Diagnostic and prognostic value of the BEX family in lung adenocarcinoma

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Abstract. Previous studies have demonstrated that members of the brain-expressed X-linked (BEX) family participate in a wide range of biological functions in normal and tumor tissues. However, their role and clinical significance in lung adenocarcinoma (LUAD) remains unclear. The present study investigated The Cancer Genome Atlas data and revealed that the BEX family was downregulated in LUAD tissues compared with adjacent non-cancerous tissues. Additionally, analysis of LUAD cohorts from the Oncomine database revealed similar results. Furthermore, the expression of BEX members was significantly decreased in several LUAD cell lines compared with normal lung epithelial cells *in vitro*. The aforementioned data mining and *in vitro* results suggested that the BEX family may be involved in the development of LUAD. Furthermore, receiver operating characteristic curve analysis revealed that BEX members exhibited high sensitivity and specificity for the diagnosis of patients with LUAD. The low expression levels of BEX1, BEX4 and BEX5 were associated with certain pathologic features, particularly in advanced LUAD. Survival analysis demonstrated that BEX members, particularly BEX4, were involved in the prognosis of patients with LUAD at early and late clinical stages. The results obtained in the current study suggested that BEX members may serve as potential tumor biomarkers for the diagnosis and prognosis of patients with LUAD.

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Abbreviations: BEX, brain-expressed X-linked; LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; ROC, receiver operating characteristic; AUC, area under the curve; AJCC, American Joint Committee on Cancer; OS, overall survival; CNV, copy number variations

Key words: brain-expressed X-linked family, lung adenocarcinoma, tumor biomarker, prognosis, diagnosis

Introduction

Lung cancer has emerged as the most common malignant tumor and accounts for ~25% of cancer-associated mortalities worldwide according to the latest cancer statistics report (1). The main pathological types of lung cancer include small cell lung cancer and non-small cell lung cancer, the latter of which predominantly comprising of lung adenocarcinoma (LUAD) cases (2). Despite advances in diagnostic tools, surgical approaches and chemotherapy, the 5-year survival rate of patients with lung cancer is reported to be <10%, potentially due to the lack of effective biomarkers (3,4). Therefore, the identification of novel and reliable diagnostic and prognostic biomarkers is required.

The human brain-expressed X-linked (BEX) family consists of five members, BEX1-5, located on the Xq22 chromosome (5). Members of the BEX family are widely expressed in several types of tissues, and are closely associated with the development of neurons (6). Previous studies have demonstrated that BEX members play important roles in regulating important cellular processes, including the cell cycle and apoptosis (7-9). Increasing evidence revealed that BEX members are aberrantly expressed in different types of cancer, suggesting that they may serve roles in tumorigenesis (10-22). BEX1 is implicated in leukemogenesis, and aberrant down-regulation of BEX1 has been observed in acute and chronic myeloid leukemia (10,11). Furthermore, BEX1 is associated with the development of other types of cancer, including pediatric intracranial ependymoma and esophageal squamous cell cancer (12,13). BEX2 may serve as an oncogene in different types of cancer, including glioma, breast and colorectal cancer (14-16). By contrast, BEX3 inhibits the formation of breast tumors and serves a pro-apoptotic role (17,18). Previous studies revealed that BEX4 serves as a tumor suppressor in ovarian cancer and as an oncogene in oral squamous cell carcinoma (19,20). To the best of the authors' knowledge, the role of BEX5 in cancer remains unreported. The aforementioned studies highlighted an association between the BEX family and various types of cancer. However, few studies have investigated the association between the BEX family and

lung cancer. A previous study reported that high expression levels of BEX2 and BEX4 were significantly associated with a favorable prognosis in patients with LUAD (21). Furthermore, overexpression of BEX4 may promote LUAD cell proliferation and growth *in vitro* (22). The potential significance of the BEX family in LUAD therefore warrants further investigation.

The present study investigated the specific expression patterns of BEX members as well as their clinical significance in LUAD by mining databases and performing *in vitro* experiments. The results obtained in the present study revealed that the BEX family was downregulated in LUAD samples and tumor cell lines compared with normal samples and human bronchial epithelial cells, respectively, and may be involved in LUAD pathogenesis. The association between the expression levels of BEX members and the clinicopathological parameters of patients with LUAD was therefore further investigated and the diagnostic and prognostic values of the BEX family in patients with LUAD were assessed.

Materials and methods

Data mining. Publicly available LUAD gene expression data and the corresponding The Cancer Genome Atlas (TCGA; cancergenome.nih.gov/) clinical data were obtained from the University of California Santa Cruz Xena repository (xenabrowser.net). The expression data was obtained from the files entitled 'TCGA_LUAD_sampleMap/HiSeqV2/PANCAN'. Additionally, the clinical phenotype information was selected from 'TCGA_LUAD_sampleMap/LUAD_clinicalMatrix'. Based on the criteria, the gene expression data from the TCGA was transformed by log2(x+1) and normalized across all cohorts, then extracted and matched with the clinical data as reported in (23).

