CELL CYCLE
2018, VOL. 17, NO. 17, 2164–2174
https://doi.org/10.1080/15384101.2018.1520558

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Elevated expression of NFE2L3 predicts the poor prognosis of pancreatic cancer patients

Hui Wang*[a], Ming Zhan*[a], Ruimeng Yang[b], Yongheng Shi[c], Qiang Liu[c], and Jian Wang[a]

[a]Department of Biliary-Pancreatic Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China; [b]The Core Laboratory in Medical Center of Clinical Research, Department of Endocrinology, Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; [c]Department of Pathology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

ABSTRACT

The highly malignant feature and difficulties for early diagnosis are the key reasons contributing to the poor prognosis of pancreatic cancer (PC) patients. NFE2L3 is a nuclear transcription factor, which has been reported an important biomarker of several tumors. But the role of NFE2L3 in PC remained undefined. Herein, through qPCR and immunohistochemistry, we found a significantly increased NFE2L3 in PC tissues as compared with adjacent non-tumor tissues. While reducing NFE2L3 expression suppressed the invasion abilities of PC cells, elevated NFE2L3 was found associated with lymph node metastasis (P = 0.001; HR = 3.95; 95% *CI*: 1.70 – 9.17) and advanced TNM stages ($P < 0.001$; HR = 4.06; 95% *CI*: 1.74 – 9.46). Consistently, data from both our two cohorts and the TCGA database revealed that higher NFE2L3 PC carriers had worse outcomes than those lower NFE2L3 expressers. Lastly, we confirmed the regulatory role of NFE2L3 on VEGFA, an important player involved in tumor angiogenesis. Collectively, our investigations suggested the oncogenic role of NFE2L3 in PC development and provided the rational for future adding NFE2L3 for the risk assessment of PC patients.

Abbreviations: NFE2L3: NF-E2-related factor 3; UHMK1: U2AF homology motif kinase 1; VEGFA: vascular endothelial growth factor A; GEO: gene expression omnibus; TCGA: The Cancer Genome Atlas; HPDE: human pancreas duct cells; OS: overall survival; IHC: immunohistochemistry; FFPE: formalin-fixed and paraffin-embedded; SEM: standard error of mean

ARTICLE HISTORY
Received 22 May 2018
Revised 29 July 2018
Accepted 28 August 2018

KEYWORDS
Pancreatic cancer; NFE2L3; VEGFA; prognosis; biomarker

Introduction

As one of the most aggressive diseases, pancreatic cancer (PC) represents the fourth highest cause of cancer deaths worldwide, with a 5-year survival rate of 7%, and only 10% – 20% cases who have radical resection can survive 5 years [1–3]. Most PC patients once diagnosed, are often at advanced stages and thus loss the opportunities for curative surgery [4,5]. Therefore, further investigations into understanding the molecular mechanisms and biological behaviors of PC are urgently needed. Moreover, identifying novel biologic markers for early diagnosis and prognosis prediction would be helpful for future developing new targeted therapy drugs.

NFE2L3 (NF-E2-related factor 3) is a member of the cap 'n' collar family of basic-leucine zipper transcription factors, which played critical roles in regulating the expression of developmental related genes [6–8]. While sequestrated in the endoplasmic reticulum under physiological conditions, NFE2L3 would then translocate into the nucleus and heterodimerize with small Maf proteins for activating target genes expression [9]. Recently, increasing evidences suggested the involvement of NFE2L3 in differentiation, inflammation, and carcinogenesis [10–13]. For instance, a tumor suppressor role of NFE2L3 in resisting lymphomagenesis was identified via *in vivo* mouse model [13]. Elevated NFE2L3 in Hodgkin lymphoma, non-Hodgkin cell lineages, as well as Mantle cell lymphoma specimens were also found [14–16]. Aside from hematopoietic malignancies, NFE2L3 mRNA levels were also increased in breast cancer cells [17,18]. It was suggested that NFE2L3 could promote cell proliferation through modulating the expression of the cell cycle regulator U2AF homology motif

CONTACT Jian Wang dr_wangjian@126.com
*Equal contributors

© 2018 Informa UK Limited, trading as Taylor & Francis Group

kinase 1 (UHMK1) [19]. Although the biological and pathological significance of NFE2L3 are coming into sight, the clinical significance and prognostic value of NFE2L3 in PC patients remain largely unknown and require further investigations.

In this study, through combing our sample set with the expression data obtained from the online databases, we retrospectively analyzed the levels of NFE2L3 in PC and the adjacent non-tumor tissues. Moreover, the relationships between NFE2L3 expression and clinical features as well as prognosis were also examined among PC patients. NFE2L3 was positively correlated with VEGFA in PC samples. Collectively, our investigations suggested the involvement of NFE2L3 in PC development, which could be a favorable marker for predicting the prognosis of PC patients.

Results

Elevated NFE2L3 expression in PC tissues

For investigating the function of NFE2L3 in PC, we first examined its expression in four independent microarray data sets from gene expression omnibus (GEO) database. Interestingly, all the databases show consistently increased NFE2L3 in PC as compared with normal pancreatic tissues (GSE16515: $P < 0.01$; GSE15471: $P < 0.001$; GSE55643: $P < 0.05$; GSE28735: $P < 0.001$) (Figure 1(a-d)). Likely, elevated NFE2L3 in PC was also detected in the cancer genome atlas (TCGA) database (Figure 1(e)). This result was also confirmed in our 46-paired fresh PC and their corresponding adjacent non-tumor tissues (Renji cohort) as assayed by qPCR ($P < 0.001$) (Figure 1(f)). Further assessing the protein and mRNA level of NFE2L3 in various pancreatic cell

