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Original Article

Genomic alterations and protein expression of STAT4 in pancreatic cancer: a study of bioinformatics based on public data and immunohistochemistry validation with 241 tissue samples

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Abstract: Introduction: Signal transducers and activators of transcription (STATs) are members of conserved transcription factors family, and deregulated STAT family members are closely associated with a variety of tumorigenesis. However, the clinical value of STAT family members in the initiation and development of pancreatic cancer has not been clarified. Thus, the present study was aimed to investigate the genomic alterations of STAT family and further focus on STAT4 in pancreatic cancer tissues based on public data and clinical validation. Methods: The genomic alterations of STATs and patients' clinical data were downloaded and analyzed from The Cancer Genome Atlas (TCGA) via the website of CBioPortal. The compendia expression profiles of STATs were assessed with Gene Set Enrichment Analysis (GSEA). Immunohistochemistry was performed to evaluate the expression of STAT4 in 157 cases of pancreatic cancer, 66 cases of para-cancer pancreatic tissues and 18 cases of non-tumor pancreatic tissues. The relationship between STAT4 protein expression and clinicopathological characteristics was further investigated. Moreover, the expression of STAT4 mRNA was detected by microRNA microarray and displayed by using the database of the Gene Expression Omnibus (GEO). Results: TCGA data showed that patients with STATs alterations had significantly better disease free survival (DFS) and overall survival (OS). Immunohistochemically, the positive ratio of STAT4 in pancreatic cancer tissues was remarkably higher than that of non-cancerous pancreatic tissues ($P<0.001$). In addition, the expression of STAT4 was correlated with tumor size ($r=0.538$, $P<0.001$), TNM stage ($r=0.414$, $P<0.001$) and lymph node metastasis ($r=0.395$, $P<0.001$) with clinical cases. Conclusion: The alterations of STAT family members might play a vital role in the tumorigenesis and progression of pancreatic cancer. STAT4, as a family member of STATs, might have the potential to act as a biomarker to diagnose and predict the disease deterioration of pancreatic cancer.

Keywords: STATs, STAT4, pancreatic cancer, TCGA, immunohistochemistry, GEO

Introduction

Pancreatic cancer (PC) is a malignant tumor with the highest malignant degree and mortality rates [1]. The mechanism of pathogenesis and development of pancreatic cancer is complex and remains unclear [2, 3]. Signal transducers and activators of transcription (STATs) are members of conserved transcription factors family, and activate the transcription of various target genes by directly binding to DNA. Accordingly, deregulated STAT proteins are closely associated with a variety of tumorigenesis [4-6]. However, the clinical value of STAT family members in the initiation and development of pancreatic cancer has not been clarified. Thus, the present study was aimed to investigate the genomic alterations of STAT family and further focus on STAT4 in pancreatic cancer tissues based on public data and clini-

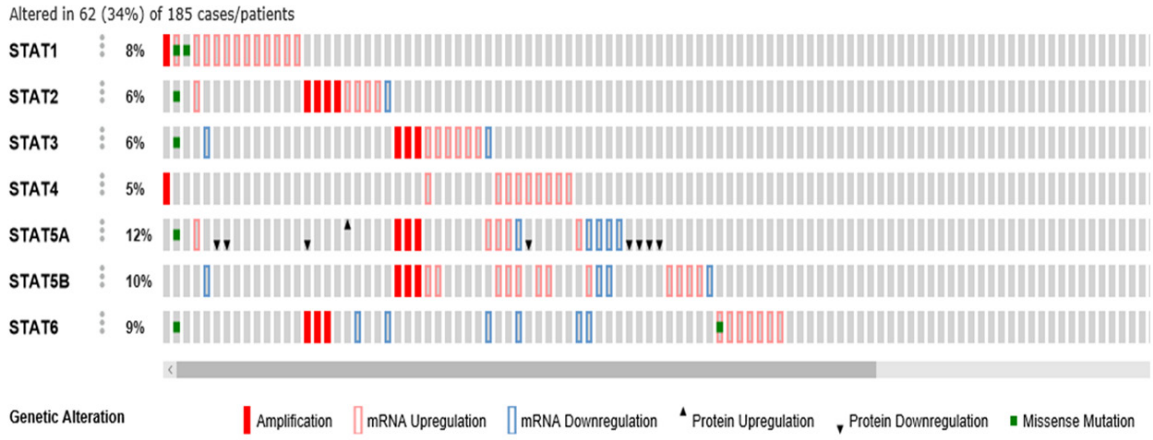

Figure 1. Genetic alterations of STAT family members in pancreatic adenocarcinoma (TCGA, provisional). Genetic alteraions, including amplification, mRNA upregulation, mRNA downregulaion, protein upregulation, protein downregulation and missense mutation, were exhibited in pancreatic adenocarcinoma for STAT family members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) by using OncoPrints from CBioPortal (www.cbioportal.org). Only part of the cases were shown representatively in the current figure.

cal validation on 241 cases of pancreatic tissues with immunohistochemistry.

Materials and methods

TCGA data analysis by using CBioPortal source

CBioPortal (www.cbioportal.org) [5, 6] was used to find out the percentage of genetic alterations of STAT family members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6). The relevant genetic alterations included amplification, mRNA upregulation, mRNA downregulation, protein upregulation, protein downregulation and missense mutation. The schematic of OncoPrints was generated for visualizing the alterations directly from CBioPortal in Pancreatic Adenocarcinoma (TCGA, provisional) containing 185 patients. The plots figures were also generated by CBioPortal based on several key clinicopathological parameters, including Neoplasm Histologic Grade, Neoplasm Disease Stage American Joint Committee on Cancer Code, Disease Free Survival (DFS) and Overall Survival (OS). In order to understand better the related gene network of STAT family, the gene network was produced by CBioPortal, as well as by Gene Set Enrichment Analysis (GSEA) (http://software.broadinstitute.org/gsea/index.jsp) [7-9].

