RESEARCH ARTICLE

Association of Promoter Methylation Patterns with Expression of *MAPK14* in Tissue of Papillary Thyroid Cancer Patients

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Abstract

   Background: Thyroid cancer is one of the most prevalent malignancies worldwide. Genetic and epigenetic alterations are one of the main causes of thyroid tumor that is responsible to the activation of oncogenes as well as the inactivation of tumor suppressor genes. This research aimed to investigate the relationship of promoter methylation patterns with the expression of *P38α* in Iranian patients with thyroid cancer. **Methods:** We collected 40 thyroid tumor samples and 40 adjacent normal thyroid samples from 40 Iranian patients with papillary thyroid cancer. The promoter methylation pattern of *P38α* gene was investigated by methylation-sensitive high-resolution melting (MS-HRM) method. Moreover, mRNA expression of P38α was investigated by Real-Time PCR method. Further validation of the obtained results was performed by the Cancer Genome Atlas (TCGA) dataset. **Results:** The obtained results indicated that the expression of the *P38α* (*MAPK-14*) gene in the thyroid cancer sample was considerably higher than tumor margin sample. Also, *P38α* gene promoter methylation was higher in thyroid margin tissue as compared to tumor tissue. These results were additionally confirmed by TCGA analysis. The receiver operating characteristic (ROC) curve analysis showed a high accuracy of *P38α* gene expression as a diagnostic biomarker for thyroid malignancy. **Conclusion:** Our study demonstrated that the *P38α* expression level gene was associated with thyroid cancer pathogenesis among the Iranian population. We suggested that this gene expression might be used as a biomarker for diagnosis of thyroid tumor.

   Keywords: Thyroid cancer- *P38α*- *MAPK14*- Methylation

Introduction

Thyroid tumor is the 5th most prevalent malignancy among females, with low 5-year survival rate, especially after tumor surgery in advanced stages (stage IV). Commonly, thyroid malignancy is 3 to 4 times more prevalence in female than male (Cabanillas et al., 2016). The papillary thyroid cancer (PTC) is the most prevalent kind of thyroid malignancy and its prevalence has increased in the last three decades in the world (Crepeau et al., 2023; Pizzato et al., 2022). Thyroid cancer is a multifactorial complex disease that happens as a result of environmental factors, genetic, and epigenetics (Chmielik et al., 2018; Vu-Phan and Koenig, 2014). Knowledge of the underlying genetics and molecular mechanisms of this cancer can cause to emergence of novel therapeutic approaches and identification of markers for early diagnosis and prognostic.

   Epigenetic alteration such as DNA methylation can lead to uncontrolled function of cancer-related genes. Change in DNA methylation profile is one of the most common epigenetic mechanisms in initiation of tumorigenesis and progression of cancers. Impaired change in DNA methylation can cause tumor suppressor genes low expression as well as upregulation of oncogenes, which might lead to tumorigenesis (Lu et al., 2020; Koch et al., 2018). In recent years, aberrant downregulation of cancer-related genes expression such as DNA repair genes, metastasis inhibitor genes, and tumor suppressor genes has been detected in patients with thyroid tumors (Hu et al., 2006). Therefore, the identification of cancer-related gene methylation status may potentially be used as prognosis and diagnosis markers.

   Accordingly, the identification of genetic biomarkers for early prognosis and diagnosis of thyroid tumor patients can impact on treatment and management of the disease (Aftabi et al., 2021; Saffar et al., 2013). One of the most prevalent epigenetic changes in thyroid tumors is the upregulation of metabolic pathways such as mitogen-associated protein kinase (MAPK) (Zarredar et al., 2019b; Zarredar et al., 2018). Four distinct genes encoded by *p38* MAPK, *p38δ (MAPK13/SAPK4), p38β (MAPK11),*

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p38γ (MAPK13), and *p38α (MAPK14)*. These pathways plays a critical role in transducing stress signals from the environment, and regulates several cellular functions, like differentiation, cell proliferation, apoptosis, and inflammation. In addition, the p38 MAPK family shows a vital role in the modulation of cell invasion and metastasis (Martínez-Limón et al., 2020; Zarredar et al., 2019a). *P38α* could act as an oncogene or tumor suppressor that related to the kind of tissue and cancer (García-Hernández et al., 2022)

For example, Abnormal expression of *p38α* MAPK is correlated with short survival and advanced stages of patients with cancer (e.g., breast, lung, prostate, liver, bladder, and thyroid cancer) (Martínez-Limón et al., 2020, Koul et al., 2013). In this research, we intended to investigate the association of promoter methylation patterns with an expression of the *P38α* gene in the tissue of thyroid cancer in Iranian patients. Moreover, we investigated the potential values of the *P38α* gene for early diagnosis and prognosis of patients with thyroid tumors.

