Germline copy number variation in the \textit{YTHDC2} gene: does it have a role in finding a novel potential molecular target involved in pancreatic adenocarcinoma susceptibility?

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\textbf{Objective}: The vast majority of pancreatic cancers occurs sporadically. The discovery of frequent variations in germline gene copy number can significantly influence the expression levels of genes that predispose to pancreatic adenocarcinoma. We prospectively investigated whether patients with sporadic pancreatic adenocarcinoma share specific gene copy number variations (CNVs) in their germline DNA.

\textbf{Patients and methods}: DNA samples were analyzed from peripheral leukocytes from 72 patients with a diagnosis of sporadic pancreatic adenocarcinoma and from 60 controls using Affymetrix 500K array set. Multiplex ligation-dependent probe amplification (MLPA) assay was performed using a set of self-designed MLPA probes specific for seven target sequences.

\textbf{Results}: We identified a CNV-containing DNA region associated with pancreatic cancer risk. This region shows a deletion of 1 allele in 36 of the 72 analyzed patients but in none of the controls. This region is of particular interest since it contains the \textit{YTHDC2} gene encoding for a putative DNA/RNA helicase, such protein being frequently involved in cancer susceptibility. Interestingly, 82.6\% of Sicilian patients showed germline loss of one allele.

\textbf{Conclusions}: Our results suggest that the \textit{YTHDC2} gene could be a potential candidate for pancreatic cancer susceptibility and a useful marker for early detection as well as for the development of possible new therapeutic strategies.

\textbf{Keywords}: copy number variations, germline alteration, pancreatic cancer susceptibility, \textit{YTHDC2} gene

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\end{center}

1. Introduction

Pancreatic cancer accounts for 3\% of all new cases of cancer and it is the fourth leading cause of cancer death, with an overall 5-year survival rate of < 5\%. This incidence has not changed in nearly 50 years \cite{1}. Pancreatic cancer is one of the most lethal human cancers and its prognosis has not been improved despite advances in diagnostic and therapeutic strategies \cite{2}. Environmental factors such as cigarette smoking \cite{3-5} or diseases such as diabetes \cite{6,7}, obesity \cite{8,9} or chronic pancreatitis \cite{10,11} can predispose to pancreatic cancer \cite{12}. Heritable genetic predisposition is also sometimes involved \cite{13,14}. The lifetime risk of pancreatic cancer is...
4.7% for first-degree relatives of patients with pancreatic cancer and the familial risk of pancreatic cancer increases with each affected family member [15,16]. Pancreatic cancer can also be inherited as part of a multi-cancer syndrome such as cancers associated with BRCA2 mutations [17,18], Peutz-Jeghers syndrome [19], Fanconi anemia syndrome [20] and familial atypical multiple mole-melanoma syndrome [21]. In these cases, the family pedigree reveals the occurrence of other cancers such as breast or intestinal tumors, or melanomas, in addition to pancreatic tumors. However, most pancreatic cancers are sporadic. The molecular mechanism by which they occur involves the same alterations in somatic gene expression as in other cancers, which include KRAS2, BRCA2 and TP53 mutations, telomere shortening, p21 (WAF1/CIP1) and cyclin D1 upregulations, expression of BRCA2 and inactivation of p16INK4/CDKN2A (WAF1/CIP1) and cyclin D1 upregulations, expression of and BRCA2 variations (CNVs) lead to peculiar alterations of gene expression that predispose to pancreatic adenocarcinoma [28].

Furthermore, rare mutations in the YTHDC2 gene have been described in these cells are largely unknown [29]. In a recent work, Tanabe et al. showed that YTHDC2 plays an important role in cancer cell growth, the activation/recruitment of ATF-2 and c-Jun to the promoter region is necessary for the transcription of YTHDC2, and that histone deacetylase activity is required for the efficient expression of YTHDC2 in both hepatocyte and hepatocellular carcinoma cells [29]. Furthermore, rare mutations in the YTHDC2 gene have been identified in individuals with autism spectrum disorders [30].

