Potential of blood exosomal ENAH, SEPT9, EGF, MMP-9 and CXCL8 for the early screening of breast cancer

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Abstract. Exosomal contents have been recognized as candidate biomarkers for cancer screening and prognosis. The current study aimed to evaluate the potential of the expression levels of exosomal enabled homolog (ENAH), septin 9 (SEPT9), epidermal growth factor (EGF), matrix metalloproteinase-9 (MMP-9) and C-X-C motif chemokine ligand 8 (CXCL8) in the blood for the early screening of breast cancer. Therefore, exosomes were extracted and purified from the peripheral blood of 47 patients with breast cancer, 63 disease controls (DCs) and 33 healthy controls (HCs). Subsequently, the exosomal mRNA expression levels of ENAH, SEPT9, EGF, MMP-9 and CXCL8 were detected by reverse transcription-quantitative polymerase chain reaction. The results showed that the exosomal levels of ENAH and EGF were significantly higher in patients with breast cancer compared with DCs and HCs (both $P<0.001$). In addition, receiver operating characteristic curves revealed that exosomal ENAH was able to discriminate patients with breast cancer from DCs [area under the curve (AUC), 0.841] and HCs (AUC, 0.859). However, exosomal EGF was only able to discriminate patients with breast cancer from HCs (AUC, 0.776). Furthermore, the levels of exosomal SEPT9 were lower in patients with breast cancer compared with DCs and HCs ($P=0.021$), and exosomal SEPT9 expression levels exhibited good potential in the discrimination of patients with breast cancer from DCs (AUC, 0.717) and HCs (AUC, 0.830). However, no significant difference was detected in exosomal levels of MMP-9 and CXCL8 among the three groups, and these RNAs showed no discriminative ability. In addition, in patients with breast cancer, the exosomal levels of ENAH were associated with molecular subtypes ($P=0.010$), while those of MMP-9 were associated with a Ki-67 index of $\geq30\%$ ($P=0.011$). In conclusion, the exosomal levels of ENAH, SEPT9 and EGF in blood samples were able to identify patients with breast cancer, thus providing a novel approach for the early screening of breast cancer.

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Abbreviations: ENAH, enabled homolog; SEPT9, septin 9; EGF, epidermal growth factor; MMP-9, matrix metalloproteinase-9; CXCL8, C-X-C motif chemokine ligand 8; PB, peripheral blood; HCs, healthy controls; DCs, disease controls; TNM, tumor-node-metastasis; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROC, receiver operating characteristic; IQR, interquartile range; CI, confidence interval; AUC, area under the curve; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor

Introduction

Breast cancer is the most prevalent type of cancer globally, and accounts for >648,000 cancer-associated deaths annually (1,2). In China, it is estimated that ~416,000 patients are diagnosed with breast cancer every year, which results in >117,000 deaths annually (3,4). Currently, the overall prognosis for patients with breast cancer is unsatisfactory, partially due to a significant proportion of patients being diagnosed with this cancer at an advanced stage (5-7). In response to this, the identification of novel biomarkers for the risk prediction and early screening of breast cancer is of great importance.

Exosomes are spherical particles released by cells that can carry a variety of molecules derived from the cells, including DNAs, RNAs and proteins (8). Due to their stability and ability to remain unaffected by the surrounding environment, it has been suggested that the contents of exosomes exhibit potential as biomarkers for breast cancer screening (9-11).

With the development of molecular biology, several specific genes involved in the pathogenesis and progression

of breast cancer have been identified. For instance, previous studies showed that enabled homolog (ENAH), an actin regulatory protein of the enabled/vasodilator-stimulated phosphoprotein family, promoted the proliferation, invasion and epithelial-mesenchymal transition of breast cancer cells when overexpressed (12,13). Also, other studies showed that septin 9 (SEPT9), an oncogenic protein, was dysregulated in patients with breast cancer with lymph node metastases and regulated the migration of breast cancer cells via ras homolog family member A/focal adhesion kinase signaling (14,15). Additionally, epidermal growth factor (EGF) has been reported to interact with its receptor to regulate the carcinogenesis and malignant behavior of breast cancer cells (16,17). Moreover, matrix metalloproteinase-9 (MMP-9) has been revealed to critically increase the migration and invasion abilities of breast cancer cells, thus reflecting the aggressiveness of breast cancer (18,19). C-X-C motif chemokine ligand 8 (CXCL8) has also been shown to promote breast cancer progression and to be involved in the immunosuppressive tumor microenvironment. Therefore, CXCL8 is considered as a potential therapeutic target for breast cancer (20-22). Accordingly, ENAH, SEPT9, EGF, MMP-9 and CXCL8 are critical genes for the pathogenesis and/or progression of breast cancer (12-22). The aforementioned findings indicate that these genes could be used in the early screening of breast cancer.