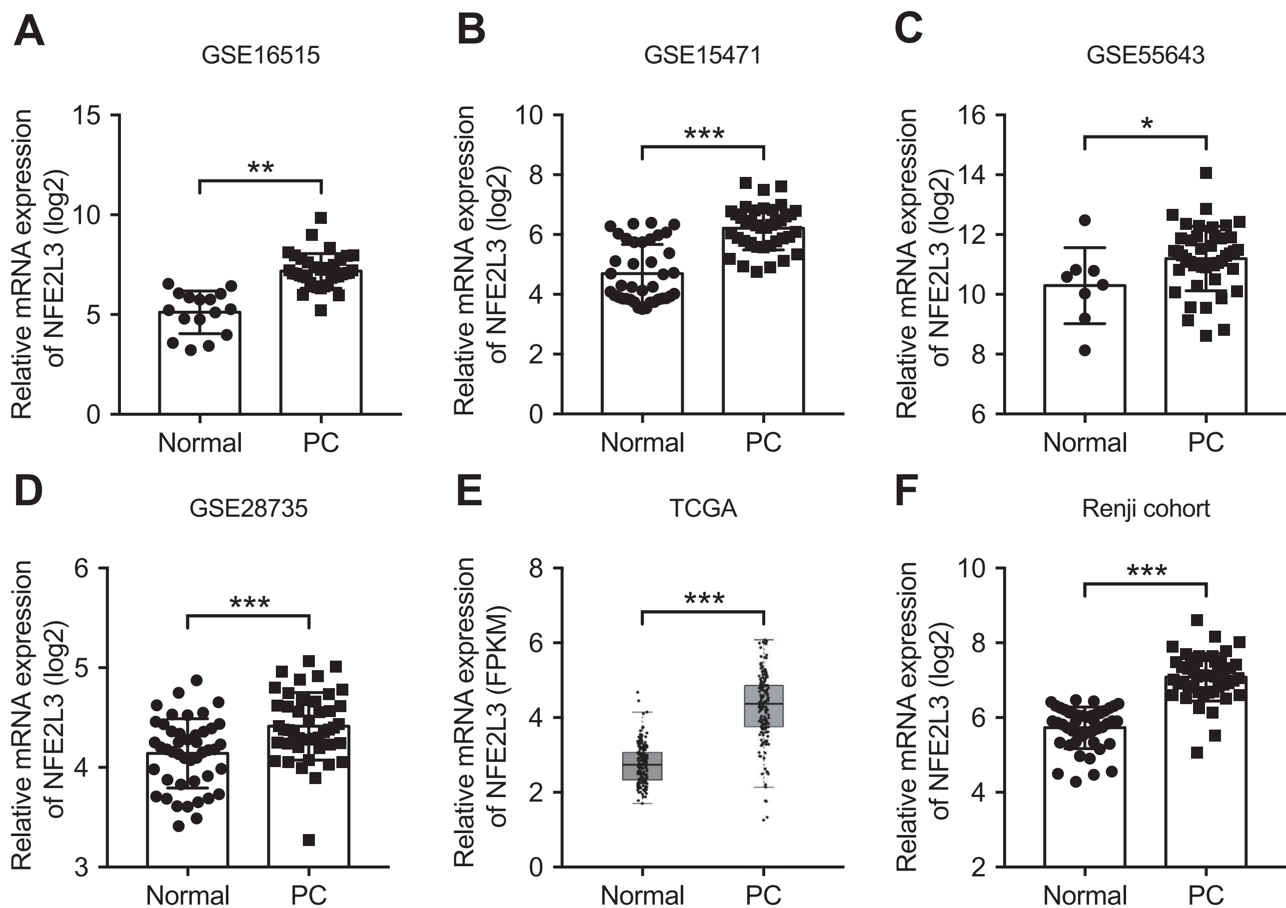

Figure 1. Elevated NFE2L3 mRNA level in PC tissues. (A-D) The mRNA levels of NFE2L3 in PC and adjacent nontumor tissues examined in four GEO databases. Consistently increased NFE2L3 in PC were found in GSE16515 (A); GSE15471 (B); GSE55643 (C) and GSE28735 (D). (E) Upregulated NFE2L3 in PC tissues as detected via the TCGA database. (F) qPCR assessment of NFE2L3 expression of the 46 pairs of PC and adjacent nontumor tissues in the Renji cohort. bar, SEM, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Student's t test; FPKM: fragments per kilo-base of exon per million fragments mapped.

lines, we found that while NFE2L3 was barely undetectable in human pancreas duct cell line (HPDE), it was otherwise obviously increased in the five PC cell lines (Figure 2(a,b)). We then performed two PC tissue microarrays for examination the protein level of NFE2L3. Significantly increased proportions of PC tissues accompanied with positive NFE2L3 expression as compared with adjacent non-tumor tissues were observed in both the two cohorts (verification cohort: 41% vs. 19%, $P < 0.01$; Renji cohort: 46% vs. 15%, $P < 0.01$) (Figure 2(c-e)). Hence, above results showed that both the mRNA and protein level of NFE2L3 were significantly increased in PC tissues.

Correlations between the NFE2L3 expression and the clinical features of PC patients

In order to validate the clinical significance of increased NFE2L3 expression in PC tissues, the relationship between NFE2L3 levels and various important clinicopathologic parameters like tumor size and TNM classification were assessed in the verification cohort. We found that higher NFE2L3 carriers are prone to have lymph node metastasis ($P = 0.001$) and at advanced TNM stages ($P < 0.001$) (Table 1). However, the correlations between NFE2L3 and other factors including tumor size, nerve invasion and distant metastasis were not detected (Table 1). To study the potential biological function of NFE2L3 in PC, MTS and Matrigel transwell assays were conducted to estimate the role of NFE2L3 in modulating cellular proliferation and metastasis. Compared with the group transfected with control siRNA, NFE2L3 siRNA treated PANC-1 and SW1990 cells manifested greatly inhibited invasion ability (Figure 2(f)). Cell proliferation rate was otherwise not affected with altered NFE2L3 expression (Figure 2(g)).

Higher NFE2L3 expression correlates with the poor prognosis of PC patients

For evaluating whether NFE2L3 could be used for predicting the prognosis of PC patients, we first examined the correlation between NFE2L3 expression and the overall survival (OS) rate as well as the disease free survival rate using data from the TCGA database. The results revealed that PC patients with higher NFE2L3 expression often accompanied with poor prognosis as show by both reduced cumulative survival rate ($P = 0.0001$) and disease free survival rate ($P = 0.0002$) (Figure 3(a,b)). Supporting this, similar results were obtained from both data of our Renji cohort and the verification cohort, reinforcing the notion that NFE2L3 functioned as an oncogene in PC (Figure 3(c,d)).

