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Original Article

ENPP4 overexpression is associated with no recovery from Barrett's esophagus

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Abstract: Early diagnosis and treatment of precancerous conditions of the esophagus is important to improve overall survival. Barrett's esophagus is the most common precancerous condition of the esophagus, and patients with Barrett's esophagus may develop tumor, maintain a precancerous condition, or recover. We analyzed miRNA and mRNA expression profiles from esophageal adenocarcinoma tissue and normal esophageal tissue in GEO database. We identified DEGs and DE_miRNAs from GEO2R online tools and used Venn software were used to detect the common DEGs and DE_miRNAs. We used Enrichr, an online bioinformatic tool, to perform the gene ontology (GO) analysis including BP, MF, and CC. We analyzed Mirdb.tsv, mirtarbase.tsv, and targetscan.tsv files and identified miRNA targeting genes. We analysed the data of RNA sequencing expression retrieved from the GEPIA website on the basis of thousands of samples from the GTEx projects and TCGA. There were three miRNA (has-mir-205, has-mir-203, has-mir-18) and one DEG (ENPP4) that were associated with the recovery from Barrett's esophagus. ENPP4 promotes coagulation, hemostasis, wound healing, and participates in neutrophil degranulation, neutrophil immune activation and its mediated immunity, contributes to the composition of some membrane particles and tertiary particles, and is related to nucleotide diphosphatase activity. ENPP4 overexpression was not conducive to Barrett's esophagus recovery.

Keywords: Barrett's esophagus, precancerous lesion, bioinformatics analysis, ENPP4

Introduction

The past ten years have seen an increased survival rate of tumor and decreased morbidity and mortality rates, thanks to the promotion of tertiary cancer prevention and advancement of early diagnosis and treatment [1]. Accurate diagnosis and appropriate treatment at an early stage of cancer or even at precancerous stages are the best ways to increase the likelihood of survival [2-5]. Esophageal cancer, especially precancerous lesions of the esophagus, benefit the most from early diagnosis and treatment among different kinds of tumor [5]. Barrett's esophagus (BE) is the most common precancerous condition of the esophagus, and is significantly associated with the development of adenocarcinoma, especially at the gastroesophageal junction where normal stratified squamous epithelium cells get replaced by columnar epithelium cells. However, BE can return to normal, maintain the condition, or progress to intraepithelial neoplasia as other precancerous conditions, and less than 5% of the patients develop tumor eventually [6, 7]. Therefore, regularly monitoring BE patients' prognosis and studying the molecular mechanisms of esophageal precancerous conditions have become the keys to improve the survival of patients with esophageal cancer.

Ectonucleotide pyrophosphatase-phosphodiesterases 4 (ENPP4) is an upregulated protein expressed on the Bacillus Calmette-Guerin-activated macrophages' surface [8, 9]. ENPP4 consists of a single catalytic domain. Isoform ENPP4 is a type-1 transmembrane protein with a short intracellular C-terminus and a small extracellular region that contains only a phosphodiesterase motif [10]. Although its func-

tions are not yet well understood, the related physiologic and pathologic roles of ENPP family members, including their regulation of extracellular pyrophosphate levels, cell motility, migration, angiogenesis, and tumor cell invasion, have recently become the focus of intense research [11-14].

Previous studies have suggested that esophageal inflammatory diseases, especially reflux esophagitis, cause an increase in inflammatory mediators that damage the esophageal mucosa. Normal squamous epithelium cells get replaced by columnar epithelium cells and patients progress to BE [15]. Sustained inflammation causes abnormal expression of miRNA and mRNA, which affects the synthesis of some important tumor-related molecules, further leads to the abnormal activation or inhibition of cell signal transduction pathways, and eventually progresses to tumor [15-17]. However, this does not fully explain the prognosis of patients who have BE but not esophagitis, and researchers claim that BE is caused by columnar epithelium cellsthat are not completely replaced with squamous epithelium cells during human embryonic development [18]. There still has no consensus on the origin of BE and the molecular mechanism of its development. Recent studies have shown that miRNA and its related gene expression changes may play an important role in the occurrence and development of the precancerous conditions of the esophagus including BE, and it's important to assess the level of risk and predict the trend of development [19, 20].

