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Association between a polymorphic variant in the *CDKN2B-AS1/ANRIL* gene and pancreatic cancer risk

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Abstract

Genes carrying high-penetrance germline mutations may also be associated with cancer susceptibility through common low-penetrance genetic variants. To increase the knowledge on genetic pancreatic ductal adenocarcinoma (PDAC) aetiology, the common genetic variability of PDAC familial genes was analysed in our study. We conducted a multiphase study analysing 7745 single nucleotide polymorphisms (SNPs) from 29 genes reported to harbour a high-penetrance PDAC-associated mutation in at least one published study. To assess the effect of the SNPs on PDAC risk, a total of 14 666 PDAC cases and 221 897 controls across five different studies were analysed. The T allele of the rs1412832 polymorphism, that is situated in the *CDKN2B-AS1/ANRIL*, showed a genome-wide significant association with increased risk of developing PDAC (OR = 1.11, 95% CI = 1.07-1.15, $P = 5.25 \times 10^{-9}$). *CDKN2B-AS1/ANRIL* is a long noncoding RNA, situated in 9p21.3, and regulates many target genes, among which *CDKN2A* (p16) that

Abbreviations: GWAS, genome-wide association study; JaPAN, Japan Pancreatic Cancer Research Consortium; lncRNA, long noncoding RNA; MAF, minor allele frequency; PanC4, Pancreatic Cancer Case-Control Consortium; PANDoRA, Pancreatic Diseases Research Consortium; PanScan, Pancreatic Cancer Cohort Consortium; PCA, principal component analysis; PDAC, pancreatic ductal adenocarcinoma; PNET, neuroendocrine pancreatic cancer; SNPs, single nucleotide polymorphism.

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frequently shows deleterious somatic and germline mutations and deregulation in PDAC. Our results strongly support the role of the genetic variability of the 9p21.3 region in PDAC aetiopathogenesis and highlight the importance of secondary analysis as a tool for discovering new risk loci in complex human diseases.

KEYWORDS
association study, genetic susceptibility, pancreatic ductal adenocarcinoma, single nucleotide polymorphisms

What's new?

Pancreatic ductal adenocarcinoma (PDAC) has a very low survival rate, yet little is known about the factors that contribute to its onset. Here, the authors analysed SNPs in 29 familial PDAC genes, which can carry high-penetrance mutations for PDAC, looking for possible low-penetrance mutations that contribute to PDAC susceptibility. They found a novel PDAC risk variant in *CDKN2B-AS1/ANRIL*, a long non-coding RNA that regulates the expression of several other genes.

1 | INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of exocrine pancreatic cancer. It is a relatively rare disease, with a 9% to 10% survival 5 years after diagnosis and an incidence rate approaching mortality.[1] One of the reasons for this very low survival is the lack of preventive strategies partially due to the lack of knowledge on causative environmental and lifestyle factors since only a small number have been unequivocally determined.[2,3] Compared to more common solid tumours also the genetic susceptibility of PDAC has only partially been explored with a very limited number of high-penetrance mutations identified in genes commonly mutated in cancer.[4]

Genome-wide association studies (GWAS) and more focused studies arising from large international consortia have identified several frequent polymorphisms with low penetrance that alone or in combination play a role in PDAC susceptibility.[4-6] However, the proportion of hereditability that remains to be discovered remains high, suggesting that more susceptibility loci need to be identified.[7] Secondary analysis of published GWAS data, followed by a replication in one or more independent populations is an increasingly popular and successful approach to identify regions associated with complex traits. For example, in PDAC secondary analyses have identified regulatory variants associated with the disease that have also helped in elucidating the molecular biology of PDAC.[8-10]

It has been observed that common, low-penetrance genetic polymorphisms in or near genes harbouring high-penetrance germline mutations are associated with the genetic susceptibility to develop cancer. For example, the *CHEK2* and *BRCA1* genes for breast cancer,[11] the *TERT* gene for melanoma,[12] the *CDKN2A/2B* genes for PDAC and pancreatic neuroendocrine tumours[13,14] and the *p53* gene for multiple cancers have both high-penetrance and low-penetrance variants that contribute to the disease risk.[15-18] Even though this phenomenon is not widespread, at least according to the current literature, exploring common variants in genes known to harbour high-penetrance variants in PDAC could still be valuable for understanding the risk of developing PDAC.