Moreover, our univariable analyzes displayed that NFE2L3 expression, along with TNM stage, lymph node metastasis, and pathological grade are all important prognostic factors in predicting the OS rate of PC patients (Table 2). Meanwhile, multivariable analysis also suggested that NFE2L3 and the pathological grade were independent predictors of OS in PC patients (Table 2). Taken together, these data demonstrated that NFE2L3 level might be a potential marker for predicting the prognosis of PC patients.

Increased NFE2L3 correlated with positive VEGFA expression in PC tissues

Previous study mentioned the importance of NFE2L3 in the manipulation of stem cell differentiation towards vascular lineage [20]. Increasing vascular formation is a well-known factor often utilized by cancer cells for further invasion and distant metastasis [21,22]. VEGFA, originally known as vascular permeability factor, is a signal protein produced by cells that could stimulate the formation of blood vessels [23]. Interestingly, we found that the mRNA level of NFE2L3 and VEGFA are positively correlated in both the TCGA ($P = 0.004$) and our Renji cohort ($P < 0.001$) (Figure 4(a,b)). Consistently, immunohistochemistry (IHC) assay of the samples in the verification cohort further confirmed their positive relationship in the protein level (Figure 4(c,d)). In supporting a direct regulatory role between NFE2L3 and VEGFA, we found that reducing NFE2L3 in both PANC-1 and SW1990 cells led to decreased VEGFA expression (Figure 4(e,f)). Moreover, combining NFE2L3 and VEGFA expression we further stratified the prognosis of PC patients into four groups, which result show that both NFE2L3 and VEGFA higher carriers revealed the worst outcome (Figure 4(g)). Thus our investigations provide the possibility that NFE2L3 might be involved in promoting PC

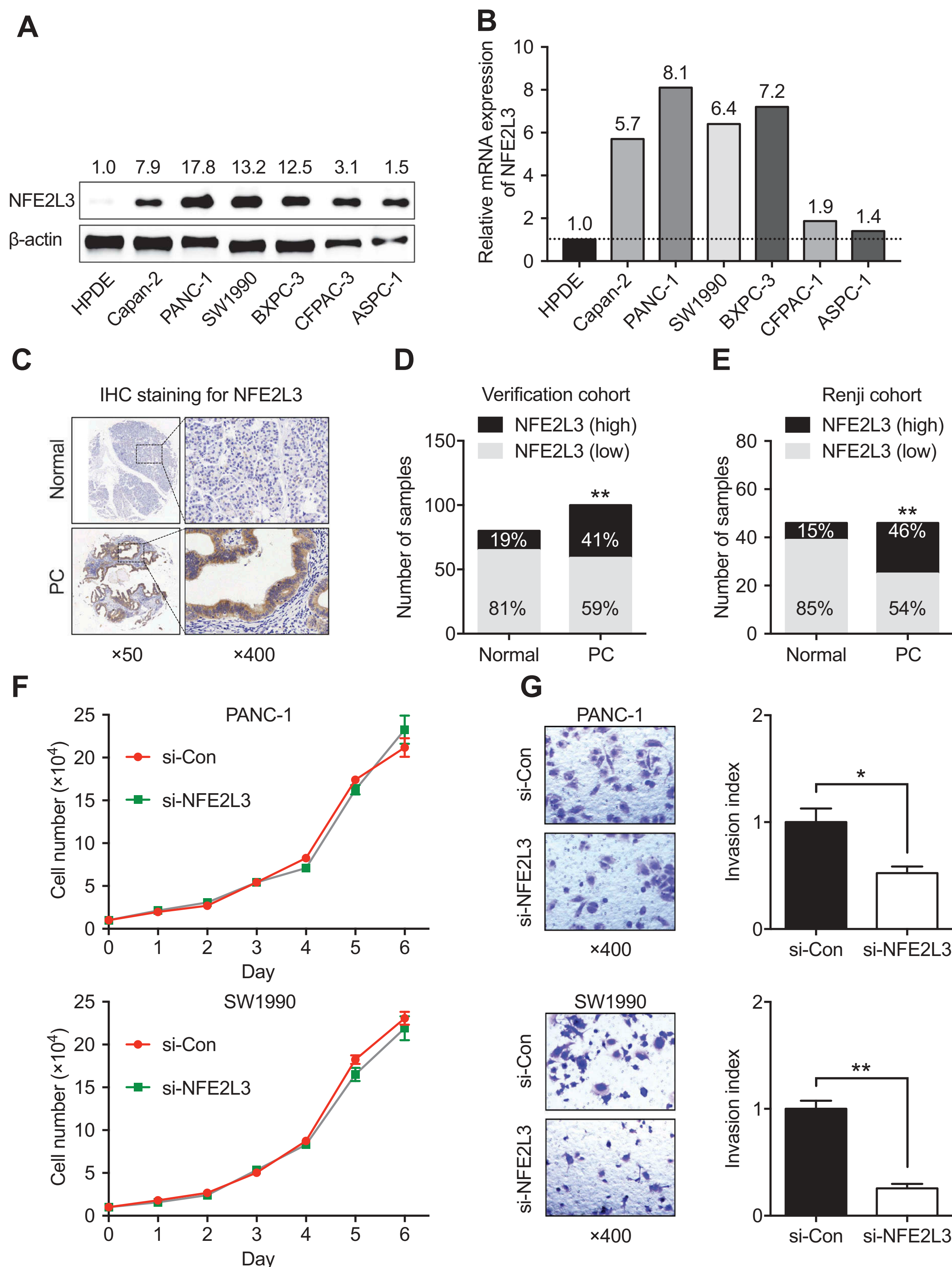

Figure 2. The expression levels of NFE2L3 in PC cell lines and tissues. (A-B) Western blot and qPCR examination show that increased NFE2L3 protein and mRNA levels in six PC cell lines compared with control pancreatic epithelial cell. (C) representative image of IHC staining reveals that while NFE2L3 was barely undetectable in normal tissues, it was instead significantly increased in PC tissues. (D-E) statistical quantification of the number of samples classified by NFE2L3 expression level in normal and PC group respectively. Both Renji and the verification cohort show that obviously increased PC patients with high NFE2L3 expression. (F) PANC-1 and SW1990 cell lines were transfected with NFE2L3 siRNA or a negative control, and cell proliferation was measured by MTS assay. (G) Matrigel transwell assays were used to assess the invasion ability of PC cells transfected with NFE2L3 siRNA or negative control. *$P < 0.05$; **$P < 0.01$; χ^2 test or Student's t test.