Samples collection

In this study, 241 cases of tissue samples were collected including the following three groups: 1) 157 pancreatic cancer tissue samples which included 153 cases of adenocarcinomas, two cases of anaplastic carcinomas and two cases of adenosquamous carcinoma; 2) 66 para-tumor pancreatic tissues; 3) 18 non-tumor normal pancreatic tissues. Among all the tissue samples, 63 samples were tissue microarray products from Fanpu Biotech, Inc (PAC481 and PAC961, Guilin, China), including 53 cases of adenocarcinomas and one case of adenosquamous carcinoma; as well as nine non-tumor pancreatic tissues. Another cohort (n=22) was collected in the Affiliated Hospital of Guangxi University of Science and Technology between January, 2010 and November, 2014, including 12 cases of pancreatic adenocarcinomas, as well as seven para-tumor pancreatic tissues and three non-tumor pancreatic tissues. The rest samples (n=156) were gathered in the First Affiliated Hospital of Guangxi Medical University between January, 2010 and February, 2015, including 88 cases of adenocarcinomas, two cases of anaplastic carcinomas and one case of adenosquamous carcinoma; as well as 59 para-tumor pancreatic tissues and six non-tumor pancreatic tissues. All the formalin-fixed and paraffin embedded samples were routinely prepared. The diagnosis of pancreatic cancer was confirmed by three experienced pathologists (Dan-Ming Wei, Lin Shi and Gang Chen). The study was approved by the Research Ethics Committees of the Affiliated Hospital of Guangxi University of Science and Technology and the First Affiliated

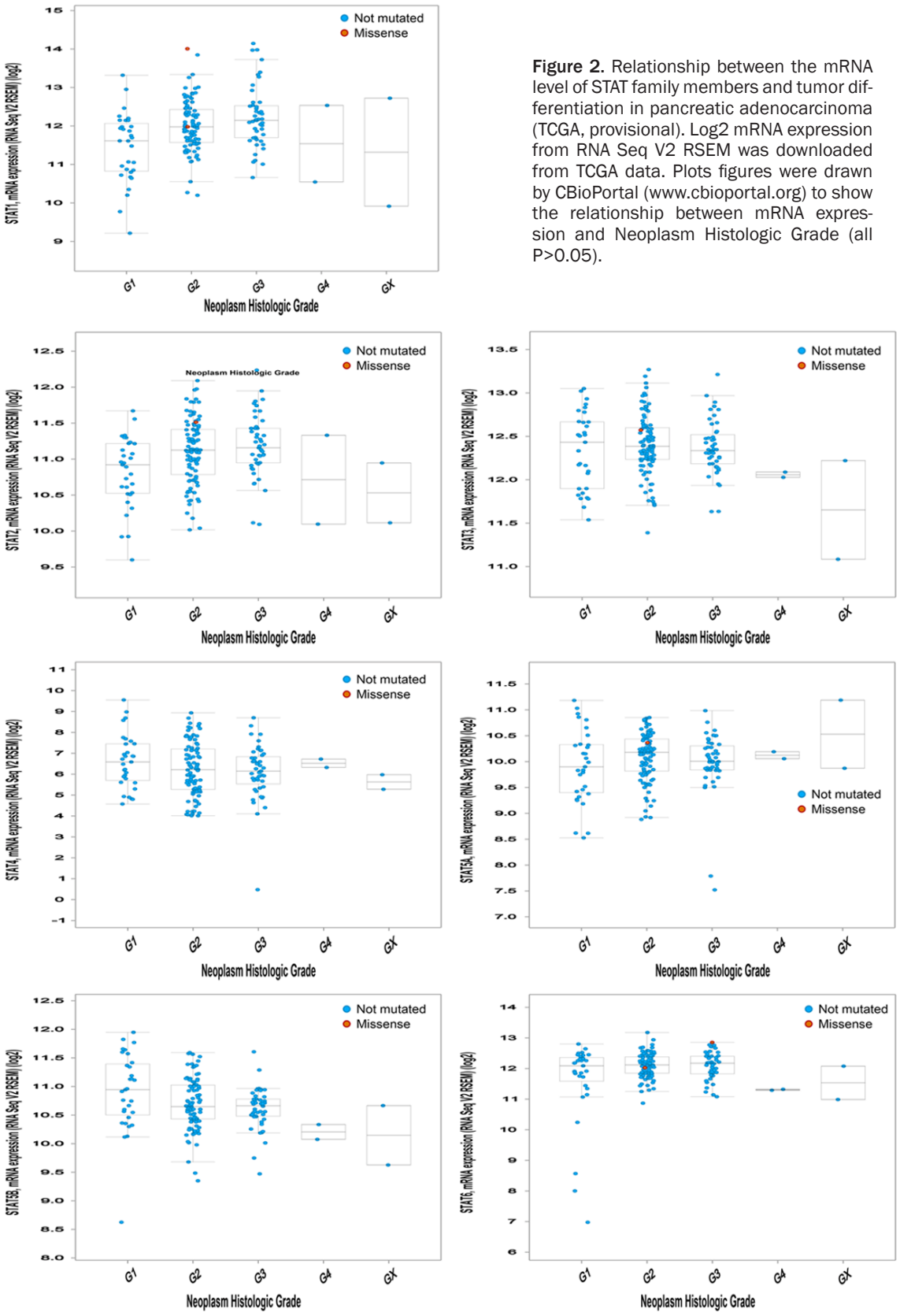

Figure 2. Relationship between the mRNA level of STAT family members and tumor differentiation in pancreatic adenocarcinoma (TCGA, provisional). Log2 mRNA expression from RNA Seq V2 RSEM was downloaded from TCGA data. Plots figures were drawn by CBioPortal (www.cbioportal.org) to show the relationship between mRNA expression and Neoplasm Histologic Grade (all $P>0.05$).