The current study aimed to evaluate the association between the exosomal levels of ENAH, SEPT9, EGF, MMP-9 and CXCL8 in the blood and the risk of breast cancer, as well as the clinical characteristics of patients with breast cancer.

Materials and methods

Subjects. Blood samples from 31 (first batch; age range, 30-87 years) and 16 (second batch; age range, 32-68 years) female patients with breast cancer were collected between January 1 and June 30, 2021 at Huashan Hospital Affiliated to Fudan University (Shanghai, China). The inclusion criteria were as follows: i) Patients diagnosed with breast cancer based on pathological tissue and imaging examinations; ii) aged >18 years; and iii) willing to voluntarily participate in the study and provide peripheral blood (PB). The exclusion criteria were as follows: i) Patients with other primary solid tumors or malignant hematological disorders; and ii) female patients diagnosed with breast cancer during pregnancy or breastfeeding. During the same period, 36 (first batch; age range, 15-85 years) and 27 (second batch; age range, 23-85 years) patients with benign breast disease were also enrolled as disease controls (DCs). Additionally, a total of 14 (first batch; age range, 26-73 years) and 19 (second batch; age range, 28-70 years) healthy subjects were recruited as healthy controls (HCs). The present study was approved by the Ethics Committee of Huashan Hospital, Fudan University (Shanghai, China) and all patients or the guardian for the patient who was <18 years old provided written informed consent prior to enrollment.

Data documentation. The clinical data of patients with breast cancer, including age, menopause status, histological type, molecular subtype and tumor-node-metastasis (TNM) stage were recorded. The molecular subtype of each patient was

determined according to the Chinese Anti-Cancer Association Breast Cancer Diagnosis and Treatment Guidelines and Standards (2021 edition) (23). The patients received the appropriate treatment based on disease stage, patient preferences and physician recommendations, which were not affected by the study. All treatments were also recorded.

Sample processing. A total of 4 ml PB was collected from each subject in an EDTA tube. Plasma was then isolated from each sample using Ficoll-Paque Plus Reagent (Cytiva) diluted with PBS at a ratio of 1:1, followed by centrifugation at 12,000 x g at 4°C for 15 min. Subsequently, bind-elute size exclusion chromatography columns (HiScreen Capto Core 700 column; Cytiva) connected to the ÄKTA Pure 25 chromatography system (Cytiva) were used to capture and purify exosomes from 1.5 ml plasma at room temperature. The columns were equilibrated with sterile PBS. The flow rate was 25 ml/min according to the manufacturer's instruction. Following exosome capture, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was carried out to assess the mRNA expression levels of ENAH and SEPT9 in the exosomes derived from the first batch of subjects (31 patients with breast cancer, 36 DCs and 14 HCs) and EGF, MMP-9 and CXCL8 in exosomes derived from the second batch of subjects (16 patients with breast cancer, 27 DCs and 19 HCs).

RT-qPCR. Total RNA was extracted from the exosomes using the RNeasy Micro Kit (Qiagen GmbH) and then reverse transcribed into cDNA using the ReverTra Ace® qPCR RT Kit (Toyobo Co., Ltd.). The conditions for reverse transcription comprised one cycle of 37°C for 15 min and 98°C for 5 min. Subsequently, qPCR was carried out using the KOD SYBR® qPCR Mix (Toyobo Co., Ltd.). The thermocycling conditions for qPCR comprised 1 cycle of 98°C for 2 min followed by 40 cycles of 98°C for 10 sec and 61°C for 30 sec. The relative mRNA expression levels were calculated by the $2^{-\Delta\Delta Cq}$ method (24). The internal reference genes were β-actin for ENAH and SEPT9, and glyceraldehyde-3-phosphate dehydrogenase for EGF, MMP-9 and CXCL8. The primer sequences are listed in Table SI.

Statistical analysis. SPSS 26.0 (IBM Corp.) and GraphPad Prism 7.01 (GraphPad Software Inc.) software were used for statistical analysis and graph plotting, respectively. Differences among groups were compared by Kruskal-Wallis H rank-sum and Wilcoxon rank-sum tests. The ability of ENAH, SEPT9, EGF, MMP-9 or CXCL8 to distinguish individuals from different groups was assessed using receiver operating characteristic (ROC) curves. The normalized partial area under the curve (AUC) was calculated as previously described (25). Associations between exosomal genes and age, menopause, hormone receptor status, HER2 and Ki-67 were analyzed using Wilcoxon rank-sum tests. Associations between exosomal genes and histological type and molecular subtypes were analyzed using Kruskal-Wallis H rank-sum tests. Associations of exosomal genes with TNM stage were analyzed using Spearman's rank correlation test. Clinical characteristics between the two batches were compared using an unpaired Student's t-test for age and a Chi-square or

Table I. Clinical characteristics of the study participants.