Materials and Methods

Study population

In this study, we enrolled 40 patients (24 women and 16 men) with thyroid cancer who were referred to the Department of Surgery at Alzahra Hospital, Tabriz University of Medical Sciences, from January 2019 to August 2021. Standard clinical, pathological, and histological parameters were used for the diagnosis of the patients with papillary thyroid tumors. Patients with previous chemotherapy, hormonal therapy, or radiotherapy as well as patients with other malignancies and acute medical conditions were omitted from this research. All age-matched patients were registered from the same ethnicity and geographical area from East Azerbaijan province, Iran. The demographic information and clinical features of all patients was collected (Table 1). Consent form Declaration of Helsinki ethical standards were signed by all participates (ethical code: IR.TBZMED. REC.1398.1262).

Tissue samples preparation

We collected 80 tissue samples (40 thyroid tumor samples and 40 margin healthy samples) from all patients during surgery. The tumor and margin normal tissues were confirmed by a pathologist as non-cancerous tissues. Serial thick paraffin sections (5 μm) from all tissues were exposed to routine eosin and hematoxylin staining to investigate the clinical stage of the tumor, histological type of tumor, and metastasis to the lymph node. All obtained tissue samples were stored at -80°C until experiments.

Methyl Specific High resolution Melting (ms-HRM)

All frozen tumor tissue samples were cut on dry ice and frozen material by a scalpel. Extraction of DNA from all samples was conducted using a Blood and Tissue Kit conferring to the manufacturer's instruction (Qiagen, United States). The quantity and purity of the extracted DNA samples were detected via electrophoresis on agarose gel and Nano-Drop spectrophotometers, respectively. The genomic DNA samples (1 μg) were bisulfite modified by Bisulfite Kit (Zymo Research, United States) conferring to the manufacturer's protocol. The MS-HRM assays method was conducted to investigate methylation levels of the *P38α* gene promoter by methylated primers in presence of control samples (Table 2). Amplification was conducted in 10 μL total volume containing each primer (0.25 μL), DNA samples (0.5 μL), Master Mix (5 μL), and deionized sterile water (4.0 μL). The temperature cycle of Real-Time PCR was as follows: 1 cycle of initial denaturation (at 94°C for 1 minute), 40 cycles of denaturation (at 94°C for 20 seconds), annealing (at 59°C for 30 seconds), and extension (at 72°C for 30 seconds). MS-HRM analysis was performed based on melt curve patterns.

RNA Extraction, cDNA Synthetize and real-time PCR

We used RNeasy® FFPE Kit to extract total RNA from formalin-fixed paraffin-embedded tissue samples conferring to the manufacturer's protocol. Also we used Thermo Fisher kit (United States America) to synthesize cDNA conferring to the manufacturer's protocol. *P38α* mRNA expression level were evaluated by quantitative Real-Time PCR (Bio Rad, United States America) and specific primers (Table 2). The PCR reaction was performed in a 10 μl total volume: 1 μl cDNA, 5 μl PCR pre-Mix, 1 μl forward and reverse primers. The *GAPDH* gene was used as a housekeeping gene.

Data validation using TCGA datasets

We analyzed DNA methylation status and mRNA expression in the Cancer Genome Atlas (TCGA) database. The obtained data were retrieved and analyzed in UCSC Xena Functional Genomics Explorer (https://xenabrowser. net/). In addition, receiver operating characteristic (ROC) curve analysis was shown to investigate the potential of *P38α* gene methylation pattern and expression level as a diagnostic biomarker for thyroid cancer. The Graph Pad 6 Prism software was used to investigate the area under the curve (AUC) at a confidence interval equal to 95%.