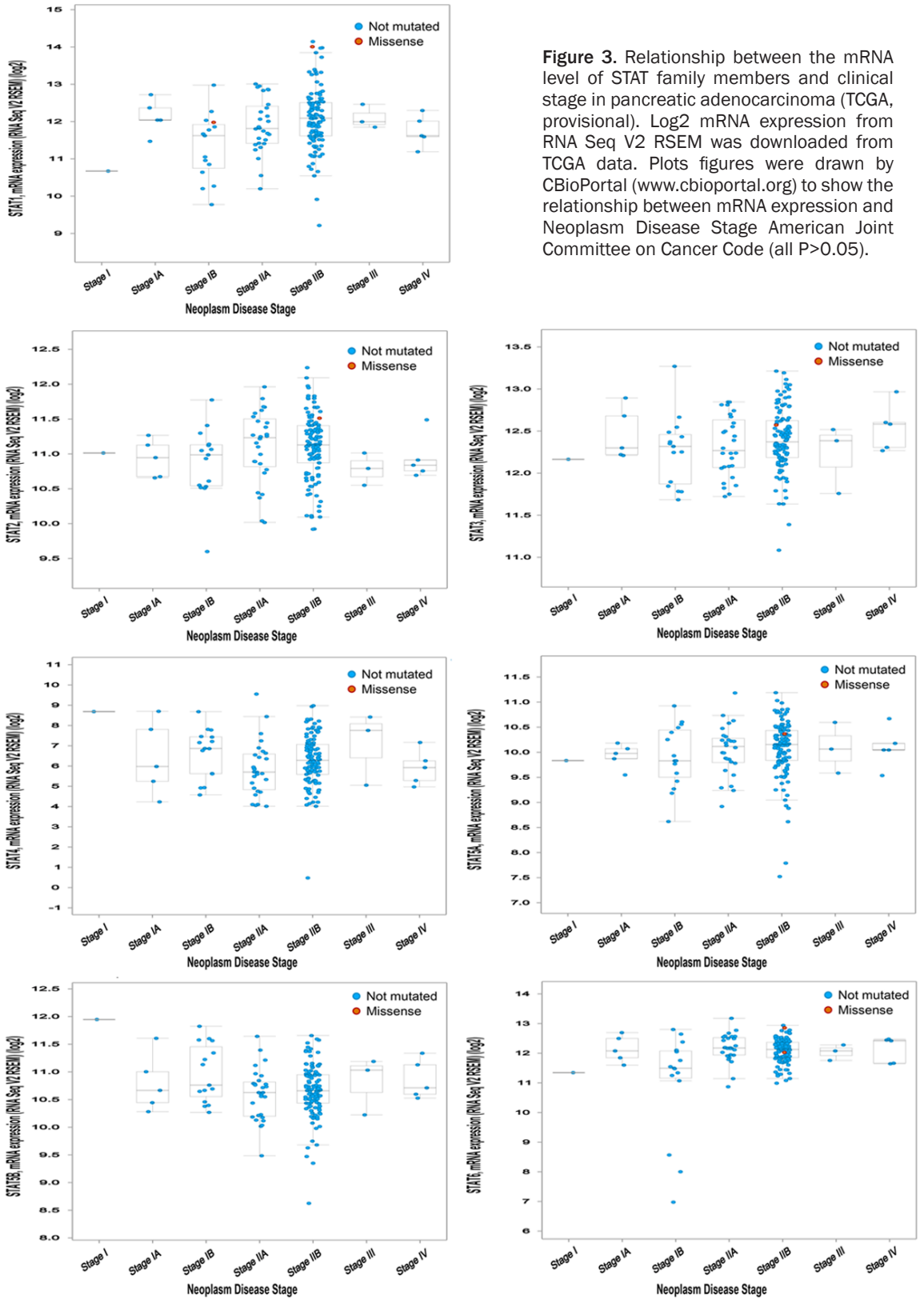

Figure 3. Relationship between the mRNA level of STAT family members and clinical stage in pancreatic adenocarcinoma (TCGA, provisional). Log2 mRNA expression from RNA Seq V2 RSEM was downloaded from TCGA data. Plots figures were drawn by CBioPortal (www.cbioportal.org) to show the relationship between mRNA expression and Neoplasm Disease Stage American Joint Committee on Cancer Code (all P>0.05).

Hospital of Guangxi Medical University, China. Informed written consents were obtained from all patients who participated in the current study.

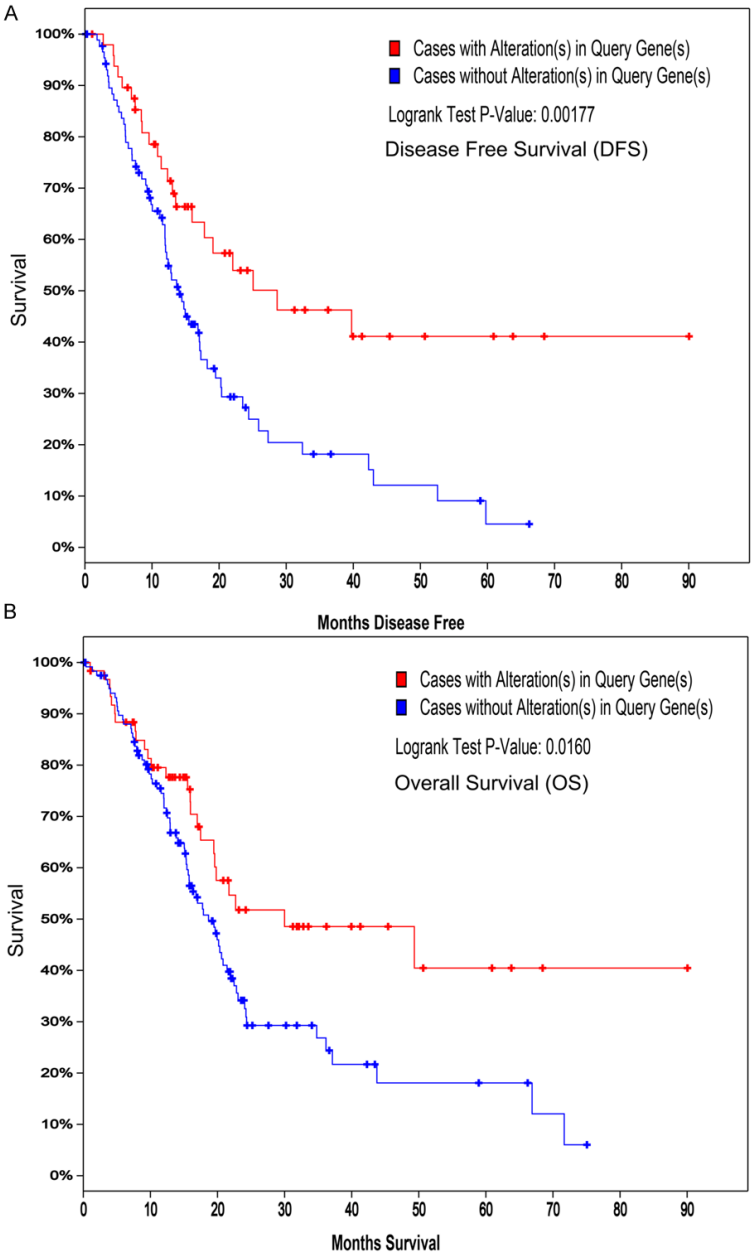

Figure 4. Relationship between the alterations of STAT family members and survival in pancreatic adenocarcinoma (TCGA, provisional). A. Disease free survival (DFS): 22 cases relapsed of 50 cases with alterations and the median time of disease free was 28.65 months, while 62 cases relapsed among 91 cases without alterations, whose median disease free time was 14.03 months; B. Overall survival (OS): 25 cases were deseased in 62 cases with alterations and the median survival time was 29.99 months. 74 cases were deceased in 122 cases without alterations, whose median survival time was 18.66 months. The survival was analyzed by Kaplan-Meier Estimate provided by CBioPortal (www.cbioportal.org).