2. Patients and methods

2.1 Sample collection
Written informed consent was obtained from all participants. We prospectively collected 72 DNA samples from the peripheral leukocytes of 72 patients with a diagnosis of sporadic pancreatic adenocarcinoma. Patients with a familial pancreatic cancer history were excluded from the study. Diagnosis of adenocarcinoma was confirmed by histologic analysis. All clinical information for each enrolled patient was recorded anonymously and coded. All DNA samples were of sufficient quality to be genotyped. Patients were European (32 from Brussels, 23 from Palermo, 11 from Barcelona and 6 from Leipzig, as self-reported in the presence of the physician). Of these patients, 39 were men and 33 were women, with a mean age of 66 years. Sixty DNA samples from individuals of European origin were used as controls. We used the HapMap database as a reference [23].

2.2 CNV analysis
DNA was extracted from whole blood using the QIAmp mini kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer’s instructions. The DNA yields and purity were determined spectrophotometrically by measuring the absorbance of aliquots at 260 and 280 nm. DNA was prepared for microarray hybridization using the GeneChip Mapping Assay Protocol (Affymetrix, Inc., Santa Clara, CA, USA) as previously described by Pugh et al. [31]. The raw images were analyzed using the GeneChip Operating Software (GCOS Ver1.4.1) and GTYPE (Ver4.1) software (Affymetrix). We excluded samples with a genotype call rate < 93% (http://www.biosstat.jhsph.edu/~iruczins/teaching/misc/gwas/papers/affymetrix2006.pdf) [32,33]. To assess CN alterations we used CNAT (Ver4.0.1) software.

We set the genomic smoothing at 0.01 Mb and kept default parameters for the other variables. CN estimates were obtained using data from 172 HapMap samples (available online) as a reference. Chromosome X was not analyzed to avoid gender-related complications [34]. Reproducibility of the method was assessed by analyzing six DNAs from patients with pancreatic adenocarcinoma in duplicate, the second analysis confirming > 96% of CNVs obtained in the first analysis.

2.3 Data analysis
Using SAS software and ad hoc programs, individual tables generated by CNAT (CNATv4.0.1) were merged and probes were ranked according to their CN value. Two lists containing the probes with CN gains (3 or 4 copies) or loss (1 or 0 copies) were generated. However, to avoid false-positive number variations due to random noise in signal intensity, we retained only the probes that showed the same condition, that is gain or loss, in all 72 patients. Selected probes were merged with their respective gene annotations and physical positions according to the NCBI human genome sequence using the NetAffx web server (Affymetrix).

2.4 Multiplex ligation-dependent probe amplification
Multiplex ligation-dependent probe amplification (MLPA) [35] was performed on genomic DNA isolated from peripheral
blood leukocytes using an MRC Holland’s EK1 kit, according to
the manufacturer’s protocol, using self-designed synthetic
MLPA probes. The MLPA reaction was performed in five main steps: i) DNA denaturation and hybridization of
MLPA probes; ii) ligation reaction; iii) polymerase chain
reaction (PCR); iv) separation of amplification products by electro-
phoresis; and v) data analysis. Following MRC-Holland
recommendations, we designed seven sets of synthetic MLPA
probes (Invitrogen, Carlsbad, CA, USA) to detect deletions
in several regions of the
YTHDC2
gene. To test the quan-
tity of the ‘home-made’ probes and performed MLPA reactions,
as well as facilitate data analysis, we used ‘SALSA MLPA kit
P200-A1 Reference probemix 1’ (MRC-Holland) as a refer-
ence internal control (data not shown). Synthetic probe mix
was obtained by combining 0.8 µl of each 1 µM oligo (half-
probe) solution in a final volume of 200 µl of Tris–EDTA.
For each MLPA reaction, we used 1 µl P200 + 0.5 µl synthetic
probe mix + 1.5 µl MLPA buffer. Ligation products were
amplified by PCR. The PCR conditions were 30 sec at 95°C,
30 sec at 60°C and 1 min at 72°C for 35 cycles; 20 min at
72°C. The resulting amplification products (from 130 to
480 nt in length) were separated and analyzed by capillary
electrophoresis using ABI 3100 Genetic Analyzer (Applied
Biosystems). GeneMapper® v3.5 software (Applied Biosys-
tems) was used to determine peak heights and areas, and frag-
ment sizes in base pairs (bp). Specific peaks corresponding
to each tested region of the YTHDC2 gene were identified accord-
ing to their migration in relation to size standards.

