ORIGINAL PAPER

S100A4 and ERBB2 as Co-Factors in Pancreatic Cancer

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ABSTRACT

Background: Pancreatic ductal adenocarcinoma (PDAC) remains one of the most aggressive tumors with dismal prognosis and survival rates. Identifying early molecular diagnostic markers could contribute in improving survival in this intractable disease. Chromosomal rearrangements and altered expression of the gene have been implicated in PDAC progression to metastasis. S100A4 protein promotes invasion and metastasis by binding to several intracellular target proteins and modulating their function. ERBB2 is marker of poor prognosis in multiple cancer types. This study examined the potential clinical significance of S100A4 and ERBB2 expression in PDAC.

Materials and methods: Pairs of non-tumoral and tumoral tissues from a total of 73 patients with PDAC were included in our study to assess the gene expression of S100A4 by two-step qPCR using TaqMan hydrolysis probes. ERBB2 gene expression was assessed by SYBR Green chemistry. S100A4 protein expression was evaluated by immunohistochemistry.

Results: Gene expression of S100A4 was increased at advances stages of PDAC (p-value= 0.02) and with tumor grading. Kaplan-Meier survival distributions showed a significant difference in patient's survival outcome according to their S100A4 expression by immuno-histochemistry (lower or higher than 10%. p-value=0.043). Multi-variate Log Rank (Mantel-Cox) test confirmed that resection type and S100A4 protein expression has a significant value in correlation with PDAC. Similarly, significant correlations were observed between ERBB2 gene expression and TNM tumor stage (p-value= 0.0048) and tumor grading (p-value=0.048). Additional analysis showed a statistically significant correlation between S100A4 and ERBB2 gene expression levels (p-value=0.0094).

Conclusions: Our results showed that both S100A4 and ERBB2 are associated with poor outcomes in PDAC, and might be valuable prognostic biomarkers.

Key words: PDAC, S100A4, ERBB2

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth most lethal cancer (1) and is expected to become one of the top three cancer killers among lung and liver cancer by 2030 in US only (2). PDAC is responsible for 7% of all cancer-related deaths in both men and women, as its treatment remains a major challenge despite multi-modality approaches. The five-year survival is dismal due to late diagnostic, presence of metastasis at the time of diagnostic and resistance of cancer cell to chemotherapeutics. Improving detection and prognostication could decrease the morbidity caused by this disease and increase survival.

The majority of all exocrine pancreatic tumors are pancreatic ductal adenocarcinoma (PDAC). Most of PDAC cases (60-70%) occurs preponderantly in head of the pancreas (3) and are characterized by the presence of a dense stroma made of fibroblast and inflammatory cells termed desmoplasia (4). In this hypoxic microenvironment thus created, the stroma is the component that is believed to supports tumor growth and metastatic spread (5,6).

S100A4 is a pro-metastatic gene and more recent studies indicated that S100A4 could be a key factor in promoting EMT (7) we choose to study its role in PDAC tissue patients and to investigate its correlation with clinico-pathologic features.

Also, in an orthotopic model of bladder cancer, the overexpression of S100A4 lead to development of lymph nodes and lung metastasis (8). Moreover, transfection studies using different cell systems have shown that S100A4 is involved in cell motility (9,10).

Overexpression of ERBB2, which is one of the four members of EGFR family (11) has been reported in 7-61% of the PDACs (12, 13, 14, 15, 16). In addition to being an oncogenic driver, the ERBB2 receptor may also mediate intracellular pathways related to chemo-resistance in PDAC (17).

In addition, American Society of Clinical Oncology (ASCO) highlights that S100A4 is one potential biomarker, which has the ability to predict patient survival after surgery for PDAC (18). Transcription of the pro-metastatic S100A4 gene is controlled by both positive and negative regulatory elements located within the first intron, which bind several transcription factors. ERBB2 directs S100A4 expression via a signaling network that includes PI3k, AKT1 and ERK1/2. Activated AKT1 phosphorylates p21Cip/WAF1 cell cycle inhibitor resulting in increased cell proliferation. S100A4 promoter contains an ERBB2 response element (19).

