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ORIGINAL ARTICLE

Bioinformatic Analysis of Expression Pattern and Prognostic Value of Oxidoreductase ERO1L in Pancreatic Cancer

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ABSTRACT

Objective: As people continue to succumb to the progression of various forms of cancer, the extreme lethal nature of pancreatic cancer in particular suggests that new therapeutic targets and novel regulatory mechanisms need to be explored.

Materials and Methods: We examined ERO1L expression in different cancer types using cBioPortal and Oncomine exploration tools. Next, we analyzed ERO1L levels in pancreatic cancer and healthy tissues via online public databases. The prognostic value of ERO1L and its correlation with clinopathological features were investigated using the UCSC, TNMplot and cBioPortal databases. The correlation analyses were then performed using data obtained from GEPIA, cBioPortal and the Gene Expression Omnibus.

Results: The enzyme ERO1L was found to be highly expressed in pancreatic cancer and elevated in tumor compared to healthy tissue. Its levels correlated with the hypoxia level and ER stress activation status of the pancreatic cancer tissues. ERO1L and VEGFA levels were also found to be correlated exclusively in tumor tissue, thus underlying its pro-oncogenic nature.

Conclusion: Oxidoreductase ERO1L is a potential prognostic marker and its oncogenic effects might be regulated via hypoxia/ER stress/ERO1L/VEGFA axis in pancreatic cancer.

Keywords: ERO1L, hypoxia, pancreatic cancer, ER stress, VEGFA

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INTRODUCTION

Cancer is a worldwide health problem causing the death of millions of people each year [1]. Pancreatic cancer accounts for about 7% of these deaths while its intense lethal character results in a 5 year survival rate of less than 9% [2]. Only a few newly diagnosed patients are eligible for surgical resection [3] and the prevalent detection of distant metastasis and local recurrence renders the use of systemic chemotherapy (i.e gemcitabine, FOLFILINOX), or radiation therapy as the main treatment options. However, the poor response rate indicates that there is an urgent need for understanding the molecular mechanisms of pancreatic cancer progression and finding novel therapy targets.

The endoplasmic reticulum(ER) is an organelle in a cell where newly synthesized proteins are folded to be delivered to their final destination. The formation of both intermolecular and intramolecular disulfide bonds play a key role in this folding process [4]. Protein disulfide isomerase (PDI), by interacting with endoplasmic reticulum Oxidoreductase 1(ERO1), is the enzyme responsible for the formation of these bonds [5]. During this process, PDI is reduced

upon oxidizing proteins and which leads to the formation of disulfide bridges. ERO1 reoxidizes PDI thus preparing it for an upcoming cycle of protein folding. Perturbations in this machinery causes the formation of misfolded proteins which results in disrupted ER homeostasis and organelle stress.

Ero1 exists in two isoforms being Ero1-alpha(ERO1L) and Ero1-beta(ERO1LB). Interestingly, while the transcription of both isoforms can be regulated by ER stress(ERS), only ERO1L shows responsiveness to hypoxia which is a well known pathological hallmark of cancer thus making the ERO1L an interesting candidate for cancer research [6-8]. It has been reported that ERO1L is amply expressed in a variety of tumors and its expression shows association with poor prognosis [8-11]. ERO1L was found to promote angiogenesis by regulating vascular endothelial growth factor A (VEGFA) at the both transcriptional and post-translational level [12]. This regulation also plays a significant role in the metastatic potential of breast cancer[13]. The ERO1L/VEGFA axis also modulates key oncogenic features in hepatocellular carcinoma [11]. The profound presence of two ERO1L promoting insults, being hypoxia and ERS [14], and increased VEGFA levels [15] in pancreatic cancer prompted us to speculate that hypoxia/ERS/ERO1L/VEGFA might be an axis regulating pancreatic cancer progression.

In the present study, we evaluated the role of ERO1L in pancreatic cancer progression using in silico analysis tools. We primarily investigated if ERO1L level is increased in pancreatic cancer compared to other cancers and further upregulated in tumor compared to healthy tissue. We then examined ERO1L's association with prognostic and clinopathological parameters and investigated a potential pathway that might facilitate ERO1L-driven carcinogenesis.

MATERIALS AND METHODS

ERO1L expression analysis among different cancer types

The expression levels of ERO1L among different cancers were analyzed using publicly available exploration tools namely cBioPortal (http://www.cbioportal.org/index.do) [16,17] and Oncomine (https://www.oncomine.org/resource/login.html) [18]. The Tumor Cancer Genome Atlas Pan Cancer data set (TCGA Pan Can) and Bittner Multi-cancer data set, Ramaswamy Multi-cancer data set were used in cBioPortal and Oncomine respectively to analyze ERO1L transcript levels in different tumor types.

Examination of gene transcript levels across Normal and Tumor tissue

Comparison of the transcript level of ERO1L between normal and tumor tissue was undertaken using data obtained from the Oncomine database [18]. Statistical analysis of the data was performed using Students' t-test with threshold search criteria of fold change>2, p-value<0.01 and gene ranking: 1% [19]. The TNMplot online database (https://www.tnmplot.com/) [20] online database contains omics data from various sources. The distribution of ERO1L expression in diversified cancers and corresponding healthy tissues was examined using this bioinformatic tool. To verify differential expression in TNMplot, the Mann-Whitney U test was used and significant differences with $p<0.05$ were marked with the (*) sign. An ERO1L, HIF1A, DDIT3 and HSPA5 expression profile between pancreatic cancer and its complementary normal tissue was also obtained using the Gene Expression Profiling Interactive Analysis (GEPIA2) website (http://gepia2.cancer-pku.cn/) [21]. The GTEx (Genotype-Tissue Expression) and TCGA data were then matched using the ANOVA differential method and the analysis was performed using the default threshold settings. Differentially expressed genes (DEGs) in the pancreatic cancer dataset GSE28735 from GEO (http://www.ncbi.nlm.nih.gov/geo/) were determined using GEO2R and utilizing the following threshold criteria: $|\log FC|\geq 1$, $p<0,05$. It is known that GSE28735 contains 45 samples of pancreatic tumor and their adjacent non-tumor tissue [22].

