Single nucleotide polymorphisms and risk factors predictive of pancreatic adenocarcinoma

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RESEARCH HIGHLIGHT

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Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease progressing asymptomatically until death within months after diagnosis. Defining at-risk populations should promote an early diagnosis and efficient follow-up, therefore avoiding its development. Single nucleotide polymorphisms, or SNPs, constitute the most abundant form of genetic variation in the human genome. SNPs are markers of diverse populations or individuals, yet also can be associated with differences in susceptibility or severity of certain diseases and/or individual responses to drugs. Many SNPs have previously been identified in studies of healthy subjects and patients with different alleles of a given gene. To date, around forty SNPs from the human genome have been correlated with predisposition to PDAC by pan-genomic studies or genome wide association studies (GWAS). However, parts of the human genome located within the GC-rich repeated domain of chromosomes are unsuitable for GWAS. Unfortunately, of those forty SNPs none are currently used in routine clinical protocols as potential biomarkers for PDAC. Exon 11 of the bile salt-dependent lipase gene (BSDL) plays a key role in pancreatic disease and encodes variable number of tandem repeat (VNTR) sequences, therefore Martinez *et al*. [Oncotarget. 2015; 6: 39855-39864.] hypothesized that a genetic link exists between mutations in VNTR loci and predisposition to pancreatic cancer (PC). Authors reported that the c.1719C>T transition (SNP rs488087) present in BSDL VNTR may be a useful marker for defining a population at risk of developing PC (occurrence: 63.90% in the PC group versus 27.30% in the control group). Notably, the odds ratio (OR) of 4.7 for the T allele was larger than those already determined for other SNPs suspected to be predictive of PC. Further studies on tumor pancreatic tissue suggested that the T allele may favor Kras G12R/G12D somatic mutations which represent negative prognostic factors associated with reduced survival. Furthermore, a robust method using probes for droplet digital (dd)-PCR was designed to specifically discriminate the C/C major from C/T or T/T minor genotypes. Altogether, Martinez *et al*. propose that detection of the T allele in rs488087 SNP should lead to an in-depth follow-up of these patients, particularly if associated with other potential risk factors of PC.

Keywords: SNP; bile salt-dependent lipase; pancreatic cancer; pancreatic ductal adenocarcinoma

Introduction

Pancreatic cancers (PC) represent 10% of all digestive cancers with over 338,000 new cases worldwide in 2012, among which 90% were pancreatic ductal adenocarcinoma (PDAC), (www.wcrf.org; http://globocan.iarc.fr). The survival rate is dramatically low with a case-fatality ratio of about 0.9. PDAC could be the second cause of mortality by cancer by the year 2030 [1]. The 5-year survival rate of PDAC is less than 4% in western countries [2]. The poor prognosis of this cancer is mainly due to its lack of response to currently available therapies [3, 4]. Its curative resection rate is very low (15% of patients) due to unspecific symptoms, the lack of early biological markers, delayed diagnosis and metastasis formation. Patients diagnosed with advanced or metastatic disease are not elected for surgery (85% of patients) and show median survival rates ranging from 6 to 9 months [5, 6]. For those patients with inoperable cancer, the main treatment remains standard chemotherapy. Precancerous lesions as well as PDAC occur as a result of alterations affecting genes essential for maintaining cellular functions. These changes can be explained by mutations, deletions or epigenetic modifications, responsible for either a gain or loss of gene function. They can either be inherited (familial cancers, predispositions) or acquired, although in both cases each stage is associated with one or more types of alteration, their evolution and accumulation increasing the invasiveness of lesions.

Single nucleotide polymorphisms (SNPs) are a one-time bi-allelic polymorphism at one base pair on a given locus in a chromosome, constituting the most abundant form of genetic variation in the human genome. Comparing two random human genomes, 99.9% of the DNA sequence is identical. The remaining 0.1% contains sequence variations including SNPs. SNPs are stable, abundant and evenly distributed throughout the genome. In fact, SNPs occur on average, once every 300 nucleotides. This translates to a potential 10 million SNPs in the human genome. Although SNPs are markers of diverse populations or individuals, they also can be associated with differences in susceptibility or severity of certain diseases and/or individual responses to drugs. Many SNPs have previously been identified in studies of healthy subjects and patients with different alleles of a given gene. Currently, pan-genome association studies, or genome wide association studies (GWAS), are one of the most frequently used analytical tools for multifactor diseases such as cancer. GWAS are used to compare the frequency of hundreds of thousands of genetic variants distributed across all chromosomes between a group of cancer patients and a control group, using high-throughput genotyping technologies. This is an "agnostic" approach, in the absence of a hypothesis based on a gene(s) of interest, unlike the genetic type of candidate gene association studies. GWAS aim to identify genetic variants with relevant predisposition to specific diseases by genotyping up to a million SNPs. Although these studies do not require prior knowledge of the functional significance of the variant studied, very large groups of samples are necessary, usually thousands of cases plus paired controls.