Items	HCs (n=33)	DCs (n=63)	Patients with breast cancer (n=47)
Age (years), mean±SD	54.3±12.0	44.5±15.5	54.6±11.5
Menopause, n (%)			
No	25 (75.8)	23 (36.5)	36 (76.6)
Yes	8 (24.2)	40 (63.5)	11 (23.4)
Histological type, n (%)			
Ductal carcinoma *in situ*	-	-	3 (6.4)
Invasive ductal carcinoma	-	-	34 (72.3)
Invasive lobular carcinoma	-	-	4 (8.5)
Others	-	-	6 (12.8)
Molecular subtypes, n (%)			
Triple-negative	-	-	2 (4.3)
Luminal A	-	-	7 (14.9)
HER2-negative luminal B	-	-	10 (21.3)
HER2-positive luminal B	-	-	17 (36.2)
HER2-enriched	-	-	11 (23.4)
Hormone receptor status, n (%)			
ER negative and PR negative	-	-	13 (27.7)
ER positive and/or PR positive	-	-	34 (72.3)
HER2, n (%)			
Negative	-	-	19 (40.4)
Positive	-	-	28 (59.6)
Ki-67, n (%)			
<30%	-	-	34 (72.3)
≥30%	-	-	13 (27.7)
TNM stage, n (%)			
0	-	-	4 (8.5)
I	-	-	13 (27.7)
II	-	-	22 (46.8)
III	-	-	6 (12.8)
IV	-	-	2 (4.3)
Surgical type, n (%)			47 (100.0)
Modified radical mastectomy	-	-	24 (51.1)
Sentinel lymph node biopsy	-	-	18 (38.3)
Radical mastectomy	-	-	15 (31.9)
Breast-conserving surgery	-	-	5 (10.6)
Neoadjuvant therapy, n (%)	-	-	9 (19.1)
Adjuvant therapy, n (%)	-	-	44 (93.6)

HCs, healthy controls; DCs, disease controls; SD, standard deviation; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; TNM, tumor-node-metastasis.

Fisher's exact test for categorical data. P<0.05 was considered to indicate a statistically significant result.