Peak heights and areas of each fragment were compared to
to those of 10 non-pancreatic cancer control samples and poten-
tial heterozygous deletions were suspected when peak height
and area differed by approximately 50%. Furthermore,
duplicate assays were performed to check the accuracy of the
MLPA analysis data.

3. Results

3.1 Germline DNA analysis reveals specific CNVs in
patients with pancreatic adenocarcinoma

In this work, we used the Affymetrix platform to genotype
500,000 unique probes in patients with pancreatic adenocarcin-
omia to investigate whether patients shared specific CNVs
associated with the disease that were not detected in individuals
without pancreatic cancer. Several DNA regions that showed
different CNV profiles in patients and controls were selected.
One of these, where the CNV profile was associated with
sporadic pancreatic cancer with high significance (p values
from 2.25E-11 to 6.87E-08 depending on the probe set),
was found particularly interesting. In this region, one allele
was deleted in 36 (50%) of the 72 analyzed patients, but not
in the controls. The deleted region is located on chromosome
5 (112,872,760 to 112,962,031) and its size ranges
from 85,687 to 41,608 nt (Table 2). This region contains the
YTHDC2 gene (112,877,309 to 112,958,880, total size
81,571 bp). The smallest deletions detected in patients encom-
pass at least 51% of the YTHDC2 gene, including several

Table 1. ‘Home-made’ MLPA probes and target sequences*.

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>YTHDC2 target sequence</th>
<th>Probe oligonucleotide sequences‡</th>
<th>Total probe size (bp) (LPO + RPO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GTCCAGTCAAAGCAAAAGCGGACTG</td>
<td>GACATCCCAAAAACGTTT</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>CAGTGGCCATGGCAACATGTTTTCGGAAGCTTACCAGTGTCACCATGATTTGGAGGTGCCAG</td>
<td>GCAATATGGATACATCA</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>TGCATTCACCACTTGTACGTCCGAGGAACTACGTTCGACTTCTTACCATGATTTGGAGGTGCCAG</td>
<td>GAAAGATCCTGCTGGACTA</td>
<td>104</td>
</tr>
<tr>
<td>4</td>
<td>AGGTGGAACTGCCTTACATTGCAGAAGATCCTGCTGGACTA</td>
<td>CTGGAGGTGATAGGGGTATACTA</td>
<td>112</td>
</tr>
<tr>
<td>5</td>
<td>CTGGAGGTGATAGGGGTATACTA</td>
<td>CAATTAGGTTATCA</td>
<td>116</td>
</tr>
<tr>
<td>6</td>
<td>CGGCTTGTCCGCTTACATTGCAGAAGATCCTGCTGGACTA</td>
<td>CTGGAGGTGATAGGGGTATACTA</td>
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</tr>
<tr>
<td>7</td>
<td>CGGCTTGTCCGCTTACATTGCAGAAGATCCTGCTGGACTA</td>
<td>CTGGAGGTGATAGGGGTATACTA</td>
<td>124</td>
</tr>
</tbody>
</table>

*LMPA probe sizes range from 96 to 124 bp.
‡Sequences do not include the universal primers located at the 5’ end of LPO and 3’ end of RPO.
LHS: Left hybridizing sequence; LPO: Left probe oligonucleotide; MLPA: Multiplex ligation-dependent probe amplification; RHS: Right hybridizing sequence; RPO: Right probe oligonucleotide.
exons (Figure 1). Interestingly, 19 of 36 patients showing het-

erozygous deletion in the DNA region containing YTHDC2

are Sicilian (52.8%). Therefore, 82.6% (19 of 23) of patients

from Palermo had a germline loss of one allele in that gene.

