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Pancreatic Ductal Adenocarcinoma: Implications of Epigenetic Role Related the Src Pathway

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Abstract

Epigenetic has become, in recent years, in a very important process in the control of several pathologies, including cancer. DNA methylation has been described in numerous studies as crucial for differentiation control, cell proliferation and invasion in cancer.

Pancreas ductal adenocarcinoma is one of the most deadly diseases of our society; its complexity and variability have become one of the medical challenges of this decade. Combining the potential of epigenetic with the high mortality and the limited knowledge of pancreatic ductal adenocarcinoma origin, may result in new approaches for diagnosis, treatment and monitoring, crucial in this pathology.

For this purpose, we analyzed genes involved in different cellular processes (**GSTP1, p16, RASFF1A, RARβ2, CyclinD2, HIN-1, SOCS1, TIMP3, DAPK and TWIST1**) in 61 cases of pancreatic ductal adenocarcinoma. A first approach led us to analyze the *Src* pathway, never studied in this sense, as crucial in the development of this pathology. Clusters for clinicopathological characteristics and epigenetic profiles were obtained.

The high degree of methylation of these adenocarcinomas, and the statistical relationship between Src pathway methylation and clinicopathological profile, has been postulated, for the first time. These results show the importance of this pathway in pancreatic disease, opening a new challenge for research and therapy.

Keywords: Epigenetic; *Src* pathway; Tumorogenesis; Pancreatic cancer

Introduction

Cancer is a complex disease characterized by multiple genetic and epigenetic genomic alterations [1,2]. DNA methylation is one of the most important epigenetic modifications, and plays a critical functional role in development, differentiation and progression [2]. Accumulating evidence demonstrates that cancer is associated with aberrant DNA methylation [2]. Promoter regions are usually enriched with CpG dinucleotides, known as CpG islands; hypermethylation of these islands correlates with transcriptional silencing of tumor suppressor genes.

DNA methylation of CpG islands located in the promoter regions of tumor-related genes, such as *p14, p15, p16, p73, APC, hMLH1, BRCA1, MGMT, GSTP1, CDH1, TIMP3 and DAPK-1*, is associated with silencing of such genes [1]. Conversely, increased expressions of oncogenes were associated with hypomethylation. Furthermore, tumorogenesis of several cancers types was also marked by specific methylation changes in their genomes. Therefore, it is useful to build a methylation profile to discover candidate genes, and to predict therapeutic outcomes [3], and patient survival in cancer.

Pancreatic carcinoma is the fourth most common cause of cancer-related death in the United States, accounting for approximately 30,000 deaths annually, and death rates for it closely mirroring incidence rates [4]. Aggressive invasion and early metastasis are characteristic, such that 90% of patients have surgically unresectable disease at the time of diagnosis. Current chemotherapy regimens provide only palliation benefit.

Lack of early diagnostic biomarkers and ineffectiveness therapies for this cancer are among the major factors that contribute to its low survival rate [5]. The mechanism for the regulation of genes and the significance of alterations in DNA methylation status found in metastatic tumors, during pancreas carcinogenesis is not fully understood.

Src is the *M*r 60,000 non-receptor tyrosine kinase protein product of the proto-oncogene c-*src*. *Src* family nonreceptor protein tyrosine kinases transduce signals that control normal cellular processes such cell proliferation, adhesion and motility. Normally, cellular *src* is held in an inactive state, but in several cancer types, abnormal events lead to elevated kinase activity of the protein, and cause pleiotropic cellular

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responses inducing transformation and metastasis. Ability of a cancer cell to undergo metastasis is to penetrate surrounding extracellular matrix; these processes are facilitated by the integrin family of cell adhesion molecules. *Src* signaling affects the formation of focal adhesions and the extracellular matrix, mainly due to interaction with integrins [6].

c-src in human cancer plays a critical role in the control cascade of cell growth. Accumulating evidence implicates *src* as an important determinant of tumorogenesis, invasion, and metastasis. All these observations link *src* to multiple processes that determine the clinical outcome of a tumor, becoming a promising target for drug discovery and targeting. *Src* is overexpressed in over 70% of pancreatic carcinoma cell lines, and *src* kinase activity is often increased. Overexpression of activated *c-src* has been reported to stimulate proliferation, cell migration and down-regulate *E-cadherin* expression in human pancreatic adenocarcinomas cell lines [7]. However, relatively little is known regarding the effects of *src* and its partners on tumor development or chemoresistance.

