Expression analysis of FOXC1 & FOXCUT genes in patients with gastric cancer

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Title: Expression analysis of *FOXC1* & *FOXCUT* genes in patients with gastric cancer

Running Title: *FOXC1* & *FOXCUT* genes in GC

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Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide and has the second rank in death-leading cancers of both sexes(1). High morbidity and mortality rate of gastric cancer is the reason of its significance for public health (2). Despite recent developments in cancer medicine, GC prognosis and treatment is encountered undesirable obstacles (3). Gastric cancers are consisted as a heterologous group of malignancies which are caused by various etiological factors. It is considered as a multifactorial malignancy that may be hereditary or more commonly, sporadic. Recognized etiologic agents of GC are *helicobacter pillory* and some viral infections, dietary factors such as high salt, meat rich and low fruit/vegetable diets, alcohol consumption and smoking. Genetic polymorphisms also play a key role in incidence and prognosis of GC (4).

Identification of relevant biomarkers could be a critical item in early detection, prediction, prognosis and appropriate treatment of GC. However, due to heterogeneous molecular profile, no reliable molecular markers are available for GC diagnosis. Therefore, the studies are utilizing recent technologies such as NGS (next generation sequencing), microarray and etc. to determine DNA, RNA and protein biomarkers of GC (5).

The *FOX* family (Forkhead box gene family) is a group of conserved genes that encode *FOX* proteins including *FOXA*, *FOXC*, *FOXM*, *FOXO* and *FOXP*. FOX proteins are transcription regulators which are related to cellular development; recent studies have proven the correlation between FOX members and different malignancies. *FOXC1* is one of the most vital members of *FOX* family that regulates gene expression by DNA binding. The gene encoding FOXC1 is located on 6p25 and

encodes the single exon 533 amino acid residues protein. The expression level and mutations of *FOXC1* are associated with different cancers (6).

While a small part of human genome is protein coding, an extensive part of it is transcribed to non-coding RNAs (ncRNAs). ncRNAs are a group of divers RNA molecules which fail to be translated to proteins. These RNAs are classified into two major groups based on the length of the molecule including small RNAs (consisting of less than 50 nt (nucleotides)) and large RNAs (more than 50 nt) (7). Although they are known as junk or garbage DNA molecules for decades, now it is believed that non-coding regions of genome (that are transcribed to ncRNAs) play an important role in the regulation of gene expression at different levels (8). Long non-coding RNAs (lncRNAs) are a group of large ncRNAs that are consisted of more than 200 to 10^4 nucleotides which have various activities in cell functions. They are precursors of other non-coding RNAs, one of the known regulators of gene expression and cell signaling pathways and influencers on protein function. Recent studies have shown the association of expression levels of lncRNAs with different cancers, as they are known to be influential regulators of oncogenes, tumor suppressing and signaling pathways. However, the profile of lncRNAs is unknown in most cancers (3, 9).

The gene coding for *FOXCUT* ia an LncRNA which is located on upstream of *FOXC1* promoter and may participate in regulation of FOXC1 expression. Several studies have shown the relation between expression levels of *FOXCUT-FOXC1* and different cancers including breast cancer, nasopharyngeal and squamous cell carcinomas (10-13). According to investigation of new biomarkers for GC, the present study was designed to analyze the relationship between *FOXCUT-FOXC1* expression level and gastric cancer lesion development in tissue samples of GC patients attending to Imam Reza hospital, Tehran, Iran.

Patients/Materials and Methods

Population

Seventy-eight Gastric tissue samples (39 Cancerous and 39 adjacent non-cancerous tissue (ANCT)) were collected from patients who referred to the gastroenterology department at Imam Reza Hospital (Tehran, Iran) from 2016 to 2018. The consent form and the protocol were approved by the Ethics Committee at the AJA Cancer Epidemiology Research and Treatment Center (AJA-CERTC), AJA University of Medical Sciences, Tehran, Iran (project code 32746A). Endoscopy was performed by expert gastroenterologists and the diagnosis of GC was confirmed by pathology unit. Demographic characteristics and clinicopathologic features including age, gender, smoking status, disease stage and grade, and tumor size were registered. The samples were immediately snap-frozen in liquid nitrogen and stored in −70 °C until being used for RNA extraction.

