnostic BC biomarker that can be easily measured in blood samples. It may also serve as an effective target for immunotherapy in patients with the most aggressive phenotype and histological high-grade BC.

Although the small sample size of the present study is a limitation, HVEM expression in the 16 samples for each group was significant, where the power of the sample was shown to be 0.81 (data not shown). The most obvious limitation is that the study used a small cohort of patients with BC without predicting the power of the samples. This is because patient samples had to be obtained before treatment and at short notice, which, together with the excessive cost of the experiment, made it difficult to expand the number of patients in this study. Although the calculated post-hoc power of the HVEM expression was strong (0.81), it can only be associated with this experimental design and should generally be considered as suggestive power, which may also be biased as it is entirely determined by the P-value. Therefore, the present study should be followed-up with future studies, taking care to use larger samples to obtain statistically significant results. Further studies are underway to elucidate the underlying HVEM mechanism and to determine the diagnostic and prognostic value of HVEM in BC. This will involve comparing HVEM expression in BC tissues and the blood of patients with BC, which could provide insights into the poor prognosis. Furthermore, conducting phenotypic analysis of blood cells using flow cytometry may provide insights into the use of markers and their mechanisms in BC for the development of more effective therapies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding authors.

Authors' contributions

MH, AA, KAS, and FB designed and coordinated the experiments. KAS and AA obtained ethical approval, patient consent, and blood sample collection. MH performed the molecular experiments and JA, KZ and PNP performed the Multiplex immunoassay. MH, PNP and FB analyzed the data. AA, JA, PNP, and KAS contributed to laboratory facilities and project funding. FB, MH and KAS drafted the manuscript. KAS, AA, PNP, and KZ edited the manuscript. AA, MH, FB, and KAS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. HA-02-J-008) by the Biomedical Ethics Research Committee of King Abdulaziz University Hospital (KAUH; Jeddah, Saudi Arabia). All patients signed a consent form to participate in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing of interests.

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