The aim of this study was to investigate S100A4 and ERBB2 gene expression levels as well as the protein level of S100A4 as potential regulatory factors in PDAC oncogenesis.

MATERIAL AND METHODS

Patients' selection and sample collection

The study prospectively enrolled patients diagnosed with PDAC and scheduled for curative surgery of their disease were prospectively included in the present study. All patients signed an informed written consent prior to samples collection. During the surgical intervention, a sample or normal (non-tumoral) and tumoral tissue were collected in RNALater (Sigma, St. Louis, MO) for RNA and DNA extractions, kept at 4°C for 24 hours and then snap-frozen in liquid nitrogen.

RNA extractions

Total RNA was isolated from normal and tumoral tissue samples by means of TRI reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions, followed by further purification using RNeasy Mini Kit (QIAGEN, Valencia, CA). The quantity and quality of the total RNA were assessed by spectrophotometry (Nano Drop 1000; Thermo Scientific, Arlington, TX) and by lab-on-a-chip Agilent 2100 technology (Agilent Technology, Santa Clara, CA). Samples with a 260:280 nm ratio greater than 1.8, a 28S: 18S ratio greater than 1.5 and an RNA integrity number greater than 7 were used for gene expression profiling of S100A4.

Real-time qPCR

Gene expression of S100A4 wad determined by using hydrolysis probes labeled with 6-carboxyfluorescein (Hs00243202_m1) using 18S as a reference gene (Hs99999901_s1) while gene expression for ERBB2 was determined with FastStart Universal SYBR Green Master (ROX) (Roche Molecular Biochemicals, Mannheim, Germany) having GAPDH as housekeeping gene. The GAPDH and ERBB2 sequences used were:
GAPDH: F: 5'-CCATCTTCCAGGAGCGAGATCCCT-3',
R: 5'-TGAGCCCCAGCCTTCTCCATGGT-3',
ERBB2: F: 5'-TAGGGTTAAGGGAAGGCGGA-3',
R: 5'GTGGTACTTCAATTGCGACTCA-3'.

The final DNA concentration was 10 ng/µl in a final volume of 25 µl. For all genes fold changes were determined in tumoral tissues compared with paired normal tissue. Data were analyzed either with SDS 1.4 software (for S100A4) or manually (ERBB2) and the fold changes were determined by using the comparative Ct method [$2^{\wedge}$ (-delta delta Ct)].

Immunohistochemistry

Paraffin tissue sections were adjusted to the size of 4 mm and were subsequently deparaffined and hydrated in xylene and graded ethanol. Epitopes were revealed by 10mM citrate buffer with a pH 6.0 and 1 mM EDTA. For staining we used an anti-human S100A4 polyclonal rabbit antibody (Dako) at a final dilution of 1:150. The staining of S100A4 was evaluated as either low (≤10%) or high (>10%).

Statistical analysis

To examine differences in the clinical data between patients and controls, either the Student's t-test (when values were normally distributed) or the non-parametric Mann–Whitney test (when the distribution normality test failed) were used. For subgroup analyses and the comparison of genes expression levels Kruskal–Wallis test followed by Dunn's Multiple Comparison post hoc test was used. A p-value <0.05 was considered statistically significant. The median and interquartile ranges are reported unless stated otherwise. All calculations were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Patient's characteristics

A total number of 73 patients were included in our study. The available clinical and pathological features are shown in *table 1*.

The patients were relatively equal distributed between gender sex ratio male:female - 56.2:43.8. The median age at the surgery was 60 years old. Most of the patients (45.2%) were diagnosed in IIB stage of disease and patients presented in a higher percentage (52%) G1 differentiation degree.

Fifty-seven percent of patients presented lymph node metastasis, and 46.6% received preoperative chemotherapy. Median tumor size was 3 cm and 80.8% of patients underwent R0 resection type.