RNA Isolation and Quantitative Real-time PCR

RNA was purified from Tissues using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Germany) (Cat No./ID: 80224) based on the kit protocol. The quality and quantity of RNA was assessed by spectrophotometric optical density measurement (at 260 and 280 nm) (Thermo Scientific, USA). Total RNA was used for cDNA synthesis by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) (Cat No: K1691). For the evaluation of *FOXCUT* and *FOXC1* genes expression, Rotor-Gene ® Q real-time PCR system (QIAGEN, Germany) was used with Takara SYBR Master mix instructions (Shiga, Japan). The primer sequences are presented in Table 1. All primer pairs were used in a single reaction with the thermal following

profile: 95℃ for 5 minutes as denaturation step followed by 38 cycles of 95℃ for 15 seconds, 60℃for 40 seconds, and 72℃for 20 seconds.

Statistics

Unfortunately, we cannot provide any biostatistics review certificate since our team members are expert with biostatistics analysis, so we analysed the data (The $2^{-\Delta\Delta CT}$ method) using REST software and GraphPad Software.

To measure the expression changes of *FOXCUT* and *FOXC1* genes among the patients and control group, the Relative Quantitative (RQ) qPCR technique was used. The levels of mRNAs in the samples were standardized to the B2M mRNA levels and were compared between tumor and normal samples.

The $2^{-\Delta\Delta CT}$ method was applied to analyze the real-time quantitative PCR. Values less than 0.5 meant a decrement in comparison with the calibrator and $0.5 < RQ < 1.9$ depicted no change. Values higher than 2 illustrated an increment in the expression of the target genes compared to the control. All the data are represented as the mean ± S.D (standard deviation) and executed using GraphPad Prism5 software (GraphPad Software, Inc., USA) (https://www.graphpad.com/scientific-software/prism). The independent *t* test was used to evaluate the association between variables. Statistical significance was determined with $P < 0.05$.

Results

Demographic and clinical data of patients

Patients' information including age, gender and tumor characteristics was taken from questionnaires and pathological tests. Demographic and clinical data of patients are summarized in table 2.

Expression of *FOXC1* and *FOXCUT* in patients' samples

After analyzing the results of quantitative RT-PCR, it was observed that the expression of *FOXC1* and *FOXCUT* genes were significantly up-regulated in tumor tissues compared with adjacent non-cancerous tissues ($p<0.05$). (Fig 1)

Correlation between *FOXC1* and *FOXCUT* expressions and clinical characteristics

To further discover the role of mentioned genes in gastric cancer, the association between their expression levels and patients' clinical characteristics was evaluated. A significant relationship was found between *FOXCUT* expression and gender parameter. There was also a significant relationship between *FOXC1* expression and tumor stage. No significant correlation was found between mentioned genes expression and other clinicopathological variables. (Fig 2 and 3)

Discussion

Gastric cancer is a high prevalence neoplastic malignancy and the third cancer in world that causes death in developing countries (14). Prevalence of GC depends on geographic areas and it is also a common disease in Iranian population (15, 16).

In this study, we presented *FOXC1* gene that belongs to Forkhead family (17). FOX proteins are divided to 19 subgroups from A to S (18). Recent studies showed that *FOXC1* has a critical role in several types of cancer such as GC (6). According to previous studies, gastric cancer has high mortality among vicious disease (19). Additionally, we consider a non-coding gene from Forkhead family called *FOXCUT* which is located on the upstream of *FOXC1* gene in Chromosome 6 (20). The relation between *FOXC1* and *FOXCUT* has been confirmed by previous studies (21). Also *FOXC1* leads to an increase of cell invasion, proliferation, metastasis and migration in many cancer types (6). *FOXC1* and *FOXCUT* genes substantial prevented cell proliferation, migration and invasion abilities in esophageal squamous cell carcinoma (ESCC) (18, 22). Nowadays, the role of noncoding RNA regulation in many diseases is approved (23). Referred to accomplished studies, lncRNA molecules play a critical role in oncogenesis and progression of tumors to cancer (21).