Furthermore, very similar results were found when segmen-
tation analysis was performed in parallel, using the Partek
Genomics Suite (Partek GS) (data not shown).

3.2 YTHDC2 may be a potential susceptibility gene

for sporadic pancreatic adenocarcinoma

The YTHDC2 (YTH domain containing 2) gene encodes for

a 1430-amino acid protein with a theoretical molecular

weight of 160,248 Da. The YTHDC2 protein contains seven

well-conserved domains, including an R3H domain (which

binds to ssDNA or ssRNA in a sequence-specific manner), a

DEAD-like helicase superfamily domain (with helicase

activity), an ANK-repeat domain (which mediates protein–

protein interactions), another DEAD-like helicase superfamily

domain with helicase activity, a HA2 domain found in

various helicases, potentially involved in nucleic acid binding,

a DUF1605 domain systematically found toward the C-

terminus of the DEAD box-containing helicases and a YTH

domain, which likely modulates alternative splice site selection

in a concentration-dependent manner. Although its function

Table 2. Patients with sporadic pancreatic cancer showing heterozygous deletion of a DNA region containing YTHDC2

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Start position</th>
<th>End position</th>
<th>Sample identification</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Smoking status</th>
<th>Length (bp)</th>
<th>CNV mean</th>
<th>zMarkers</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>112872760</td>
<td>112958447</td>
<td>210_M_13</td>
<td>F</td>
<td>77</td>
<td>F</td>
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<td>15</td>
<td>1.59E-04</td>
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<td>112956131</td>
<td>PBCN-4</td>
<td>F</td>
<td>60</td>
<td>C</td>
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<td>0.95</td>
<td>14</td>
<td>4.14E-06</td>
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<td>112956131</td>
<td>E4224</td>
<td>M</td>
<td>58</td>
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<td>83371</td>
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<td>112950168</td>
<td>210_M_16</td>
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<td>0.91</td>
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<td>1.14E-04</td>
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<td>112950168</td>
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<td>73</td>
<td>C</td>
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<td>13</td>
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<td>C</td>
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<td>96</td>
<td>N</td>
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<td>1.03</td>
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<td>5.93E-03</td>
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<td>112956131</td>
<td>210_M_22</td>
<td>F</td>
<td>83</td>
<td>N</td>
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<td>1.06</td>
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<td>1.06E-04</td>
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<tr>
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<td>112956131</td>
<td>PBCN6</td>
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<td>112956131</td>
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<td>F</td>
<td>66</td>
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<td>1.11</td>
<td>11</td>
<td>7.99E-03</td>
</tr>
<tr>
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<td>112956131</td>
<td>Paca6</td>
<td>F</td>
<td>66</td>
<td>C</td>
<td>71951</td>
<td>1.11</td>
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<td>69844</td>
<td>1.13</td>
<td>11</td>
<td>1.96E-04</td>
</tr>
</tbody>
</table>

*Chromosome = Chromosome where the segment is located; Start position = Base pair position on the chromosome at which the first marker in the segment begins (going from top of the p-arm to the bottom of the q-arm of the chromosome); End position = Base pair position on the chromosome at which the last marker in the segment begins; Sample identification = Sample name; Gender = Male (M) or Female (F); Smoking status = Current (C), Former (F), Never (N); Length (bp) = Size of the segment of copy number change; CNV mean = The mean value of CNV; zMarkers = Number of SNPs + CNV markers within the segment; p-value = p-value of CNV region.

bp: Base pairs; CNV: Copy number variation.
has not yet been studied, its primary structure strongly suggests that it is an ssRNA- or ssDNA-binding helicase. The fact that in Drosophila the *maleless* gene product, which has a similar organization (Figure 2), is a well-established helicase supports this hypothesis [36,37]. Therefore, YTHDC2 protein is expected to have several molecular functions such as ATP binding, ATP-dependent helicase activity, hydrolase activity, nucleic acid binding and nucleotide binding. Figure 3 shows some putative interactions of YTHDC2.