In the current analysis, we analyzed data of monitoring BE. We obtained differential miRNAs and differential genes and considered them to be associated with BE development. We first analyzed the miRNA datasets (GSE20099 and GSE24839) and obtained the differential miRNA (DE_miRNA), then put the DE_miRNA into the dataset miRDB, miRTarbase, and Targetscan for comparison to locate the target differential gene (Target_mRNA). Afterwards, we analyzed gene dataset (GSE36223 and GSE39491) for differential genes (DEGs). Finally, we intersected the Target_mRNA with DEGs, and identified the core gene ENPP4. We also verified ENPP4 in the GSE26886 dataset. This bioinformatics research provided useful biomarkers for BE patients.

Methods

Microarray data information

We obtained the miRNA expression profile of GSE20099 and GSE24839 and gene expression profile of GSE36223, GSE39491, and GSE26886 from precancerous lesions and normal esophagus tissues from NCBI-GEO public database. Non-coding RNA data of GSE20099 and GSE24839 were all on account of GLP8871 OSU_CCC v4.0 [condensed version] which included 14 normal tissues and 26 precancerous lesion tissues, 10 normal tissues and 20 precancerous lesion tissues, respectively. Microarray data of GSE36223 and GSE39491 were all on account of GLP571 [HG-U133A_2] Affymetrix Human Genome U133A 2.0 Array which included 23 normal tissues and 23 precancerous lesion tissues, 40 normal tissues, and 40 precancerous lesion tissues, respectively. GSE2886 data were from GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array, including 20 normal tissues and 19 precancerous lesion tissues.

Data processing of DEGs and DE_miRNA

DEGs and DE_miRNAs between precancerous lesion specimen and normal esophagus specimen were identified by GEO2R online tools [21] with $|\log FC| > 2$ and adjusted P value < 0.01 in DEGs and $|\log FC| > 1$ and adjusted P value < 0.05 in DE_miRNAs. Then, the raw data in TXT format were checked in Venn software online (http://bioinformatics.psb.ugent.be/webtools/Venn/) to detect the common DEGs or DE_miRNAs among these datasets.

Gene ontology enrichment analysis

Gene ontology analysis (GO), including biological processes (BP), molecular function (MF), and cellular component (CC), is a commonly used approach for defining genes and their RNA or protein product to identify unique biologic properties of high throughput transcriptome or genome data [22]. We used Enrichr, an online bioinformatic tool designed to identify a large number of genes or proteins function, [23] to visualize DEG enrichment of BP, MF, and CC ($P < 0.05$).