Survival analysis

RNA-seq expression values for ERO1L and the clinical data of pancreatic cancer patients were obtained from TCGA-PAAD data set. Analysis of Kaplan-Meier overall survival, disease specific survival, disease free interval undertaken using data recorded in the UCSC Cancer Genomics Browser (https://genome-

cancer.ucsc.edu) [23]. A log-rank test (test statistics and p-value) was then conducted for the statistical analysis of ERO1L high and and low groups. p-value<0.05 indicates statistical significance.

Analysis of ERO1L expression in tumors having different clinopathological features

The expression data of ERO1L in tumors and clinopathological features (recurrence, KRAS status and survival status) of the corresponding patients was obtained from the TCGA-PAAD data set and analyzed using cBioPortal. Results were presented as mean ± SD. Student's t-tests were performed to compare the difference between two groups. A violin plot showing the correlation between ERO1L and histological grade was produced using data sourced via the UCSC Cancer Genomics Browser. One-way ANOVA test was used to determine differential expression of ERO1L among three different histological grades. The expression of ERO1L in normal, cancerous and metastatic tissues were compared using TNMplot and the Kruskal Wallis test was used to assess statistical significance. The differences being considered statistically significant if p<0.05.

Hypoxia Score and ERO1L expression analysis

Winter hypoxia scores, buffa hypoxia scores and an expression heatmap showing ERO1L expression z-scores from the TCGA-PAAD dataset were obtained from cBioPortal. Hypoxia scores were determined for the $ERO1^{high}$ group (expression z-score>1) and $ERO1^{low}$ group (expression z-score<-1). Student's t-test was then used to determine statistical significance between groups.

Correlation analysis

The correlation between ERO1L, HIFA vs. ER stress signature genes (a gene set comprised of 113 genes obtained from GSEA Molecular Signatures Database HALLMARK_UNFOLDED_PROTEIN_RESPONSE) and ERO1L vs. VEGFA was analyzed using the GEPIA2 database. Correlation analysis among aforementioned parameters were performed by Pearson's correlation test. ERO1L expression levels and winter, buffa hypoxia scores of corresponding tumors in the TCGA-PAAD dataset were obtained using cBioPortal. The correlation between ERO1L vs. Winter Hypoxia Score and ERO1L vs. Buffa hypoxia score was then analyzed using Pearson's correlation test. Likewise, the correlation between VEGFA and ERO1L expression in pancreatic non-tumor and tumor tissues in the GSE28735 dataset was identified by Pearson's correlation tests.

RESULTS

The transcript levels of ERO1L is increased in pancreatic tumors

The unique expression profile of cancer tissues is used for several aims including biomarker discovery, cancer subtype identification, survival prediction and novel therapeutic target determination [24]. To establish if ERO1L has a possible role in cancer progression, we first aimed to determine its differential expression pattern in numerous cancers. Analyses using several databases revealed that ERO1L is highly expressed in tumors and pancreatic cancer is among the ten cancer types with highest ERO1L expression (Figure 1A). To further support this notion, different datasets were also examined using in silico analysis tools and ERO1L was found to be expressed significantly higher in pancreatic cancer compared to other cancer types (Figure 1B-C).

Next, we wanted to ascertain if the increasing transcript levels of ERO1L are unique for tumor tissue thus we compared ERO1L levels in tumor and normal tissues using ONCOMINE. Pan-cancer analysis results displayed that ERO1L is upregulated in 43 datasets associated with the bladder, brain and CNS, breast, colorectal, gastric, kidney, lung, lymphoma, ovarian, pancreatic, prostate cancer whereas 16 datasets showed opposite results (Figure 2A). The increased ERO1L expression in tumor tissues including pancreatic cancer was also verified using the TNMplot (Figure 2B) and GEPIA (Figure 2C) databases. Last but not least, differential gene expression analysis of 45 matching pancreatic cancer and healthy tissues from GSE28735 supported lower levels of ERO1L in normal tissue (Figure 2D). Overall, these results suggest that ERO1L might play a specific role in pancreatic cancer.

