The prognostic significance of ING4 expression on gastric gastrointestinal stromal tumors by immunohistochemistry

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OBJECTIVE: *Inhibitor of growth 4 (ING4) is a novel tumor suppressor gene that is reported to be down-regulated in various tumors including gastrointestinal stromal tumors (GISTs) originated from different locations, recently. Herein, we aimed to evaluate ING4 expression and its prognostic significance on gastric GISTs in order to add further data to the current literature.*
MATERIAL AND METHODS: *ING4 was evaluated in samples of gastric GISTs from 62 patients, by immunohistochemistry. The association between ING4 expression and clinicopathological features related with prognosis and overall survival (OS) were analyzed statistically.*
RESULTS: *There was statistically significant inverse correlation between ING4 expression and risk groups according to both NIH and AFIP, Ki67 index, tumor diameter, and mitotic count by univarite analysis (p=0.000, p=0.000, p=0.08, p=0.01, and p=0.028, respectively). The negative association between ING4 expression and risk groups according to both NIH (p=0.002, β=-0.263, t=-3.166) and AFIP (p=0.016, β=-0.244, t=-2.492) was supported by multivariate analysis. There was statistically significant direct correlation between low levels of ING4 expression and shorter OS by univariate (p=0.000) and multivariate analysis (p=0.000, β=0.769, t=9.798), as well as Kaplan-Meier method (p=0.035).*
CONCLUSIONS: *The low ING4 expression level was found to be related with unfavorable prognosis. Thus, we suggest that loss of ING4 expression might play a role in the progression of GISTs and might be used as a potential prognostic tool. Additionally, this is the first study that has evaluated the association of ING4 expression on gastric GISTs, to the best of our knowledge. Therefore, we claim that more comprehensive future studies including higher number of patients and longer follow-up might clarify the potential role of ING4 on pathogenesis and prognosis of GISTs.*

KEY WORDS: Clinicopathological features, Gastrointestinal stromal tumor, ING4, Immunohistochemistry.

Introduction

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal neoplasm in gastrointestinal tract, and constitutes 1% of all gastrointestinal neoplasms [1-2]. It is considered to be originated from the interstitial cells of Cajal [3]. The most common site that GISTs are originated is stomach (40-60%), followed by small bowel (30-40%), duodenum (5%), large bowel, and esophagus in a descending order [2,4]. Most of GISTs have mutation of c-KIT [CD117] proto-oncogene, a transmembrane tyrosine kinase receptor, at exon 11, 9, 13, or 7 [3,5-6]. However 5-7% of GISTs show mutations in the platelet-derived growth factor receptor-alpha (PDGFRA) [3,5-6]. GISTs are potentially agressive tumors. The clinical course of GISTs range from benign to malignant. Thus, some risk assesment systems have been recommended for predicting the prognosis and malignant potential of GISTs [7-9]. In 2002, National Institutes of Health (NIH) of the United States proposed a risk assesment system based on mitotic count and tumor size [8]. In the latter literature,

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tumors located in different anatomical localizations have been demonstrated to have different clinical courses. For instance, gastric GISTs have been documented to have better prognosis than small intestine GISTs [9]. Therefore, Armed Force Institute of Pathology (AFIP) has recommended another risk stratification based on mitotic count, tumor size, and also anatomic location, in 2006 [9]. High Ki67 proliferation index, tumor necrosis, tumor rupture, and nuclear pleomorphism have been claimed to be the poor prognostic parameters for GIST [10-17]. Additionally, mutation status has been claimed to be a prognostic parameter in some studies [1,18]. However, there is still no reliable prognostic marker to predict the clinical behaviour of GISTs.

Definitive surgery is the main treatment approach for localised resectable GISTs. Nevertheless, rate of postoperative recurrence is reported to vary from 40% to 90% [4,18]. Imatinib mesylate-a tyrosine kinase inhibitor that blocks KIT and PDGFRA, is mainly used for the advanced GISTs as adjuvant or neoadjuvant therapy [4]. However, resistance to imatinib mesylate is seen in some patients and the studies are being carried out to create effective alternative drugs for imatinib-resistant GISTs [4]. Thus, observation of novel biomarkers for targeting therapy as well as some reliable predictive factors for the malignant behaviour, tumor progression, recurrence and resistance to therapy are essential.