Table 1. Correlations between the expression of NFE2L3 and the clinicopathologic features of PC patients in the verification cohort.

Clinicopathological feature	Total	NFE2L3 expression Low (n = 59)	High (n = 41)	*P* value[a]	OR(95%*CI*)
Age (years)					
< 65	37	22(37.3)	15(36.6)	0.868	0.93(0.41–2.11)
≥ 65	63	37(62.7)	26(63.4)		
Gender					
Male	40	24(40.7)	16(39.0)	0.943	1.03(0.45–2.35)
Female	60	35(59.3)	25(61.0)		
Tumor location					
Head	59	36(61.0)	23(56.1)	0.623	1.22(0.55–2.75)
Body/tail	41	23(39.0)	18(43.9)		
Vascular/nerve invasion					
Absent	58	33(55.9)	25(61.0)	0.615	0.81(0.36–1.83)
Present	42	26(44.1)	16(39.0)		
Pathological grade					
Stage I-II	31	18(30.5)	13(31.7)	0.899	1.06(0.45–2.50)
Stage III-IV	69	41(69.5)	28(68.3)		
Tumor size					
≤ 3 cm	40	21(35.6)	19(46.3)	0.281	0.64(0.28–1.44)
> 3 cm	60	38(64.4)	22(53.7)		
T classifitation					
T1 + T2	32	17(28.8)	15(36.6)	0.413	0.7(0.30–1.64)
T3 + T4	68	42(71.2)	26(63.4)		
Lymph node metastasis					
Absent	56	41(69.5)	15(36.6)	0.001	3.95(1.70–9.17)
Present	44	18(30.5)	26(63.4)		
Distant metastasis					
Absent	98	58(98.3)	40(97.6)	0.794	1.45(0.09–23.87)
Present	2	1(1.7)	1(2.4)		
TNM stage[b]					
Stage I-II	54	40(67.8)	14(34.1)	< 0.001	4.06(1.74–9.46)
Stage III-IV	46	19(32.2)	27(65.9)		

OR: odds ratio; CI: confidence interval
[a]Chi square test
[b]Tumor stage was defined according to the American Joint Committee on Cancer (AJCC) TNM staging system (AJCC 7th edition).

progression through modulating VEGFA level, which would be validated by future studies.

Discussion

In this study, through combined analyzing the GEO database and our PC sample set, we found that remarkably elevated NFE2L3 mRNA in PC as compared with adjacent non-tumor tissues. NFE2L3 protein was also increased in both PC cell lines and PC tissues. Moreover, elevated NFE2L3 were found associated clinical features including lymph node metastasis and advanced TNM stages. We also found that NFE2L3 expression could be potentially used in predicting the prognosis of PC patients. Lastly, the oncogenic role of NFE2L3 might be fulfilled through modifying VEGFA expression.

The NFE2L3 transcription factor has been found implicated in various cellular processes, including carcinogenesis, stress response, differentiation, and inflammation [10–13]. Mice deficiency of NFE2L3 was predisposed to carcinogen-induced lymphomagenesis, suggesting a suppressor role of NFE2L3 in hematopoietic malignancies. However, NFE2L3 transcripts were otherwise found increased in tumors like breast cancer. So defining the precise role of NFE2L3 in cancer development was confounded the fact that mRNA levels could not reflect the final output of functional proteins. Hence, in our study, both the mRNA and protein levels of NFE2L3 in PC and adjacent non-tumor tissues were assessed. In fact, while NFE2L3 protein was barely undetectable in pancreatic epithelial cell line and adjacent non-tumor tissues, it was otherwise remarkably increased in various PC