In this study we analyzed epigenetic alterations in crucial pathways, mainly those derived from *Src*, in order to demonstrate the important role of epigenetic control and *src* pathway in pancreatic cancer development.

Materials and Methods

Tumor samples

Primary Pancreatic Ductal Adenocarcinoma (PDA) tumors were collected from Santiago de Compostela tumor bank. Inclusion criteria of newly diagnosed PDA cancer patients were based on the histopathologic information, covering from initial to advanced stages. An initial series of 61 paraffin-embedded pancreatic tumors, including a subset of paired normal tissue, were used to analyze methylation rates and evaluate the association of gene methylation with clinicopathologic variables. Demographic information is reflected on Table 1.

DNA samples

Genomic DNAs from paired normal and tumor pancreas samples were obtained from 61 patients. Genomic DNA from human amygdale was used as a control for unmethylated DNA, and the CpGenome Universal Methylated DNA (Chemicon), for methylated DNA. Genomic DNA was modified using EZ DNA Methylation Kit (Zymo Research) and by standard procedures of sodium bisulphite treatment according to [8]. DNA was purified using the Wizard DNA Clean-up system (Promega, Madison, WI), according to manufacturer's protocol.

Distribution of aberrant promoter methylation

Methylation-specific polymerase chain reaction (MSP) was carried out following the method developed by Herman et al. [8]. CpG islands were identified using the CpGPlot program (*EMBOSS: http://bioweb.pasteur.fr/seqanal/interfaces/cpgplot.html*)

Fragments of the human promoter of selected genes, containing the transcription start site, was selected from the National Centre for Biotechnology Information (NCBI) database. These sequences were analyzed with the CpGPlot software program (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) using the following settings: observed/expected CpG ratio of 0.6, minimum length island of 200 nt, and minimum G+C content of 50%.

MSP analysis was used to determine the methylation status in two independent experiments for all samples: **GSTP1, p16, RASFF1A, RARβ2, CyclinD2, HIN-1, SOCS1, TIMP3, DAPK and TWIST1** genes, as previously described [9]. Primer sequences and PCR conditions are shown in Table 2. Bisulphite sequencing was performed in 5 samples for process control, as previously described [10]. Placental DNA treated *in vitro* with **Sss I** methyltransferase (New England BioLabs, Beverly, MA) was used as positive control for methylated alleles, and DNA from normal amygdale was used as negative control for methylated alleles. Controls without DNA were performed for each set of polymerase chain reaction.

Parameters	Value	N	%
Gender	M	36	59
	F	25	41
Tumor size	<2 cm	9	14.8
	>2cm	52	85.2
pTNM			
pT	pT1	6	9.8
	pT2	7	11.5
	pT3	48	78.7
pN	pN0	26	42.6
	pN1	35	57.4
pM	pM0	58	95.1
	pM1	3	4.9
Clinical stage	IA	5	8.2
	IB	1	1.6
	IIA	19	31.1
	IIB	33	54.2
	IV	3	4.9
Resection margins	R0	36	59
	R1	24	39.4
	R2	1	1.6

Table 1: Clinicopathological characteristic of patients.

PCR was performed in a Perkin Elmer 9600 DNA thermal cycler. Amplifications were carried out in a 25-µl volume containing 200 µm each of dATP, dCTP, dGTP, and dTTP; 0.2 µm each of forward and reverse primers; 10 mm Tris (pH 8.4); 50 mm KCl; 1.5 mm $MgCl_2$; and 0.5 unit of Taq polymerase (Invitrogen). PCR products were separated in 8-12% nondenaturing acrylamide-Tris-HCl (pH 8.8)-buffered gels or 2% agarose gels, silver stained or with ethidium bromide and visualized under UV illumination.

Statistical analysis

Hierarchical clustering was performed using Gene Cluster 3.0 and visualized using Treeview 3.0 (both from http://rana.lbl.gov/eisen) or PHYLIP: PHYLogeny Inference Package (http://evolution.genetics.washington.edu/phylip.html). Statistical analyses were performed by using SPSS (Version 10.1; SPSS, Chicago).

Results

CpG island promoter methylation analysis of the investigated genes

Clinico-pathological characteristics of the patients appear on Table 1.