Inhibitor of growth (ING) family is a tumor suppressor gene family including ING1, ING2, ING3, ING4 and ING5 [19]. These tumor suppressor genes play crucial roles in transcriptional activity of p53, cell proliferation, apoptosis, cell death, contact inhibition, DNA repair, and angiogenic inhibition [19]. It is known that ING4 is expressed in normal human tissues. However, it has been described in the literature that ING4 expression decreases markedly in some tumors such as gliomas, head and neck squamous cell carcinomas, malignant melanomas, breast carcinomas and lung carcinomas [19-24]. Additionally, Nanding et al. have recently reported that low expression of ING4 affects prognosis adversely in GISTs for the first time in the literature [19]. They have evaluated ING4 expression by immunohistochemistry in 41 cases of GISTs from different locations using the risk stratification system of NIH. In the light of their study we designed this study to investigate the prognostic significance of ING4 expression on gastric GISTs of 62 cases by using the risk assesment sytem of both NIH and AFIP in order to rule out the possible difference originated from the anatomic locations and risk stratification systems, and add further data about the association of ING4 and GIST to the current literature.

Material and methods

After obtaining Bozok University Ethic Committee approval, 62 consecutive cases of gastric GIST diagnosed between 2008 and 2014 by using immunohistochemical panel of CD117, CD34, SMA, desmin, S100 and Ki67 were included in the study. Paraffin blocks of 60 cases were retrieved from the archives of Department of Pathology, Gazi University School of Medicine, and 2 cases were retrieved from the archives of Department of Pathology, Bozok University School of Medicine. The clinicopathological features [age, gender, risk group, mitotic count in 50 high power fields (HPFs), tumor diameter, tumor location, cellularity, nuclear pleomorphism, tumor cell type, surgical/biopsy procedure] were achieved from the original pathology reports. Risk-groups were established and adopted according to both NIH's [8] and AFIP's [9] risk assesment systems.

Paraffin blocks were cut into 4-μm sections, deparaffinized and dehydrated according to standard protocols. Then, immunohistochemistry was performed using the streptavidin–biotin-peroxidase method for ING4 antibody (1:150 dilution, ab113425, rabbit polyclonal antibody, abcam, USA) in an automatised stainer (Leica Bond-Max, Leica Biosystems, United Kingdom). Nuclear staining was considered positive for ING4. ING4 stained slides were evaluated for both extent and intensity of staining. Five random HPFs were examined to count immunoreactive cells under light microscope (BX53F, Olympus, Tokyo, Japan). Extent of staining was scored as: score 0=no staining, score 1=<10%, score 2=10-50%, score 3=51-75%, score 4=75-100%. Intensity of staining was scored as follows: score 0=no staining, score 1=weak (light yellow staining), score 2=moderate (yellow-brown staining), score 3=strong (brown staining). Then, an immunostaining index (ISI) was calculated by multiplying the scores of staining extent and intensity similar to the study of Nanding et al (ISI=extent X intensity scores) [19]. The ISI of ING4 ranged from 0 to 12. ISI<6 was considered as "*low*" while ISI ≥ 6 was considered as "*high*" xpression of ING4.

Ki67 stained slides used at the initial diagnosis of GIST were re-evaluated for establishing proliferation index. Nuclear staining was considered positive for Ki67. The percentage of positively stained nuclei was calculated by counting 10 randomly selected microscopic fields under high-power magnification. The cases were divided into two groups according to Ki67 proliferation index as <10% (low index) and ≥10% (high index).

A single referral pathologist (S.S.) reviewed the slides and performed scoring blinded to the study groups.

ISI of ING4 was correlated with clinicopathological parameters (age, gender, risk group, tumor location, tumor size, mitotic count, cell type, cellularity, nuclear pleomorphism, necrosis, hemorrhage, ulceration, and growth pattern), and Ki67 index statistically.

Follow-up and survival data were retrieved from the hospital records. Patients with severe disesases during follow-up were excluded from the survival data.