Since only a small percentage of the heritability of PDAC has been identified so far, our study aimed to systematically analyse the common genetic variability of PDAC familial genes (ie, genes that present rare, high-penetrance mutations in familial pancreatic cancers), to identify whether low-penetrance variants in those genes contribute to the genetic susceptibility of this disease.

2 | MATERIALS AND METHODS

The study was designed in three phases: selection, discovery and replication. For the discovery and replication phases four distinct GWAS and additional cases and controls from the Pancreatic Diseases Research (PANDoRA) consortium were used (details below). The final sample size was 14 666 PDAC cases and 221 897 controls.

2.1 | Identification phase

At first, 29 genes harbouring high-penetrance PDAC-associated germline mutations, either in sporadic or familial cases, were selected based on a recent review[4] (Table S1). We included in the selection also the *CDKN2B-AS1* since the genomic context of that region on 9p21.3 is complex, as is the regulation of the genes at the locus. To include regulatory regions, we also added 5 kb at the 5′ and 3′ end of each gene. Then, all SNPs in the chosen loci were selected, resulting in 8987 SNPs covering all the common genetic variability (minor allele frequency, MAF >0.01) of the 29 identified genes.

2.2 | Discovery phase

In the discovery phase, the genotypes of 8769 cases and 7322 controls obtained from Pancreatic Cancer Case-Control Consortium (PanC4) and the Pancreatic Cancer Cohort Consortium (PanScan) I-III

were downloaded from the database of Genotypes and Phenotypes (dbGaP, study accession nos. phs000206.v5.p3 and phs000648.v1.p1, project reference #12644). After download of the data imputation was performed using the Michigan Imputation Server (https://imputationserver.sph.umich.edu) and the genotypes of the Haplotype Reference Consortium (HRC, V.r 1.1) as reference panel. Before imputation, quality control filters were applied. The subjects with call rate <0.9, minimal or excessive heterozygosity (>3 SD from the mean), gender mismatches, or cryptic relatedness (PI_HAT >0.2) were excluded from the datasets. To estimate the population substructure, principal component analysis (PCA) was performed using PLINK 2.0 (www.cog-genomics.org/plink/2.0/) including genotypes from all the populations of phase 3 of the 1000 Genome project. We selected the minimal number of principal components that explain more than 98% of the global variation of the genetic data based on the eigenvalue calculated during the calculation of the principal components. Individuals not clustering in the PCA with Europeans of 1000 Genomes were excluded from further analysis. Rare variants with the MAF <0.005, call rate <0.9 or evidence for violations of Hardy-Weinberg equilibrium ($P < 1 \times 10^{-6}$) were excluded. All datasets were imputed separately considering that different genotyping arrays were used in the original publications. After imputation, the datasets were merged and the SNPs with INFO score $r^2 < .7$, MAF <0.005 or in LD among them ($r^2 > .8$) were excluded. After all the filters a total number of 7745 SNPs belonging to the candidate genes was analysed.