	Forward primer 5´>3´		Reverse primer 5´>3´	
GSTP1	M	TTCGGGGTGTAGCGGCGTC	GCCCCAATACTAAATCACGACG	**57°**
	U	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAACA	
P16	M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	**60°**
	U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	
CyclinD2	M	GGCGGATTTTATCGTAGTCG	CTCCACGCTCGATCCTTCG	**57°**
	U	AGAGTATGTGTTAGGGTTGATT	ACATCCTCACCAACCCTCCA	
RAR 2	M	GAACGCGAGCGATTCGAGT	GACCAATCCAACCGAAACG	**55°**
	U	GGATTGGGATGTTGAGAATGT	CAACCAATCCAACCAAAACAA	
RASSF1A	M	GTTGGTATTCGTTGGGCGC	GCACCACGTATACGTAACG	**57°**
	U	GGTTGTATTTGGTTGGAGTG	CTACAAACCTTTACACACAACA	
HIN-1	M	GGTACGGGTTTTTTACGGTTCGTC	AACTTCTTATACCCGATCCTCG	**57°**
	U	GGTATGGGTTTTTTATGGTTTGTT	CAAAACTTCTTATACCCAATCCTCA	
SOCS1	M1	GTT GTA GGA TGG GGT CGC GGT CGC	CTA CTA ACC AAA CTA AAA TCC ACA	**63°**
	U1	GTT GTA GGA TGG GGT TGT GGT TGT	CTA CTA ACC AAA CTA AAA TCC ACA	
	M2	TTGTTCGGAGGTGGATTT	ACTAAAACGCTACGAAACCG	
	U2	TTTTTTCCTCTTCGTTTGGAGGTTGGATTTT	AAAACAAAACAATAAACTAAAACACTACAAAACCA	
TWIST	M	TTT CGG ATG GGG TTG TTA TC	AAA CGA CCT AAC CCG AAC G	**56°**
	U	TTT GGA TGG GGT TGT TAT TGT	CCT AAC CCA AAC AAC CAA CC	
TIMP3	M	CGTTTCGTTATTTTTTGTTTTCGGTTTC	CCGAAAACCCCGCCTCG	**59°**
	U	TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	CCCCCAAAAACCCCACCTCA	
DAPK	M	GGA TAG TCG GAT CGA GTT AAC GTC	CCC TCC CAA ACG CCG A	**57°**
	U	GGA GGA TAG TTG GAT TGA GTT AAT GTT	CAA ATC CCT CCC AAA CAC CAA	

Table 2: MSP-primers sequences for selected genes. M: Methylated; U: Unmethylated. Melting temperature are shown.

Methylation-specific PCR amplification of different genes promoter within the CpG island was performed on a set of paired normal and tumor pancreas samples.

We found a significant proportion of aberrant methylation in the promoter region of tumor suppressor genes selected: *RARβ2* (32.31%), *RASSF1A* (56.92%), *GSTP1* (38.46%), *p16* (44.61%), *CyclinD2* (32.31%), *HIN-1* (44.61%), *DPK* (23.5%), *TWIST1* (80%), *SOCS1* (98%) and *TIMP3* (83%) Figure 1A. Illustrative examples of the MSP are shown in Figure 1B.

Analysis of sequence-specific DNA methylation

Methylation status of specific genomic DNA sequences was established by bisulphite genomic sequencing as previously described by Fraga et al. [10]. **GSTP1, p16, RASFF1A, RARβ2, CyclinD2 and HIN-1** were automatically sequenced to measure the methylation status of every single CpG dinucleotide for subsequent statistical analysis.

We performed high-resolution bisulphite genome sequencing of all CpG sites within the CpG island identified. Five PDA and control samples were selected for this assay, attending to the different intensities of PCR products in previous MSP amplifications. Tumor samples were highly methylated in most of the CpG sites examined (70-90%). By contrast, their paired normal tissues were much less methylated (20-40%) Figure 1C.

Promoter CpG island hypermethylation leads to gene inactivation

CpG island promoter hypermethylation was found between 23% and 98% in PDA samples analyzed. All normal tissues analyzed, including lymphocytes and Amygdale, were mostly unmethylated.