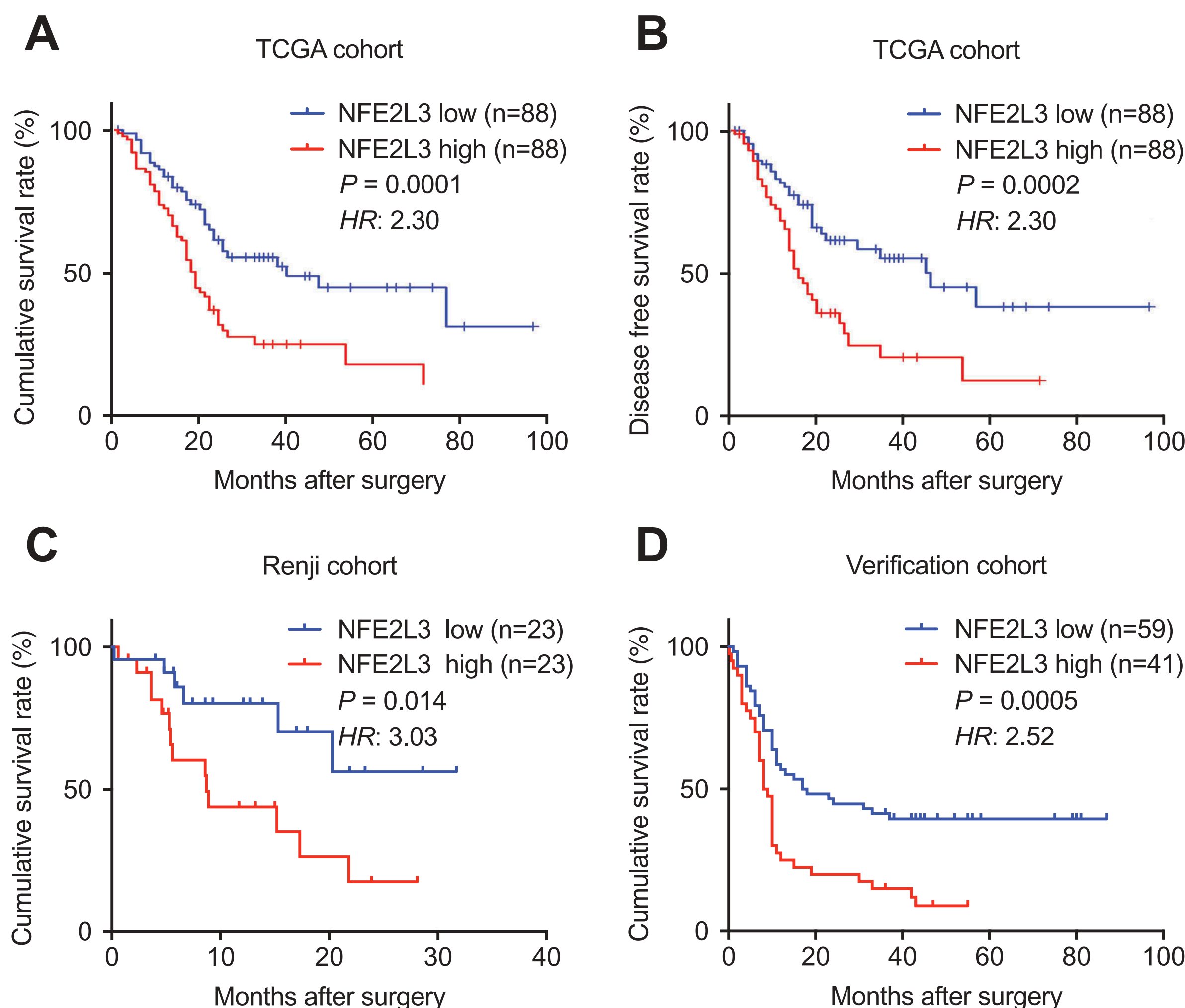

Figure 3. Assessment of the prognosis predicting values of NFE2L3 among PC patients. (A-B) the TCGA database shows that higher NFE2L3 carriers had significantly reduced cumulative survival rate (A) and disease free survival rate (B) than patients with lower NFE2L3 expression. (C-D) the correlations between increased NFE2L3 expression and the poor prognosis of PC patients were also identified in our Renji and the verification cohort.

Table 2. Univariable and multivariable Cox regression analyzes of the associations between the prognostic parameters and OS among the PC patients in the verification cohort.

	Univariate analysis			Multivariable analysis		
Prognostic parameter	β	HR	*P* value	β	HR	*P* value
Age	0.18	1.19	0.466	/	/	/
Gender	0.19	1.21	0.445	/	/	/
Tumor location	0.12	1.12	0.622	/	/	/
Vascular/nerve invasion	0.23	1.25	0.337	/	/	/
Pathological grade	0.62	1.87	0.011	0.90	2.46	0.001
Tumor size	−0.10	0.90	0.666	/	/	/
T classifitation	−0.03	0.97	0.894	/	/	/
Lymph node metastasis	0.85	2.33	0.001	0.42	1.51	0.586
Distant metastasis	0.56	1.76	0.433	/	/	/
TNM stage	0.89	2.44	0.001	0.59	1.80	0.432
NFE2L3 expression	0.78	2.18	0.001	0.70	2.02	0.004

HR: hazard ratio

cell lines and PC tissues samples. In supporting of the oncogenic role in PC development, the associations between NFE2L3 and several important tumor-related clinical parameters were examined. The results show that elevated NFE2L3 correlated with lymph node metastasis

