AFAP1 and its naturally occurring antisense RNA are downregulated in gastric cancer samples

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Abstract. Actin filament-associated protein 1 (*AFAP1*) encodes a protein which is an SRC proto-oncogene, non-receptor tyrosine kinase binding partner. This protein alters actin filament integrity in reaction to cellular signals. A long non-coding RNA, namely *AFAP1*-antisense RNA 1 (*AS1*), is transcribed from the antisense strand of this gene and potentially regulates its expression. In the present study, the expression levels of these two genes were evaluated in 30 gastric cancer samples and adjacent non-cancerous tissues (ANCTs) to identify their importance in this type of human malignancy. These two genes were significantly downregulated in gastric tumor samples compared with ANCTs (expression ratio 0.26 and 0.36, P=0.001 and P=0.04 for *AFAP1* and *AFAP1-AS1*, respectively). Relative expressions of these two genes were associated with the location of primary tumor, in that *AFAP1* and *AFAP1-AS1* were significantly downregulated in all cardia tumor types compared with their paired ANCTs (P=0.04 and P=0.001, respectively). There were indications of a significant association between the expression levels of *AFAP1* and peritoneal invasion and smoking history (P=0.05). Additionally, a lower expression level of *AFAP1* was detected in younger patients and in high grade tumor types compared with olders and low grade tumors respectively (P=0.01 and P=0.04, respectively) and significantly higher expression levels of *AFAP1-AS1* in patients with lymphatic/vascular invasion compared with those without lymphatic/vascular invasion (P=0.01). Furthermore, significant pairwise correlations were identified between the transcript levels of these genes in tumoral tissues and ANCTs (P values<0.05). The diagnostic power of *AFAP1* and *AFAP1-AS1* in gastric cancer was calculated as area under the curve (AUC) 0.75 and 0.67, respectively. The combination of the transcript levels of these two genes significantly enhanced the diagnostic power compared with diagnostic power of each gene (AUC, 0.76; P<0.001). The present study demonstrates the dysregulation of *AFAP1* and *AFAP1-AS1* in gastric cancer tissues in association with the clinicopathological data of patients and demonstrates the potential of these genes as diagnostic biomarkers.

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Introduction

Actin filament-associated protein 1 (*AFAP1*) encodes a motor fiber-associated protein, which organizes a network for linking other proteins, including SRC proto-oncogene, non-receptor tyrosine kinase (Src) and protein kinase C (1). This network alters the structure and function of actin filaments, thus contributing to cytophagy, cell motion, invasion and metastasis (2). This gene has been demonstrated to be over-expressed in the human breast cancer cell line MDA-MB-231 where its silencing resulted in a deficiency in actin stress fiber cross-linking and diminished linkage with fibronectin (3). The contribution of *AFAP1* in the carcinogenesis process has been further emphasized by the observed over-expression of the encoded protein in prostate carcinoma types despite the absence or low level of expression of this gene in normal prostatic epithelium and benign prostatic hyperplasia (4). The effect of *AFAP1* knockdown on the repression of cell proliferation has been documented *in vitro* and *in vivo* (4). One long non-coding RNA (lncRNA), namely *AFAP1*-antisense RNA 1 (*AS1*) is transcribed form the antisense strand of this gene and may potentially regulate its expression (5). Numerous studies have reported the dysregulation of this lncRNA in human malignancies in association with numerous pathological tumor features (5-7). A previous meta-analysis has demonstrated that *AFAP1-AS1* overexpression is indicative of poor patient survival and the malignant behavior of a tumor (8). Previously, the expression of *AFAP1* and *AFAP1-AS1* was evaluated in breast cancer samples and the upregulation of *AFAP1-AS1* in

tumor tissues compared with adjacent non-cancerous tissues (ANCTs) was detected (9). *AFAP1-AS1* expression has also been revealed to be elevated in gastric cancer tissues and cells compared with noncancerous gastric tissues and control cells (6,10). However, there is no data regarding the expression of *AFAP1* in gastric cancer. In the present study, the expression levels of *AFAP1* and *AFAP1-AS1* were assessed in gastric cancer samples and ANCTs to verify results of previous studies on *AFAP1-AS1* expression and to investigate the function of *AFAP1* in gastric cancer pathogenesis.

