American Journal of Internal Medicine
2018; 6(6): 144-151
http://www.sciencepublishinggroup.com/j/ajim
doi: 10.11648/j.ajim.20180606.11
ISSN: 2330-4316 (Print); ISSN: 2330-4324 (Online)

The Expression and Prognostic Value of NOX4 in Gastric Cancer

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To cite this article:

Lu Qiao, Ning Xie, Yuru Bai, Bin Qin, Baicang Zou, Yongquan Shi, Yan Chen, Na Liu, Jinhai Wang. The Expression and Prognostic Value of NOX4 in Gastric Cancer. *American Journal of Internal Medicine*. Vol. 6, No. 6, 2018, pp. 144-151. doi: 10.11648/j.ajim.20180606.11

Received: August 1, 2018; **Accepted**: August 22, 2018; **Published**: October 12, 2018

Abstract: Background: Gastric cancer (GC) is one of the most common malignant tumors worldwide which threaten the health of human. A lot of work has been done in tumor pathogenesis in recent years, while modest progress in diagnosis and treatment of gastric cancer have been made. Methods: Multiple databases including The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) were explored to identify the expression pattern of Nicotinamide Adenine Dinucleotide Phosphate Oxidase 4 (NOX4), the main source of reactive oxygen species, in gastric cancer. We also analyzed the correlation between the expression level of NOX4 and gastric cancer patients' clinical features. The prognostic value of NOX4 was explored in TCGA and K-M Plotter. Last, we utilized TCGA and DAVID databases to uncover the underlying molecular mechanisms of NOX4 with TCGA and DAVID databases. Results: We found that *NOX4* was upregulated in tumors compared with adjacent non-tumor tissues and overexpression was correlated with tumor invasion and TNM stage in gastric cancer. Furthermore, *NOX4* level could be an independent prognostic marker for GC, unacted on the choice of therapy or HER2 expression. Besides, we investigated the potential mechanisms of *NOX4* in gastric cancer. Conclusions: Our findings proved that *NOX4* may be a new prognostic factor or therapeutic marker for gastric cancer.

Keywords: NOX4, Prognostic Values, Gastric Cancer

1. Introduction

Gastric cancer is the third leading cause of cancer-related deaths in worldwide with an estimated 951,600 cases and 723,100 deaths per year [1, 2]. Surgical treatment and adjuvant chemotherapy are the main therapeutic approaches for gastric cancer. However, due to low early detection rate, the great majority of gastric cancer patients presents with advanced-stage disease, resulting in unsatisfactory 5-year survival rates and poor quality of life. Despite tremendous efforts in cancer researches, only modest improvements in early detection and survival are seen. The identification of therapeutic targets and prognostic indicators is therefore highly desirable.

NADPH oxidases 4 (NOX4), belongs to NADPH family, which consists of membrane-bound enzymes and can convert oxygen to superoxide or hydrogen peroxide [3]. Reactive oxygen species (ROS) stand for a class of extracellular metabolic product, which may react with cellular constituents to generate an array of oxidative lesions compromising genome stability which is critical for long-term cellular homeostasis [4]. NADPH family, a major source of ROS, has been proved to participate in occurrence and development of multiple kinds of tumors. NOX1/2/5 were reported to be strongly correlated with genes associated with cancer cell proliferation and metastasis, while NOX4 and DUO1 played an opposite role in liver cancer [5]. Depletion of NOX1 can induce apoptosis through attenuating the AKT signaling

pathway in oral squamous cell carcinoma cell lines [6].

Researches have revealed that *NOX4* levels are altered in pancreatic cancer [7], colorectal cancer [8], hepatocellular carcinoma [9], and thyroid cancer [10]. NOX4 can proliferate TGF-β-induced chemokinesis in pancreatic adenocarcinoma cells through a ROS/p38 MAPK cascade [11]. In addition, inhibition of NOX4 can induce apoptosis in pancreatic cancer cells via the AKT–ASK1 pathway [12]. Nevertheless, its role in gastric cancer and the underlying mechanisms are not clear.