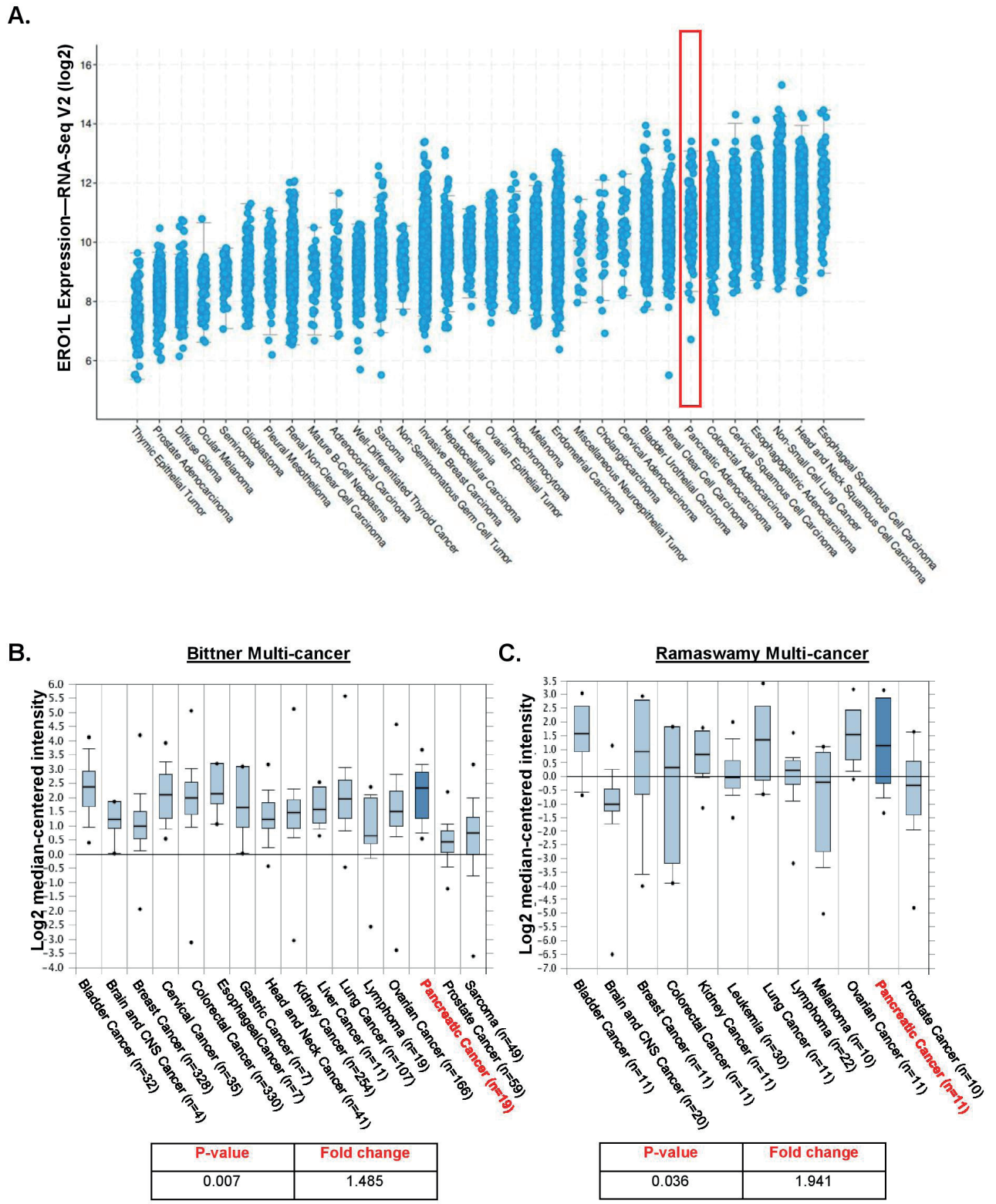

Figure 1. Expression pattern of ERO1L across cancers. A) Abundance of ERO1L was analyzed using the cBioPortal database. Cancer types aligned based on their median ERO1L transcript level. Gene expression profiling of ERO1L in B) Bittner and C) Ramaswamy multi-cancer datasets from Oncomine indicate remarkably high ERO1L expression in pancreatic cancer compared to other types of cancer. p<0,05 shows statistical significance.

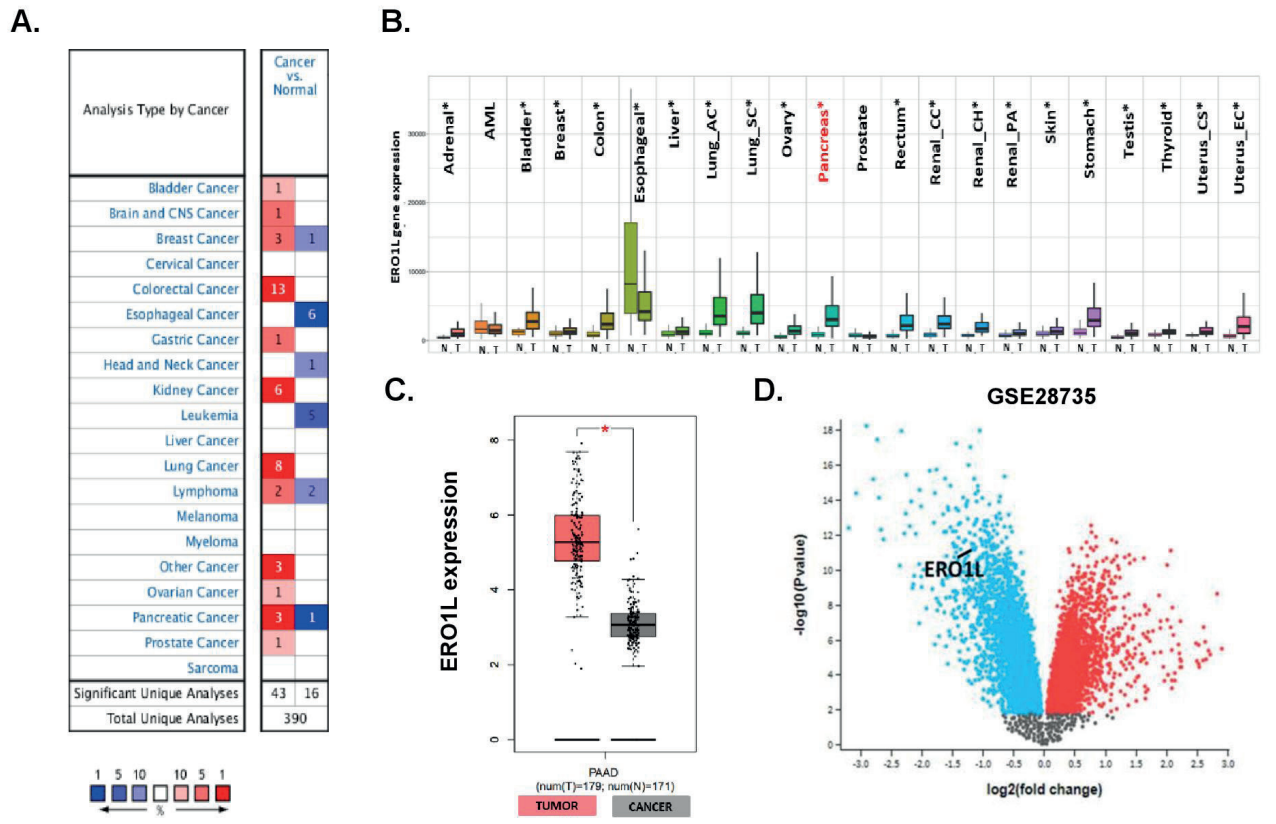

Figure 2. Differential expression of ERO1L in pancreatic normal and tumor tissues. A) The ERO1L expression levels were analyzed between normal and tumor tissues using the Oncomine database. The plot shows the numbers of datasets with higher (red) or lower (blue) ERO1L levels in cancer tissue compared to corresponding normal tissue. B) Relative ERO1L expression was investigated between healthy and cancer tissue using TNMplot. The data illustrates a universal upregulation of ERO1L in tumor tissues. C) The expression pattern of ERO1L between tumor (TCGA) and normal (GTEX) pancreatic tissue was investigated by GEPIA2 using ANOVA differential method. * indicates p-value<0.01. D) ERO1L was found to be among DEGs between pancreatic tumor and non-tumor tissue in GSE28735 and was shown lower expression in normal counterpart (p value: 5,58e-12, log FC: -1,166).