Statistical analysis

The ordinal and nominal data were presented as percentages, as well as the interval data was conferred as mean value ± standard deviation (SD). Theanalysis of amplification results and drawing of graphs was conducted by Graph Pad 6 Prism. Mann-Whitney test, Wilcoxon matched-pairs signed-rank test, and paired and unpaired t-test were used to statistically analyze differences of genes promoter methylation status between tumor tissues and margin tissues (P value< 0/05). The Spearman method was used to analyze the correlation between our studied variants.

Results

Methylation levels

In this study, we assessed promoter methylation of the *P38α* gene in both the thyroid tumor sample and margin normal thyroid sample. Our results demonstrated that *P38α* methylation level in tumor tissues was less than marginal healthy tissues (p-value = 0.269) (Figure
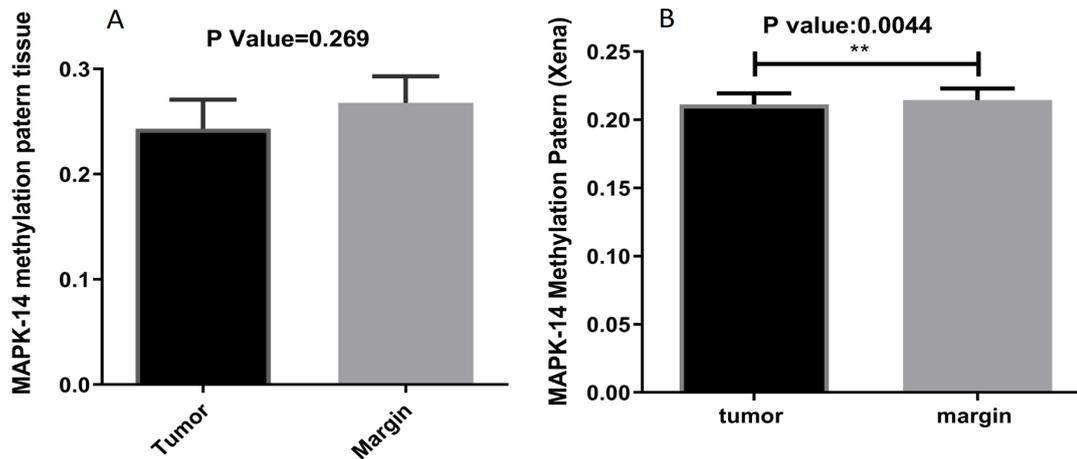
Figure 1. MAPK-14 Methylation Pattern in Tissue Samples (A), MAPK-14 methylation pattern in TCGA (B).

Table 1. Clinicopathological Features of Patients with Papillary Thyroid Carcinomas

Number of Samples	40	16 sample<50
		24 sample>50
Age mean		52.2
Sex	Female	24
	Male	16
Involve lobe	Right	18
	Left	16
	Both	6
Capsular involvement	Negative	34
	Positive	6
	Negative	13
Lymphoid metastasis	Negative	13
	Positive	27
Stage of cancer	Stage I	3
	Stage II	14
	StageIII	17
	StageIV	6
Histology	Papillary thyroid carcinomas	40
	Follicular thyroid carcinomas	0

1A). The achieved data from TCGA datasets about *P38α* methylation indicated that promoter methylation of *P38α* gene significantly increased in margin normal tissues as compared thyroid tumor samples (p-value = 0.0044)

(Figure 1B). Also, we found that there is not significant association between promoter methylation of *P38α* gene and clinical stage, lymph node metastasis, involved lobe, and capsular involvement of thyroid tumor tissues (Table 3).

Gene expression

We also investigated mRNA expression of the *P38α* gene in the thyroid cancer samples and margin healthy samples. The obtained results indicated that mRNA expression of the *P38α* gene in the tumor sample was significantly higher than tumor margin sample (P value = 0.0042) (Figure 2A). Also, the obtained data from TCGA datasets indicated that expression of *P38α* gene significantly increased in thyroid tumor tissues as compared to margin normal tissues (P value = 0.0116) (Figure 2B).

Methylation-expression correlation

In the current study, we investigated the *P38α* methylation-expression correlation in thyroid cancer tissues and methylation data and mRNA expression data from TCGA datasets. Our results indicated that the methylation level of the *P38α* is inversely associated with mRNA expression (r = 0.4160, R square = 0.1731, P value = 0.0076) (Figure 3A). However, there was no significant correlation between promoter methylation and mRNA expression of *P38α* gene in the current study (r = 0.03897, R square = 0.00151, P value = 0.32) (Figure 3B).