Rapid developments in next-generation sequencing and bioinformatics have provide us a tool to understand molecular characterization of cancers comprehensively and rapidly. In our study, we analyzed the expression level of *NOX4* with multiple databases, including one mRNA expression data set from GEO (http://www.ncbi.nlm.nih.gov/geo/), mRNA sequencing (mRNA-seq) data from TCGA and Oncomine databases. Additionally, we studied the correlation between expression of *NOX4* and characteristic features in gastric cancer. We identified its prognostic value with multiple databases and explored the co-expression genes to investigate the underlying mechanisms.

2. Materials and Methods

2.1. Gene Expression and Characteristics of TCGA Dataset

The gene expression quantification including 57288 genes from 374 tumor tissues and 32 adjacent normal tissues of gastric cancer was obtained from TCGA. Their clinical information dataset was downloaded from the TCGA data portal (March 2017). The R package 'edgeR' was used to analyzed mRNA-seq data to discover the differentially expressed genes (DEGs). DEGs were selected significantly with the criterion of combined adjusted $P < 0.001$ and logFC >1.5. After excluding 8 cases without clinical survival data, we finally got 367 cases, including 133 females and 234 males. There were 157 patients who were younger than 65 years old, while the other 210 were older than 65. Among them, 49 patients were diagnosed TNM stage I, 110 were stage II, 152 were stage III, 40 were stage IV, and the remaining 16 patients were not clear. The median follow-up period was 440 days (ranging from 0 to 3720 days).

2.2. Gene Expression Data in GEO

The microarray data from GEO (accession number GSE 79973) were explored. There were 10 pairs of GC tissue and adjacent non-tumor mucosa in GSE79973. The R package 'limma' was used to identify DEGs. DEGs were selected significantly with the criterion of combined adjusted $P < 0.001$ and logFC >1.5.

2.3. Oncomine Database Analysis

Oncomine (http://www.oncomine.org), an online microarray database, was utilized to examine the mRNA expression difference of *NOX4* between tumor and normal tissues GC. Cancer type, fold change, t-test value, p-value and sample sizes were obtained from studies that showed statistically significant differences.

2.4. Kaplan-Meier Plotter Database Analysis

The KM Plotter (http://kmplot.com/analysis/), which is capable to assess the effect of 54,675 genes on survival using 1065 gastric cancer patients was applied to evaluate the prognostic values of *NOX4* in GC. Patients were split into high and low expression group by the median values of mRNA expression. Then the desired probe ID was separately entered into the database. After that, survival analyses were carried out to achieve Kaplan-Meier plots. P-value < 0.01 was considered to indicate a statistically significant result. Survival outcome, HRs, 95% CIs and p-values were summarized from the KM plotter webpage; some representative plots were also displayed.

2.5. Linked Omics Database Analysis

We made use of LinkedOmics database (http://www.linkedomics.org) to explore TCGA to discover all the associated genes with *NOX4*. We select mRNA expression data from TCGA, and Pearson test was utilized. Gene symbols, pearson correlation coefficient and P value were obtained from the LinkFinder. The association figures were downloaded from LinkFinder.

2.6. Functional Enrichment Analysis

We performed functional enrichment analysis for associated genes through the Database for Annotation, Visualization and Integrated Discovery (DAVID) to uncover biological processes involved in *NOX4*. Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with p-value were considered as significantly enriched. 4 of the maximum hits were presented.

2.7. Statistical Analyses

The correlation between *NOX4* level and characteristic features was analyzed with χ^2 test. OS (Overall survival) was measured from the date of the initial operation until the date of death or last follow-up. Cancer patients were divided into high and low expression group by the median values of mRNA expression and survival curves were created by R 3.3.3. The Kaplan-Meier method and log-rank test were applied to assess OS. Univariate Cox regression proportional hazards analysis was used to assess clinicopathologic characteristics significantly related to OS with HRs and 95% CIs. Multivariate Cox proportional hazards analysis was performed to determine whether the gene expression is an independent prognostic factor. A two-sided p value of < 0.05 was considered statistically significant.