S100A4 gene expression in PDAC tissues

The gene expression analysis was done using the formula $2^{-\Delta\Delta Ct}$. We performed a double normalization. First compared to the housekeeping gene and then with the paired normal samples.

We observed an overexpression of S100A4 in tumor tissue compared to normal tissues. Further analysis of S100A4 showed an increase trend of gene expression of

Table 1 - Clinicopathological features of the study group

Parameters	N	%
Gender		
F	32	43.8
M	41	56.2
Age at surgery		
Average ± SD	61±9	
Median (range)	60 [35-77]	
TNM stage		
I	19	26
IIA	9	12.3
IIB	33	45.2
III+IV	11	15.1
unknown	1	1.4
Differentiation degree		
G1	38	52
G2	28	38.5
G3	7	9.5
pN		
N0	29	39.7
N1	42	57.5
unknown	2	2.8
LNR		
<0.16	43	58.9
≥ 0.16	26	35.6
unknown	4	5.5
Preoperative chemotherapy		
Yes	34	46.6
No	15	20.5
unknown	24	32.9
Tumor diameter		
Average ± SD	2.89±1.4	
Median (range)	3[0.3-8]	
Resection type		
R0	59	80.8
R1	3	4.1
other	2	2.7
unknown	9	12.3
Survival (months)		
Average ± SD	15.78±17.69	
Median (range)	10 [0-104]	

S100A4 with increased tumor stage, tumor differentiation degree, number of lymph nodes as well as with the lymph node ratio (*table 2*).

We found that S100A4 gene expression associated with the severity of disease (p = 0.02 for tumor staging) and with the degree of tumor differentiation (*fig. 1*). Correlations between subgroups of patients based on clinicopathological parameters were studied with long-rank test. The results of the univariate analysis are present in *table 3*.

These parameters were further analyzed for correlations with outcome by Log Rank Mantel-Cox in a multivariate analysis. The resection type and S100A4 protein expression were the only factors that showed a significant correlation with survival (*table 4*).

Table 2 - Log$_{10}$ n-fold gene expression of S100A4 in patient's groups

Parametes		S100A4 median (range)	P value
Gender			
	M	0.75 [-0.39-2.58]	0.64
	F	0.81 [-0.65-2.68]	
TNM stage			
	I	0.59 [-0.65-2.58]	0.02
	II	0.84 [0.25-2.17]	
	III, IV	1.59 [0.87-2.68]	
Tumor differentiation degree			
	G1	0.74 [-0.65-2.68]	0.5
	G2, G3	0.81 [-0.33-2.58]	
pN			
	pN0	0.62 [0-0.65-2.68]	0.09
	pN1	0.91 [-0.31-2.17]	
LNR*			
	<0.16	0.74 [-0.65-2.68]	0.21
	≥ 0.16	0.91 [-0.32-2.17]	
Diabetes			
	Yes	0.66 [-0.33-2.13]	0.61
	No	0.80 [-0.65-2.68]	

* the 0.16 cut-off represents the arithmetic mean of LNR for the total patients group included in the study