2.3 | Replication phase

For the replication, the summary statistics of the Japan Pancreatic Cancer Research (JaPAN) consortium[19] and FinnGen studies were used, for a total of 2644 cases and 206 598 controls. More details on data filtering and quality control procedures are given in the original publications or in the respective websites (www.aichi-med-u.ac.jp/JaPAN and www.finngen.fi). In addition, the SNPs showing a statistically significant association ($P < .05$) in all datasets were also genotyped in cases and controls form PANDoRA. The consortium has been previously described in detail. Briefly it consists if studies mainly from European countries (Greece, Italy, Germany, Netherlands, Denmark, Czech Republic, Hungary, Poland, Ukraine, Lithuania and the United Kingdom) and Brazil. For each participant, information on sex, age at recruitment for controls, age at diagnosis for the cases was collected. The controls were enrolled among the general population, blood donors or hospitalised individuals not affected by cancer, chronic pancreatitis or diabetes.[20] For our study, 4707 individuals (1686 cases and 3021 controls) were used in the replication analysis (Table S2). Genotyping in PANDoRA was performed using TaqMan technology using 384-well plates and adding to each plate negative controls and duplicate samples (~8%) to guarantee the correctness of the laboratory procedures. To determine the genotypes QuantStudio 5 Real-Time PCR system (Thermofisher, USA) was used. Concordance rate of genotypes of duplicate samples was higher than 99%.

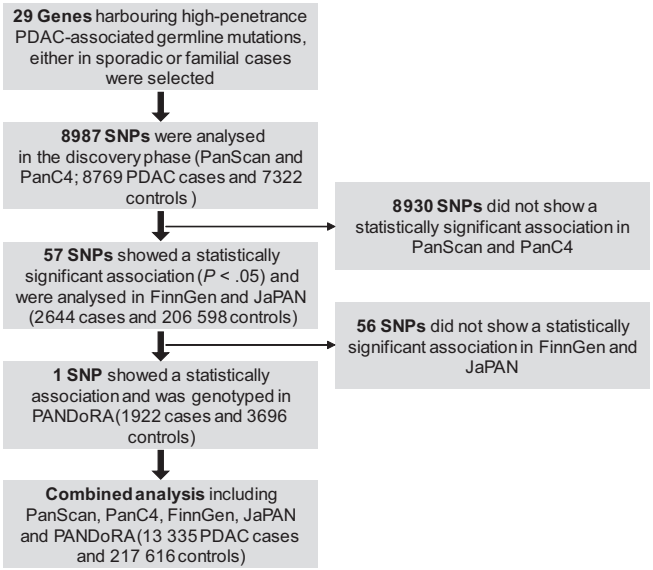

FIGURE 1 flowchart of the SNP selection

2.4 | Association analysis

The genotypes were analysed with unconditional logistic regression in the discovery phase, adjusting for sex, age, and the best eight principal components. All SNPs that showed a statistically significant ($P < .05$) association in PanC4, PanScan I-III and the pooled dataset were selected to be further analysed. In the replication phase, all SNPs significantly associated also in JaPAN and FinnGen were also analysed in PANDoRA, using logistic regression adjusting for sex, age (at recruitment for controls, at diagnosis for cases) and country of origin, since GWAS data were not available, thus principal component analysis was not feasible in PANDoRA. Finally, a meta-analysis considering all the populations together was performed for all the variants that showed a statistically significant association in PanC4, PanScan I-III JaPAN and FinnGen. In this meta-analysis, a study conducted in a sample of Chinese individuals comprising 1567 PDAC and 4956 controls was also included. To calculate the threshold for statistical significance corrected for multiple testing we used the set of 7745 independent SNPs ($r^2 < .8$) tested in the discovery phase. The significance threshold was set to $P = 6.46 \times 10^{-6}$ (0.05/7745). A schematic representation of the workflow is presented in Figure 1.

2.5 | Functional characterisation of the results

For the SNPs that showed a statistically significant association with PDAC risk a functional characterisation was carried out using three datasets. The Genotype-Tissue Expression (GTEx) project[21] data were used to analyse the SNPs in relation to gene expression to determine if they are expression quantitative trait loci (eQTLs). HaploReg[22] and RegulomeDB[23] were used to assess to the regulatory potential of the SNPs.