This study was the first report of evaluation of *FOXC1* and *FOXCUT* genes expression in tissue adjacent to gastric cancer. According to our results, the expression of these genes (lncRNA-mRNA gene pair) showed significant up-regulation in cancerous tissues compared to adjacent non-cancerous tissues. Furthermore, we founded a correlation between *FOXCUT* and *FOXC1* and overexpression of *FOXCUT* might cause upregulation of *FOXC1*.

According to Yuan Xu report in 2014, *FOXC1* has overexpression in GC. Our investigation showed clearly that *FOXC1* levels were significantly up-regulated in tumoral tissue in contrast with adjacent normal tissue. Additionally, in our study, there was a significant correlation between tumor stage and *FOXC1* expression which can be supported by their results (18).

Accumulating evidences in current study indicated that *FOXC1* and *FOXCUT* up-regulation in tumor tissues compared with adjacent non-cancerous tissues could be utilized as a biomarker for gastric cancer patients. Also it is clear that *FOXC1* is influenced by *FOXCUT* and up-regulation of *FOXCUT* can be the cause of *FOXC1* up-regulating.

Overall, we strongly recommend further studies on *FOXC1* protein expression using Western blot and immunohistochemistry methods in order to better understanding of development to this type of cancer.

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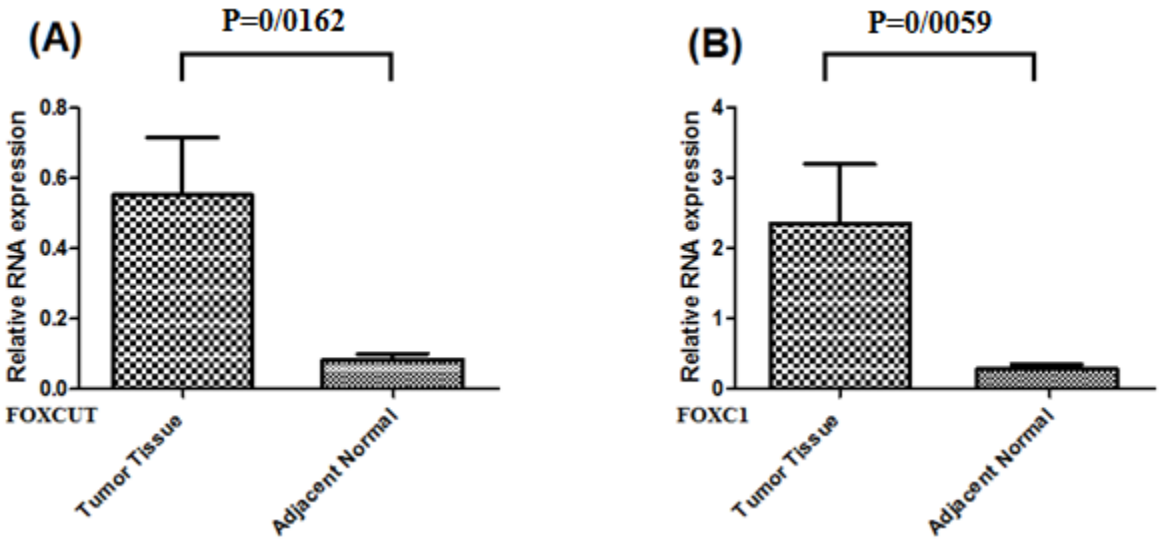


Fig 1. Comparison of expression levels between Tumor tissues and their ANCTs. (A) FOXCUT relative expression levels, (B) FOXC1 relative expression levels.