Table 3 - Results of the univariate Kaplan Meier analysis

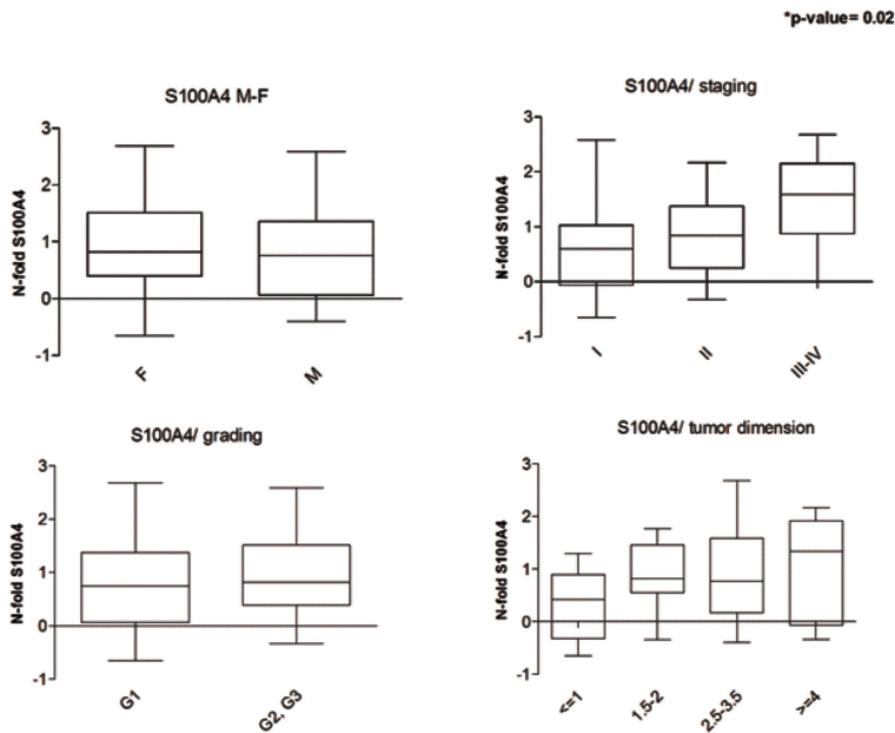| Parameters	p-value
T (tumor extension)	
T1&T2	
T3&T4	0.18
N (lymph node invasion)	
N0	
N1	0.28
M (metastasis)	
M0	
M1	0.08
TNM stage	
IA, IB&IIA	
IIB	0.21
III&IV	
Tumor differentiation	
G1. G2	
G3	0.46
LNR	
LNR (cut-off 0)	0.22
LNR (cut-off 0.16)	0.06
Resection type	
Yes	
No	0.17
R0	
R1	0.003
CA19-9	
≤ 1000	
>1000	0.05
S100A4 protein expression	
≤10%	
>10%	0.043

Figure 1 - S100A4 gene expression levels based on gender, staging, tumor grading and tumor size

Table 4 - Results of the multivariate Kaplan Meier analysis

Parameter	Sig.	Exp(B)	95% CI min.	max.
LNR	0.42			
LNR (cut-off 0)	0.86	0.90	0.28	2.86
LNR (cut-off 0.16)	0.20	1.78	0.73	4.32
Resection type (R0/R1)	0.007	33.00	2.58	421.21
Metastasis (M0/M1)	0.70	1.38	0.25	7.65
CA19-9 (cut-off 1000)	0.11	2.83	0.78	10.19
Protein expression S100A4	0.05	3.56	0.96	13.18

Detection of S100A4 protein expression in PDAC tissues by immunohistochemistry

Immunohistochemistry for S100A4 showed a cytoplasmic staining of the cells (*fig. 1*).

Analysis of the correlation between S100A4 immunostaining and survival showed a significant difference between patients with high S100A4 protein expression (>10%) compared with low S100A4 protein expression (≤10%) [Log. Rank (Mantel-Cox) p = 0.043] (*fig. 2*). The median overall survival (OS) for patients with low expression (≤10%) was 25 ± 12.55 months, which was significantly longer than in patients with high expression (>10%) – 12 ± 1.89 months (p = 0.043, *fig. 3*).

ERBB2 gene expression in PDAC tissues

We next evaluated the ERBB2 gene expression in our study group. The statistical analysis with clinicopathological features is presented in *table 5*.

We found that the ERBB2 gene expression was significantly associated in III and IV disease stage (p=0.0048), and with tumor differentiation grade (p=0.0480) (*fig. 4*).

The highest ERBB2 gene expression was not significantly correlated with tumor dimension. However, an increased expression was observed in tumors larger than 4 cm.

Analysis of the correlation between ERBB2 gene expression and survival showed a difference (but not significant) between patients with high ERBB2 gene expression (>2-fold) compared with low ERBB2 gene expression (<2-fold), when we refer to normal adjacent tissue.