ERO1L gene expression is associated with poor prognosis in pancreatic cancer patients

The predictive value of ERO1L in pancreatic cancer was determined with UCSC. We used TCGA pan-cancer data to analyze survival differences between ERO1L high(red line) and ERO1L low(blue line) groups by using the ERO1L median expression level as the cutoff. Log-rank test showed that in pancreatic cancer patients, the high ERO1L expression group had a shorter overall survival (Figure 3A), disease specific survival (Figure 3B), disease free interval (Figure 3C) and progression free interval (Figure 3D). Collectively, this data indicate that pancreatic cancer patients with high ERO1L expression have a shorter survival time than those with low ERO1L expression.

ERO1L transcript levels shows positive correlation with clinical behaviour of pancreatic cancers

We also examined if ERO1L levels are associated with the clinical features of pancreatic cancers. Analysis using the TCGA dataset in cBioPortal revealed that recurring pancreatic cancer patients showed elevated ERO1L levels compared to disease-free patients (Figure 4A). We also analyzed ERO1L levels in pancreatic cancer patients expressing WT or mutated KRAS. KRAS plays a pivotal role in signaling pathways regulating cancer progression. Mutations causing constitutive activation of KRAS are commonly seen in pancreatic cancer and shown to not only promote the proliferative and migrative capacity of cells

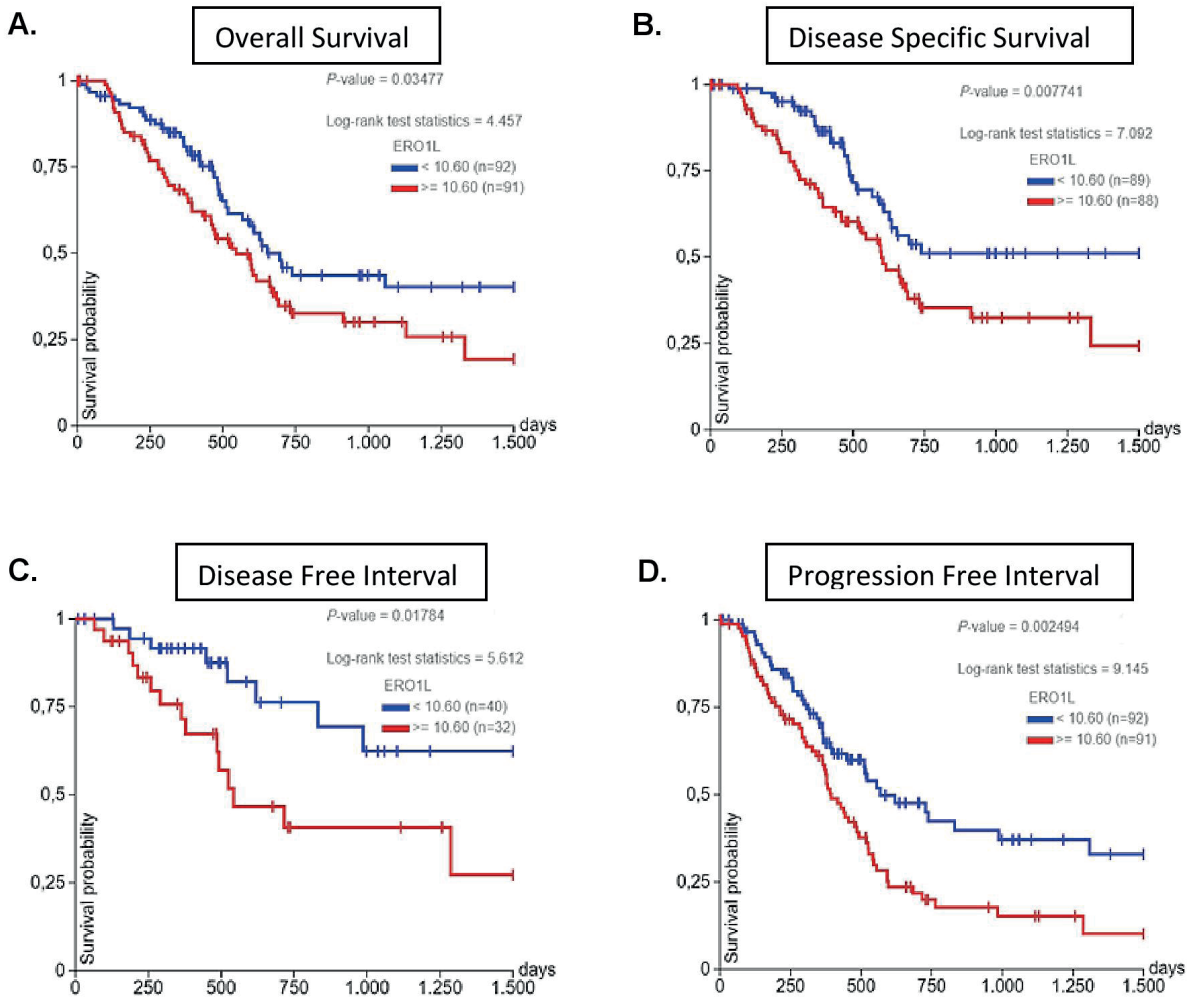

Figure 3. Prognostic value of ERO1L in pancreatic cancer. Kaplan-Meier curves comparing high and low expressions of ERO1L show that higher ERO1L expression (red line) results in shorter A) Overall survival, B) Disease specific survival, C) Disease free interval and D) Progression free interval. p<0,05 indicates statistical significance.

but also confer survival disadvantage[25,26]. Our results revealed that tumors carrying mutant KRAS also have higher ERO1L levels (Figure 4B). In addition, pancreatic tumor tissues of deceased patients showed increased ERO1L expression compared to tissues obtained from living patients (Figure 4C). To further evaluate ERO1L's correlation with clinical parameters, we used the UCSC database to investigate ERO1L's transcript levels in tumors with different histological grades. ERO1L was upregulated gradually along with the increased histological grade (Figure 4D). ERO1L levels were also higher in metastatic pancreatic tumor compared to normal tissues. Altogether, these findings strongly suggest that ERO1L has a pro-oncogenic role and hence can be used as a biomarker and therapeutic target.