Gene wide association studies (GWAS)

In recent studies of PC, particularly PDAC, many pan-genomic studies have highlighted SNPs linked to a predisposition to this dramatic disease (**Table 1**). In 2009, Amundadottir and collaborators [7] were the first to show through GWAS that some chromosome loci could predispose to PDAC. This large-scale study, (about 560,000 SNPs were analyzed), highlighted the presence of SNPs at three loci:, 7q36, 15q14 and 9q34. A study performed on an ethnically restricted population showed that four SNPs (rs6464375, rs7779540, rs6973850, rs1048768) located at the 7q36.2 locus with odds ratios (OR) close to 1.1, are associated with dipeptidyl peptidase 6 (DPP6) and could predispose to PC in the Japanese population [8]. SNPs rs8028529, rs4130461 and rs4459505 were discovered on locus 15q14. However these latter SNPs did not impact any gene. The SNP at the 9q34 locus attracted particular attention due to its highly significant association with PDAC (P-value = 5.37×10^{-8}). This SNP, referred to as rs505922, is positioned on the long arm of chromosome 9, the same locus as genes encoding the ABO blood groups. These results confirm those obtained in epidemiological studies from 1950 to 1970, which suggested that group O individuals were less prone to develop PDAC than individuals with blood groups A, B or AB [9, 10]. More recently, Wolpin and collaborators [11] tested this hypothesis in a pan-genomic study and determined which genotype of the ABO system (OO, AO, AA, AB, BO, BB blood groups) is preferentially associated with PDAC. Results revealed that there is a significantly increased risk of developing PC in individuals of A, B or AB blood groups compared to group O individuals, with close relative risk or OR of 1.38, 1.47 and 1.53 respectively. The BB genotype was the most predisposing genotype. In two large and independent populations the age-adjusted incidence rates for PDAC per 100,000 person-years were 27 for participants with blood type O, 36 for blood type A, 41 for blood type AB, and 46 for blood type B [11, 12]. However, the molecular or cellular connection between the presence of the rs505922 SNP and susceptibility to PC is still debatable. Another group demonstrated that rs505922 is associated with elevated blood levels of tumor necrosis factor alpha (TNF-α), soluble intercellular adhesion molecule 1 (sICAM-1) and alkaline phosphatase [13, 14]. TNF-α is a proinflammatory cytokine that

Table 1. SNPs predictive of pancreatic cancer

Chromosomes	Locus	Ancestry	SNP Nb	Nearest gene	OR	Ref
1	q32.1	European	37908844	NR5A2	0.77	15, 21
	p36.13	European	16861827	IGSF21	1.7	21
2	p13.3	European	1486134	ETAA1	1.14	29
3	q29	European	9854771	TP63	0.89	29
5	p13.1	European/Chinese	225280	DAB2	0.81/0.74	30,36
	p15.33	European	27336098	CLPTMIL-TERT	0.8/1.19	15
			4011681		1.06	15
6	p25.3	Japanese	9502893	FOXQ1	1.29	8
7	q32.3	European	6971499	LINC-PINT	0.79	36
	q36.2	European	167020	SHH	1.17/1.38	7
		European	172310	SHH	1.17/1.36	7
		European	288746	SHH	1.18/1.39	7
		Japanese	6464375	DPP6	1.13	8
		Japanese	7779540	DPP6	1.14	8
		Japanese	6973850	DPP6	1.1	8
		Japanese	1048768	DPP6	1.13	8
	p13	European	17688601	SUGCT	0.88	29
8	q24.21	European	1561927	PVT1	0.87	36
9	q34	European	505922	ABO locus	1.20/1.44	7
			495828		1.19/1.43	7
			657152		1.19/1.41	7
			630014		0.85/0.71	7
			488087	CEL	4.72	53
10	q26.11	European/Chinese	12413624	PRLHR	1.23	30
11	p15.4	European	12362504	SBF2	1.4	21
			10500715	SBF2	0.74	21
12	p11.21	Japanese	708224	B1CD1	1.32	8
13	q12.2	European	9581943	PVDX1	1.15	36
	q22.1	European/Chinese	9543325	KFL5	1.26	15,31
			9564966	KFL12	1.21	14
15	q14	European	8028529	none	1.14/1.31	7
			4130461	none	1.10/1.21	7
			4459505	none	1.13/1.28	7
16	q23.1	European	7190458	CTRB1/B2	1.46/0.79	36
17	p12-13	European	1042522	TP53	-	52
	q25.1		11655237	LINC00673	1.26	29
18	p11.21	European	981621	C18orf1	1.39	21
21	q21.3	European/Chinese	372883	BACH1	0.79/0.69	30
	q22.3	European/Chinese	1547374	TFF	0.79/0.79	30
22	q12.1	European	16986825	ZNRF3	1.18	36
	q13.32	European/Chinese	5768709	FAM19A5	0.23	30