3. Results

3.1. The Expression Pattern of NOX4 in Multiple Datasets

We utilized one mRNA microarray from GEO and TCGA

data to explore the expression of *NOX4* in GC. As shown in Figure 1a, *NOX4* was significantly up-regulated (logFC=1.618) in 374 tumor tissues than the 32 normal tissues from TCGA(P=1.75E-10). In 10 pairs of tumor and adjacent normal tissues of GSE79973, *NOX4* has raised 2.08 times (P=2.18E-05). In addition, to address the mRNA expression differences of *NOX4* between tumor and adjacent non-cancerous tissues in GC, we performed an analysis using the Oncomine database. In four gastric datasets (Table 1), *NOX4* were proved to be overexpressed in tumor than normal tissues. Cho's data revealed that the level of *NOX4* was 2.522 times higher in diffuse gastric adenocarcinoma than in normal gastric tissues, while study from DErrico showed that *NOX4* was up-regulated in gastric intestinal type adenocarcinoma, suggesting that in different pathological types of GC, *NOX4* was highly expressed.

Table 1. *The expression pattern of NOX4 in GC.*

Dataset	Normal(cases)	Tumor(cases)	Fold change	t test	p value
Wang Gastric	Gastric Mucosa (12)	Gastric Cancer (12)	3.836	2.815	0.005
Cho Gastric	Gastric Tissue (19)	Diffuse Gastric Adenocarcinoma (31)	2.522	6.765	1.47E-08
DErrico Gastric	Gastric Mucosa (31)	Gastric Intestinal Type Adenocarcinoma (26)	5.046	6.298	2.88E-08
Cui Gastric	Gastric Tissue (80)	Gastric Cancer (80)	2.004	2.977	0.002

3.2. The Correlation Between NOX4 Level and Characteristic Features

NOX4 has been demonstrated to be up-regulated in GC. We further proceeded to find the association between the expression of *NOX4* and clinicopathologic data. We downloaded clinical data from TCGA and discovered that *NOX4* level was related to tumor invasion (P=0. 0.00329) and TNM stage(P=0.004948) statistically. The deeper tumor invaded, the higher *NOX4* expressed. Meanwhile, GC patients who were diagnosed more advanced-stage, the higher *NOX4* was up-regulated. The expression pattern of *NOX4* was not associated with patient's age, gender, tumor metastasis and lymph node invasion (Table 2).

Table 2. *The correlation between NOX4 level and characteristic features.*

		NOX4		χ^2	P value
		low expression	high expression		
Age(y)	<65	77	80	0.131	0.718
	≥65	107	103		
Sex	female	67	66	0.005	0.945
	male	117	117		
Tumor invasion	T1	11	1	13.735	0.003
	T2	47	31		
	T3	80	86		
	T4	42	56		
Lymph node	N0	54	53	3.399	0.334
	N1	55	40		
	N2	37	38		
	N3	32	41		
Metastasis	M0	171	171	0.037	0.847
	M1	13	12		
TNM stage	I	11	1	12.861	0.005
	II	47	31		
	III	80	86		
	IV	42	56		

3.3. The Prognostic Value of NOX4 in GC

We next investigated whether *NOX4* is associated with the prognosis of GC patients from TCGA. Higher *NOX4* implied worse overall survival (OS) (P=0.01741, HR=1.48(1.07-2.06)), clearly demonstrating that high *NOX4* expression was associated with a shorter survival rate. Survival curve was presented in Figure 1b. Furthermore, univariate and multivariate Cox regression analyses were performed to confirm the possibility that *NOX4* could be useful as an independent risk factor for poor prognosis in GC from TCGA. First, we performed univariate analysis and recognized that age, TNM stage and *NOX4* expression were correlated with short overall survival time (Table 3). Thus, multivariate analyses were utilized to discover whether high *NOX4* expression could imply a poor prognosis independently. As shown in Table 3, *NOX4* could be an independent prognostic factor (P=0.01392), independent on age and TNM stage.