Diagnostic values

Our results from ROC curve analysis demonstrated

Table 2. Primer Sequencing of the Related Genes

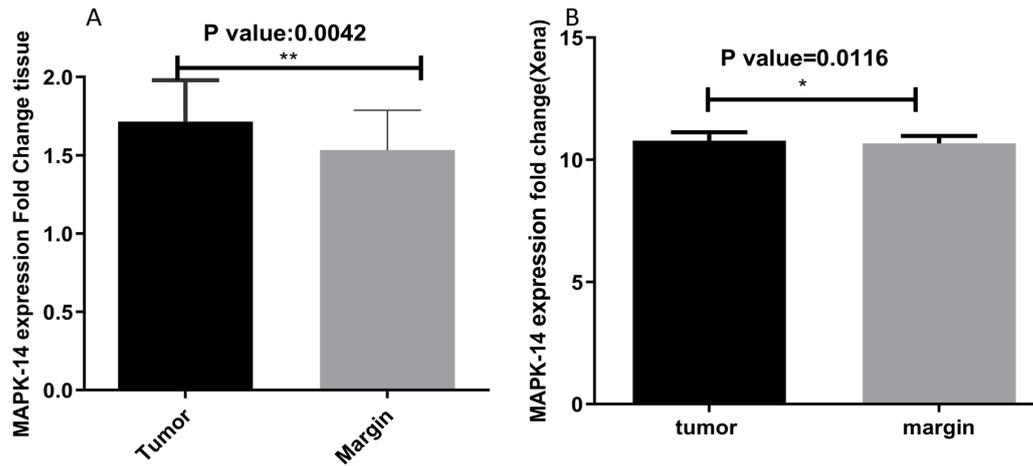|	Primer sequence	Annealing temp (°C)
Mapk-14 (p38α)	Forward 5′-GGTTACGTGTGGCAGTGAAGAAG-3′	59
	Reverse 5′-GCAGGTGTAAAAACGTCCAACAG-3′	
GAPDH	Forward: 5′-CAAGATCATCAGCAATGCCTCC-3′	59
	Reverse: 5′-GCCATCACGCCACAGTTTCC-3′	
Mapk-14 (p38α) methylation	Forward: 5′-caatcttcgtcgagaggaagcttg-3′	59
	Reverse: 5′-gaagtgcgtaggaagctgacgga-3′	

Figure 2. *MAPK-14* Expression Pattern in Tissue Samples(A), MAPK-14 Expression pattern in TCGA (B).

Table 3. Coloration of the P-38α Promotor Methylation and Expression and Clinicopathological Features of Patients

	Expression		Mean±SD	Methylation		Mean±SD
Lymphoid metastasis	P value:0.032	Positive	1.77±0.36	P value:0.053	Positive	2.33±0.51
		Negative	1.68±0.18		Negative	2.51±0.72
Involved lobe	P value:0.14	Positive	1.69±0.32	P value:0.075	Positive	2.41±0.69
		Negative	1.67±0.27		Negative	2.58±0.37
Capsular involvement	P value:0.024	Positive	1.81±0.17	P value:0.053	Positive	2.29±0.25
		Negative	1.63±0.39		Negative	2.57±0.69

that *P38α* gene expression can be a potential diagnostic target for distinguishing thyroid cancer tissue from normal tissue with AUC up to 0.67 (P value = 0.006) (Figure 4A). However, *P38α* gene methylation indicated that there is no potential diagnostic value (AUC up to 0.58 and P value = 0.13) (Figure 4B).