A total of 574 cases (515 LUAD samples and 59 normal samples from heathy controls) with BEX family expression data and 482 LUAD samples with gene expression data and clinicopathological information were included in the current study. In order to investigate the expression levels of BEX members in clinical LUAD samples, a cohort of 59 pairs of LUAD and adjacent non-cancerous tissues were taken from the 574 LUAD cases. The cancer samples were staged according to the American Joint Committee on Cancer staging system (24).

The mRNA expression of BEX members in LUAD was investigated using the Oncomine database (oncomine.org), a gene expression array database used for the analysis of the transcription levels of genes in several types of cancer. The datasets used in the present study included Garber Lung Statistics (25), Okayama Lung Statistics (26), Bhattacharjee Lung Statistics (27), Hou Lung Statistics (28), Selamat Lung Statistics (29) and Landi Lung Statistics (30). $P<0.01$ and a 1.5-fold change was set as the threshold. Genes in the present study were not limited by their rank. The final datasets collected are presented in Table I.

Data on the methylation status of BEX family was downloaded from MethHC (methhc.mbc.nctu.edu.tw/), a database of DNA methylation status and gene expression in human cancer. Data on 464 tissue samples, 435 LUAD tissues and 29 normal tissues from healthy controls, with methylation values were obtained.

Mutational and copy number variations (CNVs) analyses of the BEX family members in LUAD samples were performed using cBioPortal for Cancer Genomics (cbioportal.org/) as described previously (31). Data from the following studies were included: Lung Adenocarcinoma (Broad, Cell 2012) (32), Lung Adenocarcinoma (TCGA, Nature 2014) (33), Lung Adenocarcinoma (TCGA, Provisional) and Lung Adenocarcinoma (TCGA, PanCancer Atlas) (34).

Cell lines and culture. Human bronchial epithelial (HBE) cells and the lung cancer cell lines H520, H1975, H358, H460, A549, 95D and SPC-A-1 were obtained from the Cell Bank of the Chinese Academy of Science. HBE cells were maintained in DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and the lung cancer cell lines were cultured in RPMI-1640 medium (HyClone) supplemented with 10% FBS. All cells were maintained at 37˚C with 5% CO_2.

RNA extraction and reverse-transcription quantitative polymerase chain reaction (RT-qPCR). RNA extraction and RT-qPCR were performed as previously described (35,36). The primers used in the present study are presented in Table SI.

Receiver operating characteristic (ROC) curve analysis. The diagnostic value of the expression levels of the BEX family in LUAD was investigated by analyzing the expression data from 515 LUAD and 59 normal tissues. Specificity and sensitivity were plotted on the x- and y-axes, respectively. The area under curve (AUC) was calculated to assess the ability of the expression levels of BEX members to predict the outcome of patients with LUAD.

Survival analysis. The association between expression of the BEX family members and the overall survival (OS) of patients with LUAD was assessed using a log-rank test and Kaplan-Meier analysis. The median expression value of each gene was used as the cut-off value to divide patients into high- and low-expression groups. The multivariate Cox regression model was applied for prognostic analysis.

Statistical analysis. A two-tailed paired t-test with a Bonferroni's correction was used to analyze the gene expression differences in tumor and adjacent non-cancerous tissues. The Mann Whitney U test was used to compare tumor samples and unpaired normal control samples. The associations between the expression levels of BEX members and patient clinical characteristics were determined using a χ^2 test. One-way ANOVA followed by the Dunnett's multiple comparisons test was used to analyze the expression level of BEX members in HBE cells compared with lung cancer cell lines. SPSS software (version 20; IBM Corp.) was used to perform all the statistical analysis. $P<0.05$ was used to indicate a statistically significant difference.

Results

Expression level of BEX members is significantly downregulated in LUAD samples and tumor cell lines. The expression level of each BEX member was significantly downregulated

Table I. Changes in the BEX family transcription levels between lung adenocarcinoma and normal tissues obtained from the ONCOMINE database.

A, BEX1

Reporter	Fold change	P-value	t-test	Oncomine dataset
218332_at	-3.869	$1.53x10^{-11}$	-10.401	Okayama lung statistics
IMAGE:341706	-1.502	0.003	-3.193	Garber lung statistics
ILMN_2234697	-1.941	$1.22x10^{-10}$	-7.024	Selamat lung statistics

B, BEX2

Reporter	Fold change	P-value	t-test	Oncomine dataset
224367_at	-1.723	$2.39x10^{-4}$	-3.727	Hou lung statistics

C, BEX3

Reporter	Fold change	P-value	t-test	Oncomine dataset
ILMN_1729208	-1.508	$2.02x10^{-5}$	-4.276	Selamat lung statistics

D, BEX4

Reporter	Fold change	P-value	t-test	Oncomine dataset
40916_at	-3.440	$4.62x10^{-5}$	-4.828	Bhattacharjee lung statistics
ILMN_2351638	-2.339	$3.68x10^{-17}$	-9.910	Selamat lung statistics
215440_s_at	-1.756	$1.55x10^{-9}$	-6.562	Landi lung statistics
215440_s_at	-1.960	$4.20x10^{-7}$	-5.594	Hou lung statistics