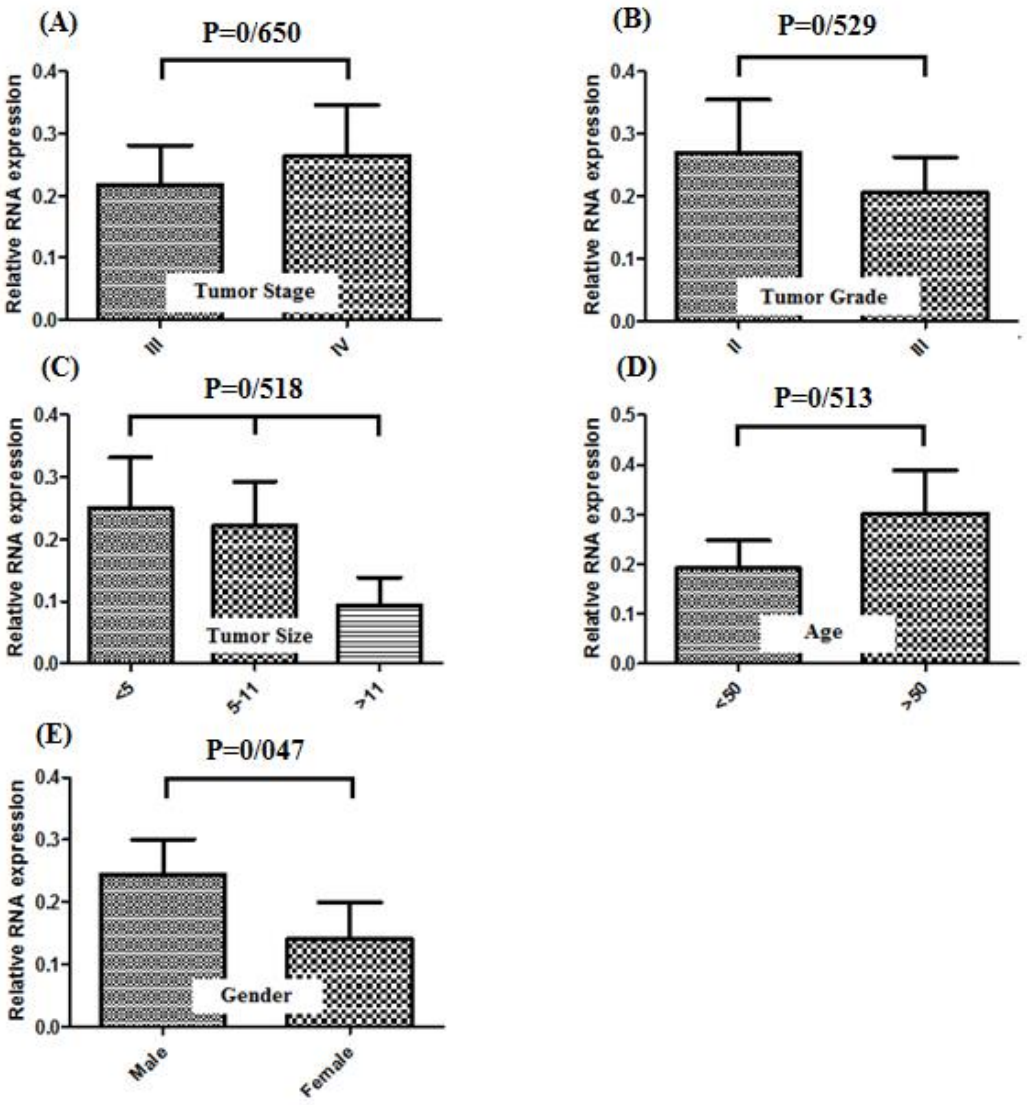


Fig 2. Comparison of FOXCUT expression levels and clinicopathological variables.