controls apoptosis of ductal cells and high levels of sICAM-1 is associated with diabetes, a well-known predisposing factor for PDAC. All these elements show that the A and B glyco-antigens may be linked to tissue inflammation susceptibility, therefore increasing the risk of developing a PDAC [7]. Overall, these studies highlight the ABO blood group as a potential marker of predisposition to PC.

Other studies have identified several SNPs as markers of susceptibility to PC. Petersen and colleagues [15] revealed 8 SNPs spread over 3 chromosome loci: 1 - The SNP rs401681 at the 5p15.33 locus which encodes both Cleft lip and palate transmembrane protein 1-like protein (CLPTM1-like protein), also known as cisplatin resistance-related protein 9 (CRR9p), and Telomerase reverse transcriptase (TERT) genes and involved in carcinogenesis. CLPTM1L plays a role in apoptosis and genetic variations are associated with many cancers including lung cancer and melanoma [16, 17]. A link has also been described between the overexpression of CLPTM1L

in cancers and resistance to cisplatin treatment [18]. Finally, genetic variations in CLPTM1L increase tumor growth and favor aneuploidy in PC [19]. This same locus also appears to be a potential marker of predisposition to PC in the Han Chinese population [20]. 2 - The locus 1q32.1 on which the nuclear receptor subfamily 5, group A member 2 gene (NR5A2) is located [15, 21] encodes a nuclear receptor (Ftz-F1) which is mainly expressed in the exocrine pancreas, liver, intestine and ovaries. NR5A2 plays a role in the homeostasis of cholesterol and bile acids as well as in cell proliferation. It is also involved in malignant transformation through interaction with β-catenin, activating a series of target genes, such as cyclin D1, c-myc, PPAR-δ (Peroxisome proliferator-activated receptor-delta) and AF17 (a fusion partner of the mixed-lineage leukemia gene) [22-26]. 3 - The 13q22.1 locus with two SNPs, rs9543325 and rs9564966, appears to be associated with susceptibility to PDAC. Indeed, these SNPs are located on a portion of chromosome 13 which encodes

Table 2. SNPs predictive of different cancers and their clinical impact

Disease	Gene	rs # and SNP location	Clinical impact
Smoking-related Cancer (lung and upper aerodigestive tract cancer)	NBS1 (Nijmegen Breakage Syndrome 1)	rs709816 : exon 10 rs1061302 : exon 14	Association with smoking status
Colorectal Cancer	ERCC1 (Excision Repair Cross-Complementing rodent repair deficiency, complementation group 1)	rs11615 : exon 4	Prediction of clinical outcome to oxaliplatine-based chemotherapy
Chronic Myeloid Leukemia	WT1 (Wilms tumor 1 or BCR-ABL fusion gene)	rs2229069 : exon 5 rs2227985 : exon 10 rs16754 : exon 7	Correlation with disease characteristics and clinical outcome
Non-Small-Cell Lung Carcinoma	EGFR (Epidermal Growth Factor Receptor)	rs2293347 : exon 27	Prediction of clinical outcome in patients treated with gefitinib
Cancers	TP53 (Tumor Protein 53)	rs111287251 : exon 27 rs1042522	Association with tumor susceptibility to cancer

Table 3. SNPs predictive of non-neoplastic diseases and their mechanistic implications