Results

Characteristics of patients with breast cancer. The mean age of the patients with breast cancer was 54.6±11.5 years, including 2 (4.3%), 7 (14.9%), 10 (21.3%), 17 (36.2%) and 11 (23.4%) patients with triple-negative, luminal A, HER2-negative luminal B, HER2-positive luminal B and HER2-enriched breast cancer, respectively. In terms of tumor stage, 4 (8.5%), 13 (27.7%), 22 (46.8%), 6 (12.8%) and 2 (4.3%) patients were diagnosed with a TNM stage of 0, I, II, III and IV, respectively (Table I). Furthermore, comparative analyses revealed that there were no differences in the demographic and disease characteristics of patients with breast cancer between the two batches (all P>0.05; Table SII). In addition, the mean age of the DCs and HCs was 44.5±15.5 and 54.3±12.0 years, respectively (Table I).
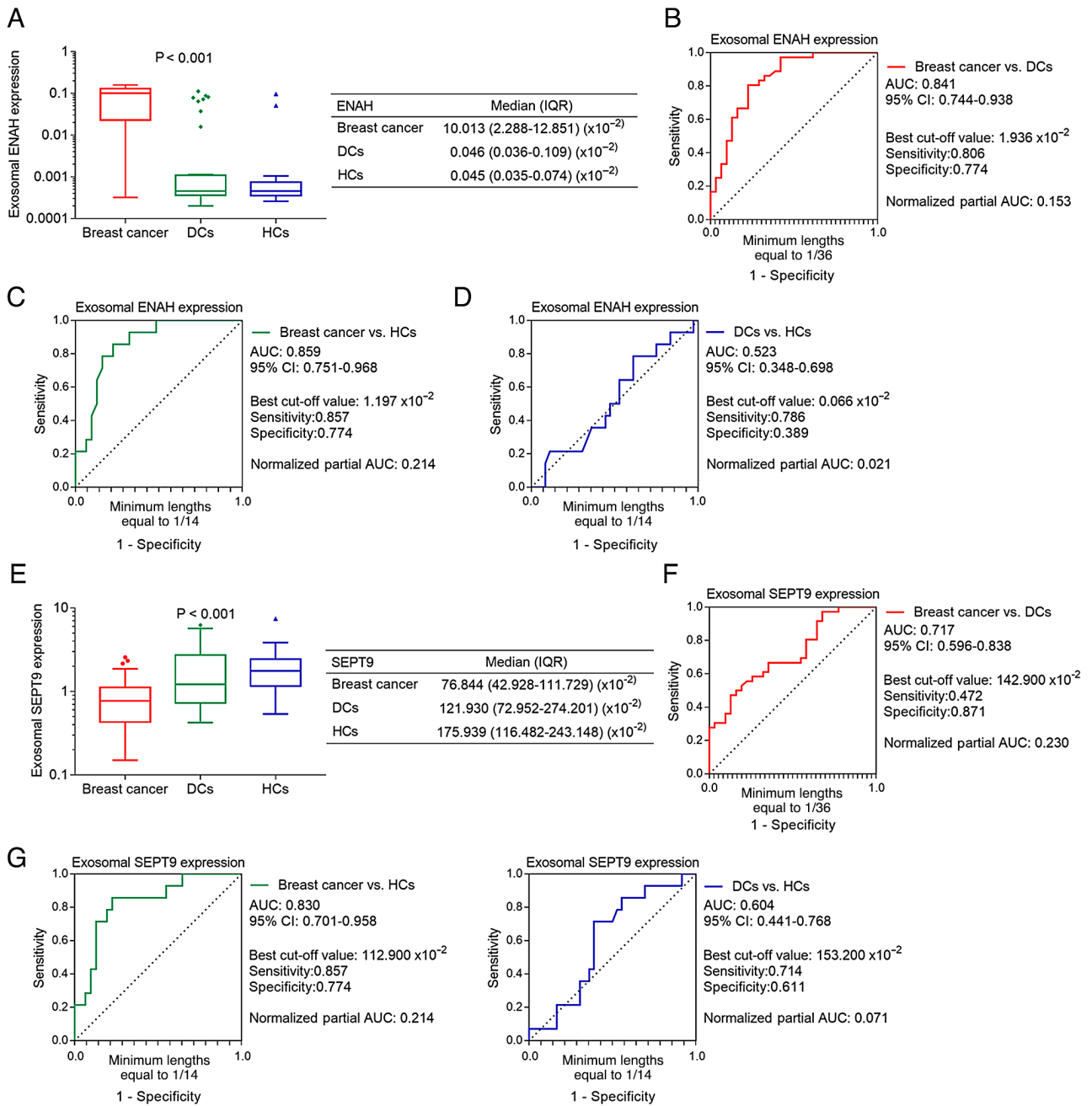
Figure 1. Differential expression of exosomal ENAH and SEPT9 among patients with breast cancer, DCs and HCs. (A) Comparison of exosomal ENAH expression. ROC curve analysis of exosomal ENAH expression for (B) patients with breast cancer vs. DCs, (C) patients with breast cancer vs. HCs and (D) DCs vs. HCs. (E) Comparison of exosomal SEPT9 expression. ROC curve analysis of exosomal SEPT9 expression for (F) patients with breast cancer vs. DCs, (G) patients with breast cancer vs. HCs and (H) DCs vs. HCs. ENAH, enabled homolog; SEPT9, septin 9; DCs, disease controls; HCs, healthy controls; ROC, receiver operating characteristic; AUC, area under the curve; IQR, interquartile range; CI, confidence interval.