E, BEX5

Reporter	Fold change	P-value	t-test	Oncomine dataset
ILMN_1806473	-1.624	$1.82x10^{-11}$	-7.327	Selamat lung statistics
229963_at	-1.868	$1.33x10^{-4}$	-3.885	Hou lung statistics

Threshold, P<0.01, fold change 1.5 and under-expression.

in 59 LUAD tissues compared with the paired adjacent non-cancerous tissues (P<0.01; Fig. 1A-E). Similarly, the expression level of BEX members was significantly downregulated in the remaining 515 tumor samples compared with the unpaired normal control samples (P<0.001; Fig. S1A-E). As presented in Table I, the expression results were successfully validated in independent cohorts from the Oncomine database. RT-qPCR revealed that the mRNA levels of BEX members were significantly decreased in the majority of the tumor cell lines, particularly in H1975, H358, SPC-A-1 and H520, compared with the HBE cells (Fig. 2A-E).

ROC analysis of BEX expression in patients with LUAD. Since the BEX family was significantly downregulated in LUAD samples compared with controls, the current study explored whether BEX members may serve as potential diagnostic biomarkers in LUAD. ROC curves and AUC analysis were performed to evaluate the overall prognostic performance. BEX1 [AUC, 0.866; 95% CI (confidence interval), 0.837-0.895], BEX2 (AUC, 0.629; 95% CI, 0.584-0.674), BEX3 (AUC, 0.732; 95% CI, 0.690-0.773), BEX4 (AUC, 0.886; 95% CI, 0.859-0.912) and BEX5 (AUC, 0.820; 95% CI, 0.779-0.860) had high sensitivity and specificity, suggesting that the BEX family may have diagnostic value in patients with LUAD (Fig. 3A-E).

Associations between the expression of the BEX family and clinicopathological characteristics of patients with LUAD. The associations between the expression levels of BEX members and the clinicopathological characteristics of 482 patients with LUAD were investigated. Low expression levels of BEX4 and BEX5 were significantly associated with an advanced American