Table I - *The associations with ING4 expression and clinicopathological features of gastric GISTs (n: 62).*

Patient Characteristics	Cumulative Population	ING4 Low expression	ING4 High expression	Univariate Analysis	Multivariate Analysis
Age (years)					
<50	9	6	3	p=0.44[a]	
≥50	53	28	25		
Gender					
Female	27	14	13	p=0.68[a]	
Male	35	20	15		
Risk groups (NIH)					
Very low-risk	8	2	6	**p=0.000[a]**	**p=0,002[b]**
Low-risk	23	7	16		B: -0.263
Intermediate-risk	14	10	4		t= -3.166
High-risk	17	15	2		
Risk groups (AFIP)					
Very low-risk	9	1	8	**p=0.000[a]**	**p=0,016[b]**
Low-risk	33	15	18		B: -0.244
Intermediate-risk	9	7	2		t=-2.492
High-risk	11	11	0		
Tumor size (mean±SD,range, cm)	5,7±3.4 (0.4-16.5)				
<2	6	1	5	**p=0.01[a]**	
2-5	21	10	11		
5-10	27	16	11		
>10	8	7	1		
Mitosis (mean±SD,range, 50 HPFs)	6.5±12.7 (0-65)				
<5/50	42	19	23	**p=0.028[a]**	
≥5/50	20	15	5		
Cell type					
Spindle	42	23	19	p=0.87[a]	
Epithelioid	4	3	1		
Mixed	16	8	8		
Cellularity					
Mild	18	8	10	p=0.43[a]	
Moderate	13	8	5		
High	31	18	13		
Nuclear pleomorphism					
Mild	48	26	22	p=0.98[a]	
Moderate	6	4	2		
High	8	4	4		
Necrosis					
Present	16	10	6	p=0.48[a]	
Absent	46	24	22		
Hemorrhage					
Present	1	0	1	p=0.27[a]	
Absent	61	34	27		
Ulceration					
Present	10	8	2	p=0.08[a]	
Absent	52	26	26		
Growth pattern					
Expansive	58	31	27	p=0.41[a]	
Infiltrative	4	3	1		
Ki67					
<10%	52	26	26	p=0.08[a]	
≥10%	10	8	2		
Overall survival (mean±SD, range, months)	47.2±22.9 (12-94)	30.2±2.1 (12-65)	69±11.8 (44-94)	**p=0.000** [a]	**p=0,000[b]** B:0.769 t=9.798

GISTs; gastrointestinal stromal tumors, HPFs; high power fields
a; Spearman correlation test, b; multivariate linear regression analysis

Statistical Analysis

All data were analyzed using PASW Statistics version 18.0 (SPSS Inc. Chicago. IL. USA). The demographic variables were detected using descriptive statistics. The compliance of data with normal distribution was evaluated with the Kolmogorov – Smirnov and Shapiro-Wilk tests. Mann-Whitney U test and chi-squared test were used to correlate ING4 expression, risk groups and other clinicopathological parameters. The correlations between two independent variables were analyzed by Spearman's rho test. Survival analysis was performed by the Kaplan–Meier method and log-rank test. The prognostic relevance was evaluated by Cox regression analysis. The effects of associated variables were studied by multiple linear regression analysis using backward method. Data were expressed as mean±standard deviation and percent (%) where appropriate. $p<0.05$ was considered statistically significant.

Results

Clinicopathological Findings

We examined specimens from 62 patients (35 women and 27 men) with a mean age of 60.33±12.18 years (range 21 to 84 years). The tumors ranged from 0.4 to 16.5 cm (mean= 5.7±3.4cm) in diameter. Mitotic count varied from 0 to 65 (mean= 6.5±12.7) per 50 high-power fields (HPFs). The risk groups of the cases according to NIH's criteria were as follows: 8 (12.9%) were *very low*, 23 (37.1%) were *low-*, 14 (22.6%) were *intermediate-*, and 17(27.4%) were *high-* risk group. AFIP's criteria were as follows: 9 (14.5%) were *very low-*, 33 (53.2%) were *low-*, 9 (14.5%) were *intermediate-*, and 11(17.7%) were *high-* risk group.
The follow-up time ranged from 12 to 94 months (median=46.0 months). Eight (12.9%) cases were deceased, and 54 (87.1%) cases were alive when the follow-up was