Kaplan-Meier analysis of OS distribution showed that the patients with the ERBB2 gene expression of n-fold>2 had a median OS of 21 months, which was higher than patients with ERBB2 n-fold<2, who had a median OS of 17 months (*fig. 5*).

The higher ERBB2 expression level the decreased survival period of time was observed, therefore an
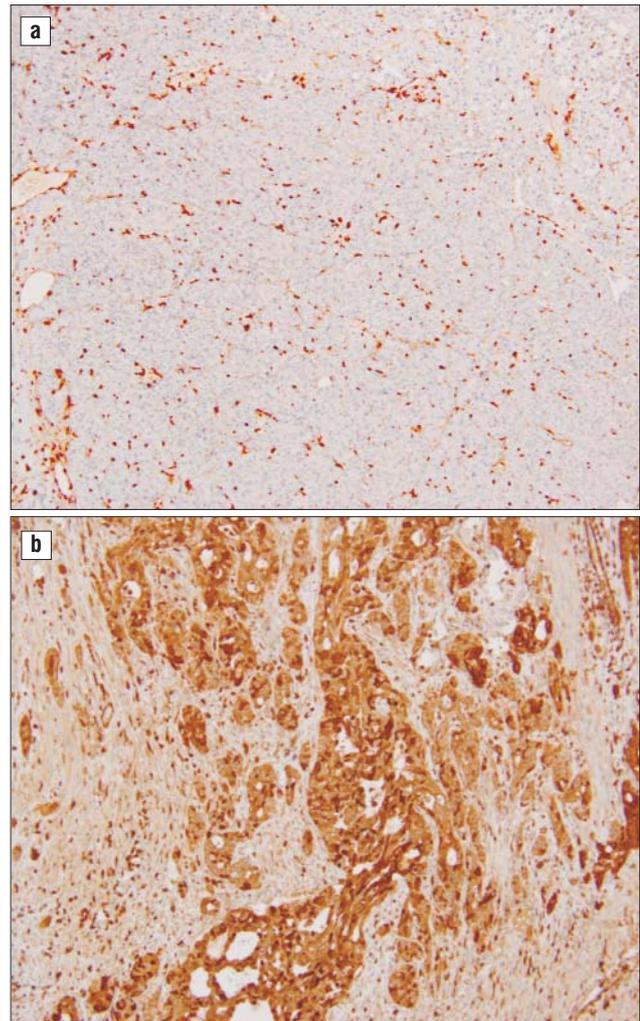
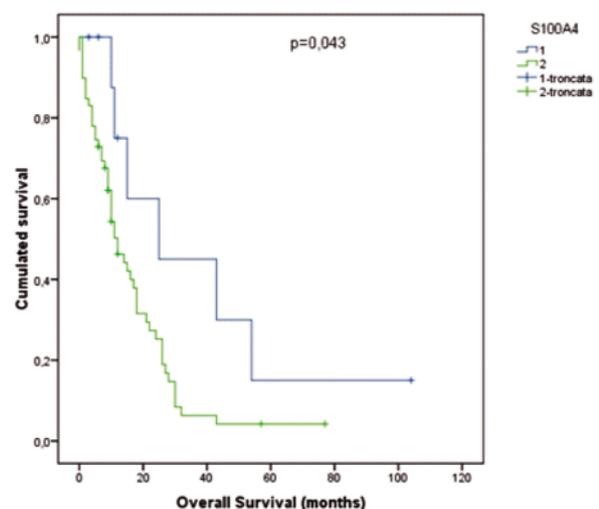**Figure 2 - Immunohistochemistry of S100A4 in (a) normal tissue and (b) tumoral tissue**

Figure 3 - Kaplan-Meier survival distributions for PDAC patients based on S100A4 staining; 1 - the group of patients with low S100A4 expression. 2 - the group of patients with high S100A4 expression

Table 5 - Gene expression of ERBB2 and association with PDAC patients' characteristics