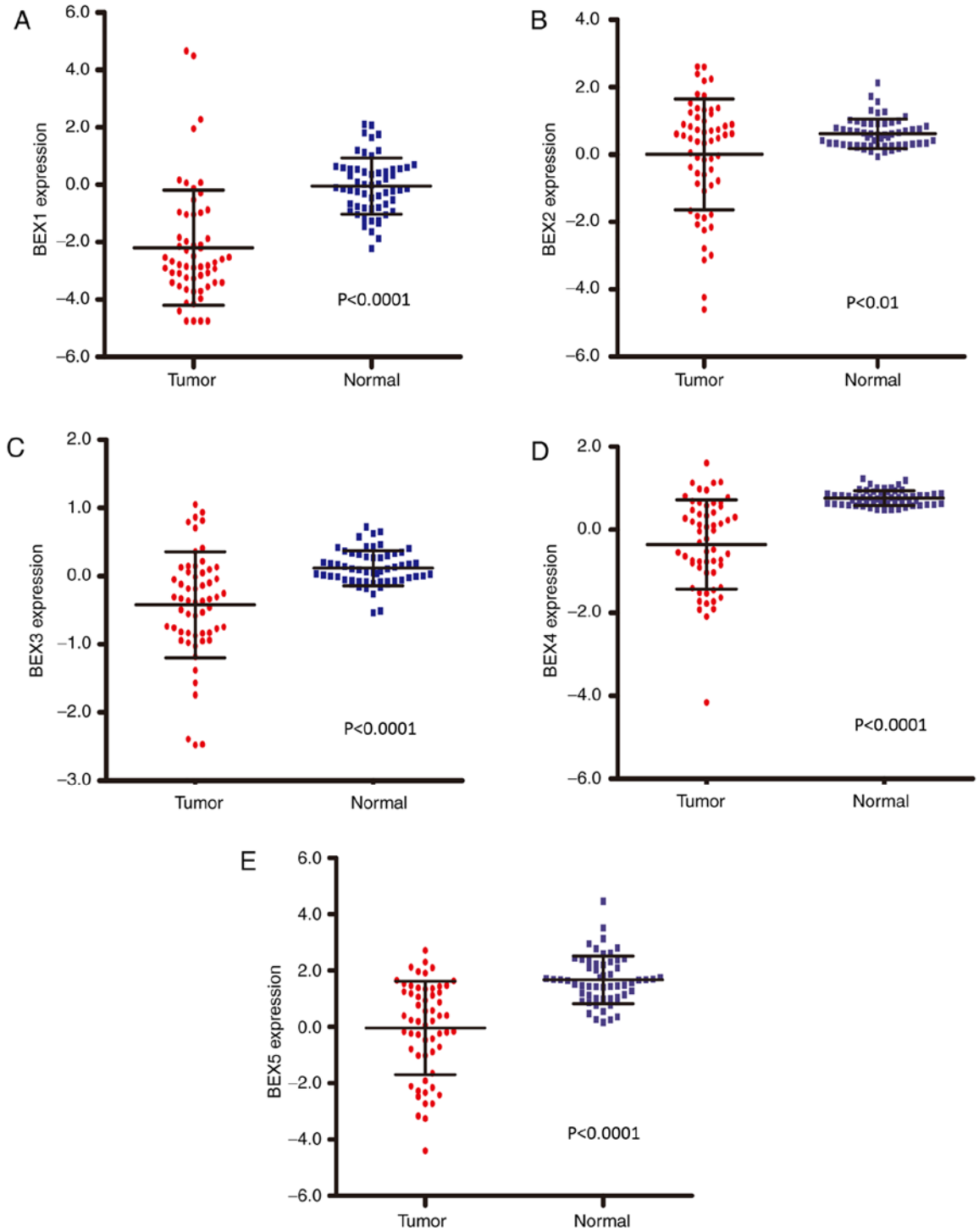

Figure 1. Expression of (A) BEX1, (B) BEX2, (C) BEX3, (D) BEX4 and (E) BEX5 was significantly downregulated in lung adenocarcinoma compared with healthy adjacent non-cancerous samples obtained from The Cancer Genome Atlas. Data were analyzed using the two-tailed paired t-test, with BEX, brain-expressed X-linked.

Joint Committee on Cancer (AJCC) clinical stage (P=0.004 and P=0.027, respectively; Tables II and III, respectively) and advanced pathological T stage (P=0.002 and P=0.003, respectively; Tables II and III, respectively). Lower expression level of BEX4 was significantly associated with pathological N stage (P=0.033, Table II). Decreased mRNA levels of BEX1 was significantly associated with advanced pathological T stage (P=0.023; Tables SII). The expression level of BEX2 and BEX4 was strongly associated with sex (P=0.003; P=0.005, respectively; Tables SIII and II, respectively). The expression level of BEX4 was significantly associated with age (P=0.019; Table II). There was no significant association between the expression of BEX3 and any clinicopathological characteristic in patients with LUAD (Table SIV).

Prognostic value of the BEX family in patients with LUAD. The Kaplan-Meier method was used to assess the prognostic significance of the BEX family in patients with LUAD in the