Expression of exosomal ENAH, SEPT9, EGF, MMP-9 and CXCL8. The exosomal mRNA expression level of ENAH was the highest in patients with breast cancer, lower DCs and the lowest in HCs (P<0.001; Fig. 1A). Additionally, ROC curve analyses showed that exosomal ENAH exhibited the ability to discriminate between patients with breast cancer and the DCs (AUC, 0.841; Fig. 1B) and HCs (AUC, 0.859; Fig. 1C). However, it could not discriminate DCs from HCs (AUC, 0.523; Fig. 1D). By contrast, the mRNA expression levels of exosomal SEPT9 were lowest in patients with breast cancer, higher in DCs and the highest in HCs (P<0.001; Fig. 1E). ROC curve analyses revealed that exosomal SEPT9 was effectively able to differentiate patients with breast cancer from DCs (AUC, 0.717; Fig. 1F) and HCs (AUC, 0.830; Fig. 1G), but not DCs from HCs (AUC, 0.604; Fig. 1H). Furthermore, the exosomal mRNA expression level of EGF was the highest in patients with breast cancer, lower in DCs and the lowest in HCs (P=0.021; Fig. 2A). ROC curve analyses demonstrated that exosomal EGF failed to discriminate patients with breast cancer from DCs (AUC, 0.664; Fig. 2B). However, it showed an acceptable ability to differentiate between patients with breast cancer and HCs, with an AUC value of 0.776 (Fig. 2C), but not DCs from HCs (AUC, 0.607; Fig. 2D). Regarding the exosomal mRNA expression levels of MMP-9 and CXCL8,
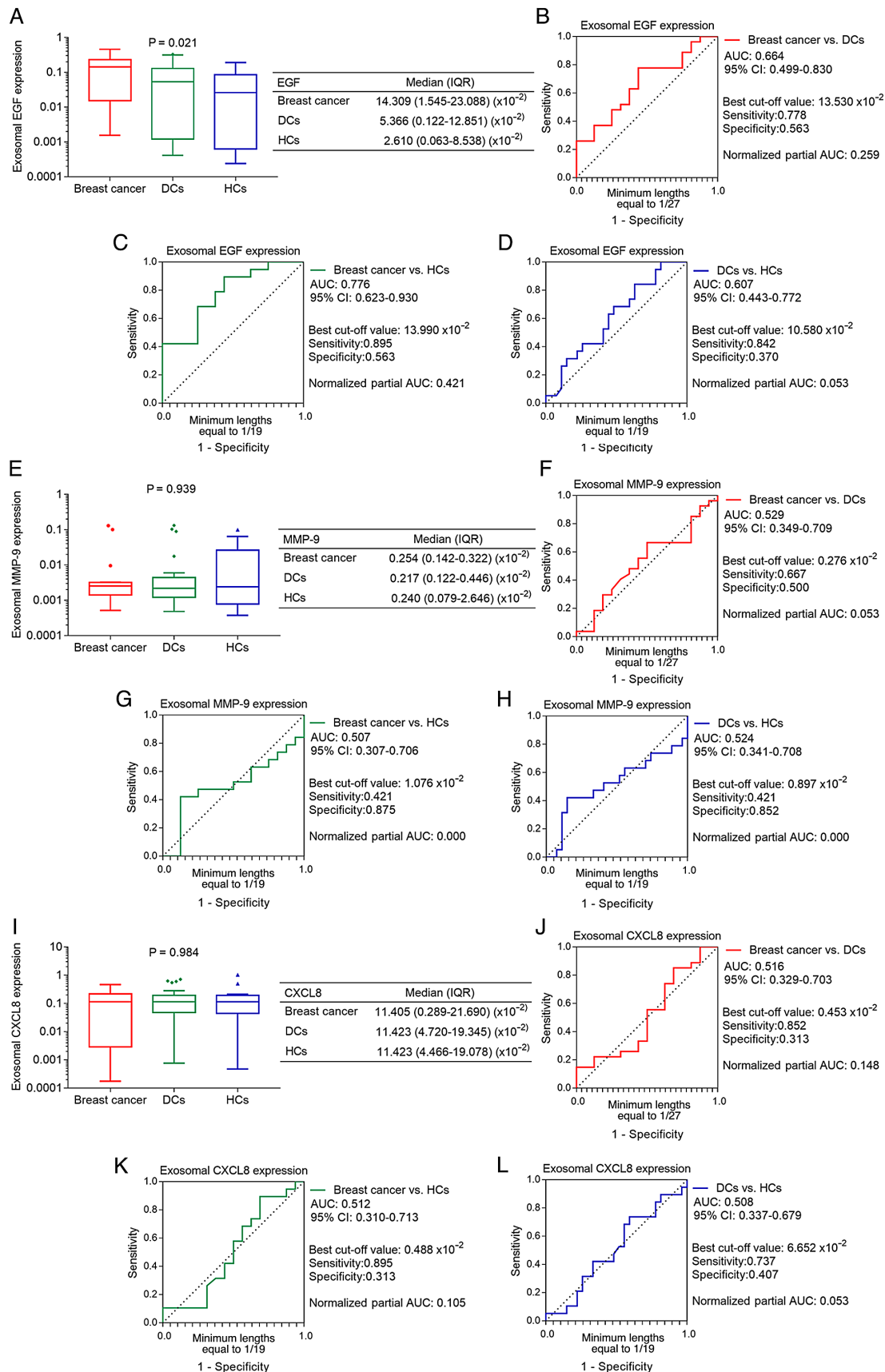
Figure 2. Differential expression of exosomal EGF, MMP-9 and CXCL8 among patients with breast cancer, DCs and HCs. (A) Comparison of exosomal EGF expression. ROC curve analysis of exosomal EGF expression for (B) patients with breast cancer vs. DCs, (C) patients with breast cancer vs. HCs and (D) DCs vs. HCs. (E) Comparison of exosomal MMP-9 expression. ROC curve analysis of exosomal MMP-9 expression for (F) patients with breast cancer vs. DCs, (G) patients with breast cancer vs. HCs and (H) DCs vs. HCs. (I) Comparison of exosomal CXCL8 expression. ROC curve analysis of exosomal CXCL8 expression for (J) patients with breast cancer vs. DCs, (K) patients with breast cancer vs. HCs and (L) DCs vs. HCs. EGF, epidermal growth factor; MMP-9, matrix metalloproteinase-9; CXCL8, C-X-C motif chemokine ligand 8; DCs, disease controls; HCs, healthy controls; ROC, receiver operating characteristic; AUC, area under the curve; IQR, interquartile range; CI, confidence interval.

Table II. Association of exosomal ENAH and SEPT9 with the clinical characteristics of patients with breast cancer.