Disease	Gene	rs # and SNP location	Implicated mechanism
Infantile Spinal Muscular Atrophy	UBE1 (Ubiquitin-like modifier activating enzyme 1)	Polymorphism in exon 15	Alteration of methylation pattern
Cystic Fibrosis	CFTR (Cystic Fibrosis Transmembrane conductance Regulator)	rs1800093 : exon 12	Modification of mRNA folding and the rate of translation
Osteoporosis	CD44	rs11033026 : exon 10	Abolishment of an exon splicing enhancer (ESE) site
Crohn's Disease	IRGM	rs10065172 : exon 2	Modification of miRNA-196 binding site and association with susceptibility to Crohn's disease
Pain perception temporo-mandibular joint disorder	COMT (Cathechol-O-Methyltransferase)	rs769223 : exon 6	Modification of structure and stability of mRNA

kruppel-like transcription factor 5 and factor 12 (KLF5 and KLF12, respectively), which are frequently deleted in a large number of cancers, particularly in PC [27]. This locus is also a potential marker for susceptibility to breast cancer 1 and 2 (BRCA1/BRCA2) non-mutated breast cancer [28]. Consequently, a particular SNP may not necessarily be specifically associated with a unique cancer type.

Another very recent GWAS study [29] highlighted 4 new loci that may predispose to PC: 1 -The 17q25.1 locus associated with long intergenic non-coding RNA protein 673 (LINC00673) and the presence of SNP rs11655237. 2 - The locus 7p13 and succinyl-CoA: glutarate CoA transferase (SUGCT) on which the SNP rs17668601 has been noted. 3 - The 3q29 locus and TP63 gene with the presence of the SNP rs9854771. 4 - The locus 2p13.3 associating ewing tumor associated antigen-1 (ETAA1) with the SNP rs1486134. This final locus may predispose to PDAC in the Chinese Han population [30]. Data presented by Low *et al.* [8] and Wu *et al.* [30], among others [21], seem to suggest that a SNP predisposing to a given pathology does not necessarily apply to only one ethnicity; other ethnicities should be examined before such a conclusion.

In 2012, Li *et al.*[31] demonstrated that three genes are involved in maturity onset diabetes of the young (MODY) which also harbor SNPs predisposing to PC: 1 - The pancreatic and duodenal homeobox 1 gene (PDX-1) involved in MODY-4 diabetes encodes a transcription factor that regulates the early stage development of the exocrine pancreas. 2 - The hepatocyte nuclear factor-1 alpha or beta genes (HNF1A and HNF1B) are involved in MODY-3 and MODY-5 diabetes, respectively [32, 33], as well as playing roles in the growth of β-cells in the islets of Langerhans and control of pancreatic organogenesis. HNF1 and PDX-1 are also associated with risk of type 2 diabetes and obesity [34]. In this context, the SNPs rs9554197 and rs9581943 in PDX-1, as well as rs7310409 and rs4794758 in HNF1A and HNF1B, respectively, could contribute to the development of PC in patients afflicted with obesity or diabetes [31, 35, 36].

SNPs in the clinic

As shown above, a number of SNPs have been experimentally validated as potential predisposition markers for PDAC. However, none of these SNPs is currently used in the clinic, most likely due to their associated low OR (generally close to 1) thus the increase in risk is limited. Tools to detect these SNPs in clinical routines are also lacking. Furthermore, no study has yet shown what functional impact these SNPs could have on cell behavior to explain the onset of PDAC. However, studies in other cancers have shown correlations between the presence of polymorphisms and their potential impact on treatment (sensitivity, resistance) (**Table 2**). The functional effects of these polymorphisms have been demonstrated in many other pathology cases (**Table 3**). SNPs resulting in a modification of the protein sequence can lead to the synthesis of truncated proteins. Although synonymous
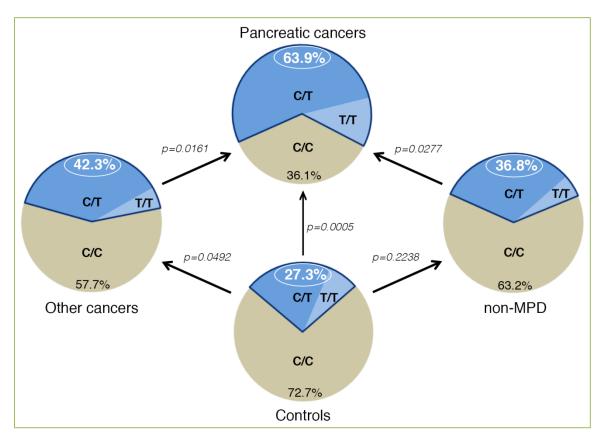
SNPs do not necessarily affect the protein sequence, many other changes in gene expression have been identified.