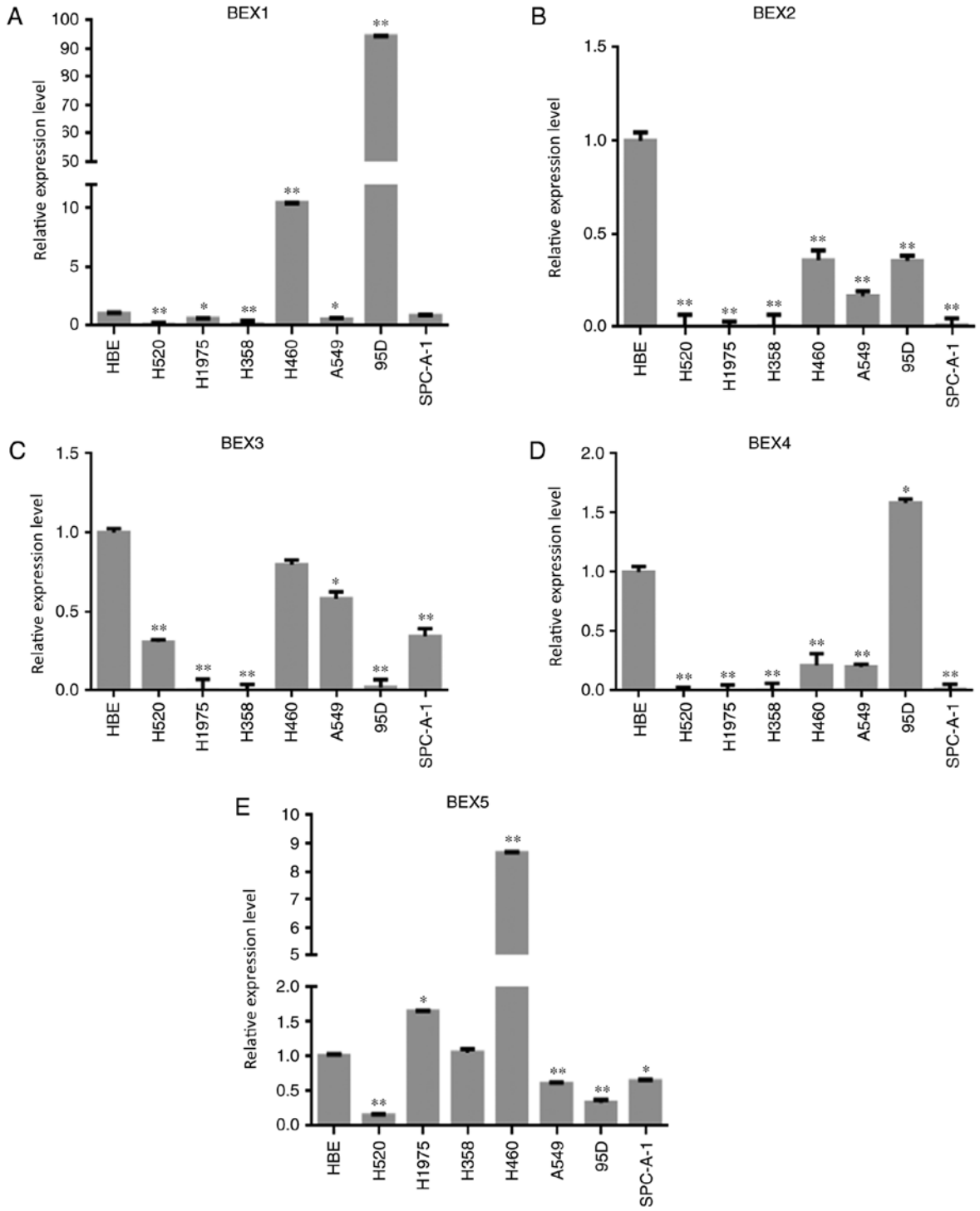

Figure 2. Expression levels of the BEX family were significantly downregulated in the majority of the lung adenocarcinoma cell lines investigated. The mRNA expression of (A) BEX1, (B) BEX2, (C) BEX3, (D) BEX4 and (E) BEX5 in HBE, H520, H1975, H358, H460, A549, 95D and SPC-A-1 cells was determined using reverse-transcription quantitative polymerase chain reaction. *P<0.05 and **P<0.01 vs. HBE cells. BEX, brain-expressed X-linked; HBE, human bronchial epithelial.

TCGA database. The expression levels of BEX1-3 were not significantly associated with OS (Fig. 4A-C). However, the downregulation of BEX4 and BEX5 expression levels were significantly associated with poor OS (P<0.001 and P=0.001, respectively; Fig. 4D-E). The association between the expression levels of the BEX family and the outcomes of patients with LUAD using clinical stage subgroup analysis revealed that BEX4 and BEX5 were associated with the OS of patients with stage I-II (P<0.001 and P=0.018, respectively; Fig. S2D-E), but not of patients with stage III-IV (P=0.261 and P=0.140, respectively; Fig. S3D-E). There was no significant difference between BEX1, BEX2 or BEX3 expression and OS of patients with stage I-II or stage III-IV (Figs. S2A-C and 3A-C).

Characteristics such as age, sex, AJCC stage and the expression level of BEX members were analyzed using a multivariate Cox regression model. In addition to clinical stage