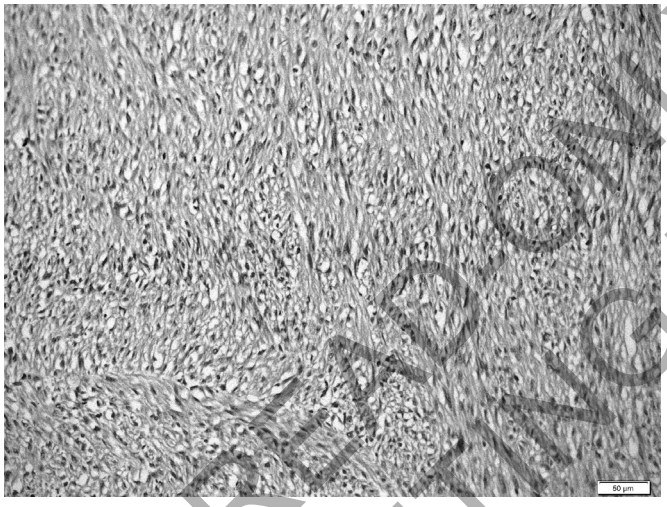

Fig. 1

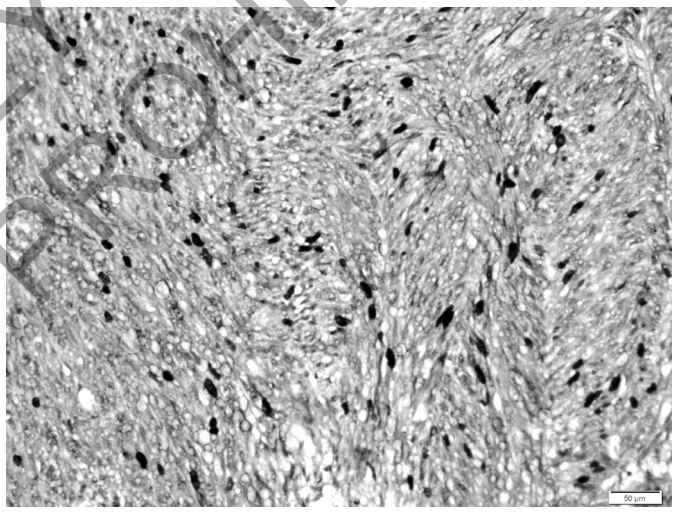

Fig. 3

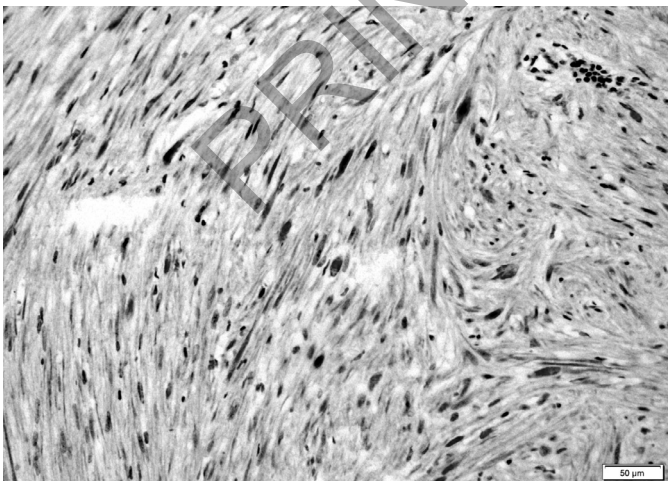

Fig. 2

Fig. 1-3: Photomicrographs of GIST. 1) The tumor composed of spindle cells (Hematoxylin&eosin, x200) 2) Immunopositivity for ING4 of tumor cells (Streptavidin-biotin-peroxidase method; x200). 3) Immunopositivity for Ki67 of tumor cells (Streptavidin-biotin-peroxidase method; x200).

finished. Overall survival (OS) ranged from 12 to 94 months (mean=47.2±22.9 months). The clinicopathological features and their correlation with ING4 expression of 62 gastric GISTs are summarized in Table I.

Immunhistochemical Findings

Correlation of ING4 expression and clinicopathological parameters

There was statistically significant inverse correlation between ING4 expression and risk groups according to both NIH and AFIP, Ki67 index, tumor diameter, and mitotic count (p=0.000, p=0.000, p=0.041, p=0.01, and p=0.028, respectively) by univarite analysis. The negatively association between ING4 expression and risk groups according to both NIH (p=0.002, β=-0.263, t=-3.166) and AFIP (p=0.016, β=-0.244, t=-2.492) was supported by multivariate analysis (Table I). The microscopic photos of demonstrative cases are given in Fig. 1-3.