SNPs and candidate gene association studies

Five hereditary syndromes are associated with an increased risk of PDAC: 1 - The Peutz-Jeghers syndrome caused by germline mutations in the STK11/LKB1 gene encoding serine/threonine kinase 11 (STK11), also known as liver kinase B1 (LKB1) or renal carcinoma antigen NY-REN-19 [29, 37]. STK11 rs741765 was associated with disease-free survival or overall survival of patients with colorectal cancer [38]. 2 - The familial atypical multiple melanoma and mole syndrome (FAMMM) caused by mutations in the cyclin dependent kinase N2A (CDKN2A) gene [29, 39, 40]. 3 - Hereditary pancreatitis is also the cause of PDAC with mutation in the cationic trypsinogen PRSS1 gene [41]. 4 - Subjects with mutations in BRCA2 or partner and localizer of BRCA2 (PALB2) [29, 42, 43]. A lower expression of BRCA2 transcript and increased pancreatic cancer risk were associated with SNP rs11571836 in the BRCA2 3'-untranslated region [44]. 5 – Finally, the Lynch syndrome often called hereditary nonpolyposis colorectal cancer (HNPCC), caused by germline mutations in DNA mismatch repair genes such as mutL homolog 1 (MLH1), mutS protein homolog 2/6 (MSH2, MSH6), and PMS1 protein homolog 2 (PMS2) appear to be linked to a slightly increased risk of developing PC [45]. SNPs in these genes were associated with overall survival in patients with locally advanced or metastatic disease [46]. BRCA1 and the ataxia telangiectasia gene (ATM) were also mutated in patients with hereditary PC [29, 47, 48]. Studies have also shown that the 8-oxoguanine DNA glycosylase gene (OGG1) is another candidate gene for PC development. Links between PC and SNPs in OGG1 are rather confusing as some SNPs are associated with either increased [49] or no risk [50] in the Japanese population, while others seem associated with a protection in Chinese populations [51]. A recent study performed on 32 patients with PDAC [52] demonstrated that the tumor protein gene (TP53) polymorphism (SNP rs1042522 at 17p12-13) is a potential genetic predisposing factor, while SNP rs2279744 located at 12q14.3-q15 in mouse double minute 2 homolog (MDM2), an E3 ubiquitin ligase that negatively regulates p53, could potentially predict survival outcome. Predictive values deduced from these genes were low with modest odds ratios. Therefore, a better genetic predictive factor is crucial in order to define an at risk population of developing PC and to increase the efficacy of follow-up of these patients. Recently, Martinez *et al*. [53] characterized the SNP rs488087 as being associated to PC. This SNP is present within the coding region of the human bile salt-dependent lipase gene (BSDL).

SNP in the BSDL gene

The human BSDL, or Carboxyl ester lipase (CEL) gene encodes a lipolytic enzyme involved in the hydrolysis of dietary cholesteryl esters and is mainly expressed by the acinar cells of the pancreas [54]. This gene locates at band 34.3 on the long arm of chromosome 9 [55, 56] and consists of 11 exons spanning 9884 base pairs (bp). The N-terminal domain of the protein, encoded by exons 1 to 10, includes bile salts binding, catalytic and N-glycosylation sites and is well conserved across evolution [54]. However, exon 11 codes the C-terminal domain with a region consisting of a variable number of tandem repeats (VNTR). Each repeat consists of GC-rich 33 bp sequence coding 11 amino acids [57] with O-glycosylation sites [58], conferring a mucin-like structure to the C-terminal domain of BSDL [59]. VNTR sequences can slightly vary and the total number of repeats differs between alleles. In Caucasian populations investigated to date, the most frequent allele has 16 repeats which results in a 722 amino-acid long polypeptide. However, VNTR number can range between 3 to 21 repeats, leading to a high level of heterogeneity in protein size both within and between individuals [60, 61]. It has been suggested that naturally occurring variable numbers of VNTR can influence BSDL function and an association between VNTR length and increased susceptibility to alcoholic pancreatitis has been suggested [62]. Furthermore, plasma levels of BSDL and Low Density Lipoprotein (LDL), (BSDL is associated with LDL, in part by a specific interaction with Apo B100 [63]), positively correlate, suggesting a role for BSDL in cardiovascular diseases [64]. Augé *et al*. [65] demonstrated that BSDL is present within atherosclerotic plaques of arterial walls where it induces proliferation of smooth muscle cells, chemotaxic migration of monocytes and oxidized LDL degradation [63, 66, 67]. Furthermore, a single base deletion in either repeat 1 or 4 within the VNTR has been associated with autosomal dominantly inherited MODY-8 with exocrine dysfunctions. Such deletions would result in truncated proteins due to a premature stop codon [68, 69]. Affected family members typically develop diabetes characterized by primary β-cell dysfunction, although transgenic mice over-expressing truncated BSDL failed to develop the MODY-8 phenotype [70]. Therefore, the pathogenic mechanisms involved are more complex than a simple loss of BSDL function. Truncated BSDL seems to be prone to form aggregates, detected both intra- and extracellularly [71, 72]. Therefore, MODY-8 and exocrine pancreatic syndrome may be caused by BSDL misfolding with a negative gain-of-function effect of truncated protein in the pancreas. Further work investigating these mechanisms demonstrated that truncated BSDL was secreted and re-internalized for degradation by lysosomes in a HEK293-T cell model [72]. Such internalization may reduce the viability of both acinar and endocrine pancreatic cells. A