Table 3. *Univariate and multivariate Cox regression test.*

Group	Num	Hazard ration (95%CI)	P value
Univariate cox model			
Sex			
Female	133	1	

Group	Num	Hazard ration (95%CI)	P value
Male	234	1.269(0.892-1.805)	0.186
Age(year)			
<65	157	1	
≥65	210	1..655(1.175-2.331)	0.004
TNM stage			
I	49	1	
II	110	1.561 (0.787-3.098)	0.202
III	152	1.046(0.128-8.576)	7.141E-3
IV	40	1.564(0.321-7.629)	1.190E-3
Tumor invasion			
T1/2	90	1	
T3/4	264	1.611(1.054-2.462)	0.028
Lymph node			
N0	107	1	
N1	95	1.646(1.012-2.677)	0.045
N2	75	1.664(0.985-2.813)	0.057
N3	73	2.708(1.669-4.396)	5.518E-5
Metastasis			
M0	342	1	
M1	25	2.196(1.263-3.818)	0.005
NOX4 expression			
low expression	183	1	
high expression	184	1.501(1.077-2.093)	0.016
multivariate cox model			
Age(y)			
<65	157	1	
≥65	210	1.935(1.351-2.772)	3.182E-4
TNM stage			
I	49	1	
II	110	1.451(0.728-2.894)	0.290
III	152	2.168 (1.139-4.127)	0.018
IV	40	4.702(2.295-9.636)	2.348E-5
NOX4 expression			
low expression	183	1	
high expression	184	1.542(1.2092-2.177)	0.014

Besides, we measured the overall survival time in different sub-groups according to treatment and HER2 expression by using Kaplan-Meier Plotter database. 631 GC patients were followed up and the overall survival rate was shown in Figure 2a. Among all these patients, 380 patients chose to have an operation only, while 34 had 5-FU adjuvant therapy. HER2 was upregulated in 153 patients. Meanwhile, HER2 was negative in 195 patients. It was found that the OS was shorter in GC patients with higher *NOX4* levels, no matter which therapy methods were chosen or whether HER2 was positive (Figure 2b-2e).