Immunohistochemistry

Tissue specimens were sliced with a thickness of four μm. Slides were deparaffinized, rehydrated, and subjected to antigen repair by high pressure. The slides were incubated by 3% H_2O_2 for 10 minutes after being rinsed by PBS solution. After that, primary STAT4 antibody (mouse monoclonal antihuman STAT4 antibody, PL-68, CA, USA, 1:300 dilution) was added to the slides at 4°C for overnight. Afterwards, the secondary antibody (Beijing Jinqiao Biological co. LTD) was applied for 20 minutes. All procedures of immunohistochemistry were carefully performed according to the instructions of manufacturers.

Evaluation of immunohistochemical staining

All slides were assessed by three experienced pathologists (Dan-ming Wei, Lin Shi and Gang Chen), who had no advanced knowledge of the patients' identities or clinical status. For discrepant opinions, the above three pathologists reviewed the slides together to reach a consensus. We counted the numbers of positive staining cells showing immunoreactivity of STAT4 in ten representative microscopic fields, and calculated the percentage of positive cells. The staining intensity (SI) was graded into four categories: 0 (no staining), 1-3 (faintly, moderately, strongly). The approximate percentage of positive (PP) cells was meanwhile subdivided into four categories: 0 (none), 1 (<10%), 2 (10-50%), 3 (51-80%) and 4 (>80%). We multiplied SI and PP to achieve a final score for each sample, and the final diagnosis of immunohistochemistry was regarded as follows: 0-2 (negative), ≥3 (positive) [10-14].

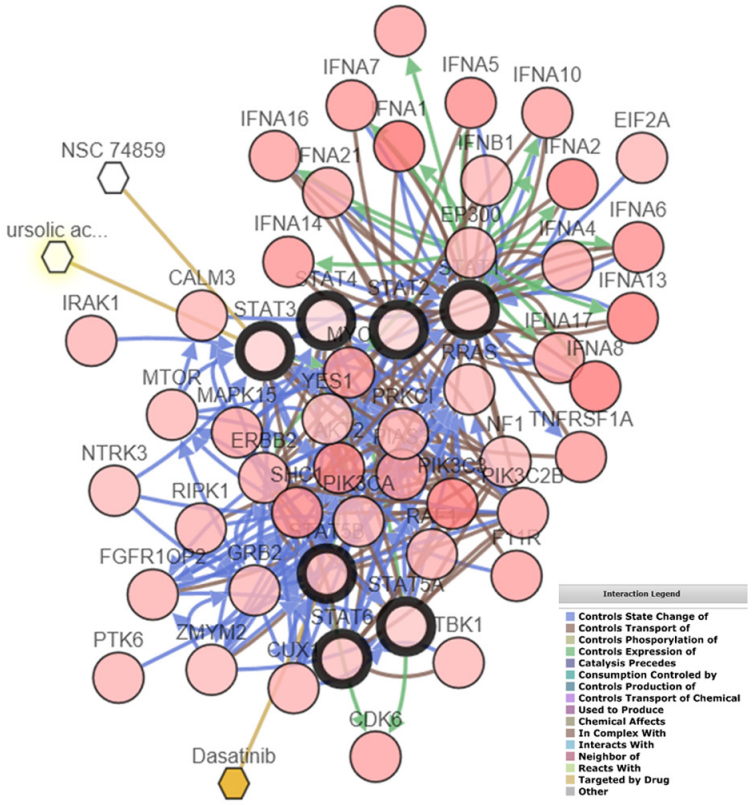

Figure 5. Drugs of specified gene network related to STAT family members in pancreatic adenocarcinoma (TCGA, provisional). The gene network in pancreatic adenocarcinoma for STAT family members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) was drawn by using CBioPortal (www.cbioportal.org). Circles indicated genes and sexangles presented drugs. Dasatnib is a FDA approved drug targeting STAT6, and STAT3 is targeted by ursolic acid and NSC 74859, which have not yet been approved by FDA.

STAT4 mRNA level based on gene expression omnibus (GEO) profiles

In order to confirm the clinical significance of STAT4 expression in pancreatic cancer with other approaches, we further delved into the public gene microarray datasets. Gene expression profiling studies were identified in the database of GEO (http://www.ncbi.nlm.nih.gov/geo/) [15-17]. Pancreatic and STAT4 were used as keywords for searching. Studies were included in the analysis if they met the following criteria: 1) Studies in agreement with the diagnosis standard of pancreatic cancer. 2) Studies accessing STAT4 mRNA level. Exclusion criteria were set as follows: 1) The data were repeated. 2) Non-human studies. The random-effects model was selected to calculate the pooled standard mean difference (SMD) [18-21]. And the funnel plot was applied to evaluate potential publication bias.

Statistical analysis

Statistical analysis was conducted by using the SPSS v22.0 software (SPSS Inc.). The associations between STAT4 expression and various clinic pathological characteristics were evaluated by using χ^2 test and Spearman correlation. Receiver operator characteristic curve (ROC) was used for analyzing diagnostic value. Survival curves were estimated with the Kaplan-Meier method. For all the tests, differences with $P<0.05$ were used to establish statistically significance.

Results

TCGA data analysis of STAT family members by using CBioPortal

OncoPrint data in the CBioPortal representing pancreatic adenocarcinoma (TCGA, provisional) showed 34% genetic alterations in a set of seven genes comprised of STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 in STAT family (**Figure 1**). We investigated the clinical value of STAT family members for several key clinicopathological parameters of pancreatic adenocarcinoma. No significant correlation was observed between the mRNA expression of STAT family members and histologic grade (**Figure 2**) or clinical stage (**Figure 3**). However, the cases with alterations showed remarkably better survival than those without alterations both for disease free survival (DFS, **Figure 4A**) and overall survival (OS, **Figure 4B**). To further explore the gene network and compendia expression profiles of STAT family members in pancreatic adenocarcinoma, CBioPortal (**Figure 5**) and Gene Set Enrichment Analysis (GSEA, **Figures 6**, **7**) were used to draw the schematics. The network also showed the molecular targeting drugs of STATs, for instance, Dasatnib, a FDA approved drug targeting STAT6, and ursolic acid and NSC 74859 targeting STAT3.