Due to paraffin embedded tissue, the reliability of gene expression study with RT-PCR or Western blotting was reduce for the samples low quality; the only alternative was Immunohistochemistry (IHQ) not for its quantitative value, but for the possibility to results comparison. We found a nice correlation between IHQ values and methylation profile in all the samples analyzed; ie, 37% of tumour samples has cyclin-D2 expression reduced by IHQ, similar to the percentage of cyclin-D2 methylation promoters samples (32.31%) (data no shown).

PDA methylation profiling

DNA methylation levels were measured in 61 PDA and control samples. Only CpGs with a standard deviation across all samples and controls >0.25 were selected for an unsupervised hierarchical cluster analysis that segregated the PDA cohort into different main groups (Figure 2A). Clusters for clinicopathological characteristics and epigenetic profiles were obtained. Overlapping both clusters, results are grouped on seven discrete and homogeneous collections with shared characteristics (Figure 2B).

Discussion

Pancreatic cancer is one of the worse prognosis tumors. Difficult access for biopsy, clinical silencing until invasion of the bile duct and others factors means late diagnosis between 50,000 and 80,000 cases per year.

Numerous studies in PDA have not yet reached a level of medical management of this disease, leaving "shadows" in almost all levels of knowledge, control and monitoring of this type of cancer.

Accumulating evidence implicates *Src* as an important determinant of tumorogenesis, invasion, and metastasis in this pathology. These observations link *Src* to multiple processes that determine the clinical outcome of a tumor, make it a promising target for drug discovery. *Src* is overexpressed in over 70% of pancreatic carcinoma cell lines where *Src* kinase activity is often increased. Overexpression of activated *c-Src* has been reported to stimulate proliferation and migration and down-regulate *E-cadherin* expression in human pancreatic adenocarcinomas cell lines [7]. There is clinical evidence that *Src* kinase expression plays a role in the development of chemoresistance, and its inhibition could enhance chemosensitivity of tumors [11]. Increased *Src*