Parametes	ERBB2 median (range)	p-value
Gender		
M	0.649 [-0.62-9.65]	0.8887
F	0.645 [-0.208-3.39]	
TNM stage		
IA. IB.	0.58 [0.01-4.49]	0.0048
IIA		
IIB	0.41 [-2.08-3.39]	
III.IV	0.71 [0.52-9.65]	
Tumor differentiation degree		
G1. G2	0.55 [-2.08-5.42]	0.0364
G3	1.7 [0.1-9.65]	
pN		
pN0	0.48 [-2.08-4.49]	0.62
pN1	0.59 [-0.62-5.42]	
LNR*		
<0.16	0.51 [-2.08-9.65]	0.61
≥ 0.16	0.67 [-0.63-5.42]	
Diabetes		
Yes	0.40 [0.014-2.52]	0.22
No	0.64 [-2.08-5.42]	

increased ERBB2 expression level can be correlated with poor prognostic.

We used Spearman correlation to evaluate a possible association between the factors (S100A4 and ERBB2). The analysis showed that these two tissue biomarkers are significantly inversely correlated in PDAC ($r2=-0.3325$ 95% CI: 0.5463, -0.07818, $p = 0.0094$) (*fig. 6*).

DISCUSSION

At the time of diagnosis, the vast majority of PDAC patients present with an advanced tumor stage. Because of the late diagnosis, the curative interventions are very limited, which leads to a poor prognosis and a dismal overall survival for these patients (less than 5% at 5 years). This study examined two candidate biomarkers – S100A4 and ERBB2 – for estimating more accurately the prognosis of surgically resected PDAC patients.
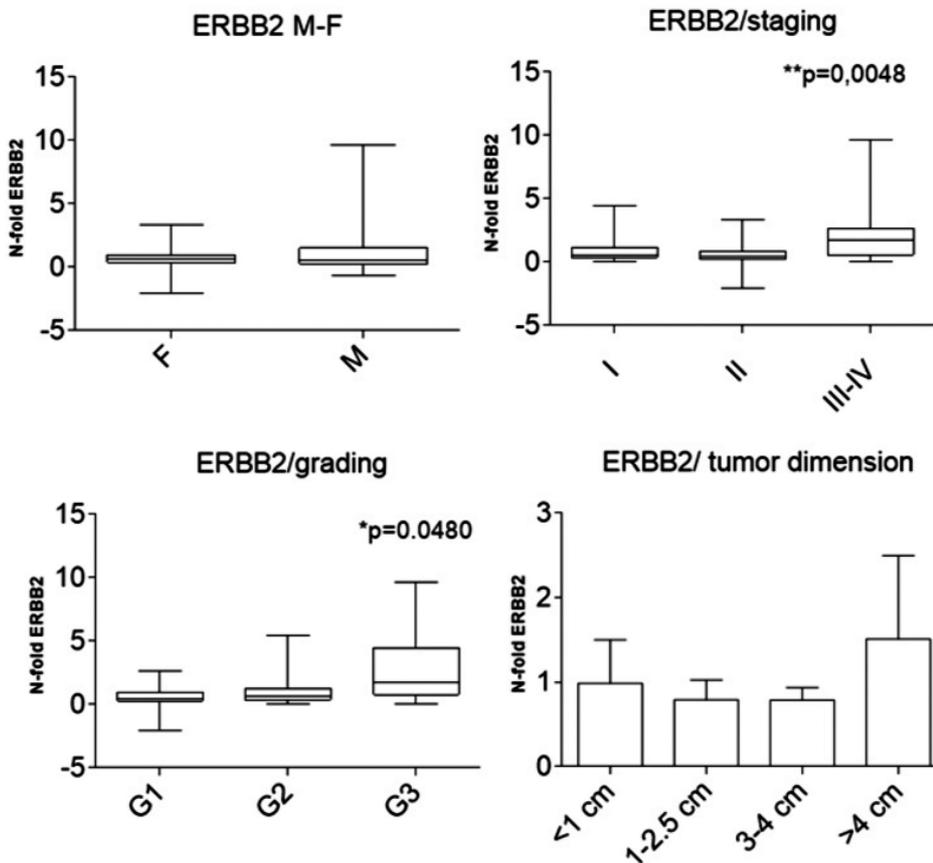