Items	Exosomal ENAH expression[a]			Exosomal SEPT9 expression[a]		
	Median (IQR)	Z/X^2/ρ value	P-value	Median (IQR)	Z/X^2/ρ value	P-value
Age (years)		-0.454	0.650		-0.186	0.853
<60	0.102 (0.035-0.128)			0.758 (0.437-0.976)		
≥60	0.067 (0.001-0.133)			0.835 (0.232-1.395)		
Menopause		-0.756	0.450		-0.071	0.943
No	0.095 (0.006-0.126)			0.766 (0.437-1.079)		
Yes	0.111 (0.074-0.129)			0.785 (0.293-1.197)		
Histological type		2.790	0.425		3.353	0.340
Ductal carcinoma *in situ*	0.123 (0.104-NA)			1.197 (0.785-NA)		
Invasive ductal carcinoma	0.074 (0.001-0.129)			0.763 (0.325-1.117)		
Invasive lobular carcinoma	NA			NA		
Others	0.125 (0.031-0.139)			0.758 (0.682-0.818)		
Molecular subtypes		13.176	0.010		1.386	0.847
Triple-negative	0.067 (0.000-NA)			0.956 (0.763-NA)		
Luminal A	0.067 (0.001-0.085)			0.835 (0.607-1.256)		
HER2-negative luminal B	0.125 (0.113-0.144)			0.758 (0.380-0.926)		
HER2-positive luminal B	0.060 (0.001-0.103)			0.655 (0.335-0.976)		
HER2-enriched	0.134 (0.090-0.145)			0.616 (0.284-1.939)		
Hormone receptor status		-1.715	0.086		-0.226	0.821
ER negative and PR negative	0.131 (0.047-0.140)			0.817 (0.334-1.687)		
ER positive and/or PR positive	0.095 (0.001-0.123)			0.768 (0.429-0.979)		
HER2		-0.360	0.719		-0.621	0.535
Negative	0.110 (0.034-0.130)			0.785 (0.588-1.133)		
Positive	0.098 (0.017-0.129)			0.655 (0.317-1.201)		
Ki-67 (%)		-0.993	0.321		-0.248	0.804
<30	0.095 (0.023-0.127)			0.763 (0.429-1.149)		
≥30	0.118 (0.012-0.142)			0.802 (0.334-0.942)		
TNM stage		-0.088	0.639		0.007	0.970
0	0.114 (0.026-0.140)			0.991 (0.691-2.225)		
I	0.092 (0.025-0.121)			0.540 (0.214-0.898)		
II	0.095 (0.034-0.129)			0.768 (0.445-1.133)		
III	0.100 (0.001-NA)			0.763 (0.253-NA)		
IV	NA			NA		

[a]Exosomal ENAH and SEPT9 were detected among the first batch patients with breast cancer (n=31). ENAH, enabled homolog; SEPT9, septin 9; IQR, interquartile range; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; TNM, tumor-node-metastasis; NA, not applicable.

no differences were observed among the patients with breast cancer, DCs and HCs (both P>0.05; Fig. 2E and I). ROC curve analyses also revealed that exosomal MMP-9 and CXCL8 could not discriminate patients with breast cancer from DCs or HCs, or DCs from HCs (Fig. 2F-H and J-L).

Association of exosomal ENAH, SEPT9, EGF, MMP-9 and CXCL8 with the clinical characteristics of patients with breast cancer. Exosomal ENAH was differentially expressed among patients with different molecular subtypes of breast cancer (P=0.010). More specifically, its expression level was increased in patients with HER2-negative luminal B and HER2-enriched breast cancer and reduced in those with triple-negative, luminal A and HER2-positive luminal B breast cancer. Additionally, exosomal SEPT9 was not found to be associated with any of the clinical characteristics of patients with breast cancer (all P>0.05; Table II). Furthermore, the exosomal expression of MMP-9 was associated with a Ki-67 index of ≥30% (P=0.011), but not with other clinical characteristics of the patients with breast cancer (all P>0.05). Furthermore, the exosomal expression levels of EGF and CXCL8 were also not found to be associated with any of the clinical characteristics of the patients with breast cancer (all P>0.05; Table III).

Table III. Association of exosomal EGF, MMP-9 and CXCL8 with the clinical characteristics of patients with breast cancer.