Discussion

MAPK signaling pathway plays an active role in various cellular functions such as development, differentiation, apoptosis, and proliferation (Rezatabar et al., 2019). Previous studies have suggested that aberrant methylation of tumor suppressor genes is one of the most important events in various tumors and malignancies (Khatami et al., 2019). The epigenetic alterations are involved in the early stages of tumorigenesis, and are emerging biomarkers in the early diagnosis and prognosis of numerous tumors (Krasteva et al., 2014). Evidence suggests that numerous environmental factors such as nutrition, alcohol drinking, smoking, stress, and lifestyle stimulate various epigenetic mechanisms, such as DNA
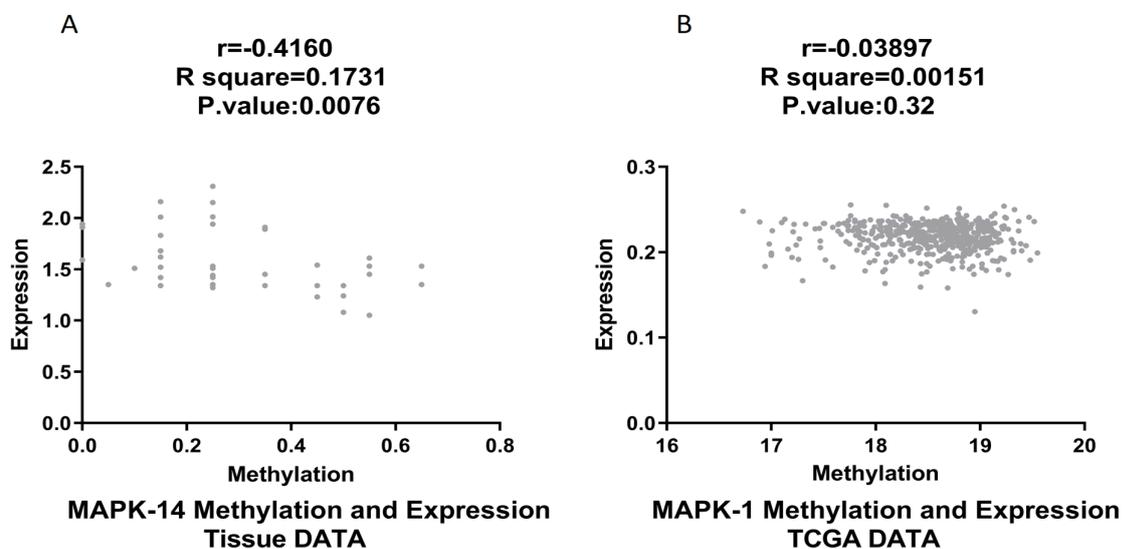

Figure 3. Correlation between *MAPK-14* Expression and Methylation Tissue Samples(A). Correlation between *MAPK-14* Expression and methylation in TCGA (B).
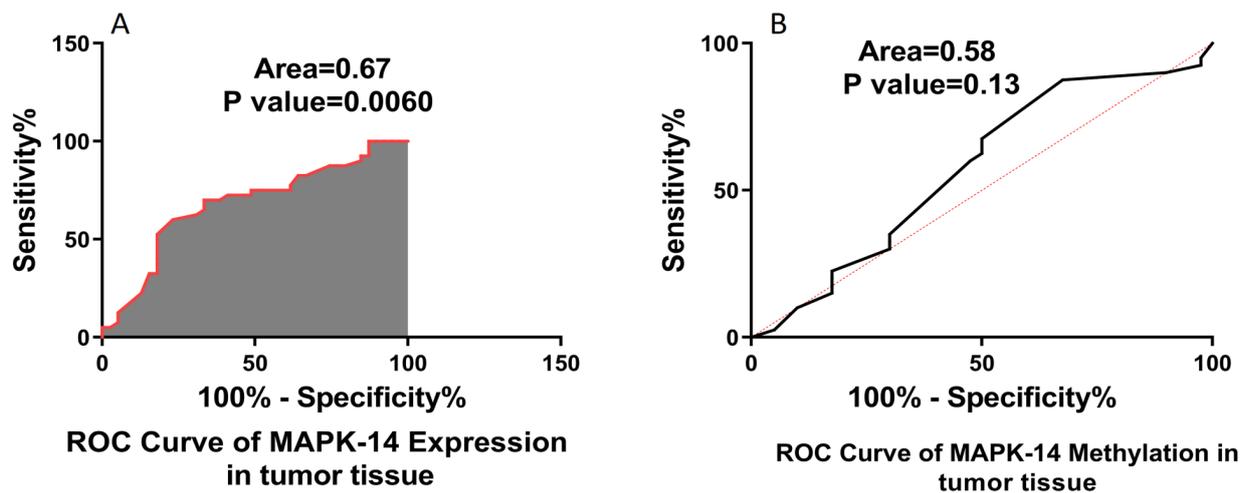
Figure 4. Roc Curve Analysis of *MAPK-14* Expression(A) and methylation(B).

methylation, which lead to malignancy (Tiffon, 2018).