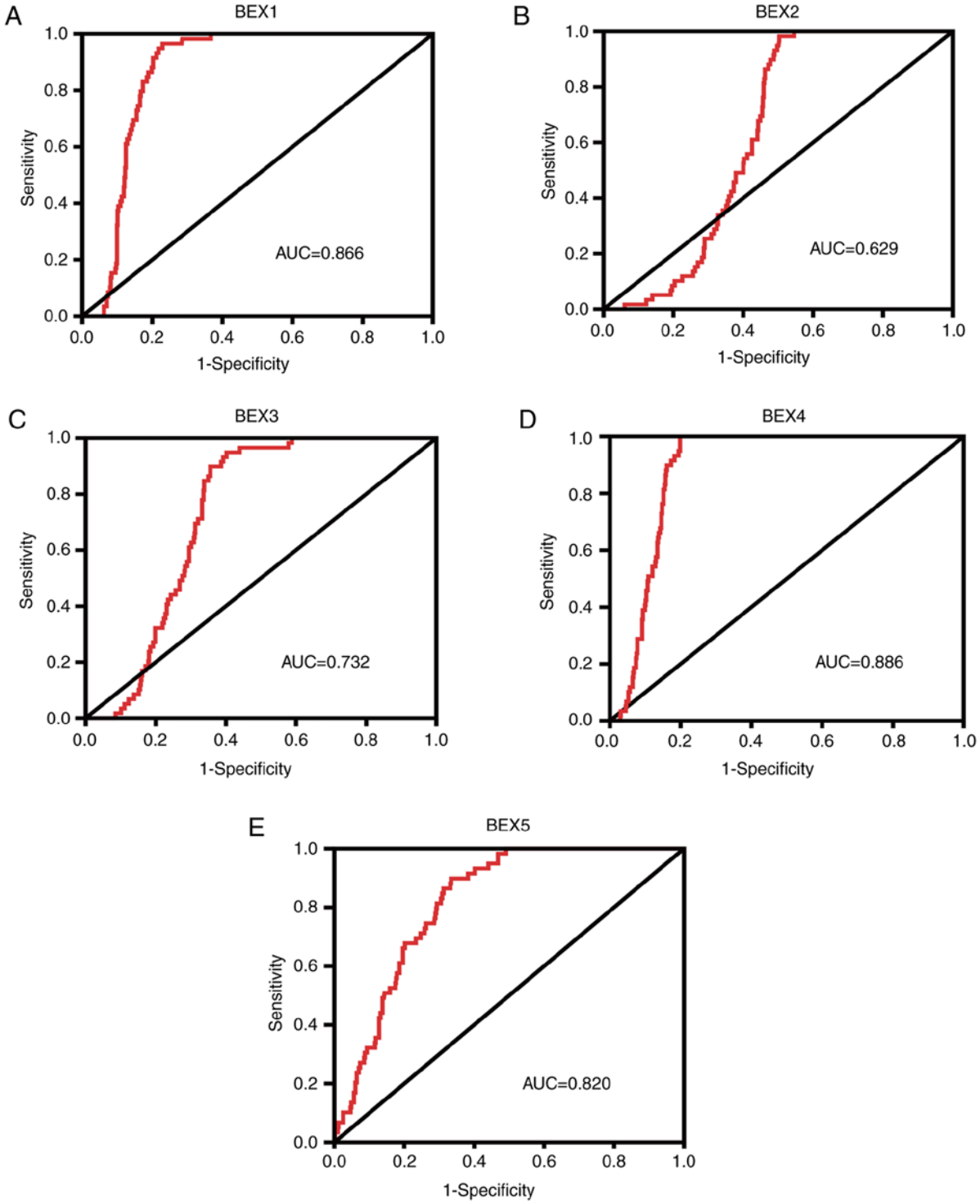

Figure 3. Receiver operating characteristic curves for estimating the diagnostic value of (A) BEX1, (B) BEX2, (C) BEX3, (D) BEX4 and (E) BEX5. The red and black lines represent the ROC curves of BEX family and the random performance, respectively. BEX, brain-expressed X-linked; AUC, area under the curve.

[hazard ratio (HR)=2.290; P<0.001], the expression level of the BEX family was an independent prognostic factor (HR=1.468 and P=0.030 for BEX3; HR=0.469 and P<0.001 for BEX4; HR=0.723 and P=0.043 for BEX5) for patients with LUAD (Table IV). In addition, as shown in Table IV, BEX4 expression was significantly associated with OS at stages I-II (HR=0.476; P=0.002), while BEX3 (HR=2.450 and P=0.014), BEX4 (HR=0.288; P=0.001) and BEX5 (HR=0.569 and P=0.047) were associated with OS at stages III-IV.

Methylation, mutation and CNV analysis of BEX family members in patients with LUAD. The total alteration frequency of gene mutations, amplifications and deletions in patients with LUAD was <2% (Fig. S4) according to the sequencing data in datasets obtained from cBioPortal. BEX1 and BEX3 were hypermethylated in tumor tissues compared with normal tissues, whereas BEX2, BEX4 and BEX5 did not exhibit any statistically significant difference in methylation (Fig. S5).

Table II. Association between the BEX4 expression level and patient clinical characteristics in The Cancer Genome Atlas lung adenocarcinoma cohort.

		BEX4 expression			
Characteristic	n	High	Low	χ^2	P-value
Age at diagnosis (years)				5.545	0.019
<60	131	54	77		
≥60	351	187	164		
Sex				8.030	0.005
Male	221	95	126		
Female	261	146	115		
Clinical stage				8.288	0.004
I+II	378	202	176		
III+IV	104	39	65		
T (Primary tumor)				15.226	0.002
T1	165	101	64		
T2	252	116	136		
T3	44	15	29		
T4	18	7	11		
TX	3	2	1		
N (Regional lymph nodes)				4.549	0.033
N0	312	166	146		
N1-3	161	69	92		
NX	9	6	3		
M (Distant metastases)				0.552	0.457
M0	319	158	161		
M1	24	10	14		
MX	139	73	66		

A total of 482 samples were analyzed, 241 with low BEX4 expression and 241 with high BEX4 expression. A χ^2 test was used for statistical analysis. BEX, brain-expressed X-linked.

Table III. Associations between the BEX5 expression level and patient clinical characteristics in The Cancer Genome Atlas lung adenocarcinoma cohort.

		BEX5 expression			
Characteristic	n	High	Low	χ^2	P-value
Age at diagnosis (years)				1.268	0.260
<60	131	60	71		
≥60	351	181	170		
Sex				3.017	0.082
Male	221	101	120		
Female	261	140	121		
Clinical stage				4.904	0.027
I+II	378	199	179		
III+IV	104	42	62		
T (Primary tumor)				14.117	0.003
T1	165	102	63		
T2	252	109	143		
T3	44	21	23		
T4	18	8	10		
TX	3	1	2		
N (Regional lymph nodes)				2.204	0.138
N0	312	162	150		
N1-3	161	72	89		
NX	9	7	2		
M (Distant metastases)				0.041	0.840
M0	319	153	166		
M1	24	11	13		
MX	139	77	62		

A total of 482 samples were analyzed, 241 with low BEX5 expression and 241 with high BEX5 expression. A χ^2 test was used for statistical analysis. BEX, brain-expressed X-linked.

Discussion

Despite improvement in diagnostic and treatment strategies, the prognosis of patients with lung cancer remains poor (37), potentially due to a lack of effective predictive markers at the early stages of the disease (38,39). Therefore, novel biomarkers for the detection and prognosis evaluation of LUAD are required. Previous studies have revealed that the BEX family may serve roles as predictive biomarkers in tumors, such as esophageal squamous cell cancer and acute myeloid leukemia (12,40). The potential clinical value of the BEX family members in LUAD warrants further research.