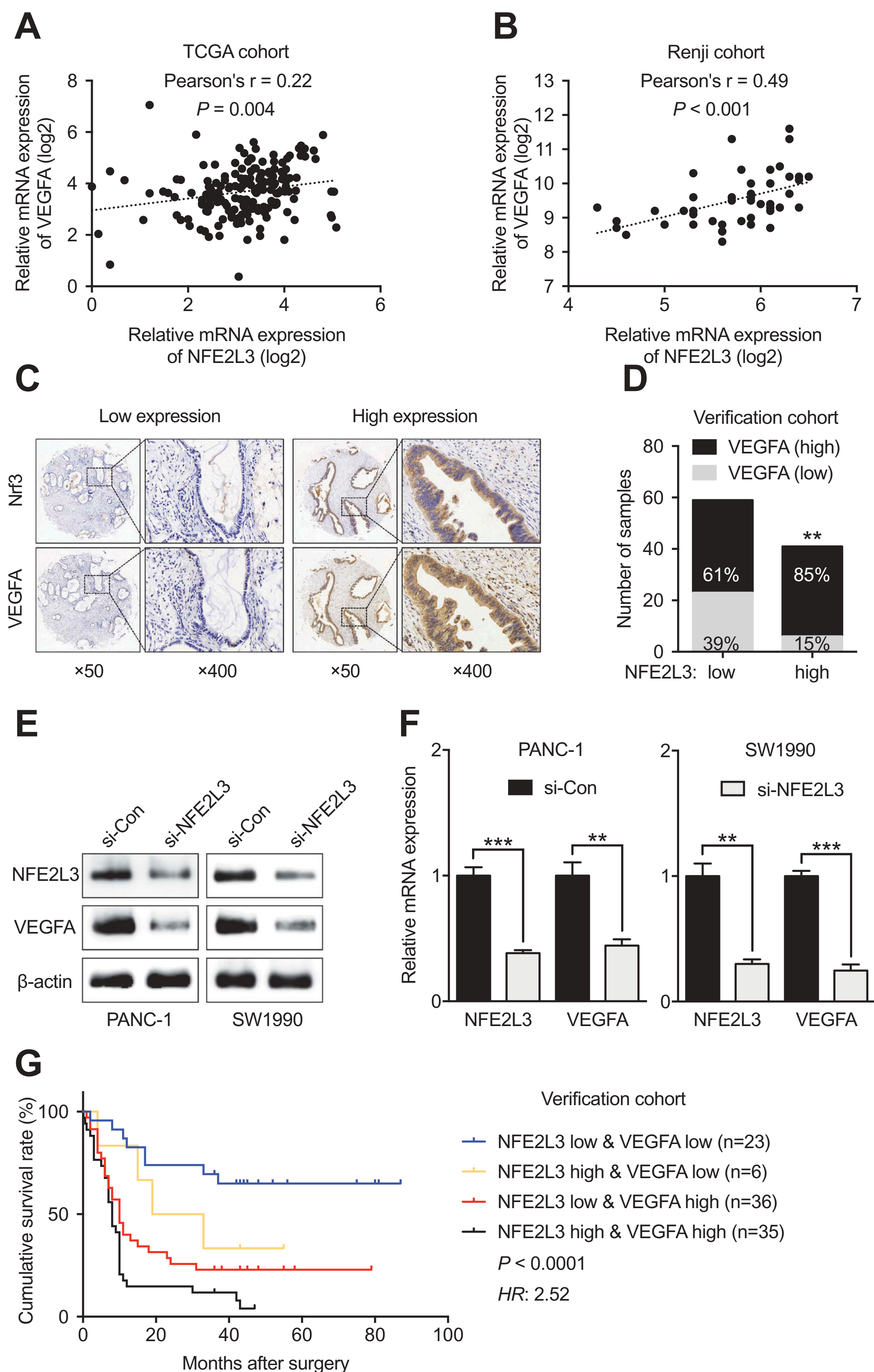

Figure 4. Examination of the association between NFE2L3 and VEGFA expression levels in PC tissues. (A) the TCGA database reveals that NFE2L3 is positively correlated with VEGFA in PC tissues. (B) positive correlations between NFE2L3 and VEGFA were also found in the Renji PC sample set. (C) representative image show the consistently elevated NFE2L3 and VEGFA expression in PC tissues via IHC. (D) Quantification of the number of patients with higher or lower VEGFA expression in the two groups classified by NFE2L3 levels respectively. (E-F) immunoblotting and qPCR analyzes of the NFE2L3 expression in PC cells after transfection with NFE2L3 siRNA or negative control. (G) Kaplan-Meier analyzes of the prognostic prediction value of NFE2L3 and VEGFA in PC patients. $^{**}P < 0.01$; $^{***}P < 0.001$; χ^2 test or student's t test.

and at advanced TNM stages, which also independently predicted the overall survival rate of PC patients. Collectively, our investigations suggested that NFE2L3 probably act as an oncogene in PC development.

NFE2L3 when translocates into the nuclear, also binds the antioxidant response element or Maf recognition elements by heterodimerizing with small Maf proteins [7]. It has also been mentioned that NFE2L3 regulates cancer cell proliferation through regulating the cell cycle regulator UHMK1 expression [19]. The roles of another member of CNC family, NFE2L2 in controlling the expression of ROS- and compound-detoxifying enzymes have been well-established [24,25]. Whether NFE2L2 and NFE2L3 harbor similar or antagonistic functions remain unknown. Indeed, the regulation of several oxidative related genes like NQO1 and NOX4 by NFE2L3 have been described [11,26]. ROS is an important regulator of cancer development through modulating processes like vascular formation. Vascular formation encompassing several processes including smooth muscle cell differentiation, angiogenesis, endothelial progenitor cells recruitment, and vascular cell migration. Recent study suggested that ROS mediated by NFE2L3 plays important role in the differentiation and phenotypic modulation of vascular smooth muscle cells [20]. Vascular development is indispensible for both cancer development and progression and VEGFA is one of the master regulators in orchestrating vascular formation. So in order to verify the possibly existed relationship between NFE2L3 and VEGFA, their expression in PC tissues were examined at the same time. Positive correlations of both proteins and mRNA between NFE2L3 and VEGFA were identified. We also found that NFE2L3 can directly regulate the expression of VEGFA in PC cells. More importantly, even worse outcomes were detected among PC patients with NFE2L3 and VEGFA double positive than those with single positive. More studies are needed in the future for validating the possibly existed regulatory role between them.

In summary, our study further demonstrated that upregulation of NFE2L3 is associated with poor survival in PC patients, indicating that NFE2L3 may serve as a valuable prognostic marker in PC and represent a potential molecular target for the treatment of PC.

Materials and methods

Clinical specimen

This study was approved by the Ethical Committee of Renji hospital, School of Medicine, Shanghai Jiao Tong University. All of the subjects were provided with written informed consent before enrollment. PC and matched adjacent nontumor tissues (> 5 cm away from the PC tissue) were obtained from 46 PC patients (Renji cohort) who underwent surgical resection at the Department of Biliary-Pancreatic Surgery (Renji Hospital) from January 2012 to December 2014. Tissue microarrays of 100 primary PC samples and 80 adjacent nontumor tissues (verification cohort) were obtained from Shanghai OutdoBiotech Ltd. The clinico-pathological features of the 100 PC patients are shown in Table 1. All PC patients underwent surgical resection without any other tumor-related therapies. Retrospectively follow-up was conducted by office visit, telephone call, or outpatient clinic visit, and OS information was acquired from all PC patients in the Renji cohort and the verification cohort. The definition of OS is the time interval between the dates of surgery and last follow up (31 December 2016) or death.

Online PC data sets acquisition and process

The data sets of GSE16515, GSE15471, GSE55643, and GSE28735 were downloaded from the public source GEO data repository (http://www.ncbi.nlm.nih.gov/geo/) [27]. The NFE2L3 and VEGFA expression data for PC tissue and the corresponding prognostic data were downloaded from TCGA (https://gdc-portal.nci.nih.gov/), which were processed and analyzed by GEPIA, a web server for cancer and normal gene expression profiling and interactive analyzes (http://gepia.cancer-pku.cn) [28].