Here, we evaluated promoter methylation and mRNA expression of the *P38α* gene in thyroid tumor tissues and margin normal tissues. Finally, the obtained results were compared with TCGA dataset. In addition, we investigated the association of the methylation status of the *P38α* with clinicopathological characteristics in thyroid cancer. ms-HRM is semi-quantitative technique to identify the methylation of a specific site. It is simple, cheap and easy to set in compression to the other quantitative techniques and according to the available facilities and available budget, it was the most suitable method for our study (Wojdacz et al., 2008). Also, we found that there is no significant association between promoter methylation of the *P38α* gene and clinical stage, lymph node metastasis, involved lobe, and capsular involvement of thyroid tumor tissues. Our results demonstrated that promoter methylation of the *P38α* gene in the thyroid tumor sample was less than normal margin sample. Moreover, mRNA expression of *P38α* gene was considerably higher in thyroid malignancy tissue as compared to normal margin tissue. Also, we found that there is an inverse correlation between methylation and mRNA expression level of the *P38α* in thyroid tissue samples. However, in our results, there was no significant association between promoter methylation of the *P38α* gene and clinical stage, lymph node metastasis, involved lobe, and capsular involvement of thyroid tumor tissues. Finally, ROC curve analysis demonstrated that mRNA expression of the *P38α* gene may be a potential diagnostic and prognostic target for thyroid cancer. But, there was no potential diagnostic value for *P38α* gene methylation in our study.

Wang et al., (2022) found that the *P38α* expression level was overexpressed in the colorectal cancer sample compared with the marginal healthy sample and the *P38α* overexpression was involved with poor prognoses and poor clinicopathological features in the colorectal malignancy array. Also, they demonstrated that *P38α* might be used as a new potential therapeutic target and prognostic biomarker for the treatment of colorectal malignancy.

In another study, Liu et al., (2020) showed that phosphorylated mitogen-activated protein kinase 14 (P-MAPK14) was upregulated in bladder tumor cell lines and tissues which stimulate the growth and metastasis of bladder tumor cells. And could be used as a possible target for bladder malignancy therapy.

Mesquita et al., (2020) suggested that suppression of MAPK14 considerably inhibit cell development, promote cell death and strongly prevents cell metastasis of gastric tumor cell (AGP-01). They also found that suppression of the MAPK14 leads to c-MYC downregulation and TP53 upregulation after SB-245392 (MAPK14 inhibitor) treatment. They proposed that MAPK14 could be a possible biomarker for advanced gastric cancer. Also, Limoge et al., (2017) confirmed that upregulation of the *p38α* gene in breast tumor promote growth, invasive, metastatic and carcinoma vascularization capacities which are related to the role of the p38 in different malignancy. In another research, Zarredat et al., (2019a) indicated that suppression of the *p38α* expression via siRNA, decreased the growth, development and induced apoptosis in the A549 lung tumor cell line.

In contrast, Zhong et al., (2014) stated that p38 expression level could be used as a diagnostic factor in Pancreatic tumors. In another research, Han et al., (2019) show that baicalein can stimulate cell death in FRO cells via stimulation of the ERK/p38 MAPK, and partially, PI3K signaling axe. Yu et al., (2007) found that DUSP26, stimulates anaplastic thyroid tumor cell development via controlling p38 MAPK activity. So, it is obvious that *p38α* has a different role (induce or suppress malignancy) in different cancers.

In the present study, we focused on the Iranian Azerbaijan population in the same geographical area, race, and ethnicity. Therefore, to achieve more knowledge on the role of *P38α* gene methylation in tumorigenesis and earlier diagnosis of thyroid cancer, we recommended to more studies on various populations and ethnicities with larger sample sizes. This evidences demonstrated that *P38α* gene expression could be used as biomarkers in early-stage diagnosis of human thyroid cancers.

In conclusion, our study suggested that decrease in DNA methylation leads to *P38α* upregulation that causes tumorigenesis of thyroid cancer.

Author Contribution Statement

M.SH. and M.R. Designed the study.H.Z. and M.A. and M.SH. Collect and/or Processing data. A.C. and A.F.A. interpreted the data. M.R. and Z.S. and A.A.K. Write the manuscript.

Acknowledgements

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restrains growth through JNK inhibition and correlates with
improved survival. *Clin Cancer Res*, **20**, 6200-11.