Items	Exosomal EGF expression[a]			Exosomal MMP-9 expression[a]			Exosomal CXCL8 expression[a]		
	Median (IQR)	Z/X^2/ρ value	P-value	Median (IQR)	Z/X^2/ρ value	P-value	Median (IQR)	Z/X^2/ρ value	P-value
Age (years)		-0.623	0.533		-0.963	0.336		-0.623	0.533
<60	0.092 (0.003-0.254)			0.003 (0.002-0.003)			0.092 (0.003-0.230)		
≥60	0.144 (0.072-0.309)			0.002 (0.001-0.052)			0.186 (0.090-0.215)		
Menopause		-1.091	0.275		-1.334	0.182		-0.121	0.903
No	0.145 (0.019-0.304)			0.003 (0.002-0.008)			0.136 (0.003-0.217)		
Yes	0.073 (0.015-0.143)			0.002 (0.001-0.003)			0.114 (0.026-0.347)		
Histological type		0.181	0.913		3.299	0.192		5.000	0.082
Ductal carcinoma *in situ*	NA			NA			NA		
Invasive ductal carcinoma	0.092 (0.003-0.321)			0.003 (0.002-0.010)			0.102 (0.003-0.186)		
Invasive lobular carcinoma	0.144 (0.142-NA)			0.001 (0.001-NA)			0.218 (0.212-NA)		
Others	0.099 (0.053-NA)			0.003 (0.002-NA)			0.046 (0.000-NA)		
Molecular subtypes		3.287	0.349		2.592	0.459		1.522	0.677
Triple-negative	NA			NA			NA		
Luminal A	0.036 (0.003-NA)			0.006 (0.003-NA)			0.117 (0.048-NA)		
HER2-negative luminal B	0.098 (0.015-0.155)			0.002 (0.001-0.003)			0.172 (0.032-0.370)		
HER2-positive luminal B	0.145 (0.073-0.356)			0.003 (0.002-0.052)			0.179 (0.048-0.338)		
HER2-enriched	0.160 (0.047-0.353)			0.002 (0.001-0.066)			0.003 (0.002-0.166)		
Hormone receptor status		-1.076	0.282		-0.623	0.533		-1.190	0.234
ER negative and PR negative	0.160 (0.047-0.353)			0.002 (0.001-0.066)			0.003 (0.002-0.166)		
ER positive and/or PR positive	0.142 (0.003-0.159)			0.003 (0.002-0.003)			0.179 (0.048-0.218)		
HER2		-1.735	0.083		-0.325	0.745		-0.434	0.664
Negative	0.061 (0.002-0.146)			0.002 (0.001-0.005)			0.156 (0.036-0.269)		
Positive	0.153 (0.070-0.337)			0.003 (0.001-0.028)			0.097 (0.003-0.217)		
Ki-67 (%)		-0.736	0.462		-2.549	0.011		-0.736	0.462
<30	0.142 (0.053-0.159)			0.002 (0.001-0.003)			0.102 (0.002-0.212)		
≥30	0.254 (0.002-0.421)			0.003 (0.003-0.115)			0.179 (0.003-0.347)		
TNM stage		-0.248	0.354		0.013	0.962		-0.215	0.423
0	NA			NA			NA		
I	0.159 (0.003-NA)			0.002 (0.001-NA)			0.230 (0.002-NA)		
II	0.144 (0.036-0.320)			0.003 (0.002-0.055)			0.102 (0.026-0.182)		
III	0.142 (0.002-NA)			0.001 (0.001-NA)			0.218 (0.001-NA)		
IV	NA			NA			NA		

[a]Exosomal EGF, MMP-9 and CXCL8 were detected among the second batch of patients with breast cancer (n=16). EGF, epidermal growth factor; MMP-9, matrix metalloproteinase-9; CXCL8, C-X-C motif chemokine ligand 8; IQR, interquartile range; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; TNM, tumor-node-metastasis; NA, not applicable.

Discussion

Breast cancer screening is a currently focus of attention, since it can diagnose patients with breast cancer at an early stage of the disease, thus providing a satisfactory overall prognosis (6,26). Mammography is recommended for the screening of breast cancer in several countries. However, some subjects may be unwilling to undergo mammography due to concerns about radiation (27). Other screening modalities include ultrasound, magnetic resonance imaging and clinical breast examination. However, the above modalities may have one or more of the following limitations: Low sensitivity/specificity, increased cost and the potential influence of demographic characteristics including age and body weight on their effectiveness (28,29). Therefore, the exploration of novel screening modalities for breast cancer is of great importance. It has been recently reported that several RNAs and proteins exert a great ability in predicting the risk of breast cancer and, therefore, these molecules could be used in the early screening of breast cancer (30-32). Among these biomarkers, exosomes and their contents are of great interest. Due to the robust bilayer lipid membrane of exosomes, their contents are protected from the surrounding environment and can therefore provide accurate information on the tumor (33-35). Thus, exosomal contents could be considered as appropriate biomarkers for the early screening of breast cancer.