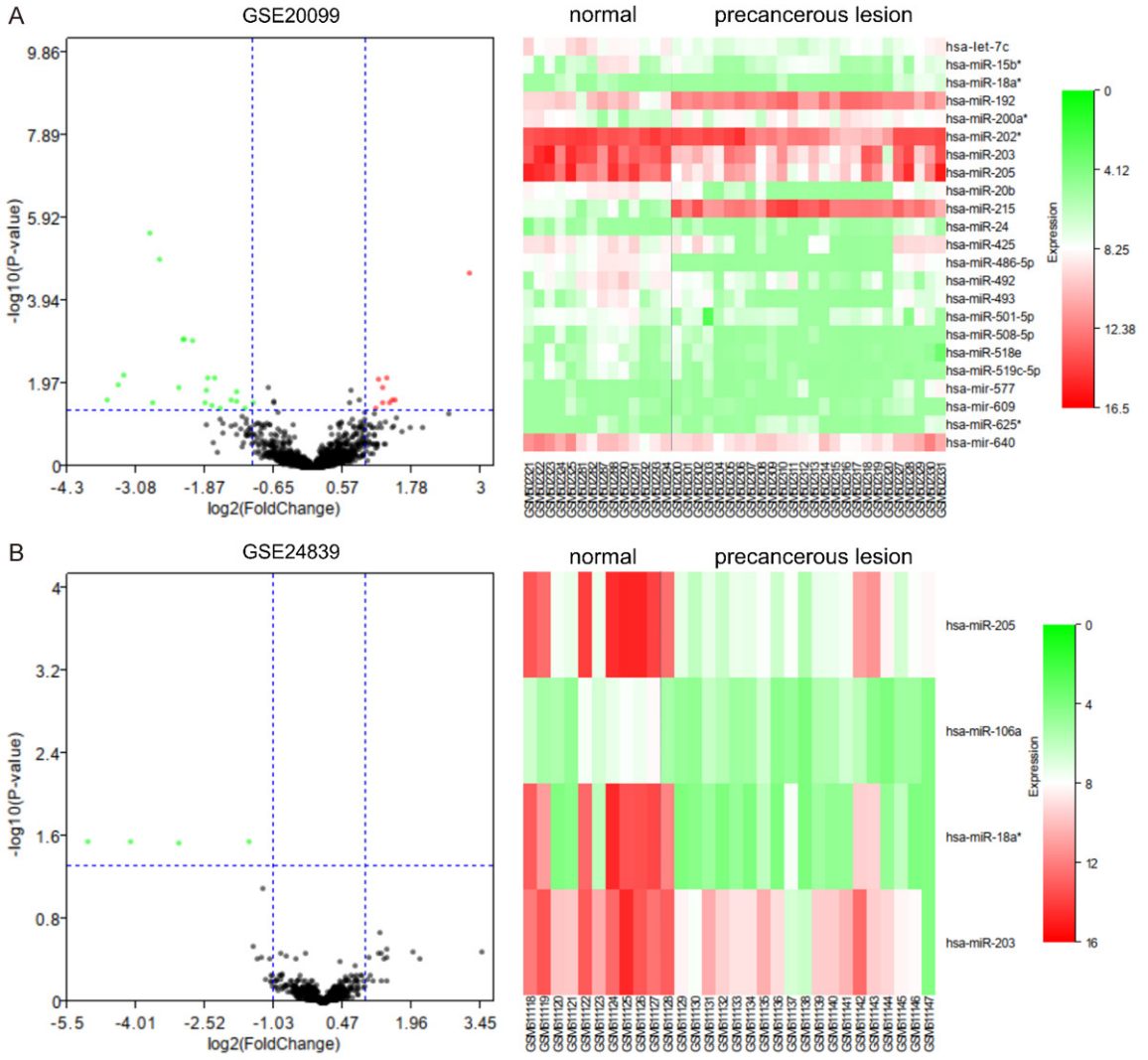

Figure 1. DE_miRNAs are showed in Volcano plot (left) and heatmap (right), Red node stands for upregulated miRNAs and green node stands for downregulated miRNAs, (A) GSE20099, (B) GSE24839.

Target_mRNA

We obtained the miRNAs for hsa-mir-18a, hsa-mir-203, hsa-miR-205 in circinteractiveome database (https://circ interactive home.nia.nih.gov/bin/mirnasearch), and constructed the miRNA-txt file. We downloaded mirdb.tsv, mirtarbase.tsv and targetscan.tsv, and identified miRNA targeting genes.

Gene expression profiling interactive analysis (GEPIA) analysis

To validate these DEGs, we applied the GEPIA website (http://gepia.cancer-pku.cn/) to analyze the data of RNA sequencing expression on the basis of thousands of samples from the GTEx projects and TCGA [24].

Statistical and bioinformatic analyses

The miRNAs and DEGs that were expressed differently in the different esophageal lesions were identified using a random-variance t-test, which is an improvement over the standard separate t-test, because it enables information on within-class variation to be shared among genes without assuming that all genes have the same variance.