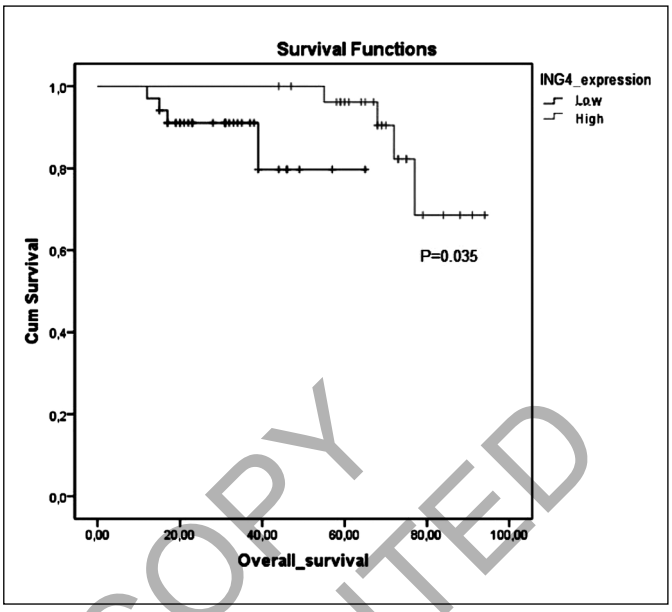

Fig. 4: Kaplan-Meier survival curves illustrating inverse correlation between ING4 expresion and overall survival (months). Cum Survival, cumulative survival

Correlation of ING4 expression and overall survival

During follow-up, we detected that 4 of 34 cases with low expression of ING4 were deceased, while remaining 30 were alive. Four of 28 cases with high expression of ING4 were found to be deceased, while remaining 24 were alive. There was no statistically significant correlation with ING4 expression and mortality (p=0.53). Mean OS was 30.2±2.1 months in cases with low expression of ING4, while it was 69.0±11.8 in cases with high expression of ING4. There was statistically significant direct correlation between loss of ING4 expression and shorter OS by univariate (p=0.000) and multivariate linear regression analysis (p=0.000, β=0.769, t=9.798), as well as Kaplan-Meier method (p=0.035) (Fig. 4). The data about the mean OS of the cases according to the ING4 expression is given in Table I.
Additionally, OS was found to be negatively correlated with risk groups according to both NIH and AFIP, tumor diameter, and mitosis (p=0.001, p=0.000, p=0.041, and p=0.006, respectively) by univariate analysis, however none of those correlations was supported by multivariate analysis (p>0.05).

Discussion

In the current literature a small number of study have been evaluated the relation between ING4 expression on some tumors [19-24]. In general, it has been demonstrated that down-regulation of ING4 might induce disease progression in various tumors originated from different tissues [25]. Garkavtsev et al. have reported that ING4 plays a role in promoting brain tumor growth and angiogenesis [21]. They have also found that expression of ING4 is significantly lower in gliomas as compared with normal human brain tissue, and the extent of reduction correlates with the progression from lower to higher grades of tumours, indicating tumor progression [21]. Li et al. have evaluated the role of ING4 in malignant melanoma pathogenesis, and demonstrated the association with reduced ING4 and poor survival [26]. Wang et al. have reported that reduced ING4 mRNA as well as low expression of ING4 by immunohistochemistry are associated with initiation and progression of lung cancer [27]. Lou et al. have documented that colorectal cancer has significantly lower levels of ING4 mRNA level compared to colonic adenoma and normal colonic tissue [28]. They have reported that reduced ING4 expression is correlated with higher clinical stage and histological grade [28]. They have claimed that ING4 may inhibit tumor growth by modulating angiogenesis and therefore reduced ING4 levels might be considered as a predictive biomarker of tumor progression [28]. Similar to Lou et al. [28], You et al. [24] have indicated that ING4 participates in colorectal cancer progression. Byron et al. have reported higher levels of ING4 expression in ductal carcinoma in situ than invasive breast carcinoma [25]. In addition, they have detected low levels of ING4 expression in higher grade and lymph node positive invasive breast cancers [25]. Recently, Nanding et al. published a study in which ING4 expression was examined in 41 GISTs [19]. They have reported that ING4 expression is inversely correlated with Ki67 proliferation index and high risk group according to NIH's similar to the present study. In addi-

tion, we have demonstrated an inverse correlation between ING4 expression and high risk groups according to NIH's as well as AFIP's risk assesment systems different from that study. Inverse correlation with ING4 expression and tumor diameter and mitosis were also found as other different results in our study. Furthermore, we have detected an inverse correlation with loss of ING4 expression and overall survival as another novel striking result. However, the molecular mechanism of ING4 down regulation in GISTs has not been clearly demonstrated in the literature [19]. Deletions, mutations and loss of heterozygosity are suggested to be responsible [19]. For this reason, further studies are needed to determine the causes of ING4 down regulation in GISTs.