Hypoxia is a master regulator of ERO1L expression level in pancreatic tumors

Next, we sought to understand the molecular basis of ERO1L upregulation in pancreatic tumors. It is known that oxygen deprivation occurs in tumors when cancer cells multiply rapidly, a phenomenon known as hypoxia. Hypoxic tumors show resistance to therapy and have a more aggressive phenotype [27]. Tumors with high ERO1L levels also show prevalent hypoxia [28] indicating a possible connection between them. This connection was verified in lung adenocarcinoma. Lung cancers with high ERO1L levels have been shown to carry a prominent hypoxic signature [29]. The severe hypoxic nature of pancreatic cancers also prompted us to investigate if this hallmark of cancer might regulate ERO1L levels in pancreatic tumors [30].

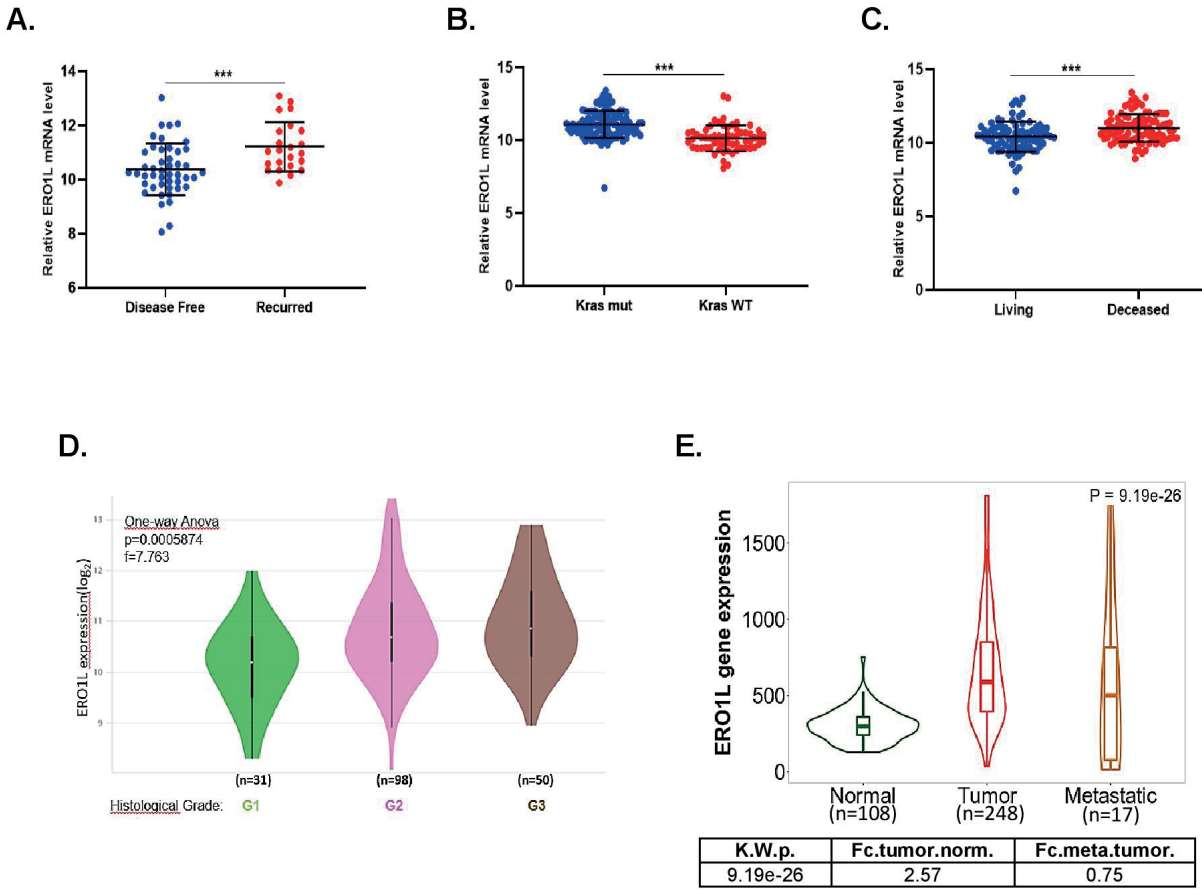

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Figure 4. The relationship between ERO1L expression and clinopathological features. Log2-transformed ERO1L mRNA expression levels in the A) recurring or disease-free B) Kras mutant or Kras WT C) deceased or living PAAD samples was examined using cBioPortal. D) Violin plot showing the association between ERO1L expression and histological grade in patients with pancreatic cancer(UCSC). E) Boxplot comparing ERO1L levels in paired normal, tumor and metastatic tissues from gene chip data at TNMplot. (*** means p<0.001)

We used the TCGA dataset in cBioPortal and explored the correlation between ERO1L levels and hypoxia. Our analyses revealed that tumors with strong ERO1L expression exhibited significantly elevated Winter (Figure 5A-B) and Buffa (Figure 5D-E) hypoxia scores. These findings were also supported by the observed positive correlation of ERO1L expression levels with Winter (Figure 5C), and Buffa (Figure 5F) hypoxia scores. Taken together, these results clearly demonstrate that hypoxia modulates ERO1L expression in the pancreatic tumor microenvironment.