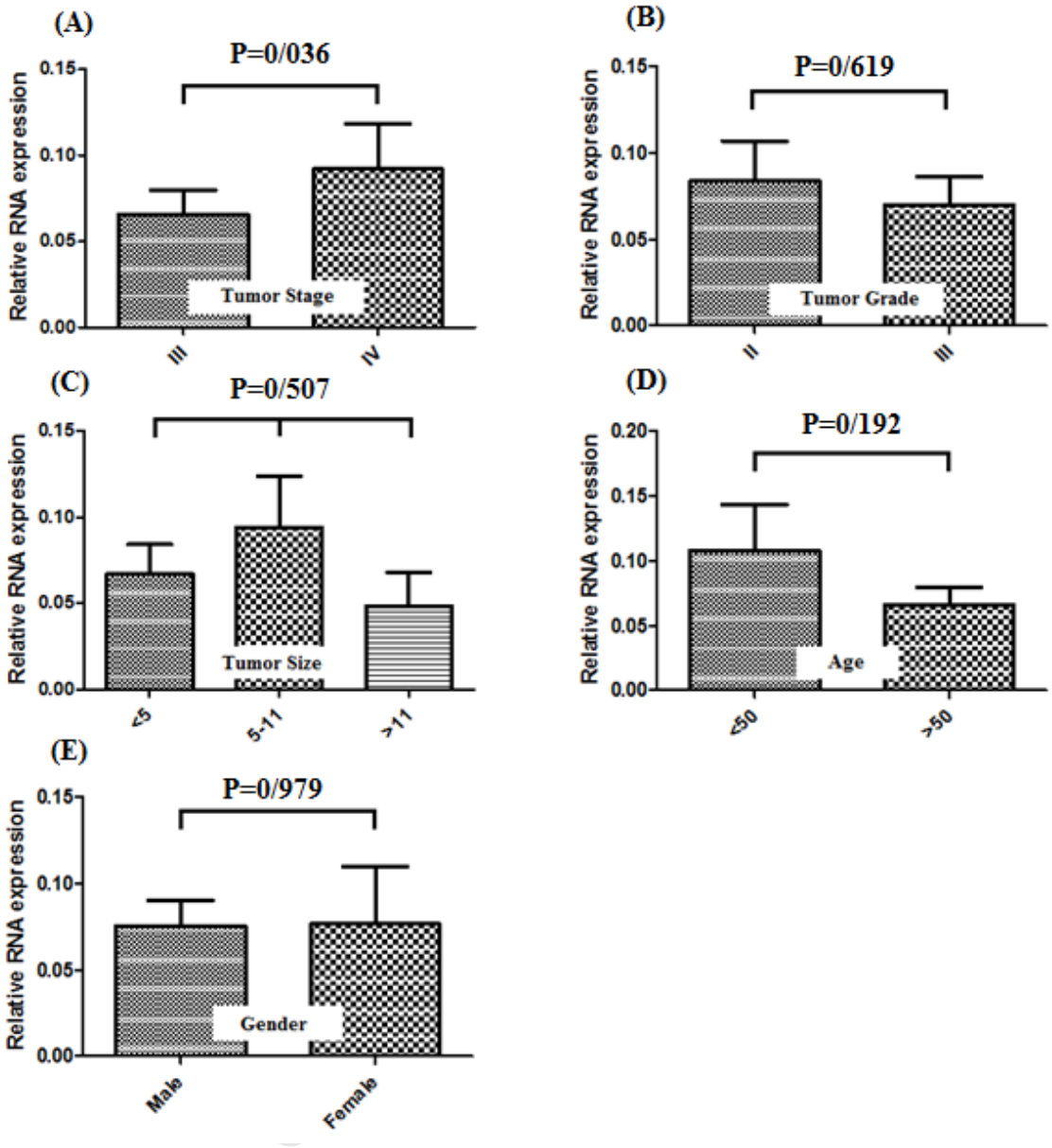


Fig 3. Comparison of FOXC1 expression levels and clinicopathological variables.

Table 1. Primers were used for quantification reactions

Gene Symbol	Primer Sequence (5'-3')	Length (bp)	GC (%)*	Product Length (bp)	Tm (°C)
FOXC1	F:5'-TGCGGGAGATGTTCGAGTCA-3'	20	55.00	110	61.25
	R:5'-GGACGTGCGGTACAGAGACT-3'	20	60.00		61.58
FOXCUT	F:5'-TAAGGACGGGGCTGAATTGG-3'	20	55.00	149	59.75
	R:5'-ACGTTAGTCATCGGTGCGAC-3	20	55.00		60.46
B_2M	F:5'-TGCTGTCTCCATGTTTGATGTATCT-3'	25	40.00	86	60.34
	R:5'-TCTCTGCTCCCCACCTCTAAGT-3'	22	54.55		61.98