Results

Identification of miRNAs in BE

We analyzed 14 patients who recovered to normal in the GSE20099 dataset and 26 patients

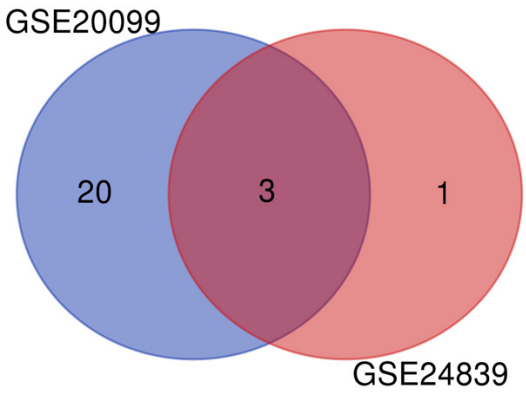

Figure 2. Authentication of 3 common DE_miRNAs in the two datasets (GSE20099 and GSE24839) through Venn diagram software. (Available online: http://bioinformatics.psb.ugent.be/webtools/Venn/). Different color means different datasets.

Table 1. DE_miRNA expression

Names	Total	Elements
Intersection	3	hsa-mir-18a*
		hsa-mir-203
		hsa-mir-205
GSE20099	20	hsa-mir-519c-5p
		hsa-mir-200a*
		hsa-let-7c
		hsa-mir-518e
		hsa-mir-192
		hsa-mir-486-5p
		hsa-mir-202*
		hsa-mir-609
		hsa-mir-501-5p
		hsa-mir-508-5p
		hsa-mir-577
		hsa-mir-15b*
		hsa-mir-215
		hsa-mir-24
		hsa-mir-493
		hsa-mir-492
		hsa-mir-425
		hsa-mir-625*
		hsa-mir-640
		hsa-mir-20b
GSE24839	1	hsa-mir-106a

who still had not recovered from precancerous conditions but did not progress to tumor, and found 23 DE_miRNAs (**Figure 1A**). Of the BE patients retrieved from the GSE24839 dataset, 10 recovered to normal and 20 remained in precancerous conditions and did not progress to tumor. We analyzed four DE_miRNAs (**Figure 1B**) and we used a Venn diagram online generator to analyze these two datasets and obtained a total of three DE_miRNAs (**Figure 2** and **Table 1**), -1 < logFC < 1, FDR = 0.05.

Identification of Target_mRNAs from DE_miRNA

We compared the DE_miRNA with the mRNAs related to DE_miRNA in the three datasets of miRDB, miRTarbase, and Targetscan, and found 48 target differential genes (Target_mRNA) (**Table 2**).

Identification of DEGs (DE_mRNA) in BE

We analyzed the GSE39491 dataset which included 40 normal tissues and 40 precancerous lesion tissues, and the GSE36223 dataset which included 23 normal tissues and 23 precancerous lesion tissues. Using GEO2R online tools and Venn diagram online software, we obtained 413 and 1431 DEGs respectively, of which 399 DEGs were the same (**Figures 3**, **4**).

Identification of core DEG-ENPP4

We used Venn diagram online generator to analyze the 399 Target_mRNAs and 48 DEGs, and finally obtained one core differential gene: ENPP4 (**Figure 5**). In addition, we analyzed differential genes in 19 normal esophageal mucosa samples and 20 precancerous lesion samples in the GSE26886 dataset, and the results showed an ENPP4 overexpression in precancerous lesions (**Figure 6** and **Table 3**). We used GEPIA to examine the level of ENPP4 gene expression in esophageal adenocarcinoma tissue and normal esophageal tissue (**Figure 7**), and the results showed that ENPP4 expression was higher in esophageal adenocarcinoma tissue.