Hypoxia regulates ERO1L levels via ERS activation

The disturbed homeostasis in the tumor environment triggers the formation of ERS activating stimuli including hypoxia [31] thus ERS activation is prevalent in tumor tissues [32]. Previous studies showed that ERS activation regulates ERO1L levels on a trancriptional level [7,8,33]. Based on these findings we hypothesized that ERS might be the intermediary modulator between hypoxia and ERO1L transcription. To verify this link we first investigated if hypoxia and ERS are upregulated in pancreatic cancer tissue. Analysis using the TCGA and GTEx datasets in GEPIA revealed that hypoxia marker HIF1A (Figure 6A) [34] and ERS activation markers DDIT3 (Figure 6B) and HSPA5 (Figure 6C) [35] are upregulated in pancreatic tumor tissues. These results imply a functional hypoxia and ERS axis in the pancreatic tumor microenvironment. We then sought to establish whether ERS is regulated by hypoxia in tumor tissue. The correlation analysis tool in GEPIA revealed that HIF1A shows a strong correlation with ERS activation signature (Figure 6D) indicating the validity of this regulation. Close correlation of ERS signature and ERO1L levels

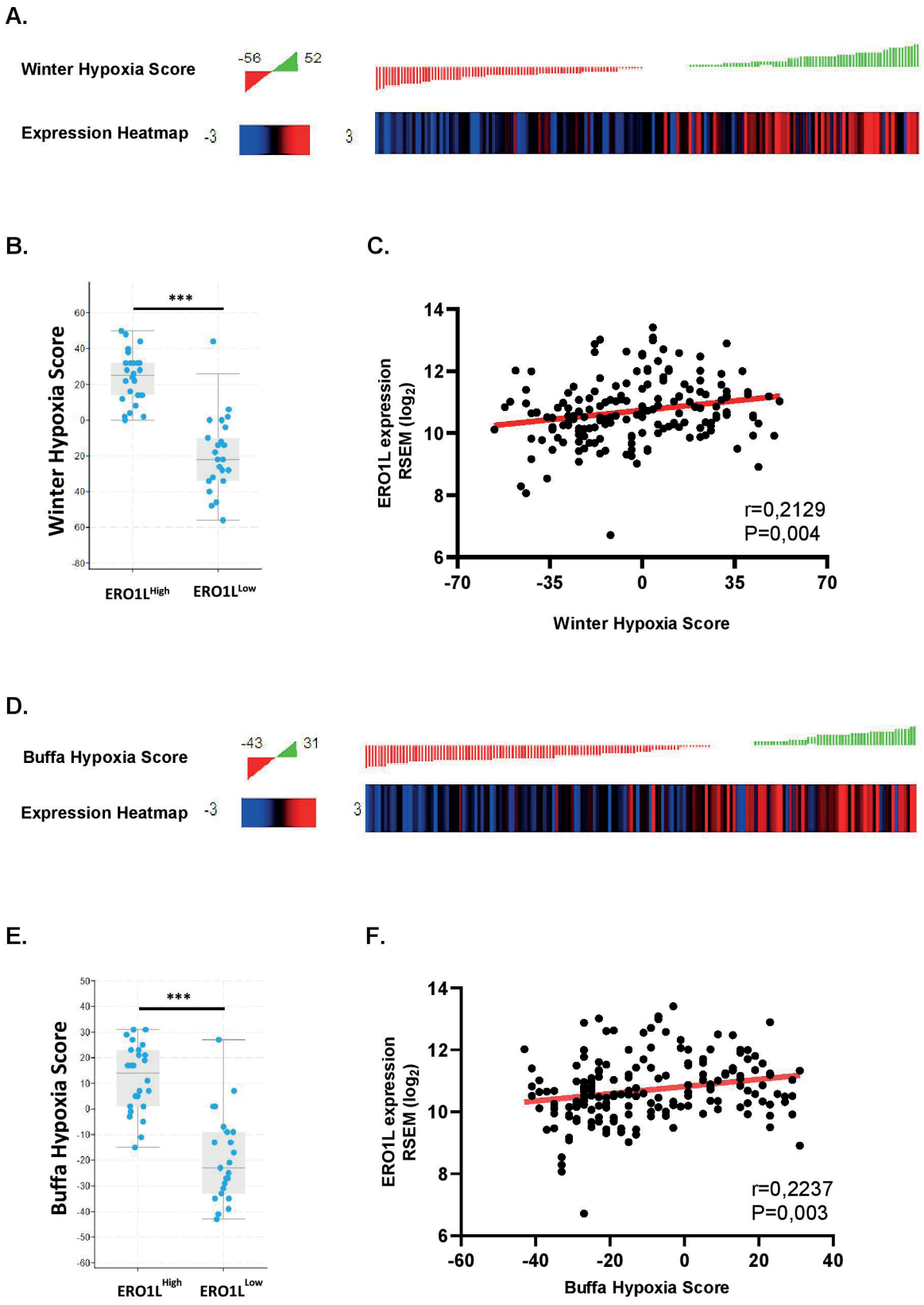

Figure 5. ERO1L is upregulated with hypoxia A) The score bar and heatmap showing winter hypoxia score and ERO1L expression z-scores respectively. B) Winter hypoxia scores in ERO1high and ERO1low tumors in TCGA-PAAD cohort. C)The correlation between ERO1L expression and winter hypoxia score in 177 TCGA pancreatic cancer tissues. D) The score bar and heatmap showing buffa hypoxia score and ERO1L expression z-scores respectively. E) Buffa hypoxia scores in ERO1high and ERO1low tumors in TCGA-PAAD cohort. F) The correlation between ERO1L expression and buffa hypoxia score in 177 TCGA pancreatic cancer tissue. p<0,05 indicates statistical significance. (*** means p<0.001)