The dysregulation of ENAH, SEPT9, EGF, MMP-9 and CXCL8 in breast cancer tissues and/or cell lines is known to be of considerable importance. For example, a study used data from the ONCOMINE database to analyze the mRNA expression levels of ENAH in breast cancer tissues and the results showed that ENAH was upregulated in breast cancer tissues compared with normal tissues (36). Furthermore, another study revealed that a high level of SEPT9 methylation is present in breast cancer cell lines and tissues (37). Additionally, the dysregulation of EGF, MMP-9 and CXCL8 in breast cancer cell lines or tissues has also been previously reported (38-40). However, to the best of our knowledge, the expression levels of the aforementioned genes in exosomes isolated from patients with breast cancer have not been previously investigated. The present study demonstrated that exosomal ENAH and EGF were notably upregulated, while exosomal SEPT9 was downregulated in patients with breast cancer. This finding suggests that high levels of ENAH and EGF as well as reduced levels of SEPT9 could facilitate the growth of breast cancer cells (12,14,16). Furthermore, breast cancer cells may encapsulate these genes into exosomes and release them into the circulatory system. This assumption is consistent with the enhanced exosomal levels of ENAH and EGF, and the reduced levels of exosomal SEPT9 observed in the current study. In addition, ROC curve analysis showed that exosomal ENAH exhibited good capacity for discriminating patients with breast cancer from DCs and HCs, whereas exosomal SEPT9 and EGF each had an acceptable capacity for this discrimination. These findings indicate the potential of these exosomal biomarkers in the early screening of breast cancer. A previous study demonstrated that ENAH was elevated in pancreatic cancer tissues compared with tissues from patients with pancreatitis or normal subjects (41). Additionally, another study revealed that the methylation of SEPT9 was increased in colorectal cancer tissues (42). Regarding MMP-9, a previous study showed that it was aberrantly expressed in osteosarcoma tissues, in which its expression was higher than that in paracancerous tissues (43). Furthermore, CXCL8 has been found to be significantly upregulated in prostate cancer tissues (44).

The results of the present study demonstrated that the exosomal levels of ENAH were partially associated with HER2-negative luminal B and HER2-enriched breast cancer A possible explanation for this could be that exosomal ENAH showed a tendency to associate with estrogen receptor (ER)- and progesterone (PR)-negative breast cancer, as well as with a Ki-67 index of ≥30%, although this tendency did not reach statistical significance. Based on the expression of ER, PR, HER2 and Ki-67, breast cancer is classified in different molecular subtypes, namely HER2-negative luminal B breast cancer, characterized by a lack of expression of PR and upregulated expression of Ki-67, and HER2-enriched breast cancer, characterized by the lack of PR and ER expression. Therefore, exosomal ENAH showed a tendency to correlate with the aforementioned breast cancer subtypes. The results of the current study also showed that exosomal MMP-9 was associated with a Ki-67 index of ≥30%. This could be due to the expression of Ki-67 reflecting the proliferation ability of breast cancer cells (45). Additionally, MMP-9 promotes the proliferation of breast cancer cells (18); therefore, it was also associated with a Ki-67 index of ≥30%.

However, the present study has some limitations. Firstly, the sample size was relatively small. Therefore, the association of the expression levels of exosomal ENAH, SEPT9, EGF, MMP-9 and CXCL8 with the risk of breast cancer should be further investigated using a larger sample size. Secondly, a validation cohort is required to verify the diagnostic value of exosomal ENAH, SEPT9 and EGF in the early screening of breast cancer. Thirdly, due to the single-center study design, there may be regional bias. Fourthly, the expression levels of exosomal ENAH and SEPT9 were detected in one batch of patients, while those of EGF, MMP-9 and CXCL8 were detected in a different batch of patients. However, the comparison of baseline characteristics revealed that the demographic and disease features were comparable between the two batches of patients, thus suggesting that there were no major confounding factors. Although the treatment strategy varied between batches, all samples were collected prior to treatment, i.e., before surgery or neoadjuvant therapy if the patients were due to receive it. Therefore, different treatment approaches could not significantly affect the main findings of the present study. Fifthly, the current study lacked follow-up, and thus the association between the expression levels of the aforementioned exosomal genes and the prognosis of patients with breast cancer requires investigation in further studies. Finally, further studies are also required to evaluate the value of the expression of other exosomal genes for the prediction of breast cancer risk.

In conclusion, the results of the present study suggest that the expression levels of exosomal ENAH, SEPT9 and EGF in blood possess the potential to identify patients with breast cancer. However, this potential was not observed for MMP-9 and CXCL8, possibly due to the small sample size. The results also indicate that detection of the exosomal levels of ENAH, SEPT9 and EGF in the blood could improve the early screening

of breast cancer. However, further validation experiments are necessary. In addition, whether these exosomal genes could serve as potential indicators for the prognosis of breast cancer merits further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QZ and SW substantially contributed to the conception and the design of the study. ZZ, HW and YJ were responsible for the acquisition and analysis of the data. CC, JB and JH contributed to interpretation of the data. FT, LY and LZ contributed to data interpretation and manuscript drafting. QZ, LY and SW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by Ethics Committee of Huashan Hospital, Fudan University (Shanghai, China). Each patient or guardian of the patient who was <18 years old signed a written informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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