Materials and methods

Patients. The present study was performed on samples obtained from 30 patients (23 males and 7 females) diagnosed with gastric cancer mean ± standard deviation (range) age 42.53±10.1 years, ranging from 14-55 years old. Patients were admitted in Imam Khomeini hospital, Tehran during February 2016 to December 2017. Tumoral and ANCTs were excised from the patients during surgical removal of the gastric tumor. Patients had no former history of chemo/radiotherapy; those with familial disease were also excluded. Inclusion criteria were availability of clinical data and appropriate tissue for RNA extraction. All tissue samples were inspected by pathologists to evaluate the existence of tumoral cells. Tumor-node-metastasis staging was performed based on the American Joint Committee on Cancer (AJCC) system, 8th edition (11). The cutoff values for the age groups were determined based on recent study (12). The study protocol was ethically approved by the ethical committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran). All patients signed written informed consent forms.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tumoral tissues and ANCTs using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Approximately 75 ng RNA samples was converted to cDNA using Applied Biosystems High-Capacity cDNA Reverse Transcription kit according to manufacturer protocols (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative expressions of *AFAP1* and *AFAP1-AS1* were quantified in the Rotor Gene 6000 Real-Time PCR Machine using TaqMan® Universal PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Transcript levels were normalized to those of hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). The sequences of primers and probes are presented in Table I. The thermocycling conditions included a primary denaturation step at 95˚C for 15 min, 40 cycles of 95˚C for 15 sec and 60˚C for 55 sec. qPCR analyses were performed using the Pfaffl method (13).

Evaluation of Helicobacter pylori (H. pylori) presence in tissues. The presence of *H. pylori* in tissues were assessed with RT-qPCR using primers against *H. pylori* 16s rRNA (forward, 5'-AGCGTTACTCGGAATCACTG-3' and reverse, 5'-CACATACCTCTCACACACTC-3') at a final concentration of 0.2 pmol/µl. Reactions were performed on 100 ng synthesized cDNA as described above. The thermocycling conditions were as follows: 95˚C for 15 min and then 95˚C for 15 sec and 60˚C for 1 min for 40 cycles followed by melting curve analysis. TaqMan® Universal PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used. qPCR analyses were performed using the Pfaffl method (13).

Statistical analysis. Demographic and clinical data were presented as the mean ± standard deviation or as percentages. Relative expression levels of genes in tumoral tissues compared with ANCTs were assessed using REST 2009 software (Qiagen GmbH, Hilden, Germany). The difference in the expression levels of genes between paired tumoral tissues and ANCTs were evaluated using a Student's paired t-test. The association between clinical data and the relative expression of genes was assessed using a χ^2 or Mann-Whitney test. The correlation between the relative expression of *AFAP1* and *AFAP1-AS1* was assessed using a regression model. $P<0.05$ was considered to indicate a statistically significant difference. The diagnostic power of transcript levels of genes in gastric cancer was assessed by plotting a receiver operating characteristic (ROC) curve.

Results

General data of the patients. Thirty patients (23 males and 7 females) diagnosed with gastric were enrolled in the study. The degree of invasiveness, histological form and the presence othe *H. pylori* infection were analyzed, as well as other features. Demographic and clinical data of the patients with gastric cancer are presented in Table II.

Relative expression of AFAP1 and ASAP1-AS1 genes in tumor tissues compared with ANCTs. The two genes were significantly downregulated in gastric tumor samples compared with ANCTs (expression ratios 0.26 and 0.36, P=0.001 and P=0.04 for *AFAP1* and *AFAP1-AS1*, respectively). Fig. 1 presents the -ΔCq values (Cq *HPRT1*-Cq target gene) in tumoral tissues and ANCTs.

Association between the expression of levels of AFAP1 and ASAP1-AS1 and tumor characteristics. Relative expression levels of the two genes were revealed to be associated with the location of the primary tumor, in that *AFAP1* and *AFAP1-AS1* were significantly downregulated in all cardia tumors compared with their paired ANCTs (P=0.04 and P=0.001, respectively). There were indications of a significant association between the expression levels of AFAP1 and peritoneal invasion and smoking history (P=0.05). However, the expression levels of neither gene were associated with the presence of *H. pylori* in samples (P=0.65 and P=0.08, respectively). Table III presents the results of the association analysis between the relative expression levels of *AFAP1* and *AFAP1-AS1* in tumoral samples compared with ANCTs and tumor characteristics.