### 3.3 MLPA analysis

Because half of the patients with pancreatic adenocarcinoma showed a germline heterozygous deletion in a region of chromosome 5 containing *YTHDC2*, seven gene regions were selected for MLPA validation to confirm data previously obtained by genome-wide CNV analysis. An MLPA assay was performed in 36 patients showing loss of one allele, using a set of self-designed synthetic MLPA probes specific for seven target sequences of *YTHDC2* (Table 1). We normalized the samples by using peak height and area values and comparing the patients with 10 healthy controls that had no CNVs (deletions or amplifications) in *YTHDC2*, as confirmed by a previous analysis (data not shown). Heterozygous deletions were detected when peak height and area of each fragment were reduced by approximately 50% with respect to controls (Figure 4). MLPA analysis indicated that *YTHDC2* target sequences showed a putative germline heterozygous deletion only in 10 of 36 (27.8%) patients with pancreatic adenocarcinoma, partially confirming the previously obtained CNV analysis data (Table S1). Interestingly, among these, six patients are from Palermo, in part confirming the relatively higher percentage of patients belonging to this geographical area showing germline loss of one allele of *YTHDC2*.
4. Discussion

Pancreatic cancer is one of the most common fatal malignant tumors worldwide with poor prognosis and frequent resistance to conventional therapies such as radiotherapy or chemotherapy [38-40]. CNV has recently gained considerable interest as a source of genetic variation as it seems to play a role in phenotypic diversity and evolution [25]. Associations between CN changes and complex diseases were discovered in whole-genome association studies. Furthermore, genome analysis provided a powerful approach to test for evidence of genetic variations within and between geographical regions and local populations [41,42]. In a recent work, Chen et al. reported that whereas the overall CN variant frequencies are similar between populations, their distribution is highly specific to the population of origin [43]. DNA samples used in this study include those from groups of individuals from different geographical areas so as to facilitate detection of possible population-specific common variants. However, this approach involves that variants that are rare and population-restricted could be not detected because the number of individuals examined in each population is small. The germline CNV identified in this study was found in 36 of the 72 analyzed patients with sporadic pancreatic adenocarcinoma, but in none of the 60 controls, indicating that this DNA anomaly is frequent in patients and rare in non-affected...
If this alteration generates a functional defect involved in the occurrence of sporadic pancreatic cancer, its absence in half of the patients should be explained. At least two possibilities can be considered. The first is that other functional defects, other than a defect in YTHDC2 gene, can trigger pancreatic adenocarcinoma. Indeed, we found other CNVs associated with pancreatic cancer with a lower frequency that might account for such defects. The second possible explanation is technical. We cannot exclude the possibility that short DNA losses or other DNA anomalies involving this region were undetectable with our approach. These include inversions, insertions and more complex rearrangements, which are found in other patients with pancreatic cancer.

Interestingly, our genome microarray analysis detected that 19 of 23 (82.6%) patients from Palermo (Italy) showed germline heterozygous deletion in the DNA region containing YTHDC2. MLPA analysis further confirmed that most of the patients showing loss of one allele in YTHDC2 (27.8%) belongs to geographical area of Palermo (Italy). Because CNVs may differ greatly among different populations, we hypothesize a putative population-specific CNV in this region. However, further investigations are needed to confirm this hypothesis. Therefore, we suggest that YTHDC2 could be a potential candidate for susceptibility to pancreatic cancer. To our knowledge, this is the first report that associates a particular CNV with susceptibility to a sporadic cancer. Interestingly, results from a microarray analysis on familial pancreatic cancer [44] showed that patients with a familial history of pancreatic cancer had a total of 56 unique germline genomic regions with CNVs that were not present in the controls, including 31 amplifications and 25 deletions. These patients did not show DNA