Cell culture

Human pancreatic ductal epithelial cell line HPDE were obtained from the American Type Culture Collection. Human PC cell lines Capan-2, PANC-1, SW1990, BXPC-3, CFPAC-3, and ASPC-1 were all preserved in the lab of biliary-pancreatic surgery at Renji hospital. All cell lines were cultured in RPMI-1640 (Gibco, Cat.

#88,365) which was supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C.

SiRNA knockdown experiment

The PANC-1 and SW1990 cells were cultured for 24 h in the medium without antibiotics, and then transfected with 40 nM siRNA using Lipofectamine 2000 (Invitrogen, Cat. #11,668,027). After transfection for 48 h, the cells were utilized for MTT and Transwell experiments. The sequences of the sense strands of the NFE2L3 siRNA duplexes as follows: 5′-CGCAAAUU GGACAUAAUUU-3′.

Cell proliferation and invasion assays

Cell proliferation was determined by MTS assays (Promega, Cat. # G3582). Briefly, PC cells were seeded in 96-well plates and cultured at 37°C for 24 h. After transfection, the MTS solution (20 µl) was added to each well of the plate at the different time points. Then, the plates were incubated at 37°C for 1 h, and the absorbance at 450 nm was measured. Cell invasion assays were conducted using 24-well transwell chambers with 8.0-µ m pore size polycarbonate membranes (Corning, Cat. #3374). Then, 50,000 cells were seeded on the top side of the membrane pre-coated with Matrigel (Corning Cat. #354,234). After incubation, the PC cells inside the upper chamber were removed with cotton swabs. The invaded cells on the lower membrane surface were then fixed and stained with a 5% crystal violet solution. Three images of ten random fields of each membrane were captured, and the number of migratory cells was counted. The invasion index is defined as the ratio of the experimental group to the control group.

RNA extraction and qpcr analysis

Total RNAs were extracted from tissues or cells using TRI reagent (Sigma, Cat. #93,289), and the cDNA were transcribed using PrimeScript RT Reagent Kit (Takara, Cat. #RR037A) according to the manufacturer's instructions. qPCR was performed by the SYBR Premix Ex Taq (Takara, Cat. #RR420A) in Applied Biosystems ViiATM 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative mRNA expression level was calculated with $2^{-\Delta\Delta CT}$ method and normalized to GAPDH mRNA. Primers for NFE2L3 and VEGFA were purchased from Sangon Biotech (Shanghai, China) and the sequences are listed as follows: NFE2L3 forward, 5′- GGGGAAGAGGAGAAGG CAC-3′, reverse, 5′-GGCTGACACCCTTTCTTCA TT-3′; VEGFA forward, 5′-GGAGGGCAGAATCA TCACGA-3′, reverse, 5′-ATCGCATCAGGGGCAC AC-3′; GAPDH forward, 5′-GAAGGTGAAGGTC GGAGTC-3′, reverse, 5′-GAAGATGGTGATGGG ATTTC-3′.

Western blot

Total proteins were extracted from cells using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Cat. #89,901). Protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Cat. #23,227). Standard western blotting techniques, and the Bio-Rad ChemiDoc MP imaging system (Hercules, CA, USA) were used according to the procedure described previously [29]. The NFE2L3 and VEGFA antibody were purchased from Sigma (Cat. #HPA055889) and Abcam (Cat. #ab1316). The anti-rabbit IgG secondary antibody was purchased from Cell Signaling Technology Inc. (Cat # 5151S).

IHC assay

The IHC staining of formalin-fixed and parrffin-embedded (FFPE) tissues were performed as described previously [29]. Briefly, FFPE tissue microarrays were cut into 3-µm sections and then deparaffinized with xylene and hydrated with graded ethanol solution. Sections were treated with citrate buffer solution at 100°C for 1 min for antigen retrieval and were permeabilized in 3% hydrogen peroxide for 10 min at room temperature. After being blocked with 10% BSA for 30 min, slides were incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies for 60 min at 37°C. Finally, 3,3-diaminobenzidine tetrahydrochloride was used as coloring reagent, and hematoxylin was used as a counterstain for nuclei. The stained fields were photographed using a light microscope equipped with a camera (Olympus, Tokyo, Japan). The primary antibodies used for IHC were as follows:

NFE2L3 (Sigma, Cat. #HPA055889), and VEGFA (Sigma, Cat. #V1253). Semi-quantitative scoring of protein expression was based upon the staining intensity (I: negative, 0; weak, 1; moderate, 2; intense, 3) and the percentage of positive-staining cells (P: 0–5%, scored 0; 6–35%, scored 1; 36–70%, scored 2; and > 70%, scored 3) to obtain a final score (Q) defined as the product of I × P. The tissues with a final score ≤ 1 were sorted into "low expression" and those with a final score > 1 were classified as "high expression." Two senior pathologists performed the scorings independently in a blinded manner. In addition, the IHC staining was also done in a blinded manner.

Statistical analysis

Data were presented as mean ± standard error of mean (SEM). Group comparisons of normally distributed data (log_2 transformed) were performed using unpaired Student's t test. Correlation between NFE2L3 expression and the categorical clinical variables in patients with PC was evaluated by χ^2 test or Fisher's exact test. Kaplan-Meier method and log-rank tests were used to determined cumulative survival and disease free survival. Univariate and multivariate Cox regression analysis were performed to identify potential factors that had significant influences on survival by Cox proportional hazards model. The Pearson χ^2 test was used to analyze the association between the expression levels of NFE2L3 and VEGFA. SPSS 17.0 software (IBM, Chicago, IL, USA) was used for all statistical analysis. Two-sided *P* values < 0.05 were considered as statistically significant.

Declarations

This study was approved by the Ethical Committee of Renji hospital, School of Medicine, Shanghai Jiao Tong University. And all patients involved in this study provided written informed consent.