The present study additionally compared the relative expression levels of each gene between the distinct categories of patients and identified a significantly lower expression of *AFAP1* in younger patients compared with older patients (P=0.01) and in high grade (grade 3 and 4) tumor types compared with a lower grade (grade type 2; P=0.04) and a significantly higher expression of *AFAP1-AS1* in patients with lymphatic or vascular invasion compared with those who did

Table I. Nucleotide sequences of primers and probes.

Gene name	Primer and probe sequence	Primer and probe length	Product length
HPRT1	Forward, 5'-AGCCTAAGATGAGAGTTC-3'	18	88
	Reverse, 5'-CACAGAACTAGAACATTGATA-3'	21	
	5'-FAM-CATCTGGAGTCCTATTGACATCGC-TAMRA-3'	24	
AFAP1	Forward, 5'-CAGAAGCAGGAGACCGCTAAC-3'	21	117
	Reverse, 5'-GAGGGAGGATGTTGGCAATGG-3'	21	
	5'-FAM-TGCCAGCCCCTCCTCAGATGCCC-TAMRA-3'	23	
AFAP1-antisense RNA 1	Forward, 5'-TCCTTGACCTCCGTCAGCTC-3'	20	141
	Reverse, 5'-GGGAGTAACGGCGTTTCTGG-3'	20	
	5'-FAM-CTGACACGCTCCGCCTCCTTCTGC-TAMRA-3'	24	

HPRT1, hypoxanthine phosphoribosyltransferase 1; *AFAP1*, actin filament-associated protein 1.

Table II. General demographic and clinical data of patients.

Variables	Values
Age [mean ± SD (range)]	42.53±10.1 (14-55)
Sex	
Male	76.6%
Female	23.3%
Site of primary tumor	
Cardia	41.4%
Antrum	31%
Body	27.6%
Histological grade	
2	37.5%
3	58.3%
4	4.2%
Lymphatic invasion	
Yes	82.8%
No	17.2%
Vascular invasion	
Yes	82.8%
No	17.2%
Peritoneal invasion	
Yes	62.1%
No	37.9%
Tumor-Node-Metastasis stage	
I	3.4%
II	31%
III	44.8%
IV	20.8%
Histological form	
Intestinal	46.7%
Diffuse	53.3%
Helicobacter pylori infection	
Positive	50%
Negative	50%
Smoking	
Never Smoker	50%
Current Smoker	13.6%
Ex-Smoker	36.4%

SD, standard deviation.

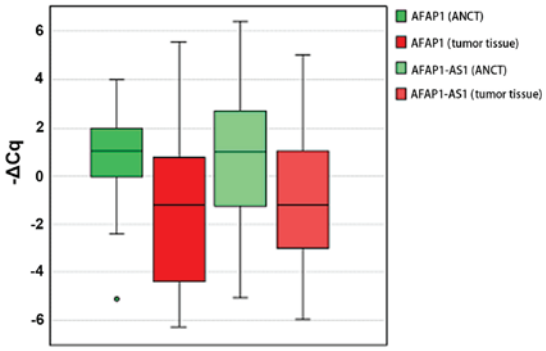

Figure 1. Relative expression levels of *AFAP1* and *AFAP1-AS1* in tumor tissues (n=30) and ANCTs (n=30) as calculated by -ΔCq values (Cq hypoxanthine phosphoribosyltransferase 1-Cq target gene). *AFAP1*, actin filament-associated protein 1; *AFAP1-AS1*, actin filament-associated protein 1-antisense RNA 1; ANCT, adjacent non-cancerous tissues.

not (P=0.01). Table IV presents the results of the association analysis between the transcript levels of genes in tumoral tissues and tumoral features.

Correlation between the expression levels of AFAP1 and AFAP1-AS1. Significant pairwise correlations were identified between transcript levels of *AFAP1* and *AFAP1-AS1* in tumoral tissues and ANCTs (R^2=0.42 and 0.31, P<0.05; Fig. 2A and B, respectively).