* Nucleotide position in codon. GC-content (or guanine-cytosine content)

Table 2. Demographic and clinical data of patients

Variable	Frequency
Age (mean±SD)	61.46±13.705 (35-81)
Tumor Size (mean±SD)	30.81±103.548 (2-505)
Gender % (N)	
Male	87.2% (34)
Female	12.8% (5)
Smoking % (N)	
No	82.1% (32)
Yes	17.9% (7)
Stage % (N)	
II	2.6% (1)
IIIA	48.7% (19)
IIIB	5.1% (2)
IV	41% (16)
missing	2.6% (1)
Grade % (N)	
II	41% (16)
III	56.4% (22)
IV	2.6% (1)

Institutional review board statement:

Seventy-eight Gastric tissue samples were collected from patients who referred to the gastroenterology department at Imam Reza Hospital (Tehran, Iran) from 2016 to 2018. The consent form and the protocol were approved by the Ethics Committee at the AJA Cancer Epidemiology Research and Treatment Center (AJA-CERTC), AJA University of Medical Sciences, Tehran, Iran (project code 32746A). Endoscopy was performed by expert gastroenterologists and the diagnosis of GC was confirmed by pathology unit.
The study was reviewed and approved by the Ethics Committee at the AJA Cancer Epidemiology Research and Treatment Center (AJA-CERTC), Institutional Review Board.

Institutional animal care and use committee statement:

All procedures involving Human samples were reviewed and approved by the Biobank of the AJA Cancer Epidemiology Research and Treatment Center (AJA-CERTC) and **no animal testing** was conducted.

Conflict-of-interest statement:

To the best of our knowledge, no conflict of interest exists.

cDNA: complementary DNA

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

RNA: ribonucleic acid
RNase: ribonuclease
rRNA: ribosomal RNA

NGS: next generation sequencing

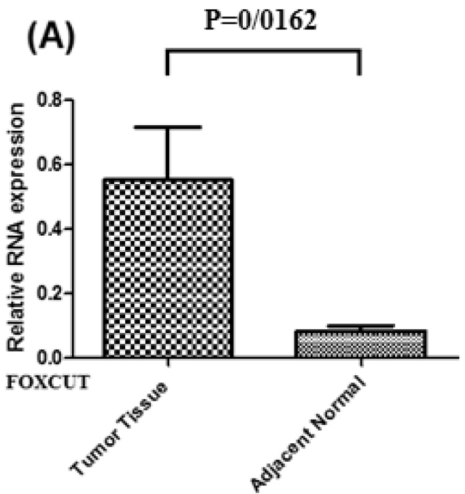
GC: gastric cancer

lncRNA: long non-coding RNA

FOX: Forkhead box family

ncRNAs: non-coding RNAs

ANCT: adjacent non-cancerous tissue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0.2
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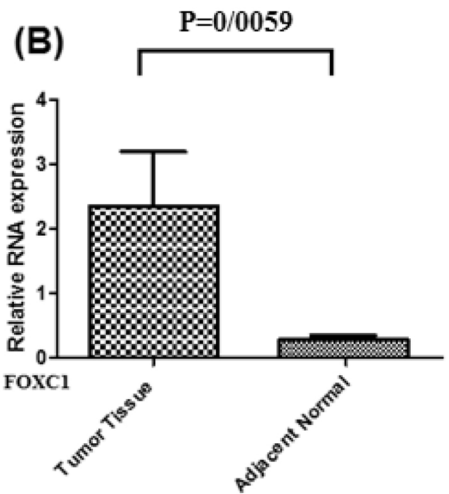
FOXCUT
Tumor Tissue
Adjacent Normal