Figure 4 - The ERBB2 gene expression levels based on gender, tumor differentiation grade and tumor size for investigated patients
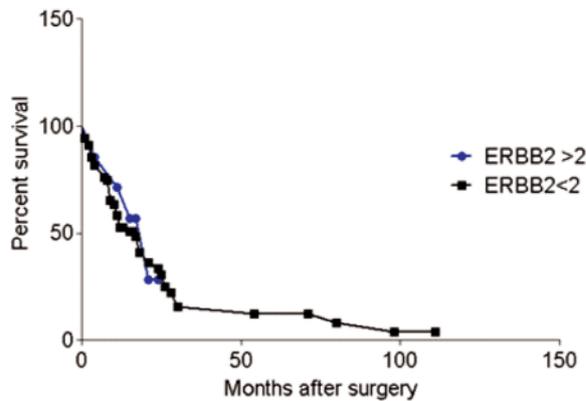
Figure 5 - Kaplan-Meier survival curve for patients based on ERBB2 gene expression

Figure 6 - Correlation between S100A4 and ERBB2 gene expression for the investigated patients

Our data indicate that S100A4 may be a valuable prognostic biomarker, consistent with its important roles in tumor progression. We observed an increased gene expression along with increased tumor stage, tumor differentiation degree, number of lymph nodes as well as with the lymph node ratio (LNR). Furthermore, high protein levels of S100A4 determined by immunohistochemistry correlated with poor OS in the PDAC patients. These results are in line with prior reports of S100A4 overexpression in PDAC and its correlation with tumor invasiveness and size, disease stage and poor prognosis (20,21).

It has been shown that S100A4 synergize with ERBB2 in the development of metastatic breast cancer (22), and ERBB2 act signaling via PI3k and ERK1/2 to activate S100A4 expression, by enhancing transcription. The expression of ERBB2 and S100A4 are concordant, also in primary human medulloblastoma. Therefore, suggesting that ERBB2 may promote the metastasis. On the other hand, the S100A4 promoter presents response element located between bp −1487 and −1099, indicating that ERBB2 may also, controls the S100A4 gene expression (23).

The mechanism that involves ERBB2 in metastasis remains to be elucidated. Its overexpression was already determined to be correlated with drug resistance and decrease patient survival time (24).

Moreover, in pancreatic cancer it is well known that a significant number of cases presents an increased level of expression of ERBB2/HER2 and this is linked with a poor prognosis and resistance to therapy (25,26).
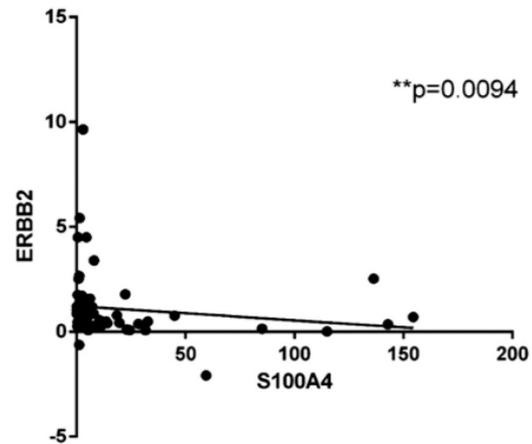
CONCLUSIONS

In our study both S100A4 and ERBB2 showed statistically significant correlation with clinicopathologic features of the studied patients` group.

Aberrant expression of S100A4 and ERBB2 at both gene and protein level stratifies PDAC patients in good and poor prognostic. Further studies in a bigger lot of patients can clarify the involvement of S100A4 in PDAC progression as well as the contribution of ERBB2 in activation of S100A4, which might have further clinical implications and the potential to improve patient selection.

Acknowledgements

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