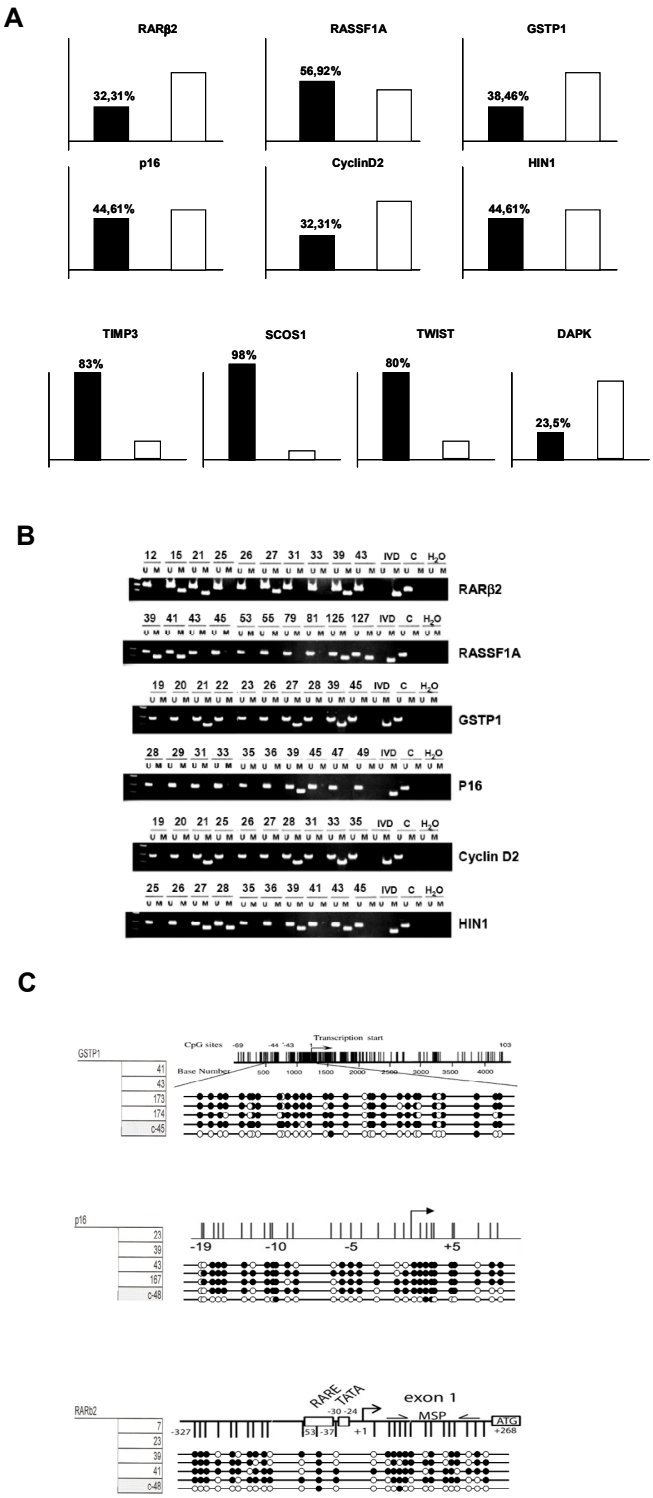

Figure 1: ***Methylation Specific PCR and Bisulfite Sequencing.*** **(A)** Graphic representation of Methylated/Unmethylated percentages for each of the studied genes: *GSTP1, p16, RASFF1A, RARβ2, CyclinD2, HIN-1, SOCS1, TIMP3, TWIST1 and DAPK.* Black columns show Methylated percentage and white ones Unmethylated. **(B)** *RARβ2, RASFF1A, GSTP1, p16, CyclinD2, HIN-1* were hypermethylated in human primary PDA. Genomic DNA was isolated from a panel of 61 PDA and left untreated or treated with sodium bisulfite. MSP was performed using PCR primers specific for the unmethylated (U) or methylated (M). A negative control for the methylated allele was done using DNA from normal human Amygdale (C). A negative control for the PCR reaction was performed in the absence of DNA (H_2O). Placental DNA treated *in vitro* with *Sss I* methyltransferase (New England BioLabs, Beverly, MA) was used as positive control for methylated alleles (IVD). The nomenclature for each primary PDA is indicated at the top of the figure. **(C)** Bisulfite sequencing. Example of five PDA and one control samples in three genes (GSTP1, p16 and RARβ2) are presented. Gene structure with transciption start site and CpG island position (vertical lines) are illustrated. CpG Methylation profiles: White circle: Unmethylated, Black circle: Methylated.