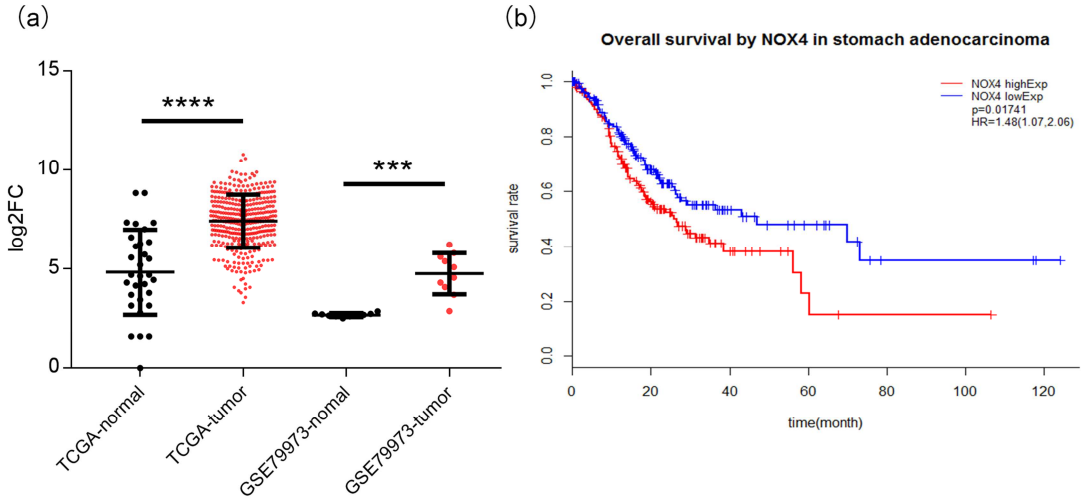


Figure 1. *The expression pattern and prognostic value from TCGA and GEO: (a) The levels of NOX4 in GC from TCGA and GSE79973, the red dots: tumor tissues, the black dots: normal tissues, ****: P value < 0.0001; ***: P value <0.001; (b) Overall survival by NOX in GC, the red line: NOX4 high expression group, the blue line: NOX4 low expression group.*

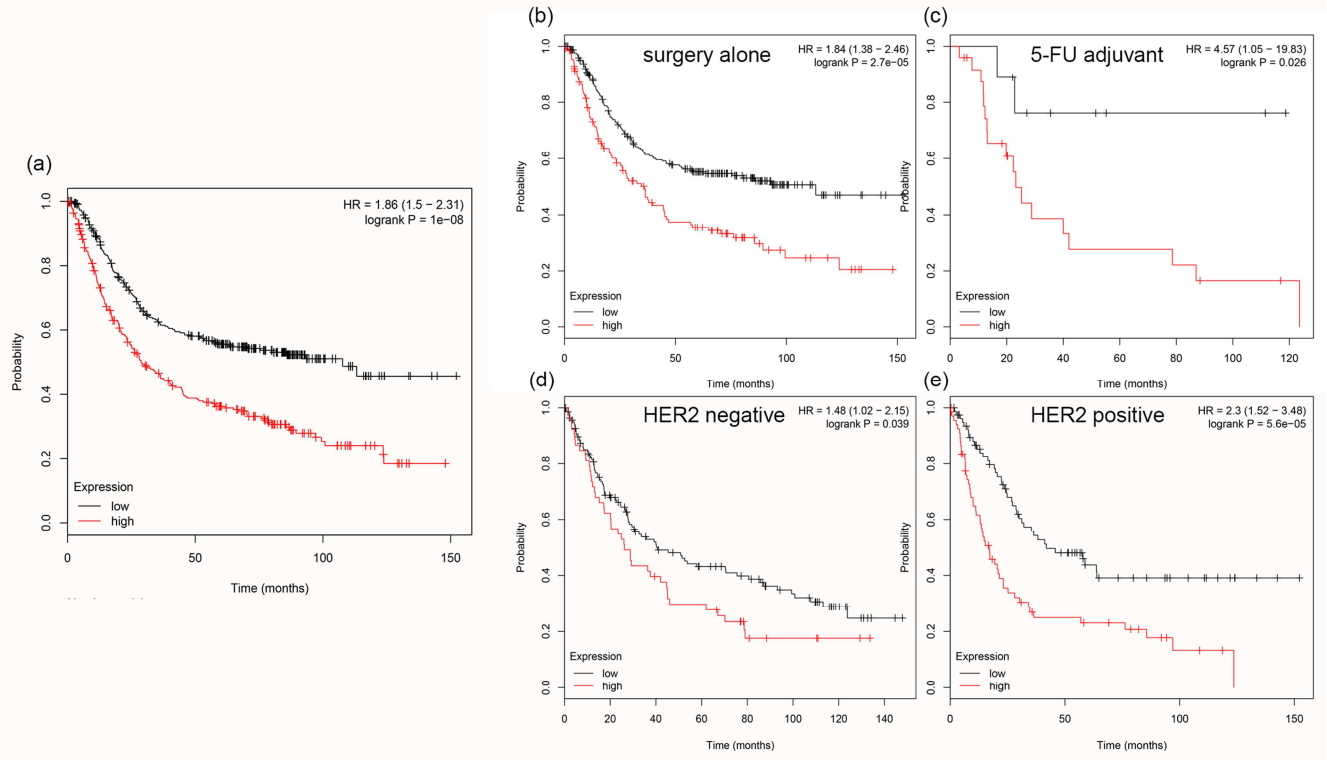
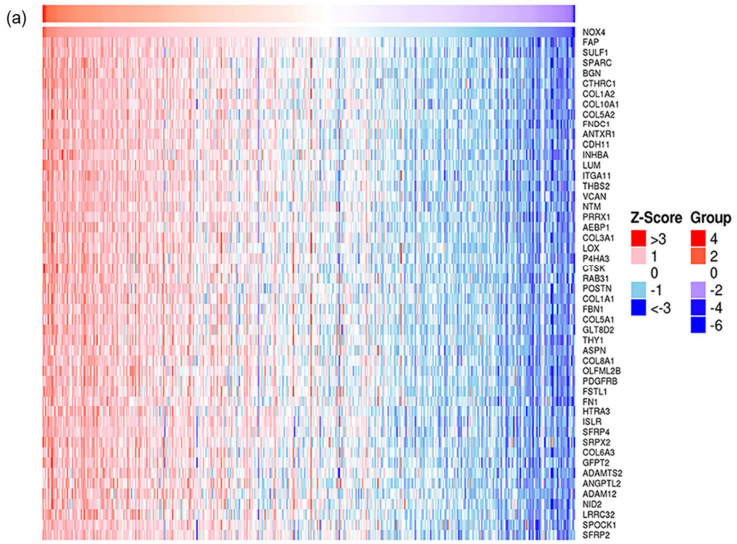