Figure 1. Schematic representation of SNP rs488087 occurrence in different groups. Non-MPD, non-malignant pancreatic diseases; Controls, cancer-free control subjects.

recent study [73] suggested that a recombined allele of BSDL, and its pseudogene BSDLP, confers susceptibility to chronic pancreatitis, an inflammatory disease of the pancreas known for predisposition to PDAC [74]. The resulting BSDL hybrid protein showed impaired secretion, prominent intracellular accumulation and induced autophagy. There are numerous links between the BSDL gene and diabetes as circulating antibodies to the C-terminal mucin-like domain of BSDL were detected in the sera of patients with type-1 diabetes [75]. Antibodies to this domain were also detected in the blood of patients affected with PDAC, establishing another link between PC and diabetes [75].

The sequencing of genes containing GC-rich tandem repeated sequences using standard methodologies can pose serious limitations [76]. Therefore, Martinez *et al*. [53] Sanger sequenced VNTR in BSDL from a restricted French cohort of patients with PC. Analysis of genomic DNA from PC tissue extracts highlighted the synonymous SNP rs488087, c.1719C>T, located in the second VNTR sequence of BSDL. The authors [53] compared the C/C major genotype and cancer-free control group to the PC cohort, revealing that the c.1719C>T transition was prevalent in PC patients (P-value = 0.0005 and an OR of 4.72). Notably, this OR for T allele-holders in the PC population is larger than any