Table III. Results of association analysis between the relative expression levels of actin filament-associated protein 1 (*AFAP1*) and *AFAP1*-antisense RNA 1 (*AS1*) in tumor tissues compared with adjacent non-cancerous tissues and tumor features.

Variables	*AFAP1* upregulation	*AFAP1* downregulation	P-value	*AFAP1-AS1* upregulation	*AFAP1-AS1* downregulation	P-value
Age			0.62			1.00
>50	4 (19.0%)	17 (81.0%)		5 (23.8%)	16 (76.2%)	
≤50	2 (28.6%)	5 (71.4%)		2 (28.6%)	5 (71.4%)	
Sex			1.00			0.28
Female	1 (16.7%)	5 (83.3%)		0 (0.0%)	6 (100.0%)	
Male	5 (22.7%)	17 (77.3%)		6 (27.3%)	16 (72.7%)	
Site of primary tumor			0.04			0.00
Cardia	0 (0.0%)	12 (100.0%)		0 (0.0%)	12 (100.0%)	
Antrum	3 (33.3%)	6 (66.7%)		6 (66.7%)	3 (33.3%)	
Body	3 (37.5%)	5 (62.5%)		1 (12.5%)	7 (87.5%)	
Histology grade			1.00			0.51
2	2 (22.2%)	7 (77.8%)		1 (11.1%)	8 (88.9%)	
3	3 (21.4%)	11 (78.6%)		5 (35.7%)	9 (64.3%)	
4	0 (0.0%)	1 (100.0%)		0 (0.0%)	1 (100.0%)	
Lymphatic invasion			1.00			1.00
Yes	5 (20.8%)	19 (79.2%)		6 (25.0%)	18 (75.0%)	
No	1 (20.0%)	4 (80.0%)		1 (20.0%)	4 (80.0%)	
Vascular invasion			1.00			1.00
Yes	5 (20.8%)	19 (79.2%)		6 (25.0%)	18 (75.0%)	
No	1 (20.0%)	4 (80.0%)		1 (20.0%)	4 (80.0%)	
Peritoneal invasion			0.05			1.00
Yes	6 (33.3%)	12 (66.7%)		4 (22.2%)	14 (77.8%)	
No	0 (27.8%)	11 (100.0%)		3 (27.8%)	8 (72.2%)	
Pathological T			0.62			0.54
T2b	0 (0.0%)	4 (100.0%)		0 (0.0%)	4 (100.0%)	
T3	3 (17.6%)	14 (82.4%)		4 (23.5%)	13 (76.5%)	
T4	2 (33.3%)	4 (66.7%)		2 (33.3%)	4 (66.7%)	
Pathological N			0.06			0.26
N0	2 (22.2%)	7 (77.8%)		1 (11.1%)	8 (88.9%)	
N1	0 (0.0%)	9 (100.0%)		2 (22.2%)	7 (77.8%)	
N2	4 (50.0%)	4 (50.0%)		4 (50.0%)	4 (50.0%)	
N3	0 (0.0%)	3 (100.0%)		0 (0.0%)	3 (100.0%)	
Tumor-Node-Metastasis Staging			0.87			0.64
I	0 (0.0%)	1 (100.0%)		0 (0.0%)	1 (100.0%)	
II	2 (22.2%)	7 (77.8%)		1 (11.1%)	8 (88.9%)	
III	2 (15.4%)	11 (84.6%)		4 (30.8%)	9 (69.2%)	
IV	2 (33.3%)	4 (66.7%)		2 (33.3%)	4 (66.7%)	
Histological form			0.37			1.00
Intestinal	4 (28.6%)	10 (71.4%)		3 (21.4%)	11 (78.6%)	
Diffuse	2 (12.5%)	14 (87.5%)		4 (25.0%)	12 (75%)	
H. pylori Infection			0.65			0.08
Positive	4 (26.7%)	11 (73.3%)		1 (6.7%)	14 (93.3%)	
Negative	2 (13.3%)	13 (86.7%)		6 (40.0%)	9 (60.0%)	
Smoking			0.05			0.64
Non-Smoker	3 (27.3%)	8 (72.7%)		3 (27.3%)	8 (72.7%)	
Smoker	2 (66.7%)	1 (33.3%)		1 (33.3%)	2 (66.7%)	
Ex-Smoker	0 (0.0%)	8 (100.0%)		1 (12.5%)	7 (87.5%)	

Table IV. Results of association analysis between the transcript levels of *AFAP1* and *AFAP1-AS1* in tumor tissues and tumor features.