Figure 2. *Survival analyses of NOX4 in gastric cancer from KM plotter: (a): Overall survival analysis of NOX4 in gastric cancer patients. (b-c): Prognosis analysis of NOX4 in patients with surgery alone and 5-FU adjuvant therapy. (d): Prognosis analyses of NOX4 in HER2 negatively expressed patients. (e): Prognosis analyses of NOX4 in HER2 positively expressed patients.*

3.4. The Associated Genes with NOX4 in GC

To uncover the underlying mechanisms of *NOX4* in GC, we explored the LinkedOmics database to identify all the genes related to *NOX4*. There were 434 positively correlated significant genes (correlation coefficient >0.5, P value <0.05), while coefficients of association of negatively correlated genes were under 0.5. As Figure 3a shown, genes with maximum correlation were labeled on the heatmap. Moreover, we performed functional enrichment analysis to determine which biological processes (BP) and pathways related genes involved in to uncover the possible mechanisms.

We detected that associated genes of *NOX4* were most likely linked to cell adhesion and extracellular matrix organization (Figure 3b), which were responsible for epithelial-mesenchymal transition and tumor metastasis. Top4 of maximum hits of Kyoto Encyclopedia of Genes and Genomes (KEGG) was presented in Figure 3c, and the associated genes were annotated to take part in kinds of classical tumor development and metastasis pathways, for example, the PI3K-Akt signaling pathways, focal adhesion, pathways in cancer and ECM-receptor interaction.