previously determined (**Table 1**). The SNP occurrence was 63.90% in patients with sporadic PC (n = 36), and decreased to 27.30% in cancer-free control subjects (n = 44) (**Figure 1**). The frequency of the T allele was 37.50% (n = 72) in the PC cohort versus 17.05% (n = 88) in the control group (P-value = 0.0017). Comparing the T allele frequency in the PC group with the rs488087 SNP data bank (UCSC genome browser, n = 1275) showed that data remained significant (37.50 % versus 23.966%, P-value = 0.0041). In addition, the T allele frequency of the control group showed no significant difference with that of the rs488087 data bank (P-value = 0.0669). Therefore, the limited size of cohorts analyzed by Martinez *et al*. [53] did not appear to impair the statistical significance of their results. Sequence analysis of patients with non-malignant disease of the pancreas (non-MPD) revealed that 36.80 % held the T allele, with no significant difference compared to the control group (P-value = 0.2238). However, occurrence of the polymorphism in the non-MPD group was statistically lower than that in the PC group (36.80% vs 63.90%, P-value = 0.0277). This result based on matched non-MPD and PC populations strongly suggests a link between the c.1719C>T transition and PC. In a cohort of non-pancreatic malignant diseases, i.e. other cancers cohort (OC) (**Figure 1**), Martinez *et al*. [53] found a significant difference between the occurrence of the c.1719C>T SNP
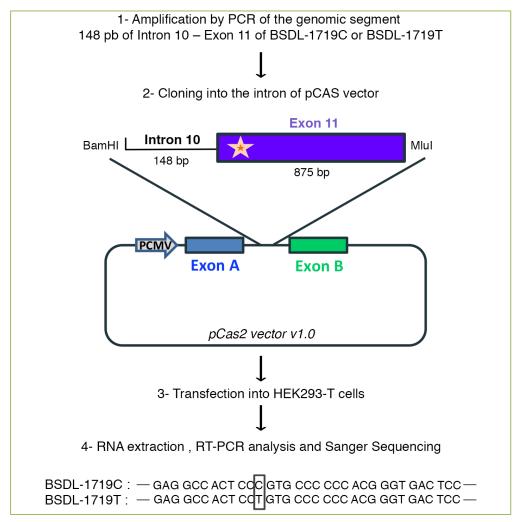
Figure 2. Representation of the functional splicing vector pCAS2. The amplicons (BSDL-1719C and BSDL-1719T) were cloned into the pCAS reporter vector, based on the pcDNA3.1 plasmid which contained a minigene composed of two exons (named A and B) separated by an intron. The splicing reporter minigene assay pCAS2 was used to evaluate the effect on splicing. After transfection into HEK-293T cells and RNA extraction, analysis of transcripts by Sanger sequencing showed no difference between the BSDL-1719C construction and BSDL-1719T construction by which an ESE sequence could be created. Grey arrows show forward and reverse primers and the star symbolizes the putative ESE sequence and the localization of the SNP rs488087.
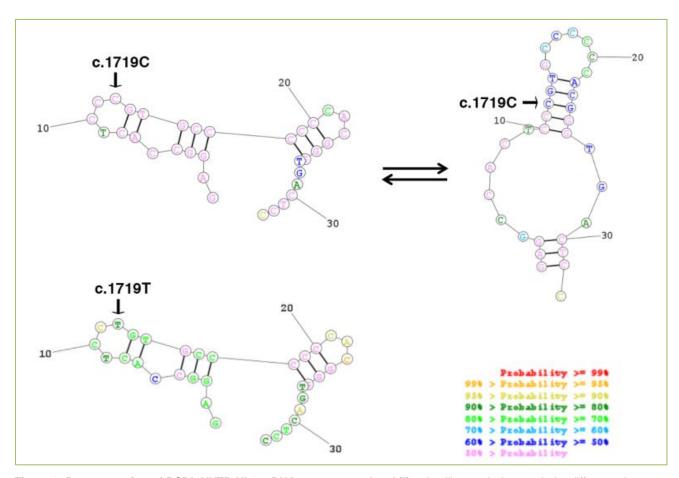
among the OC cohort (42.30%) compared to the PC group (63.90%, P-value = 0.0161). However, the SNP occurrence among the OC cohort did not differ from that of the control group. In terms of allelic frequency, Martinez *et al*. [53] found no significant difference between the OC group (23.70%) and control group (17.05%) (P-value = 0.1108), or between the OC group and the rs488087 data bank (23.70% versus 23.966%, P-value = 0.4725).

In view of the clinical interest of these results, Martinez *et al*. [53] constructed two probes to discriminate between the C and T SNP alleles of BSDL by droplet digital PCR (ddPCR). Analyses obtained on 143 patients by ddPCR matched 100% of those genotyped by Sanger sequencing. This absolute concordance demonstrates the high specificity of these probes to rs488087 SNP and their capacity to allow its simple, rapid and specific detection. Martinez *et al*. also used specific properties of this ddPCR technique [77] to count the number of copies of DNA target sequence in each of the DNA samples tested by Sanger sequencing. In the examined heterozygous samples, the fractional abundance of T/T+C was close to 0.5 (i.e. 0.45 +/- 0.07, n = 52) for all cohorts, thus confirming the germline character as the SNP of c.1719C>T transition.

Potential impact of SNP rs488087 on BSDL function/outcome

The transition c.1719C> T occurs in the third position of a codon which results in no change in terms of encoded amino

Figure 3. Representation of BSDL VNTR Nb2-mRNA structure and stability. In silico analysis revealed a difference between the two transcripts: BSDL-1719C and BSDL-1719T. Colors and percentages indicate mRNA stability.

acids (synonymous SNP), hence the protein sequence is unchanged. However, this may not translate to a non-functional effect of this SNP. Indeed, literature based on these particular polymorphisms reveal that synonymous SNPs could have effects on the splicing of pre-messenger RNA, the stability and structure of mRNA, as well as translation [78]. Pre-messenger RNA may be spliced in different ways, dependent on whether SNPs occur at donor or acceptor splicing sites, or at the level of regulatory sequences such as exonic splicing enhancer (ESE), exonic splicing silencer (ESS), intronic splicing enhancer (ISE), or intronic splicing silencer (ISS). In the case of cancer, some associations between the presence of a SNP and splicing modification have been identified. This is the case for the gene encoding cyclin D1 (CCND1) which displays an A870G silent polymorphism that alters splicing and therefore induces a susceptibility to the development of lung cancer [79]. Many SNPs can generate new splicing sites in exons of the p53 gene, thereby producing a truncated protein [80]. Since the C-terminal domain of BSDL is involved in intracellular processing of BSDL as well as its enzyme activity, [81] the authors determined whether the SNP rs488087 could affect BSDL mRNA splicing by an in silico