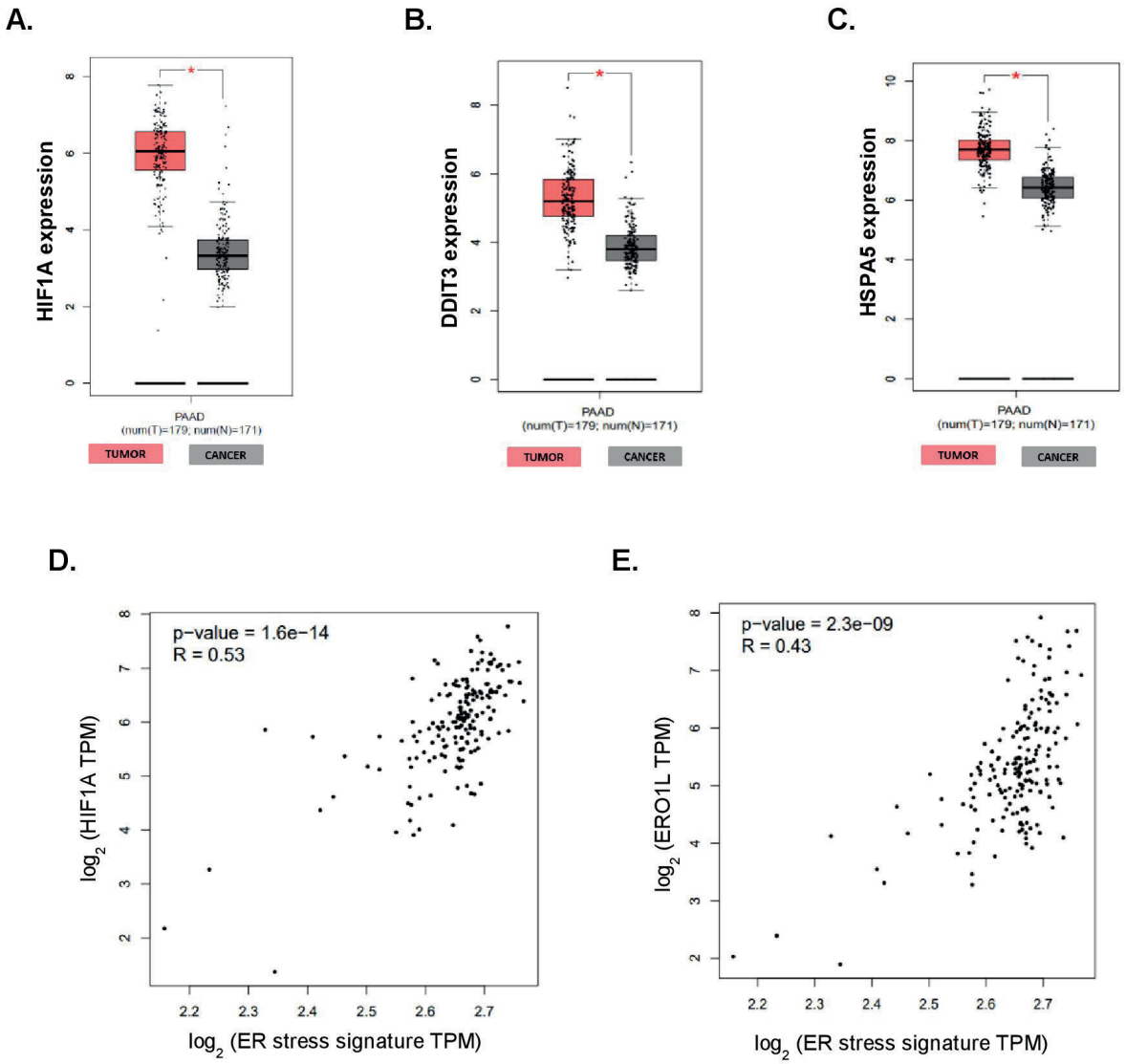

Figure 6. Hypoxia and ER stress markers' correlation with ERO1L expression. Box plots derived from GEPIA comparing the expression of A) hypoxia (HIF1A) and B,C) ER stress (DDIT3 and HSPA5) markers in PAAD(TCGA) and normal tissues(GTEx). The positive correlation of D) hypoxia marker HIF1A and E) ERO1L with ER stress related genes were analyzed by GEPIA (* means p<0.01).

(Figure 6E) strongly suggest a critical role for ERS in ERO1L upregulation. In brief, these results provide strong evidence that hypoxia driven ERS activation modulates ERO1L levels and accounts for the poor prognosis of patients with pancreatic cancer.

ERO1L and VEGFA are exclusively correlated in cancerous tissues of pancreas

Metastasis occurs when cancer cells spread from the primary site to other body parts. The low 5-year overall survival rate of pancreatic cancer patients is further reduced if their tumors are metastatic [36]. ERO1L's higher expression in metastatic pancreatic cancer (Figure 4E) and its significant contribution to poor patient prognosis (Figure 3A) led us to speculate that ERO1L might be involved in the regulation of genes that are involved in metastasis. VEGFA has been shown to stimulate tumor metastasis [37] and its transcript and secretion levels have been regulated by ERO1L especially in a hypoxic environment [38]. To better understand if this also holds true for pancreatic cancer, we analyzed the correlation between ERO1L and VEGFA expression. Our data revealed that ERO1L expression level was not correlated with VEGFA levels in healthy tissue (Figure 7A) whereas there was a strong correlation in tumor tissue