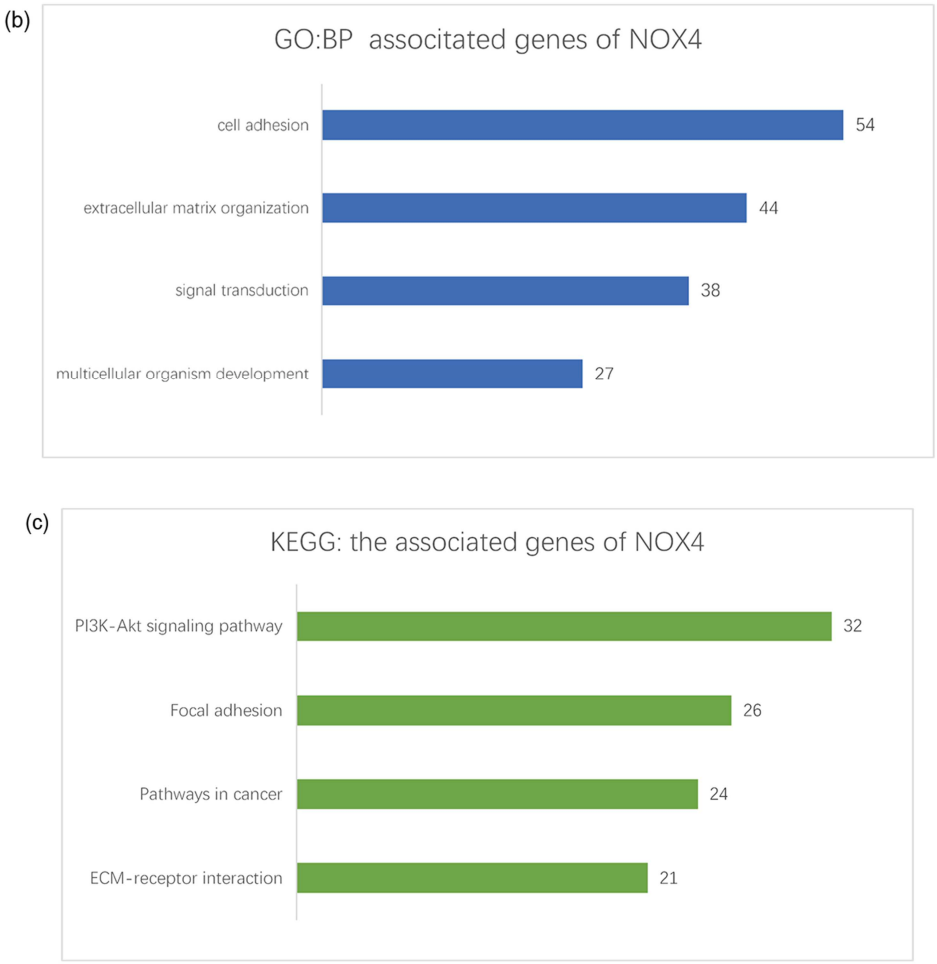


Figure 3. *Analysis of the associated genes with NOX4 in gastric cancer: (a) The heatmap of NOX4 positively associated genes. Functional enrichment analysis of the associated genes of NOX4 in GC: (b) Biological progress enrichment of NOX4 associated genes; (c) KEGG pathway enrichment of NOX4 associated genes.*

4. Discussion

NADPH oxidases family, generating ROS by transporting electrons from cytosolic NADPH across biological membranes to molecular oxygen, has been reported to have positive and negative impacts on cellular health and tumor [4]. NOX4, a member of NADPH oxidases family, have been revealed to have different effects in various types of cancers. Silence of NOX4, through pyruvate kinase-M2 isoform, can sensitize human renal carcinoma cells to drug-induced cell death [3]. Paradoxically, increased NOX4 expression was proved to be associated with genes that inhibit hepatocellular carcinoma progression [5]. Our study focused on the levels of *NOX4* in GC and its correlation with clinicopathologic data. Besides, we discussed the predicted molecular mechanisms of NOX4 in GC.