The present study aimed to investigate the role of the BEX family in LUAD. The expression levels of BEX1-5 in LUAD were examined by mining the TCGA and Oncomine databases. The results obtained in the current study revealed that the expression levels of BEX members in carcinoma tissues were significantly decreased compared with normal tissues. Similarly, experimental *in vitro* data revealed that the mRNA levels of BEX members were decreased in the majority of the lung cancer cell lines investigated compared with HBE cells. The abnormal expression of the BEX family has been implicated in various types of cancer, such as breast cancer and oral squamous cell carcinoma (16,20). The expression levels of BEX1 and BEX4 were significantly reduced in oral squamous cell carcinoma tissues compared with paired normal epithelial (20). Foltz *et al* (41) reported that BEX1 and BEX2 had differentially methylated CpG sites and increased methylation was associated with a decreased expression level in malignant glioma tissues compared with paired normal specimens. Previous studies revealed that several factors are involved in gene regulation, including mutations, copy number variations (CNVs) and DNA methylation (42-46). To further explore the results of differential expression, gene methylation status, mutation status and CNVs were analyzed. As the total alteration frequency of gene mutations, amplifications and deletions was <2%, the expression levels of the BEX family were likely not associated with mutations or CNVs. The methylation status

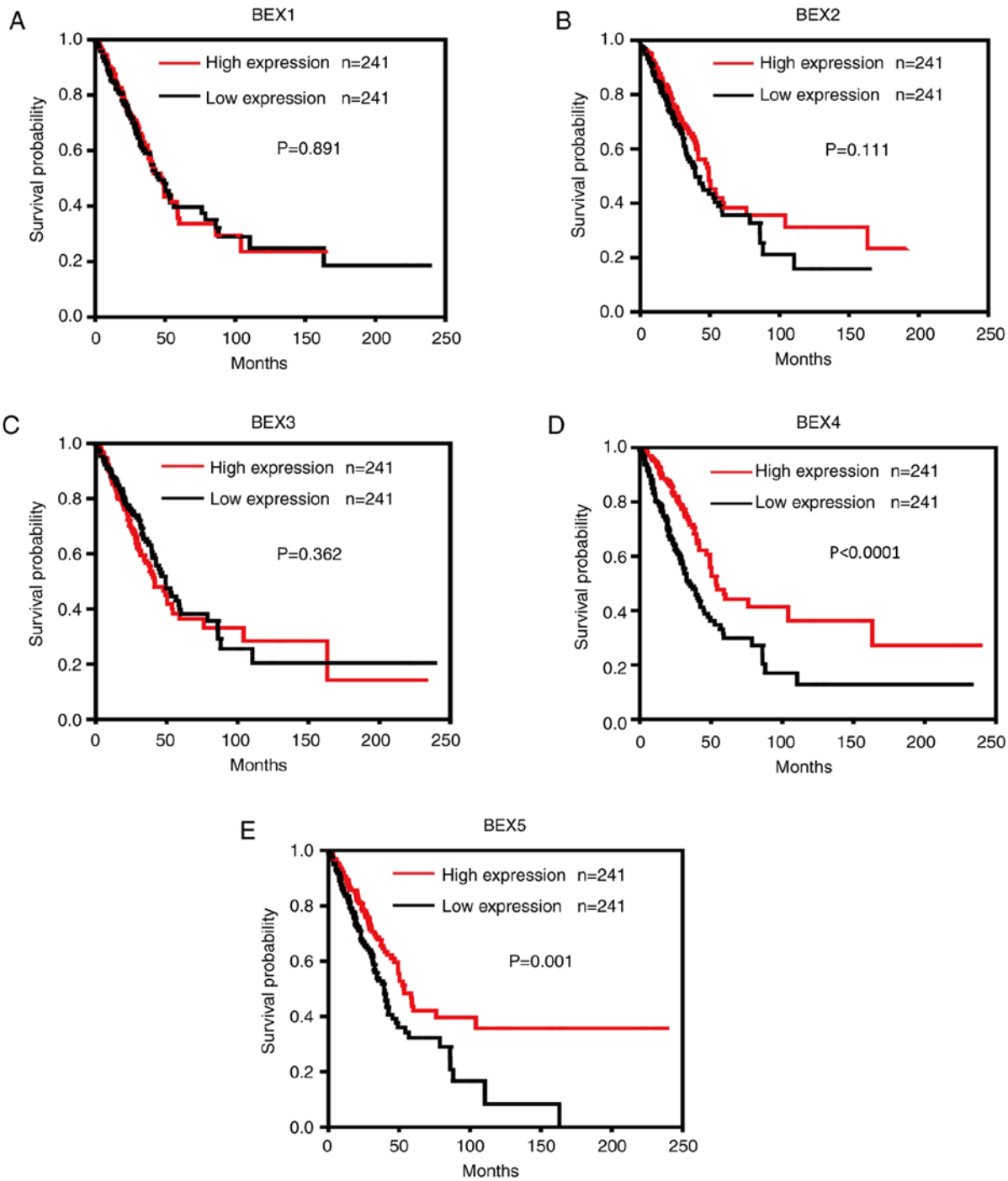

Figure 4. Kaplan-Meier survival curves for assessing the prognostic value of (A) BEX1, (B) BEX2, (C) BEX3, (D) BEX4 and (E) BEX5 in 482 patients with lung adenocarcinoma. BEX, brain-expressed X-linked.

of BEX1 and BEX3 may partially explain the low expression of them in LUAD. However, the mechanisms underlying the downregulation of BEX family requires further investigation.