study (unpublished data). Results obtained using the "Human Splicing Finder" software (http://www.umd.be/HSF/) revealed that the presence of the minor T allele induces the formation of a regulatory sequence of ESE type splicing. The authors therefore examined whether the presence of the SNP rs488087 affects splicing of BSDL mRNA. For this purpose, the cDNA presenting the C or the T polymorphism (150 bp overlapping sequence segment in intron 10 and exon 11 of BSDL-1719C or BSDL-1719T) were cloned into the pCAS2 mini-gene containing 2 artificial exons separated by an intron [82]. After transfection of plasmid constructs pCAS2-BSDL-1719C and pCAS2-BSDL-1719T in HEK293-T cells, RNA was extracted and PCR performed with primers designed to target artificial exons (**Figure 2**). Results revealed no differences in sequence (and size) between the two transcripts (**Figure 2**). Therefore, the SNP rs488087 does not appear to induce any alteration of splicing.

The second hypothesis that Martinez *et al*. investigated was whether this SNP affects mRNA stability and structure. To determine this, they conducted an in silico study using the software "RNA Structure" (http://rna.urmc.rochester.edu/RNAstructureWeb/). The

mRNA structure at the repeated sequence Nb 2 with C nt appears to be in equilibrium mainly between two stable structures, whereas the mRNA structure with T nt gains stability with a unique rigid structure (**Figure 3**). The c.1719C> T transition therefore induces a potential change in the secondary structure of BSDL mRNA and possibly impacts the processing of RNA and translation into protein. This hypothesis agrees with the cellular over-expression of BSDL in various PDAC, partly in the exocrine-PDAC subtype, despite the overexpression of genes encoding lipolytic pancreatic enzymes such as pancreatic lipase (PNLIP) and pancreatic phospholipase A2, group 1B (PLA2G1B) [83].

A third hypothesis of the potential impact of the c.1719C> T transition was a possible link between this SNP and other gene mutations which could play a role in early tumor progression in PDAC patients. Martinez *et al*. [53] examined Kras point mutations in their cohort of PDAC patients. Among the PDAC patients examined, 66.7 % were T allele holders with Kras mutations. However, the examination of exon 2 Kras mutation subtypes in T allele holders indicated that the T allele in rs488087 SNP was mainly associated (85.7 %) with either the G12D or G12R Kras phenotype. These data clearly suggest that T allele holders of rs488087 SNP favor further somatic mutations in Kras, essentially leading to the G12D or G12R phenotype. Interestingly, these two phenotypes are associated with both the worse prognostic and lowest patient survival [84, 85].

The final hypothesis is whether the presence of the transition c.1719C> T alters a binding site of a regulating miRNA. A study by Brest *et al*. [86] showed in a case of Crohn's disease that a synonymous SNP (rs10065172; c.313C> T) present within immunity-related GTPase family M protein (IRGM) (also known as interferon-inducible protein 1 (IFI1)) alters the binding of family 196 mi-RNAs (miR -196A and miR-196B) [86]. Studies are currently in progress to characterize miRNA binding sites potentially generated by the c.1719C> T transition.

Conclusions

To fully understand the functional impact of the synonymous SNP rs488087, it is essential to thoroughly test each hypothesis to clearly establish any links between the occurrences of the SNP, in part the T allele, and neoplastic diseases of the pancreas. Further investigation is required to confirm the association between the rs488087 SNP and Kras mutations. From a clinical perspective, predictive or diagnostic markers cannot be dualistic (or binary) within the field of biomarkers. More importantly, a single biomarker alone cannot provide a clinician with definitive black or white answers, therefore SNPs should be associated with other relevant biomarkers, and/or risk factors, to define particular patient populations. This is particularly appropriate for PC patients, as the sr488087 SNP can be associated to other predisposing factors such as hereditary syndromes with BRCA mutations.

Conflicting interests

The authors have declared that no conflict of interests exist.

Acknowledgments

Abbreviations

BSDL: bile-salt-dependent lipase; PDAC: pancreatic ductal adenocarcinoma; PC: pancreatic cancers.

Author contributions

D.L. and Er.M. write the article. F.S and Em.M conducted the experiment. F.S., Em.M. and Er.M. analysed the results.

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