We explored TCGA and 1 microarray (GSE79973) from GEO and discovered that mRNA expression of *NOX4* was upregulated in tumor than normal tissues, agreeing with outcomes from Oncomine. Additionally, Oncomine datasets proved to us that in different histological types of GC, *NOX4* levels were all overexpressed. Multiple databases certified that *NOX4* levels were raised significantly in cancerous tissues, implying its function in development of stomach adenocarcinoma. Furthermore, we detected that *NOX4* overexpression was corelative with tumor invasion and TNM stage statistically. Fundamentally, patients with higher *NOX4* expression had shorter survival time, compared with those with lower levels. Univariate and multivariate regression analyses illustrated that *NOX4* could be an unfavorable prognostic biomarker for GC, independent of TNM stage and age. We also analyzed other datasets from KM Plotter to demonstrate that the prognostic value of *NOX4* has no relationship with HER2 levels or treatment options, prompting its extensively potential clinical utility. Lee, J developed and validated a prognostic algorithm for GC with a microarray gene expression profiling and NOX4 was a member of gene signature in stage II GC patients [13]. We reported that *NOX4* had a significant prognostic role in GC, no matter which stage GC patients were diagnosed with. In general, our findings suggested that *NOX4* might be a cancer-promoting gene and have a crucial place in GC occurrence and progression.

To elucidate the underlying biological functions of *NOX4* in GC, we summarized all the linked genes with *NOX4* and

found that the positively related genes were enriched in cell adhesion and metastasis. Accumulating evidence has proved that *NOX4* can alter cell adhesion in various types of tumors. Silence of NOX4 with siRNA can inhibit GC cell lines MGC-803 and BGC-823 invasion [14], revealing that NOX4 can enhance GC metastasis in vivo and identifying with our results. Treatment of papillary thyroid cancer cells with NOX4-targeted siRNA downregulates induced thyroid iodide-metabolizing gene repression, inhibiting cancer cell invasion [15]. NOX4 inhibition leads to loss of cell mobility and down-regulation of epithelial mesenchymal transformation in hepatic and neuroblastoma tumors [16]. We discovered that the most likely pathway NOX4 involved in was PI3K-Akt signaling pathway with bioinformatics prediction approaches. Evidence revealed that in non-small cell lung cancer (NSCLC), there was a mutual positive regulation between NOX4 and PI3K/Akt signaling. Overexpression of NOX4 could enhance cell proliferation and invasion, and produce larger tumors, shorter survival time, and more lung metastasis in nude mice than control cells in NSCLC [17]. The product of NOX4, superoxide ROS, was reported to be key molecule in Akt-VEGF pathway in melanoma [18]. Moreover, inhibition of NOX4 can induce pancreatic cancer cells apoptosis through the Akt pathway [12, 19]. Taken together, our predictions are in accord with previous researches, implying that *NOX4* may proliferate gastric cancer cell growth and metastasis through PI3K-Akt signaling pathway.

There are few studies focusing on the role of *NOX4* in GC. One determined depletion of *NOX4* could inhibit GC cell invasion in vivo, and another recognized *NOX4* was a member of GC prognostic gene signature. Our research took advantage of multiple datasets with detailed and complete clinical features to identify biological functions of *NOX4*. However, there is need to explore pathogenesis and mechanisms of *NOX4* in GC comprehensively. We further plan to collect clinical GC cases to build our cohort to validate its prognostic significance and design experiments in vivo and in vitro to certify our bioinformatics findings.

In conclusion, this study uses high throughput data to clarify expression pattern of *NOX4* in GC and demonstrate that *NOX4* mRNA expression is an unfavorable prognostic biomarker in gastric cancer patients. It sheds a new light on better understanding the complex and crucial role of *NOX4* in gastric cancer and uncover a new target for GC diagnosis and therapy.

5. Conclusion

In our manuscript, we found that NOX4 mRNA level was upregulated in gastric cancer tissues than normal gastric mucosa. Besides, the level of NOX4 was correlated with GC patients' clinicopathologic features and The deeper tumor invaded, the higher NOX4 expressed. Furthermore, we confirmed that the expression of NOX4 could be an independent prognostic factor for gastric cancer, indicating the significant role of NOX4 in gastric cancer.

Acknowledgements

This paper is supported by the National Natural Science Foundation of China under Grant number 81370484 (Na Liu) and Nature Science Foundation of Shaanxi Province, China under Grant number 2013JC2-30 (Yan Chen). The funder had been greatly helpful in study design, decision to publish, and modification of the manuscript.

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