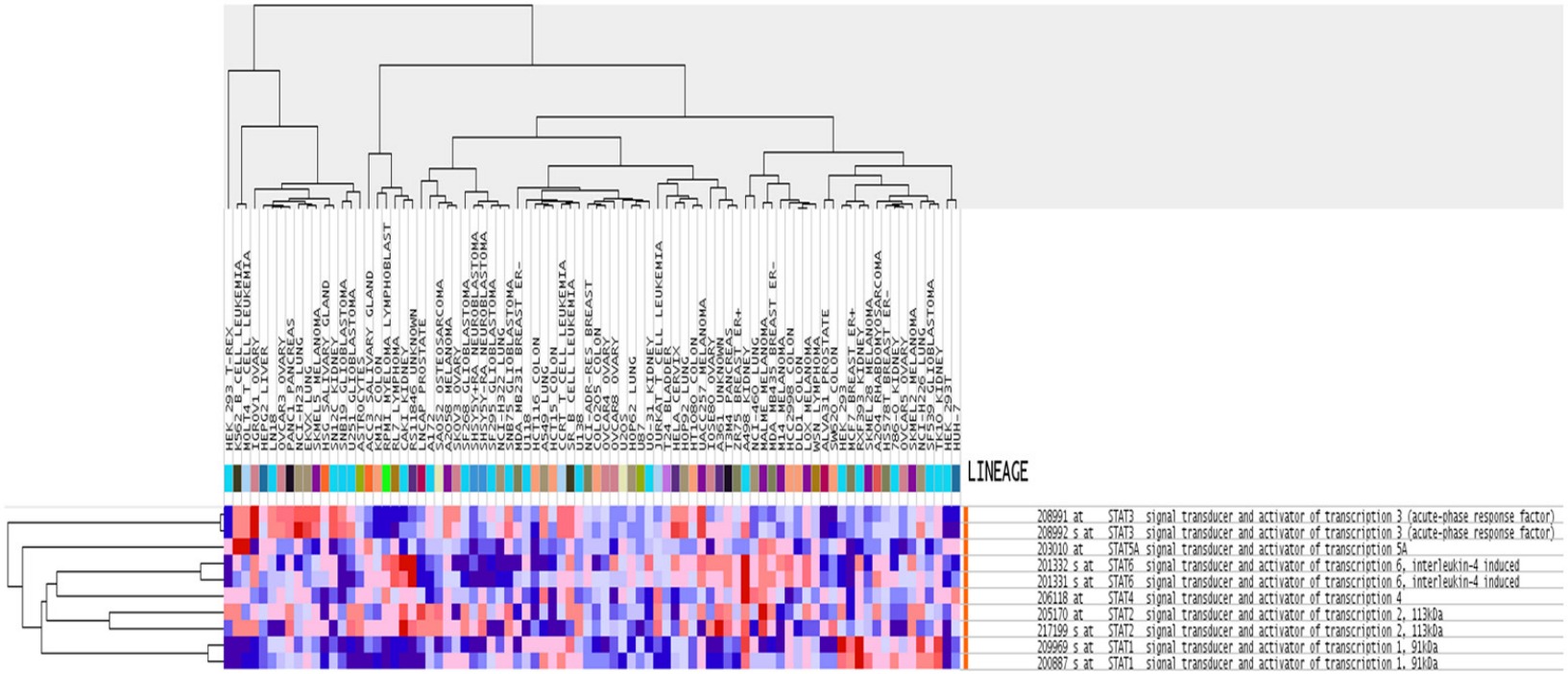

Figure 6. Compendia expression profiles of STAT family members of human tissue compendium. The compendia expression profiles of STAT family members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) were sent to Gene Set Enrichment Analysis (GSEA) for Human Tissue Compendium (http://software.broadinstitute.org/gsea/index.jsp).