(B)
P=0/0059
Relative RNA expression
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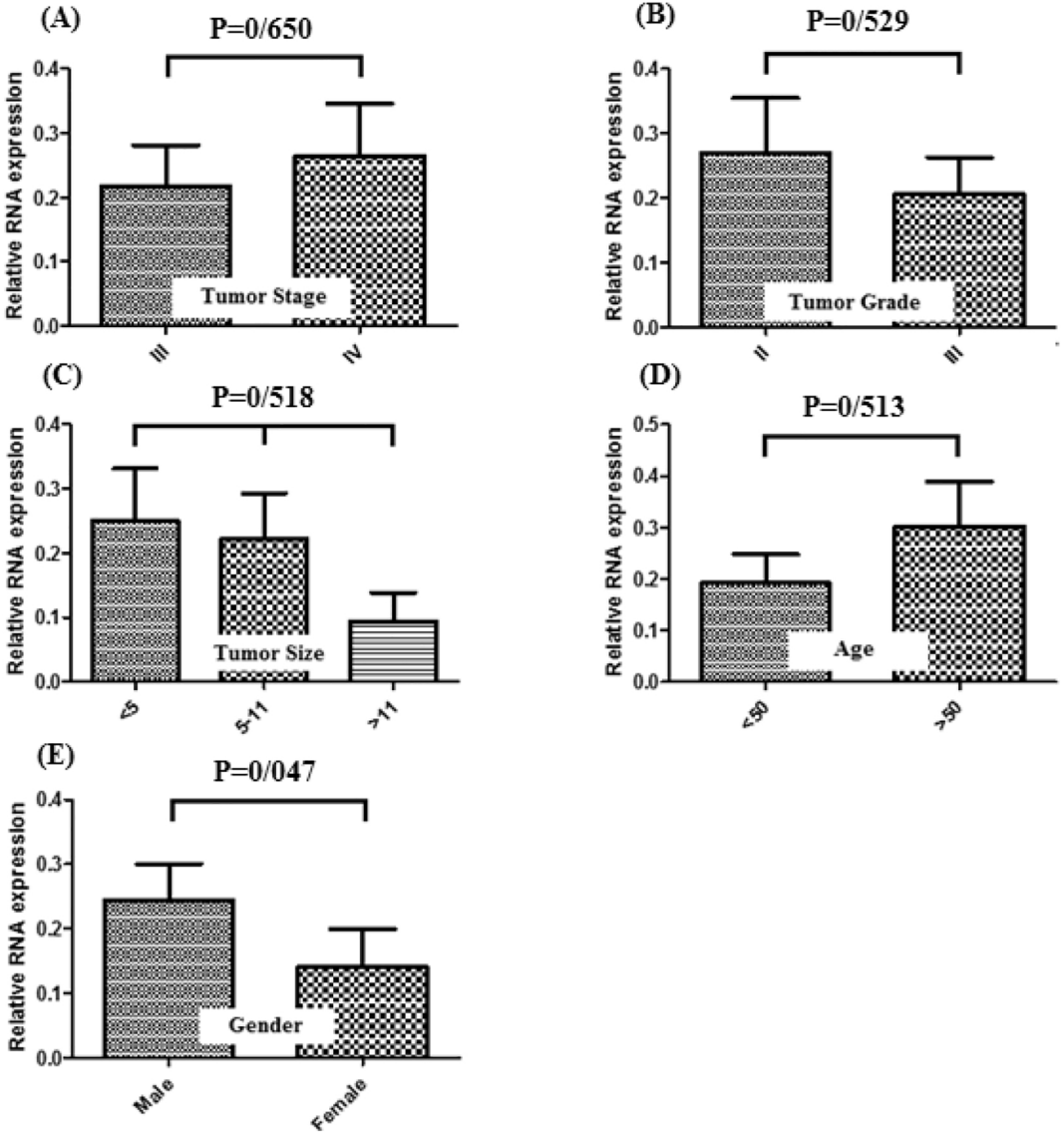
3
2
1
0
FOXC1
Tumor Tissue
Adjacent Normal