Availability of data and materials

Online PC microarray data sets GSE16515, GSE15471, GSE55643, GSE2873, TCGA-PAAD and corresponding clinical data in this study were directly downloaded from GEO database (http://www.ncbi.nlm.nih.gov/geo/) and the open access tiers of TCGA data portal (https://gdc-portal.nci.nih.gov/).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Foundation of Shanghai Shen Kang Hospital Development Center (No. 16CR2002A and 16CR3028A), National Science Foundation of China (No. 81472240 and 81773184), and Shanghai Outstanding Academic Leaders Plan (2016, JW).

References

[1] Miller KD, Siegel RL, Lin CC, et al. Cancer treatment and survivorship statistics. CA Cancer J Clin. 2016;66(4):271–289.

[2] Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. N Engl J Med. 2014;371(11):1039–1049.

[3] Siegel RL, Miller KD, Jemal A. Cancer statistics. CA Cancer J Clin. 2017;67(1):7–30.

[4] Canto MI, Hruban RH. Diagnosis: A step closer to screening for curable pancreatic cancer? Nat Rev Gastroenterol Hepatol. 2015;12(8):431–432.

[5] Heestand GM, Murphy JD, Lowy AM. Approach to patients with pancreatic cancer without detectable metastases. J Clin Oncol. 2015;33(16):1770–1778.

[6] Andrews NC. The NF-E2 transcription factor. Int J Biochem Cell Biol. 1998;30(4):429–432.

[7] Chevillard G, Blank V. NFE2L3 (NRF3): the cinderella of the cap'n'Collar transcription factors. Cell Mol Life Sci. 2011;68(20):3337–3348.

[8] Derjuga A, Gourley TS, Holm TM, et al. Complexity of CNC transcription factors as revealed by gene targeting of the Nrf3 locus. Mol Cell Biol. 2004;24(8):3286–3294.

[9] Zhang Y, Kobayashi A, Yamamoto M, et al. The Nrf3 transcription factor is a membrane-bound glycoprotein targeted to the endoplasmic reticulum through its N-terminal homology box 1 sequence. J Biol Chem. 2009;284(5):3195–3210.

[10] Braun S, Hanselmann C, Gassmann MG, et al. Nrf2 transcription factor, a novel target of keratinocyte growth factor action which regulates gene expression and inflammation in the healing skin wound. Mol Cell Biol. 2002;22(15):5492–5505.

[11] Kannan MB, Dodard-Friedman I, Blank V. Stringent control of NFE2L3 (nuclear factor, erythroid 2-like 3; NRF3) protein degradation by FBW7 (F-box/WD repeat-containing protein 7) and glycogen synthase kinase 3 (GSK3). J Biol Chem. 2015;290(43):26292–26302.

[12] Xiao Q, Pepe AE, Wang G, et al. Nrf3-Pla2g7 interaction plays an essential role in smooth muscle differentiation from stem cells. Arterioscler Thromb Vasc Biol. 2012;32(3):730–744.

[13] Chevillard G, Paquet M, Blank V. Nfe2l3 (Nrf3) deficiency predisposes mice to T-cell lymphoblastic lymphoma. Blood. 2011;117(6):2005–2008.

[14] Kuppers R, Klein U, Schwering I, et al. Identification of Hodgkin and reed-sternberg cell-specific genes by gene expression profiling. J Clin Invest. 2003;111(4):529–537.

[15] Obrador-Hevia A, Fernández de Mattos S, Villalonga P, et al. Molecular biology of mantle cell lymphoma: from profiling studies to new therapeutic strategies. Blood Rev. 2009;23(5):205–216.

[16] Willenbrock K, Kuppers R, Renne C, et al. Common features and differences in the transcriptome of large cell anaplastic lymphoma and classical Hodgkin's lymphoma. Haematologica. 2006;91(5):596–604.

[17] Almstrup K, Ottesen AM, Sonne SB, et al. Genomic and gene expression signature of the pre-invasive testicular carcinoma in situ. Cell Tissue Res. 2005;322(1):159–165.

[18] Rhee DK, Park SH, Jang YK. Molecular signatures associated with transformation and progression to breast cancer in the isogenic MCF10 model. Genomics. 2008;92 (6):419–428.

[19] Chowdhury A, Katoh H, Hatanaka A, et al. Multiple regulatory mechanisms of the biological function of NRF3 (NFE2L3) control cancer cell proliferation. Sci Rep. 2017;7(1):12494.

[20] Pepe AE, Xiao Q, Zampetaki A, et al. Crucial role of nrf3 in smooth muscle cell differentiation from stem cells. Circ Res. 2010;106(5):870–879.

[21] Dulloo I, Hooi PB, Sabapathy K. Hypoxia-induced DNp73 stabilization regulates Vegf-A expression and tumor angiogenesis similar to TAp73. Cell Cycle. 2015;14(22):3533–3539.

[22] Yu X, Sha J, Xiang S, et al. Suppression of KSHV-induced angiopoietin-2 inhibits angiogenesis, infiltration of inflammatory cells, and tumor growth. Cell Cycle. 2016;15 (15):2053–2065.

[23] Falchook GS, Kurzrock R. VEGF and dual-EGFR inhibition in colorectal cancer. Cell Cycle. 2015;14(8):1129–1130.

[24] de Vries HE, Witte M, Hondius D, et al. Nrf2-induced antioxidant protection: a promising target to counteract ROS-mediated damage in neurodegenerative disease? Free Radic Biol Med. 2008;45(10):1375–1383.

[25] Schafer M, Dutsch S, auf dem Keller U, et al. Nrf2: a central regulator of UV protection in the epidermis. Cell Cycle. 2010;9(15):2917–2918.

[26] Sankaranarayanan K, Jaiswal AK. Nrf3 negatively regulates antioxidant-response element-mediated expression and antioxidant induction of NAD(P)H: quinoneoxidoreductase1 gene. J Biol Chem. 2004;279 (49):50810–50817.

[27] Edgar R, Domrachev M, Lash AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002;30(1):207–210.

[28] Tang Z, Li C, Kang B, et al. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res. 2017;45(W1):W98–W102.

[29] Long M, Zhan M, Xu S, et al. miR-92b-3p acts as a tumor suppressor by targeting Gabra3 in pancreatic cancer. Mol Cancer. 2017;16(1):167.