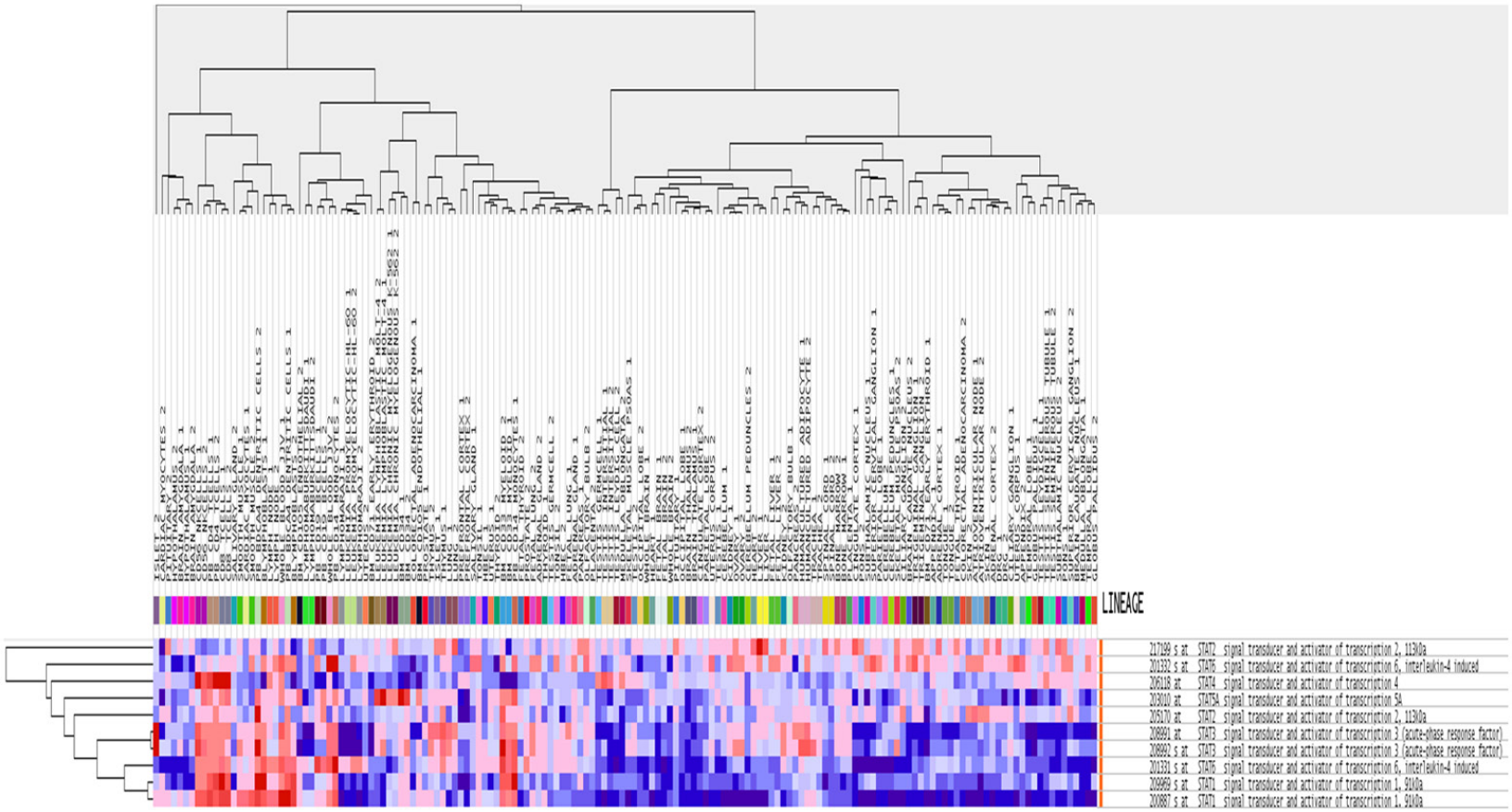

Figure 7. Compendia expression profiles of STAT family members of NCI-60 cell lines. The compendia expression profiles of STAT family members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) were sent to Gene Set Enrichment Analysis (GSEA) for NCI-60 Cell Lines (National Cancer Institute) (http://software.broadinstitute.org/gsea/index.jsp).

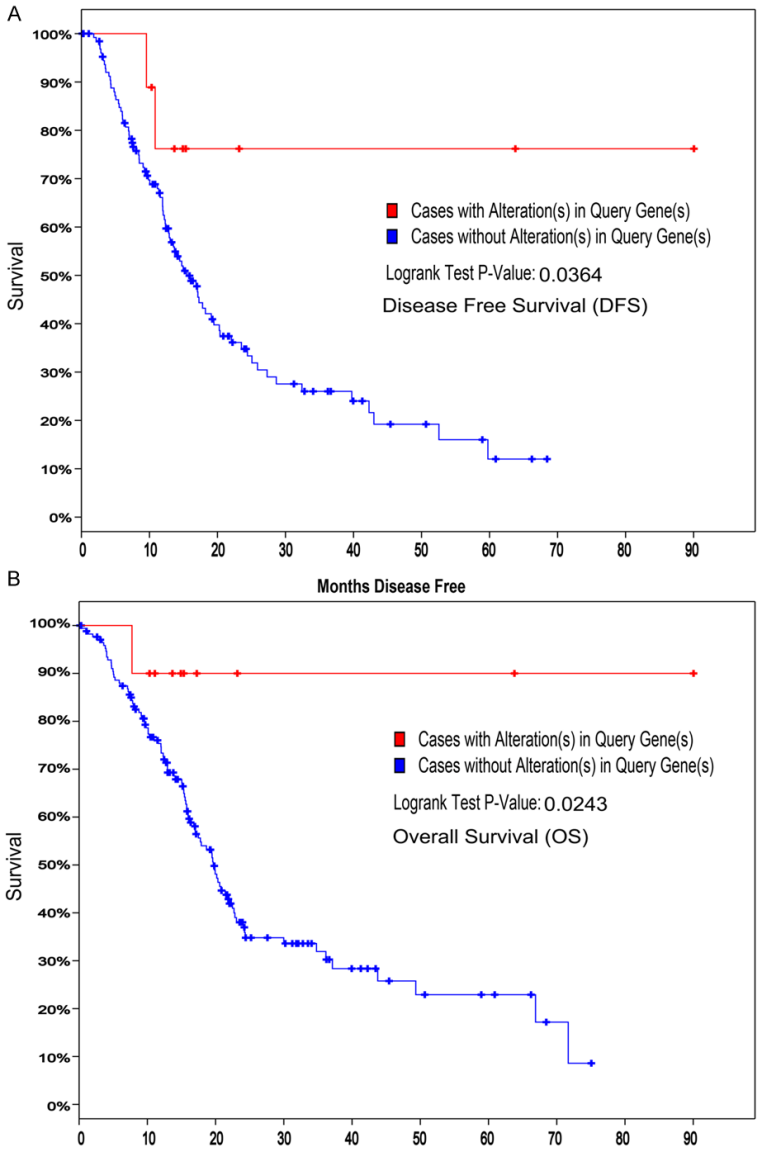

Figure 8. Relationship between the alterations of STAT4 and survival in pancreatic adenocarcinoma (TCGA, provisional). A. Disease free survival (DFS): two cases relapsed of 9 cases with alterations, while 82 cases relapsed among 132 cases without alterations, whose median disease free time was 15.51 months; B. Overall survival (OS): one case was deseased in 10 cases with alterations. 98 cases were deceased in 174 cases without alterations, whose median survival time was 19.65 months. The survival was analyzed by Kaplan-Meier Estimate provided by CBioPortal (www.cbioportal.org).

Alteration of STAT4 and survival in pancreatic adenocarcinoma (TCGA, provisional)

Previously, we have found that STAT4 was downregulated in hepatocellular carcinoma, but upregulated in breast cancer, nasopharyngeal carcinoma, cervical cancer and glioma, which indicated that the role of STAT4 might be tumor dependent [10-14]. Since the clinical role of STAT4 in pancreatic cancer remains unknown, we were curious about its clinical value. As shown in **Figures 2** and **3**, STAT4 mRNA level was not significantly related to the tumor cell differentiation or disease progression. However, the genomic alterations of STAT4 gained close relationship with patient survival, e.g., cases with alterations had significant longer DFS and OS time than those without alterations (P=0.0364, P=0.0243, **Figure 8**), respectively. Since no information of STAT4 protein expression could be achieved from TCGA data, we then detected the STAT4 protein expression with immunohistochemistry with clinical samples in house.

Expression of STAT4 protein in different pancreatic tissues with immunohistochemistry

Immunohistochemically, 97 of 157 PC tissues (62%) stained positive for STAT4 protein, which was significantly higher than that of para-cancer tissue (27%, 18/66, P<0.001) and non-tumor normal pancreatic tissue (0%, 0/18, P< 0.001). There was also a statistically significant difference of the positive rate between para-cancer tissue and non-tumor tissue (P<0.001, **Figure 9**). In addition, ROC curve indicated that STAT4 might have a moderate diagnostic value in PC. The calculated area under curve (AUC) of STAT4 was 0.783 (95% CI 0.650-0.916, P<0.001). Diagnostic sensitivity and specificity were 89.9% and 66.67%, respectively (data not shown).