Performing Ki67 is strongly recommended while diagnosing a GIST, since high Ki67 proliferation index is widely considered as an indicator of poor outcome in the literature [10-15]. Recently, some studies have suggested that Ki67 proliferation index over than 10% indicates poor outcome [10-15]. Similar to the literature, Ki67 proliferation index more than 10% was found to be associated with low OS by univariate analysis in our study. We have also found a direct association with higher mitotic count and low OS and high-risk group by univariate analysis, similar to Ki67 labeling index. Nevertheless, we have demonstrated an inverse correlation between OS and higher mitotic count, but not with Ki67 proliferation index by multivariate analysis. This result might be contributed to the fact that mitotic count reflects the M phase of mitotic cycle, while Ki67 indicates the proliferative cells in G1, S, and G2 phases [12]. Therefore, we think that higher mitotic count is still more reliable prognostic indicator than Ki67 for GIST, and future studies should be conducted to clarify this issue.

In the literature, there are some risk group classifications established for predicting the prognosis and malignant potential of GISTs [7-9, 16-17]. Risk assesment system of NIH is the older system, however AFIP's system is the novel one that is widely used and suggested to give more reasonable clues about the risk of progression rather than NIH's [7-9]. In this study, we have used the risk assesment of both NIH and AFIP. However, we have found similar correlations between those risk stratifications and clinicopathological features including ING4 expression. This result might be attributed to existence of the small cohort of cases evaluated in the study and the presence of homogenous study group including only gastric GISTs.

Conclusion

In summary, this study has showed that lower levels of ING4 expression is a poor prognostic factor for GISTs by both univariate and multivariate analysis. We strongly recommend that adding ING4 in the routine immunohistochemical panel while diagnosing GIST in order to predict the prognosis. Furhermore, we suggest that ING4 might be used as a potential target biomarker for GIST therapy. We conclude that more comprehensive future studies including higher number of patients and longer follow-up are crucial for clarifying the potential role of ING4 on pathogenesis and prognosis of GISTs.

Riassunto

ING4 è un nuovo gene inibitore di crescita tumorale accreditato per essere inattivato in vari tumori tra cui i GIST a diversa sede. Abbiamo dunque studiato l'espressione del ING4 ed il suo significato prognostico su GIST gastrici per contribuire con nuovi dati alla letteratura.

ING4 e stato studiato con l'immunoistochimica su campioni di GIST gastrici di 62 pazienti, analizzando quindi statisticamente l'espressione e le caratteristiche chiniche ed anatomopatologiche correlate con la prognosi e con la sopravvivenza globale.

Il risultato è una significativa correlazione inversa tra l'espressione del ING4 ed i gruppi a rischio sia secondo NIH che AFIP, l'indice Ki67, il diametro tumorale e la conta mitotica valutata con analisi univariata, rispettivamente (p=0.000, p=0.000, p=0.041, p=0.01, and p=0.028). L'associazione negativa tra l'espressione ING4 ed i gruppi a rischio secondo sia NIH (p=, β=, t=) e AFIP (p= 0.016, β= -0.244, t= -2.492) è supportata dall'analisi multivariata.

Vi è una significativa correlazione statistica firetta tra i bassi livelli di espressione del ING4 e una più breve sopravvivenza globale con analisi univariata (p=0.000) e con analisi multivariata (p=0.000, β=0.769, t=9.798), come pure col metodo Kaplan-Meier (p=0.035).

In conclusione si è trovato che l'espressione bassa di ING4 è dorrelata con una prognosi sfavorevole. Ciò ci induce a suggerire che la perdita di espressione del ING4 può giocare un ruolo nella prognostica dei GIST e può essere potenzialmente usato come strumento prognostico. Inoltre, per quanto a noi noto, questo è il primo studio che ha valutato l'associazione l'associazione dell'espressione del ING4 sui GIST gastrici. Quindi affermiamo che studi futuri su un più ampio numero di pazienti e con follow-up più prolungato potrebbe chiarire il ruolo potenziale del ING4 sulla patogenesi e sulla prognosi dei GIST.

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