Variables	*AFAP1*	P-value	*AFAP1-AS1*	P-value
Age				
<50 years old vs. ≥50 years old	48.76 (113.11) vs. 72.59 (176.15)	0.01	12.63 (43.63) vs. 24.67 (54.89)	0.97
Lymphatic invasion				
Yes vs. no	63.84 (136.9) vs. 0.01 (0.02)	0.08	18.24 (49.24) vs. 0.06 (0.1)	0.01
Vascular invasion				
Yes vs. no	63.84 (136.9) vs. 0.01 (0.02)	0.08	18.24 (49.24) vs. 0.06 (0.1)	0.01
Helicobacter pylori infection				
Positive vs. negative	68.22 (150.37) vs. 33.92 (94.57)	0.77	13.97 (49.91) vs. 15.32 (40.03)	0.52
Tumor grade				
Grade 2 vs. 3 and 4	186.24 (322.94) vs. 3.64 (8.85)	0.04	31.47 (39.54) vs. 1.19 (1.24)	0.41

Mean (standard deviation) values of Cq reference gene-Cq target gene are presented in the table. *AFAP1*, actin filament-associated protein 1; *AFAP1-AS1*, actin filament-associated protein 1-antisense RNA 1.

Table V. Results of receiver operating curve analysis.

Variables	Estimated optimal cutoff value for gene expression	Area under the curve	J[a]	Sensitivity	Specificity	P-value
AFAP1	>0.66	0.75	0.46	60	86.7	<0.01
AFAP1-antisense RNA 1	>-0.15	0.67	0.33	70	63.3	0.01
Combination of the two genes	>0.52	0.76	0.5	63.3	86.7	<0.0001

[a]Youden index. *AFAP1*, actin filament-associated protein 1.

ROC curve analysis. The diagnostic power of *AFAP1* and *AFAP1-AS1* in gastric cancer was calculated based on area under curve (AUC) values of 0.75 and 0.67, respectively. The combination of the transcript levels of these genes significantly enhanced the diagnostic power when compared with the diagnostic power of each gene (AUC, 0.76; P<0.001). Table V exhibits the parameters of the ROC curve analysis.

Discussion

In the present study, the downregulation of *AFAP1* and *AFAP1-AS1* in gastric cancer samples compared with ANCTs was demonstrated. This observation is in contrast with the previously reported expression pattern and function of *AFAP1-AS1* in gastric cancer (6,10). Such an inconsistency may be due to the function of ethnicity-based and environmental factors in the determination of lncRNAs expression. The latter is supported by the fact that non-coding RNAs contribute to the regulation of gene expression in response to environmental signals (14). Future studies are required to assess the function of putative environmental factors on the expression of *AFAP1-AS1* in association with gastric cancer risk. The presence of certain genomic variants within the promoter region of *AFAP1-AS1* may additionally affect expression of this lncRNA and may be responsible for the observed downregulation of this lncRNA in Iranian patients. Downregulation of these genes in cancerous

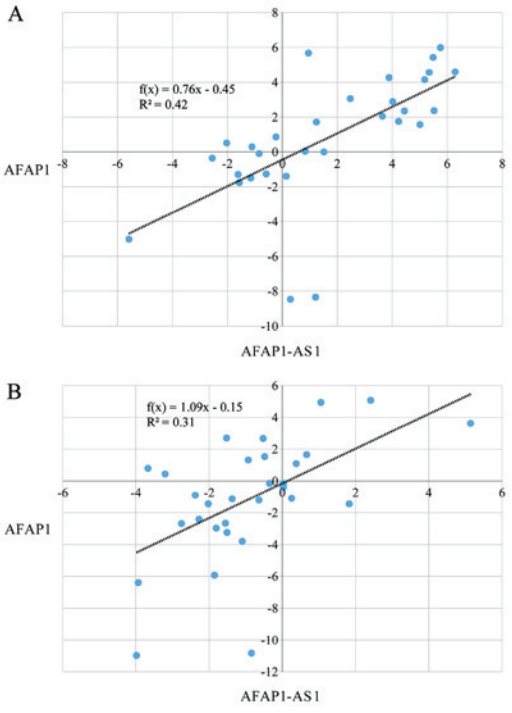

Figure 2. Pairwise correlation between the expression levels of *AFAP1* and *AFAP1-AS1* in (A) tumor tissues and (B) adjacent non-cancerous tissues. *AFAP1*, actin filament-associated protein 1; *AFAP1-AS1*, actin filament-associated protein 1-antisense RNA 1.