Gene ontology (GO) analysis of ENPP4

We used Enrichr software to conduct the gene ontology (GO) analysis for ENPP4 and the results showed that during BP, ENPP4 promoted coagulation, hemostasis, wound healing, and participated in neutrophil degranulation, neutrophil immune activation and its mediated

Table 2. DE_miRNA targeting genes

miRNA	mRNA
has-mir-205 has-mir-203 has-mir-18a*	ACSL1 ACSL4 AMOT ARPP19 B4GALT6 DDX5 DMXL2 DYNLT1 ENPP4 ESRRG ETNK1 EZR GLO1 ICK KATNAL1 LAMC1 LCOR LPCAT1 LRP1 LRRK2 LUZP2 LYSMD3 MANEA MARCKS MED1 MMD NDUFA4 NFAT5 NIN NOTCH2 NPTN NUP54 PDLIM5 PHC2 PRKCE PTPRJ PTPRM RAB11FIP1 RFX7 RPP14 RTN3 SESN3 SHISA6 SQLE STK38L TROVE2 VEGFA ZEB1

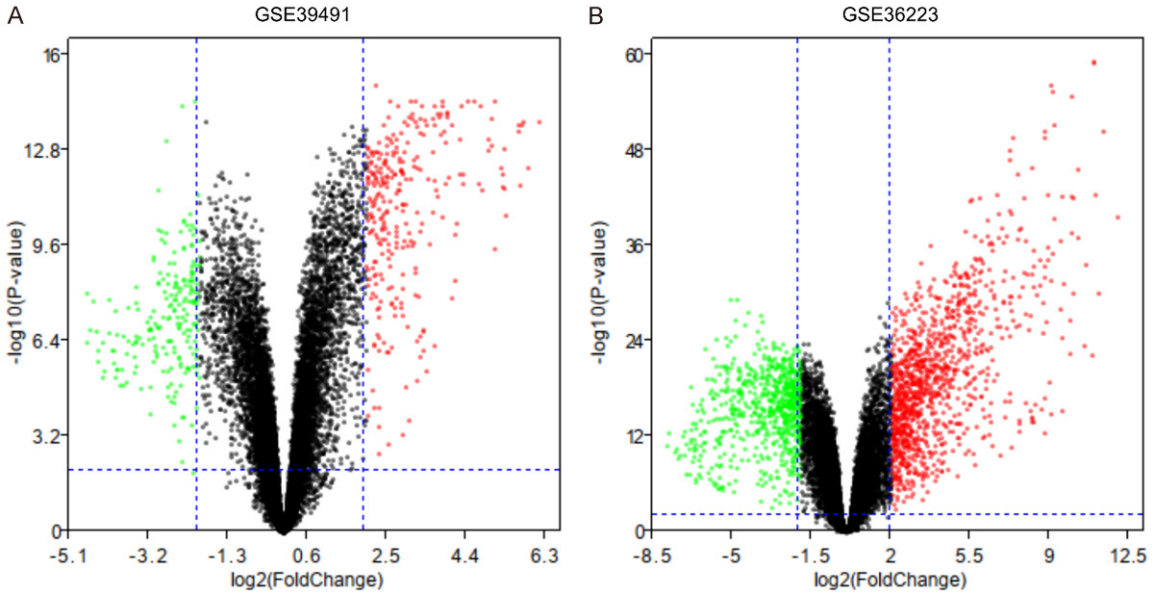

Figure 3. DEGs are shown in Volcano plot. Red node stands for upregulated miRNAs and green node stands for downregulated miRNAs, (A) GSE39491, (B) GSE26223.

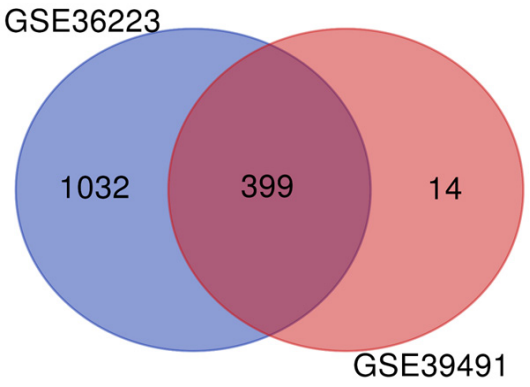

Figure 4. Authentication of 399 common DEGs in the two datasets (GSE39491 and GSE26223) through Venn diagram software (available online: http://bioinformatics.psb.ugent.be/webtools/Venn/). Different colors mean different datasets.