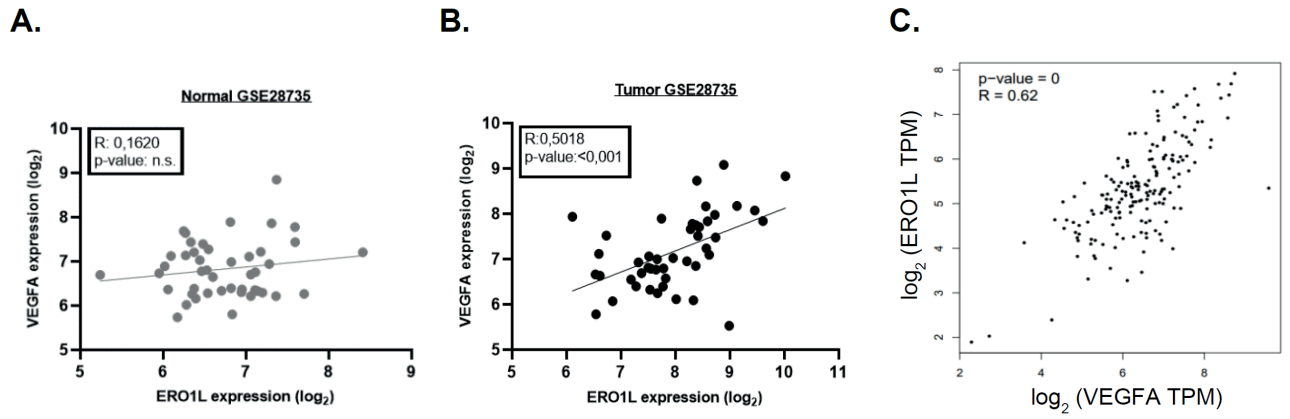

Figure 7. Correlation analysis of VEGFA and ERO1L in tumor and normal tissue A)The lack of correlation was observed between ERO1L and VEGFA in normal pancreatic tissues of PAAD patients based on the GSE28735 data set. The positive correlation of VEGFA and ERO1L was observed in pancreatic cancer tissues based on the B) GSE28735 data set and C) TCGA cohort analyzed by GEPIA. p<0,05 was used as the significance threshold.

(Figure 7B-C). Together, these results provide strong evidence that ERO1L is a principal prognostic marker in pancreatic cancer. Its levels were regulated via hypoxia-ERS axis and its contribution to tumor progression might very well be driven by VEGFA modulation in tumor tissues.

DISCUSSION

The strong proliferative nature of cancer cells is thought to accompany the high protein expression required during the formation of new cells. These proteins need to be properly folded in the ER prior to taking up their final positions in the cell. Disulfide bonds play a crucial role in the folding process and their formation is regulated by PDI/ERO1L axis. Thus, a disturbance in this pathway not only impair organelle homeostasis but also distort the protein repertoire which in turn may modulate oncogenic events in tumors [4,5]. ERO1L levels are known to be regulated by hypoxia and ERS [6,7]. Moreover, ERO1L controls VEGFA levels, a well known angiogenic molecule, transcriptionally and post-transcriptionally [12]. However, the role of the hypoxia/ERS/ERO1L/VEGFA axis in human pan-cancer has not been identified and whether ERO1L can be used as a biomarker or therapeutic target is still obscure.

In our study, we have focused on pancreatic cancer due to prominent presence of ERO1L increasing stimuli, being hypoxia and ERS [14]. Our results also verified the notable hypoxic and ERS active nature of pancreatic cancers by showing the presence of upregulated hypoxia marker HIF1A and ERS markers DDIT3, HSPA5 in tumors compared to healthy tissues. The existence of prevalent ERO1L augmenting stimuli in pancreatic cancer is supported by our data showing ERO1L's significantly higher expression levels in pancreatic cancer compared to other cancer types. Moreover, its expression profile is upregulated in pancreatic tumor compared to normal tissues. Notably, we detected a strong prognostic value for ERO1L in pancreatic cancer as well. Patients with high ERO1L levels had lower overall survival and disease specific survival. This data was also supported by the increased ERO1L levels seen in deceased patients. In addition, high ERO1L also resulted in shortened disease free interval and progression free interval. The elevated ERO1L levels in recurred patients were in line with this finding and suggested that regulating ERO1L levels might be a valuable tool for follow-up treatment. The presence of KRAS mutations in pancreatic cancer is a well known indicative for poor prognosis [39]. KRAS mutation plays a pro-oncogenic role and there are no drugs directly targeting mutant KRAS which in turn making the treatment of pancreatic cancer patients carrying KRAS mutation harder. Our results revealed increased ERO1L levels in tumors carrying mutant KRAS thus showing that ERO1L might be a used as a novel therapy target in these patients. Overall, our results showed that ERO1L was a plausible oncogenic gene with a prognostic value in pancreatic cancer. Additionally, based on the results from the Oncomine and TNMplot, ERO1L

expression is upregulated in many other cancer types including bladder, breast, lung and colorectal cancer thus it might very well be that ERO1L is involved in the progression of other cancer types as well.

Previous studies pinpointed hypoxia and ERS activation as the main regulators of ERO1L expression [6,8]. The increment in ERO1L levels with increasing hypoxia and ER stress signature scores verified those findings. Since hypoxia is a well known inducer of ERS [31], we hypothesized that ERS activation bridges hypoxia and ERO1L expression. The strong correlation between hypoxia marker HIF1A vs. ERS and ERS vs. ERO1L indicated that hypoxia-induced ERS might very well be the axis leading to ERO1L upregulation in the tumor microenvironment.

Subsequent to determination of upstream regulators of ERO1L levels, we aimed to unveil the downstream effectors that might regulate pancreatic tumor progression. Angiogenesis plays a key role in cancer progression. ERO1L was found to be involved in VEGFA, a master regulator of angiogenesis, modulation at transcriptional and post-transcriptional level [12,28]. Analysis using gene expression data series GSE28735 and GEPIA exhibited that ERO1L shows correlation with VEGFA levels exclusively in pancreatic tumor tissues. This may also explain our data showing high ERO1L expression in metastatic tumors based on the fact that angiogenesis has an essential role in metastasis. The development of resistance against the common treatment regimen, chemotherapy has led to a continuing search for novel therapeutic targets in pancreatic cancer. The increased levels of VEGF and its receptors in pancreatic cancer makes anti-angiogenic therapy a strong candidate for its treatment. In this context, bevacizumab, a recombinant humanized monoclonal antibody that binds to VEGFA, seems to be a promising target. However, high VEGFA levels have been shown to be an underlying factor for developing resistance to bevacizumab treatment [40]. Although it is tempting to speculate that decreasing VEGFA levels by blocking ERO1L might improve bevacizumab's efficacy, further studies are warranted. Taken together, our data uncover a mechanistic axis involving hypoxia, ERS, ERO1L, VEGFA and tumor progression in pancreatic cancer.

Author contribution

Study conception and design: BK; data collection: BK; analysis and interpretation of results: BK; draft manuscript preparation: BK. The author reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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