Concerning tumor stages, in advanced-stage (III/IV), STAT4 positive expression (85.1%, 57/67) was significantly higher compared to that in early stage (I/II, 44.4%, 40/90, P<0.001).

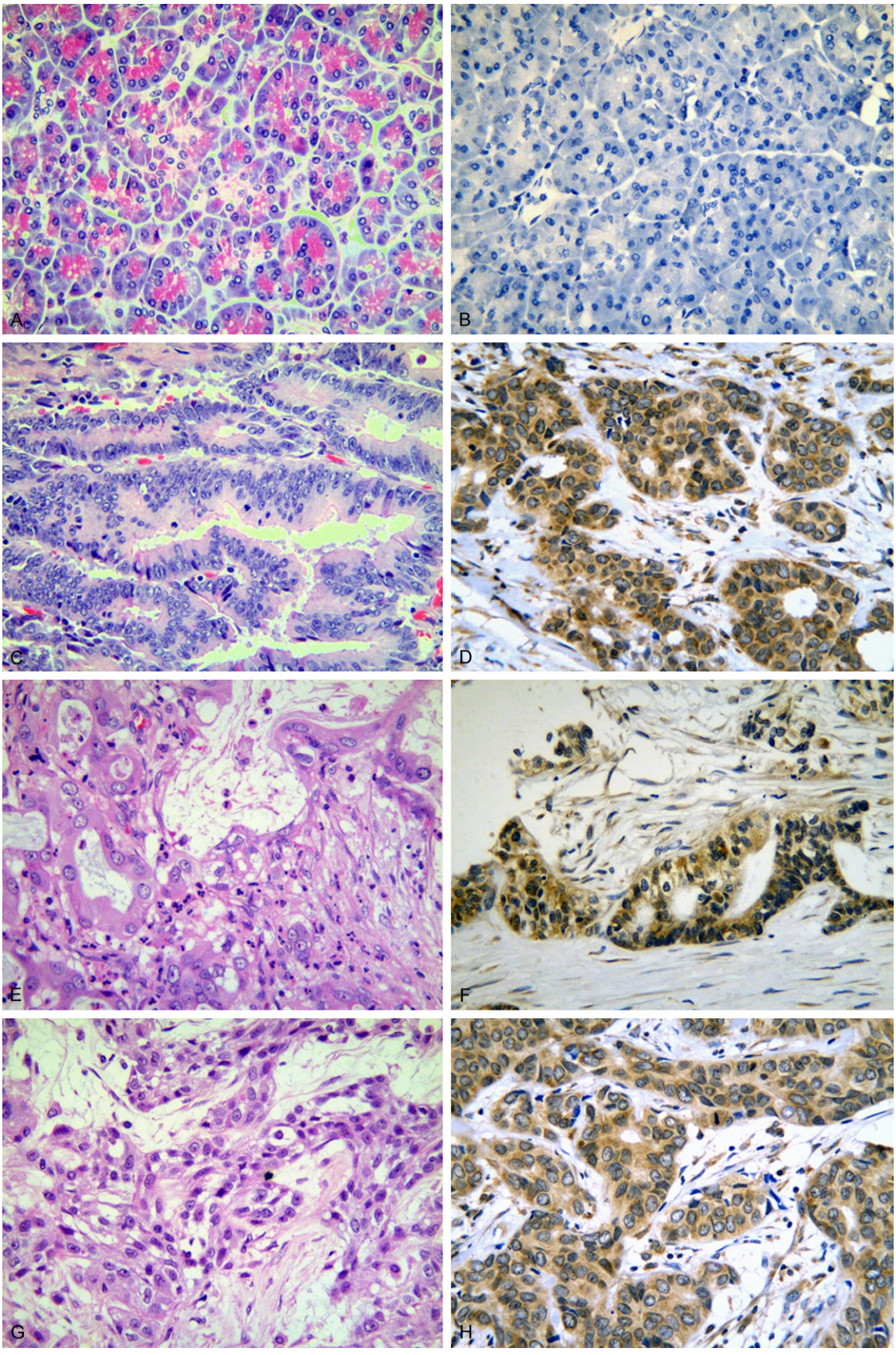
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Figure 9. STAT4 protein expression in various pancreatic tissues detected by immunohistochemistry. The STAT4 protein signaling was located in the cytoplasm of pancreatic cancer cells or pancreatic cells. Photos in the left column (A, C, E and G) showed HE-stained sections and right ones (B, D, F, H) showed immunohistochemically stained sections. (A and B) Non-tumor normal pancreatic tissues. (C and D) Pancreatic cancer of stage I. (E and F) Pancreatic cancer of stage II. (G and H) Pancreatic cancer of stage III. (400×).

Table 1. Relationship between the STAT4 expression and several clinical pathological parameters

Expression of STAT4 in Pancreatic Cancer				
Parameters		Negative cases/ total number (%)	Positive cases/ total number (%)	*P*-value
TNM stage	I and II	50/90 (55.6)	40/90 (44.4)	<0.001
	III and IV	10/67 (14.9)	57/67 (85.1)	
Lymph node metastasis	Absent	50/92 (54.3)	42/92 (45.7)	<0.001
	Present	10/65 (15.4)	55/65 (84.6)	
Distant metastasis	Absent	59/147 (40.1)	88/147 (59.9)	0.090
	Present	1/10 (10.0)	9/10 (90.0)	

Abbreviations: activator of transcription-4 (STAT4); TNM, tumor-node-metastasis. Calculated with χ^2 test.

As for lymph node metastasis, STAT4 positive expression was observed to be markedly higher in the group with lymph node metastasis (84.6%, 55/65) than that without lymph node metastasis (45.7%, 42/92, P<0.001). Further, Spearman's test revealed that the higher protein level of STAT4 in PC was consistently correlated with TNM stage (r=0.414, P<0.001) and lymph node metastasis (r=0.395, P<0.001, **Table 1**). A similar trend could also be observed between the STAT4 protein expression and the status of distant metastasis, however, the *P* value did not reach to be statistically significant (P=0.09 with χ^2 test). Either, the high expression of STAT4 was not associated with other parameters, including sex, age, tumor size, tumor position, histology, neural invasion, level of CA199 or CEA, overall survival, etc. (Data not shown).