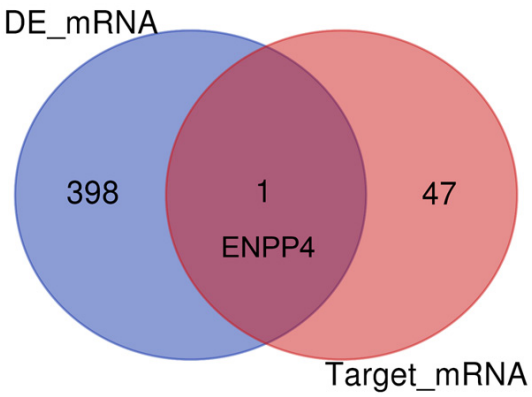

Figure 5. Screen of candidate genes. The intersection of target genes of DE_miRNAs and DEGs.

immunity. In CC, ENPP4 contributed to the composition of some membrane particles and tertiary particles; for MF, ENPP4 was related to nucleotide diphosphatase activity (**Table 4**).

Discussion

Barrett esophagus () is a precancerous condition of the esophagus and is closely related to the incidence of esophageal adenocarcinoma, therefore it needs regular monitoring [25]. Some patients with BE progress to esophageal

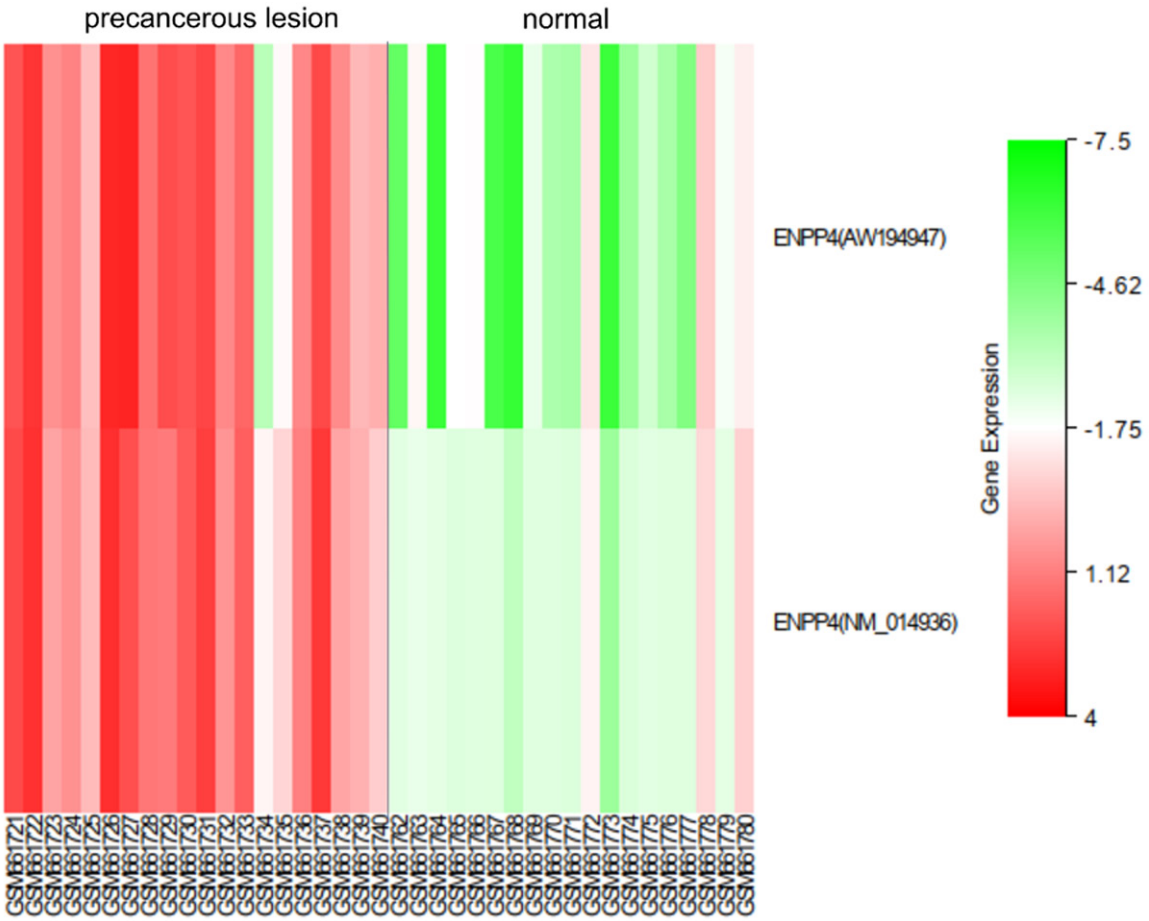

Figure 6. Heatmap shows ENPP4 in GSE26886.

Table 3. Enpp4 verified in GSE26886

miRNA	mRNA
has-mir-205 has-mir-203 has-mir-18a*	ACSL1 ACSL4 AMOT ARPP19 B4GALT6 DDX5 DMXL2 DYNLT1 ENPP4 ESRRG ETNK1 EZR GLO1 ICK KATNAL1 LAMC1 LCOR LPCAT1 LRP1 LRRK2 LUZP2 LYSMD3 MANEA MARCKS MED1 MMD NDUFA4 NFAT5 NIN NOTCH2 NPTN NUP54 PDLIM5 PHC2 PRKCE PTPRJ PTPRM RAB11FIP1 RFX7 RPP14 RTN3 SESN3 SHISA6 SQLE STK38L TROVE2 VEGFA ZEB1

cancer and others maintain the precancerous condition or recover [7]. Recent studies indicated the important role of miRNA during each stage of BE development [26]. Unlike most other mRNA molecules, miRNAs survive long in vivo and remain stable in vitro, therefore the analysis of its expression profile is more reproducible [27, 28]. Most studies on miRNA and BE focused on the malignant transformation of BE and its relationship with tumor [28-34] and few have studied factors influencing the recovery of BE patients.

We used bioinformatics analysis and identified molecular markers that affected BE recovery. Using sequencing data from the GEO public database, we found that down-regulation of has-mir-205, has-mir-203, and has-mir-18 were related to BE recovery. Previous studies have reported in breast cancer patients, that has-mir-205 could inhibit breast cancer stem cells and VEGF thus influencing its invasion and metastasis [35]. Additional studies on glioma [36], colon cancer [37], prostate cancer [38], and gastric cancer [39] indicated its role in