Figure 4. Heterozygous deletion of YTHDC2 gene. MLPA-derived amplification products were separated by electrophoresis and peak patterns were generated from a control DNA sample (A) and a patient sample (B). Relative amounts of probe amplification products, compared to a control DNA sample, reflect the relative copy number of seven target sequences. The comparison between the peak heights showed a reduction by approximately 50% in patient sample with respect to control. MLPA: Multiplex ligation-dependent probe amplification.
anomalies in the YTHDC2 gene region, suggesting that familial and sporadic pancreatic cancers develop through different pathways. In addition, a truncating germline mutation in the PALB2 gene was found in several patients with familial pancreatic cancer. PALB2 protein is a binding partner for BRCA2. PALB2 mutations have previously been reported in patients with familial breast cancer, and PALB2 is now considered to be a susceptibility gene for pancreatic cancer [45]. An attractive hypothesis is that genomic alterations associated with inherited pancreatic cancer have sufficient penetrance to trigger the disease, whereas alterations associated with sporadic pancreatic cancer require the additional influence of environmental factors [22]. In 2009, Amundadottir et al. [46] identified an SNP that maps to the first intron of the ABO blood group gene, which is significantly associated with pancreatic cancer, suggesting that people with blood group O may have a lower risk than those with groups A or B. However, the involvement of this SNP in susceptibility to pancreatic cancer remains to be established.

We cannot exclude the possibility that a specific CNV is also associated with other forms of cancer. Evaluating pancreatic specificity by repeating the analysis with a series of DNA samples from patients with other cancers was beyond the scope of this study. However, if cancer is the consequence of a given series of inherited genetic imbalances, the corresponding CNV should be found in a population of individuals chosen at random, in a proportion corresponding to cancer occurrence in humans (American Cancer Society 2007). We did not find the same proportion of CNV (~20%) in our control population, suggesting that there is no clear genetic predisposition to cancer in general, but that more specific genetic abnormalities predispose individuals to cancers of specific organs. The CNV identified in this study, associated with susceptibility to pancreatic adenocarcinoma, might be an example of such specificity.

The CNV selected in this study comprises at least 51% of the YTHDC2 gene, including several exons, indicating that one allele is inactivated in patients with sporadic pancreatic cancer. The YTHDC2 gene encodes for a putative helicase, well conserved in pluricellular eukaryotes, including early organisms. The YTHDC2 function has not yet been established, but several proteins with similar structures are DNA and/or RNA helicases. How the deletion of one YTHDC2 allele is a potential susceptibility factor for pancreatic adenocarcinoma also remains to be elucidated. However, activity loss of several helicases has been implicated in breast and prostate cancer susceptibility. Helicases are involved in DNA and RNA metabolism, including DNA repair and recombination, chromosomal stability, splicing and removal of proteins from RNA. Some may act as viral receptors, others may be involved in transcription regulation and some are involved in the initial steps of eukaryotic translational mechanism. Many functions are strongly associated with the cancer development.

5. Conclusions

This is a preliminary/pilot study performed on individuals from different geographical areas in order to identify new possible common variants in sporadic pancreatic adenocarcinoma. However, we are looking at expanding our sample size to carry out a research more extended and to further confirm obtained results. The CNV reported in this study may help increase our understanding of the physiopathology of pancreatic cancer because gene deletions generate imbalances in the corresponding mRNA and encoded protein levels. For genes and pathways critically dependent on a fixed amount of a functional product, it seems likely that CNVs could account for individual variations in disease susceptibility [47]. Also, the interaction between germline modifications and somatic mutations could influence the phenotype. For instance, the result of a somatic mutation occurring in a gene whose expression is restricted to a single allele would correspond to a knock-out. In conclusion, these data suggest that the occurrence of pancreatic cancer involves a combination of specific somatic mutations and germline alterations. Knowledge of the genes whose CN is altered in pancreatic cancer could be useful for identifying patients at high risk of developing this disease both for diagnosis and also to possibly reveal new gene targets for preventive or curative strategies.

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D Fanale and JL Iovanna contributed equally to this work.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.
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Supplementary materials available online
Table S1.