tissues may be due to epigenetic changes which have occurred during the process of tumorigenesis. Alternatively, the differential expression of transcription factors between tumoral and non-tumoral tissues may affect expression of these genes in these two sets of samples.

Feng *et al* (10) reported positive associations between the elevated expression levels of *AFAP1-AS1* in gastric cancer samples and lymph node metastasis, Tumor-Node-Metastasis stage and poor patient outcome. In line with their results, the present study demonstrated higher expression levels of *AFAP1-AS1* in patients with lymphatic/vascular invasion compared with those who did not.

The present study additionally detected the downregulation of *AFAP1* in gastric cancer tissues compared with ANCTs. *AFAP1* has been recognized as an Src binding partner. It may also modulate actin filament integrity in response to cellular cues, and may link Src family members to actin filaments (1). Based on the previously reported upregulation of Src in gastric cancer samples (15), it was hypothesized that the observed downregulation *AFAP1* in gastric cancer tissues may be due to a compensatory mechanism to lessen the effects of Src on cell proliferation. Alternatively, this observation implies the existence of a negative regulatory feedback between Src and *AFAP1*. Assessment of *AFAP1* protein levels or function is required for the verification of these hypotheses.

Notably, the present study detected significant associations between the expression levels of the two genes and the location of the primary tumor (P=0.04 and 0.001, respectively) in that *AFAP1* and *AFAP1-AS1* were downregulated in all cardia tumor types compared with their paired ANCTs. This observation is consistent with the previously reported dissimilar gene signature between cardia and non-cardia tumor types (16). Additionally, this expression pattern signifies the function of these genes as diagnostic markers for tumor types originating from the cardia region.

Furthermore, the lower expression levels of *AFAP1* were detected in younger patients compared with older patients and in high grade tumor types compared with low grade tumor types. Previous studies have revealed dissimilarities in the clinicopathological traits between younger and older patients with gastric cancer. These studies indicate a poor patient outcome in younger patients as a result of late diagnosis and a more aggressive tumor phenotype (17,18). Altogether, it may be hypothesized that there exist associations between *AFAP1* low expression levels and determinants of an aggressive tumor phenotype. Further evidence for this hypothesis has been provided by the observed trends toward an association between the expression level of *AFAP1* and peritoneal invasion. The association between smoking history and *AFAP1* expression may indicate the impact of the interaction between smoking, altered actin filament structure and cancer development. A previous study has reported the function of cigarette smoke extract in the induction of actin filament reconstitution (19). Future studies are required to assess the interactive and combinational functions of these factors in gastric cancer evolution.

The present study additionally detected significant pairwise correlations between the transcript levels of these genes in tumoral tissues and ANCTs. Future functional studies are necessary to investigate whether *AFAP1-AS1* may affect the expression of *AFAP1* at genomic, transcriptomic or protein levels.

Finally, the diagnostic power of *AFAP1* and *AFAP1-AS1* in gastric cancer were determined to be AUC=0.75 and 0.67, respectively. The combination of the transcript levels of these genes marginally enhanced the diagnostic power. Therefore, the present study indicates the potential application of these transcripts as diagnostic biomarkers in gastric cancer. However, this hypothesis should be verified in a larger cohort of patients.

The present study has a number of limitations. First, the present study did not implement mechanistic experiments to unravel the cause of the differential expression of the genes between the two sets of samples. Second, the study power was limited by the relative small size of studied samples. Taken together, the results of the present study indicates the role of *AFAP1* and *AFAP1-AS1* in gastric cancer and their application as biomarkers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FE and MT performed the experiments. MT designed the study. AN collected the data and participated in the experiments. VKO analyzed the dat. SGF wrote the manuscript and supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by ethical committee of Shahid Beheshti University of Medical Sciences. All patients provided written consent for participation in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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