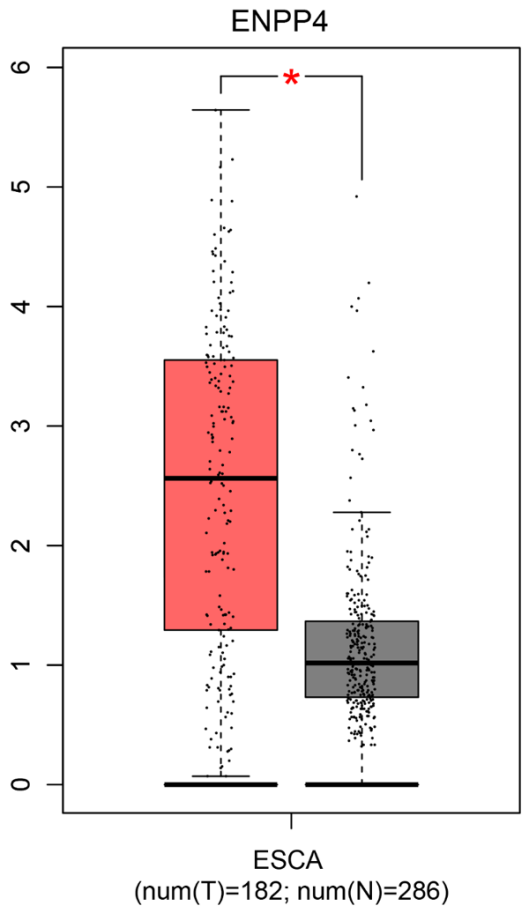

Figure 7. ENPP4 in esophageal adenocarcinoma cancer tissues (which are related to poor prognosis) compared to normal esophageal tissues, analyzed by GEPIA website, (*P < 0.05). Red color means tumor tissues and grey color means normal tissues.

DNA repair-related gene expression and inhibition of epithelial-mesenchymal transition. Has-mir-203 directly affects the expression of the p63 gene and helps to maintain the stability of squamous cells in different tissues, while its underexpression is associated with the loss of native squamous phenotype and emerging columnar morphology [40, 41]. Has-mir-18 plays an important role in promoting apoptosis [42], controlling inflammation [42, 43] and restoring glucocorticoid receptor gene expression [44]. In our study, we found an underexpression of these three miRNAs in BE patients who had not recovered when compared with those who had recovered, excluding BE patients who already developed tumor.

ENPP4 (ectonucleotide pyrophosphatase-phosphodiesterases 4) is reported to be associated with BCG-mediated activation of tumoricidal macrophage protein [45]. There have been few studies on ENNPP4 and its tumor-related biologic process. One bioinformatics analysis comparing ceRNA between smokers versus non-smokers' lung squamous cell carcinomas indicated that ENPPT was one prognostic biomarkers in smokers [46]. We aim to focus on ENPP4 in the future and intend to conduct in vivo and in vitro experiments to explore its molecular mechanism and its impact on the development of precancerous conditions of the esophagus including BE.

In conclusion, this study revealed that ENPP4 overexpression may not be conducive to Barrett's esophagus recovery, through the analysis of DE_miRNA and DEGs. ENNP4 might be a biomarker for BE patients' prognosis and contribute to understanding BE pathogenesis and relevant clinical decision-making.

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Disclosure of conflict of interest

None.

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Table 4. ENPP4 gene ontology analysis of differentially expressed genes

	Term	*P*-value	Odds Ratio	Combined Score
GOTERM_BP_DIRECT	positive regulation of coagulation (GO: 0050820)	4.50E-04	2222.2222	17125.09176
GOTERM_BP_DIRECT	positive regulation of hemostasis (GO: 1900048)	4.50E-04	2222.2222	17125.09176
GOTERM_BP_DIRECT	positive regulation of blood coagulation (GO: 0030194)	9.50E-04	1052.6316	7325.338596
GOTERM_BP_DIRECT	positive regulation of wound healing (GO: 0090303)	0.0013	769.23077	5111.85544
GOTERM_BP_DIRECT	regulation of blood coagulation (GO: 0030193)	0.0015	666.66667	4334.873554
GOTERM_BP_DIRECT	neutrophil degranulation (GO: 0043312)	0.0239498	41.753653	155.8160165
GOTERM_BP_DIRECT	neutrophil activation involved in immune response (GO: 0002283)	0.0241498	41.407867	154.1812633
GOTERM_BP_DIRECT	neutrophil mediated immunity (GO: 0002446)	0.0243498	41.067762	152.5761812
GOTERM_CC_DIRECT	ficolin-1-rich granule membrane (GO: 0101003)	0.00305	327.86885	1899.222849
GOTERM_CC_DIRECT	tertiary granule (GO: 0070820)	0.0081999	121.95122	585.8087961
GOTERM_CC_DIRECT	ficolin-1-rich granule (GO: 0101002)	0.0091999	108.69565	509.6263345
GOTERM_MF_DIRECT	nucleotide diphosphatase activity (GO: 0004551)	5.50E-04	1818.1818	13646.58026

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