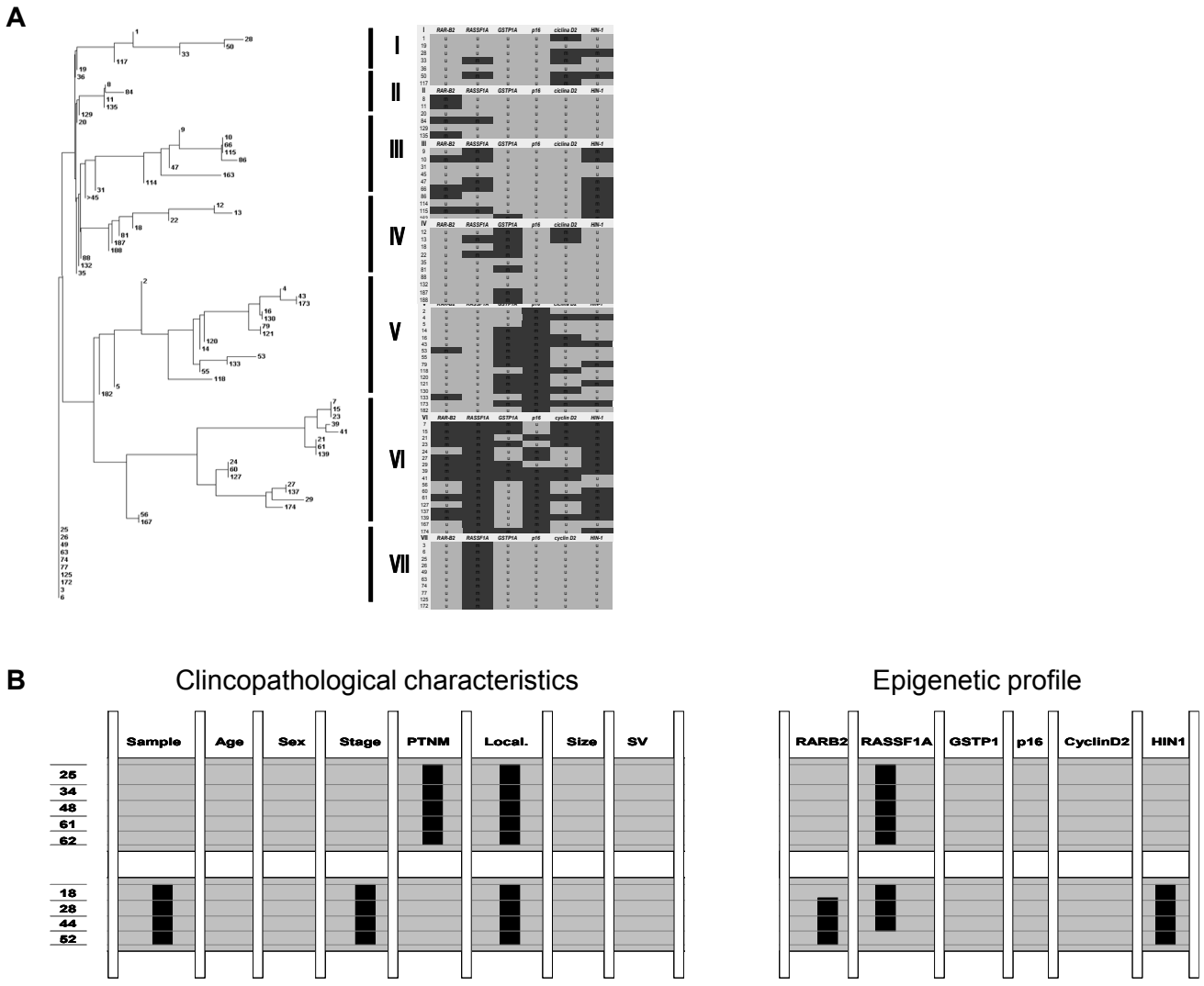

Figure 2: ***Cluster analysis. (A) Hierarchical clustering*** of DNA methylation profiles using PHYLogeny Inference Package (http://evolution.genetics.washington.edu/phylip.html). (***B) Cluster overlay diagram.*** Left panel clinicopathologycal characteristics: Sample, Age, Sex, Clinical Stage pTNM, Localization, Size and Survival. Rigth panel, epigenetic profiles of *RARβ2, RASSF1A, GSTP1, p16, Cyclin D2* and *HIN-1* genes. Left numbers are PDA anonimized samples, and black blocks correspond to shared characteristics. Figure shows two matched groups (ie, upper lines tumors with the same pTNM and Localization are defined by just, and only, methylation in *RASSF1A)*.

activity represents a cytoprotective mechanism capable of promoting chemoresistance.

Receptor signal transduction pathways are often compared to electrical circuitry networks, where the eventual outcome is dependent not only on the set of individual switches incorporated into the circuit board, but also on the combination of signals that are on or off at any one time. In this sense, *SOCS-1* suppresses the *JAK/STAT* pathway by inhibiting *JAK2* activity, and since *STAT3* is downstream of *JAK2* in the *JAK/STAT* pathway, *SOCS-1* methylation status affect the level of phosphorylation of *STAT3*. *pSTAT3* induction by *IL-6* would be more likely to occur if *SOCS-1* is methylated than if it is unmethylated. It was not observed induction of *pSTAT3* or *JAK2* by 5-Aza-dC treatment despite induction of *SOCS-1*, but is suspected that the level of induction of *SOCS-1* expression by 5-Aza-dC was insufficient to result in a change in *pSTAT3*. There was not a clear relationship between *pSTAT3* levels and *SOCS-1* expression and this observation reflects the fact that *STAT3* is regulated not only by *JAK2* but also by other factors including the insulin-like growth factor-1 receptor (*IGF-R*) and *Src* [12,13].

Src expression increases with disease progression, suggesting that *Src* may be more active in invasion and metastasis than in tumor formation [14].

Epigenetic phenomenon, by reversible, has become an interesting target for treatment. The 61 patients diagnosed with PDA, chosen for this study, represent a clinically uniform group with resectable pancreatic cancer at diagnosis. Our results agree with data regarding gender bias, classically indicating PDA prevalence in men over women by 40-50% [15].