Figure 1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Tumor Stage
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IV
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Tumor Grade
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III
(C)
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0.1
0.0
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>50
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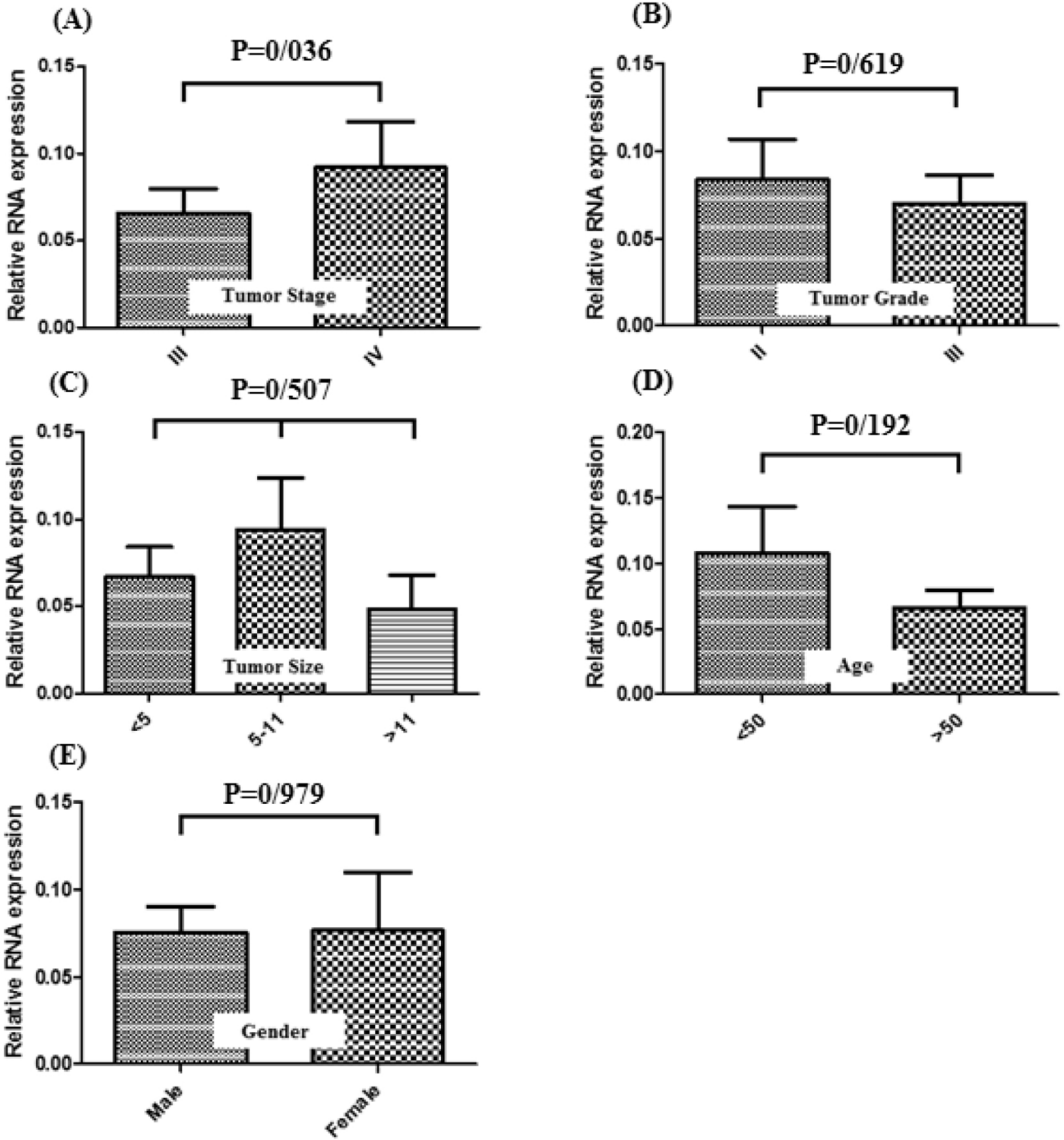
0.1
0.0
Gender
Male
Female

Figure 2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Tumor Stage
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IV
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0.00
Tumor Grade
II
III
(C)
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Relative RNA expression
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0.10
0.05
0.00
Tumor Size
<5
5-11
>11
(D)
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Relative RNA expression
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0.15
0.10
0.05
0.00
Age
<50
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(E)
P=0/979
Relative RNA expression
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0.10
0.05
0.00
Gender
Male
Female

Figure 3