Clinical value of STAT4 mRNA based on GEO data

In total, STAT4 mRNA level could be extracted from two eligible datasets including GDS3836 and GDS4103. Altogether, 46 cases of pancreatic cancer patients and 48 cases of healthy people were enrolled in the current analysis (**Table 2**). The random-effects model was selected to calculate the pooled SMD and 95% CI according to the heterogeneity test (P<0.001, I^2=96%). The result indicated that the difference was not significant (95% CI, -2.6 to 7.72; P=0.33) between pancreatic cancer and normal controls. The funnel plot was not applied to evaluate potential publication bias because of small size of included microarray data.

Discussion

Pancreatic cancer has been recognized as one of the most devastating cancers and is the fourth leading cause of cancer death in the United States [1]. But the pathogenesis of pancreatic cancer remains unclear. STAT family, which consist of STAT1~STAT6, is a group of protein binding DNA that can regulate cell proliferation differentiation and apoptosis. STATs have several domains with the same structure. They are involved in gene transcriptional regulation by the activation of numerous extracellular peptide molecules [4]. The clinical value of STAT family members has not been clarified in pancreatic cancer. Herein, we summarized all the genomic alterations of STAT family members via TCGA data and CBioPortal website. Several genomic alterations were observed, including amplification, mRNA upregulation and downregulation, as well as protein upregulation and downregulation. Interestingly, cases with genomic alterations showed significantly better DFS and OS than those without genomic alterations. But, when we picked up the mRNA level among all the alterations to investigate its clinical role separately, we failed to achieve a significant correlation between the mRNA level of STATs and the clinicopathological parameters, including tumor histological grades and disease stages. The correlation between STATs alteration and disease progression needs further verification with larger patient size.

STAT4 is a member of STATs family tissue and mainly locates in lymphoid and myeloid tissue. Since we have previously found that STAT4 may play different roles according to the cancer type [10-14], we performed immunohistochemistry

Table 2. Characteristics of the selected GEO datasets

GEO ID	Authors	Tissue	Sample size (Normal control: Pancreatic cancer)	Platform	Sample source
GSE15471	Badea L, et al. (2009)	Normal pancreatic tissues, PDAC*	78 (39, 39)	GPL570: [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	In vivo
GSE19650	Hiraoka N, et al. (2010)	Normal and neoplastic epithelial cells (normal main pancreatic duct, IPMC, and invasive carcinoma originating in IPMN)	22 (7, 9)	GPL570: [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	In vivo

PDAC: Pancreatic ductal adenocarcinoma; IPMC: intraductal papillary-mucinous carcinoma of the pancreas; IPMN: Intraductal papillary-mucinous neoplasm of the pancreas. *Only pancreatic cancer was involved in the current analysis.

to investigate the clinical value of STAT4 protein level with clinical FFPE samples of pancreatic tissues including pancreatic cancers and non-cancerous controls.

Besides the previous work of our group [10-14], STAT4 has also been well studied in other malignancies, such as colorectal cancer [22] and gastric cancer [23]. There were only several studies available, which attempted to explore the role of STAT4 in pancreatic cancer. Cotterchio M et al [24] investigated the association between variants in atopy-related candidate genes and pancreatic cancer risk with a population-based case-control study of pancreas cancer cases diagnosed during 2011-2012 (via Ontario Cancer Registry). They found 18 SNPs in 14 candidate genes, including STAT4, were significantly associated with pancreas cancer risk [24]. However, they did not study the clinical role of STAT4 expression, either mRNA or protein, in pancreatic cancer. To our knowledge, no publication was available concerning the clinicopathological value of STAT4 mRNA or protein level in pancreatic cancer. Then our current study appealed to the public data of microarray from GEO and RNA-seq from TCGA. However, neither of the two datasets from GEO (GSE15471 or GSE19650) showed no significant difference of STAT4 mRNA expression between pancreatic ductal adenocarcinoma and normal pancreatic tissues. Data from TCGA revealed the similar results that STAT4 mRNA was not related to the disease deterioration. Surprisingly, the genomic alterations of STAT4 could predict better survival of pancreatic cancer, which provides a direction for the clinical application of detection of STAT4 genomic alteration. However, this finding needs to be confirmed with larger size of samples.

Further, we continued to investigate the expression of STAT4 protein using immunohistochemistry with 241 cases of pancreatic tissue, and aimed to find out the possible role of STAT4 in pancreatic cancer. The results showed that STAT4 protein expression of pancreatic tissue was significantly higher than para-cancer and normal tissue, which indicated that STAT4 may be involved in the tumorigenesis of pancreatic cancer. And STAT4 thus has the potential to be a biomarker for the diagnosis of pancreatic cancer with the AUC of 0.783. More importantly, STAT4 protein expression was found to be closely related to the disease progression, as the patients of advanced stage or with metastasis had markedly higher level of STAT4. This finding suggests that STAT4 may play similar function in pancreatic cancer, as in CRC [22], since STAT4 protein was expressively upregulated in CRC tissues than that in adjacent non-tumorous tissues, and STAT4 protein was associated with the Duke's staging and depth of invasion in CRC patients. Knock-down of STAT4 gene could also block cell proliferation and invasion of CRC cells *in vitro*. Thus, STAT4 protein level in pancreatic cancer may be related to clinical stage and metastasis, which reflect tumor biology behavior.

Most recently, Cattaneo F and colleagues [25] reported that STAT4 could act as a novel transcriptional regulator of p66Shc in B cells. With chromatin immunoprecipitation and reporter gene assays, they found that STAT4 was able to bind to and activate the p66shc promoter. Knock-out or overexpression of STAT4 resulted in a co-modulation of p66Shc. IL-12-dependent STAT4 activation caused a coordinate increase in STAT4 and p66Shc expression, which correlated with enhanced B cell apoptosis. Furthermore, treatment with the STAT4 inhibitor lisofylline reverted partly this effect, suggesting that STAT4 phosphorylation is not essential for but enhances p66shc transcription. Additionally, reconstitution experiments

confirmed that chronic lymphocytic leukemia (CLL) B lymphocytes had a STAT4 expression defect which partly accounted for their p66Shc deficiency. Finally, p66Shc could participate in a positive feedback loop to promote STAT4 expression. These results provided new insights into the mechanism of p66Shc expression in B cells and its defect in CLL, identifying the STAT4/IL-12 pathway as a potential therapeutic target in CLL, which also provides a line of thinking for the research of pancreatic cancer.

In conclusion, the alterations of STAT family members might play a vital role in the tumorigenesis and progression of pancreatic cancer. STAT4, as a family member of STATs, might have the potential to act as a biomarker to diagnose and predict the disease deterioration of pancreatic cancer. However, the underlying biology mechanism needs further *in vitro* and *in vivo* verification.

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Disclosure of conflict of interest

None.

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