We made a prospective study about the promoter methylation role in PDA. For this purpose we select genes involved in cell cycle (p16,

CyclinD2), DNA repair (GSTP1), proapoptosis…, etc. Due to previous studies describing methylation profile in cancer types, we select for our first approach the following genes: **GSTP1, p16, RASFF1A, RARβ2, CyclinD2 and HIN-1.** Methylations percentages range between 32.3 and 56.9%. Due to describe implication of Src pathway in pancreas tumors *and although* Src alterations in cancer always goes *through* overexpression, some relatives genes up- or downstream of this pathways could be involved. For this reason we select four genes involved in **Src** cascade**:** *SOCS1, TIMP3, TWIST1 and DAPK*. Methylation profiles of these genes goes from 23.5 to 98%, clearly demonstrating implication of Src and epigenetic control in PDA.

We showed a clear prevalence of deregulation of one or more pathways in each of the seven clusters obtained grouping epigenetic profiles (Figure 2A). Group I emphasize the Cyclin D2 methylation prevalence; in group II RARβ2 gene was silenced; in group III RARβ2, RASSF1A, and HIN-1 genes are frequently methylated; in group IV GSTP1 gene is frequently methylated; the same join to hypermethylated p16 are found in group V. Special attention require group VI, the largest one (n = 16), with a high rate of aberrant methylation in almost all studied genes except GSTP1 and CyclinD2. Group VII presents aberrant methylation in 100% of cases for RASSF1A gene.

These findings, by itself, reflect the presence of epigenetic tumor subtypes with some "dominant" pathways down-regulated by promoter hypermethylation. The coexistence alterations of more than two pathways, typically divided by their targets and activation factors, such as RARβ2, RASSF1A and HIN-1 (group III) and GSTP1 and p16 (group V), are consistent with the modern hypothesis of candidate or collaborative pathways that trigger a similar functional effect (ie, tumor progression) using different cell characteristics. The high percentage of aberrant methylation in multiple genes (group VI), suggest an accumulative effect, well known from animal models, in different pathways due to methylation. So we found groups that share clinical and pathological criteria with defined epigenetic alterations.

According with these findings, locally advanced tumors that are larger than 2 cm and extend through parapancreatic adipose tissue (without involvement of celiac axis or superior mesenteric artery) have a characteristic profile of epigenetic alterations in *RAS* pathway, highlighting, once again, the involvement of this pathway as a crucial event in tumor progression of PDA (Figure 2B, upper group) .

Another group consists of 6 cases of primary tumors with clinical stage IIB [pT1, pT2 and pT3, with lymph node metastasis (pN1), but not remotely (M0)], which showed a combination of aberrant methylation of *RAR*β2 (4/6 cases), *RASSF1A* (5/6 cases) and *HIN-1* (6/6 cases). These finding evidence a nodal involvement in both primary tumors localized (pT1, pT2) and locally advanced (pT3), not dependent on tumor size.

A specific set of epigenetic alterations serves as a marker for tumor progression. The fact of the coexistence of different metabolic pathways involved in tumor progression corroborates the hypothesis of genetic and epigenetic changes collaborating in transition from a neoplasm to a metastatic disease.

As we describe above *Src* is frequently implicated in PDA. Some of our selected genes (**GSTP1, p16, RASFF1A, RARβ2, CyclinD2 and HIN-1**) are related with *Src* pathway, through cell cycle (CyclinD2) or complex pathways interactions (Plag1/CNK1/RASSF1A/SRC) [16]. For this reason, we decided to go deeply in methylation study of control of *Src* pathway. We selected genes involved or related with *Src* pathway, already described in the literature as methylated-controled genes: *SOCS1*, competitive inhibition with *Src* protein for the receptor [17]; TWIST1, directly involve in *Src* control of Epithelial Mesenquimal Transition (EMT) and ductal carcinomas specificity, mainly through *cadherins* [18]; *TIMP3* related with *Src* control of metastasis via matrix metalloproteases (*MMPs*) [19]; and *DAPK*, as key point in *Src* control of migration/invasion and apoptosis [20]. Epigenetic studies of new genes directly involved in *Src* pathway (*SOCS1, TIMP3, TWIST1 and DAPK*) results in a clear epigenetic expression control from 23.5 to 98% as mechanism implicated in development and progression of PDA.

In conclusion we demonstrated that epigenetic mechanism related with *Src* pathway could have implications in development of Pancreatic Ductal Adenocarcinomas.

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