Gene expression signatures have been used for the clinical diagnosis of various types of cancer (47). The differential expression of the BEX family in LUAD and normal tissues revealed in the present study suggested that that the BEX family may have diagnostic value in LUAD. Furthermore, the high AUC values obtained in the present study indicated that BEX1-5 exhibited good diagnostic performance. Whether the combination of BEX members provides higher sensitivity and specificity compared the individual use of each BEX family member requires further investigation.

A previous study reported that low expression of BEX1 was associated with clinicopathological characteristics such as tumor volume, T stage and clinical stage in esophageal squamous cell cancer (12). The present study investigated the association between the expression of the BEX family and the clinicopathological features of patients with LUAD. The low expression levels of BEX1, BEX4 and BEX5 were significantly associated with advanced clinical stage. The downregulation of BEX4 was significantly associated with

Table IV. Multivariate analyses of overall survival in patients with lung adenocarcinoma.

A, All patients

	Multivariate analysis		
Parameter	P-value	Hazard ratio	95% confidence interval
Age (≥60 vs.<60)	0.549	1.108	0.792-1.549
Sex (female vs. male)	0.543	1.081	0.797-1.466
Clinical stage (III/IV vs. I/II)	<0.001	2.290	1.665-3.149
BEX1 expression (high vs. low)	0.394	1.146	0.838-1.567
BEX2 expression (high vs. low)	0.452	1.167	0.781-1.745
BEX3 expression (high vs. low)	0.030	1.468	1.038-2.077
BEX4 expression (high vs. low)	<0.001	0.469	0.320-0.686
BEX5 expression (high vs. low)	0.043	0.723	0.528-0.989

B, Patients with stage I+II

	Multivariate analysis		
Parameter	P-value	Hazard ratio	95% confidence interval
Age (≥60 vs. <60)	0.216	1.321	0.850-2.051
Sex (female vs. male)	0.912	0.979	0.675-1.421
BEX1 expression (high vs. low)	0.519	1.134	0.773-1.664
BEX2 expression (high vs. low)	0.915	0.974	0.604-1.572
BEX3 expression (high vs. low)	0.341	1.220	0.810-1.835
BEX4 expression (high vs. low)	0.002	0.476	0.299-0.757
BEX5 expression (high vs. low)	0.265	0.798	0.537-1.186

C, Patients with stage III+IV

	Multivariate analysis		
Parameter	P-value	Hazard ratio	95% confidence interval
Age (≥60 vs.<60)	0.892	1.040	0.592-1.827
Sex (female vs. male)	0.126	1.587	0.878-2.869
BEX1 expression (high vs. low)	0.662	1.139	0.635-2.042
BEX2 expression (high vs. low)	0.173	1.696	0.793-3.628
BEX3 expression (high vs. low)	0.014	2.450	1.197-5.015
BEX4 expression (high vs. low)	0.001	0.288	0.134-0.618
BEX5 expression (high vs. low)	0.047	0.569	0.326-0.992

BEX, brain-expressed X-linked.

regional lymph node metastasis. These results suggested the BEX family may be involved in tumor invasion and metastasis, which may result in less favorable outcomes in patients with LUAD. Additional studies are required to investigate the effect of the BEX family on tumor metastasis.

The present study revealed that BEX4 and BEX5 were associated with the prognosis of LUAD patients. The low expression levels of BEX4 and BEX5 were associated with poor prognosis. The results obtained in the current study suggested that members of the BEX family may serve as biomarkers for predicting poor prognosis in patients with LUAD.

The present study is limited by the lack of sufficient experimental data and validation at the protein level. Therefore, further studies using large, independent clinical cancer cohorts are required to corroborate the results obtained in the present study. Additionally, the roles and underlying mechanisms of members of the BEX family in the development of LUAD

remain unclear and warrant further investigation. In summary, the present study demonstrated that the BEX family was significantly downregulated in LUAD tissues compared with normal tissues, and that this expression pattern may serve as a potential biomarker for the diagnosis and prognosis prediction of patients with LUAD.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request. The datasets generated and/or analyzed during the present study are available from The Cancer Genome Atlas (cancer.gov/tcga).

Authors' contributions

JC and WL conceived and designed the study. ZZ and ZL analyzed the data and drafted the manuscript. FH and JL participated in data interpretation and manuscript revision. HC performed the RT-qPCR. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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