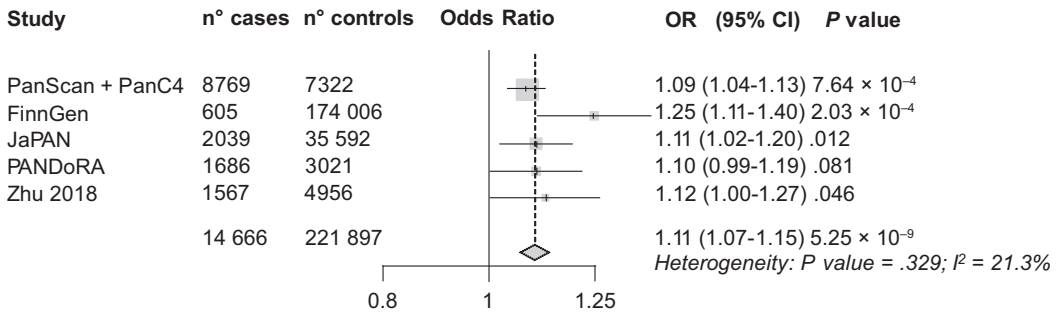

FIGURE 2 Forest plot of the meta-analysis for rs1412832-T

3 | RESULTS

In the discovery phase, 57 SNPs located in three independent loci (*CDKN2B-AS1*, *CHEK2* and *TP53*) showed a statistically significant association ($P < .05$) independently in PanScan I-III, PanC4 and the pooled dataset (Table S3). These loci are characterised by a very strong linkage disequilibrium (LD; $r^2 > .8$ in Europeans, considering the SNPs at the same locus), and all the SNPs represent the same association signal at their respective genomic region. In the replication phase, one SNP (rs1412832-T) located in *CDKN2B-AS1* showed a statistically significant association both in JaPAN (OR = 1.08, 95% CI = 1.04-1.13, $P = .012$) and FinnGen (OR = 1.25, 95% CI = 1.114-1.40, $P = 2.03 \times 10^{-4}$) and therefore was genotyped also in PANDoRA. None of the other SNPs showed any association in both datasets, but 43 SNPs belonging to *CDKN2B-AS1* showed an association in FinnGen and four SNPs in *CHEK2* were, instead, associated in JaPAN (Table S3).

CDKN2B-AS1-rs1412832-T showed a borderline association (OR = 1.10, 95% CI = 0.99-1.22, $P = .081$) in PANDoRA going in the same direction as the discovery phase. A combined analysis of this SNP, consisting of all the datasets together (PanC4, PanScan I-III, JaPAN, FinnGen and PANDoRA), showed an association between the T allele and increased risk of developing PDAC below the genome-wide significance threshold (OR = 1.11, 95% CI = 1.07-1.15, $P = 3.92 \times 10^{-8}$). Additionally, in a study focusing on Chinese individuals Zhu and colleagues investigated the 9p21.3 region, in a sample of 1567 PDAC and 4956 controls, and found numerous associations with risk of developing the disease.[24] They also have analysed rs1412832 and reported an association that is very similar to what we observed: OR = 1.12, 95% CI = 1.00-1.27, $P = .046$.[24] We performed a meta-analysis with their results and obtained an even more statistically significant association (OR = 1.11, 95% CI = 1.07-1.15, $P = 5.25 \times 10^{-9}$, $I^2 = 21.3\%$). The result of the meta-analysis is reported in Figure 2. Additionally, a visual representation of the results, using LocusZoom and divided by ancestry is given in Figures **S1 to S3**.

CDKN2B-AS1-rs1412832 is an eQTL for the *CDKN2B-AS1* gene. In detail the T allele is associated with increased expression of the gene (NES 0.28, $P = 2.3 \times 10^{-7}$).

4 | DISCUSSION

The aim of the study was to find novel low-penetrance polymorphisms associated with the risk of developing PDAC. We have analysed 29 regions known to harbour rare, high-penetrance germline mutations commonly found in familial pancreatic cancer cases. Using a tagging approach, we have investigated all the common (MAF >0.01) genetic variability in those regions. We used a secondary analysis approach and a multistep process that included, also considering the summary statistics from Zhu and colleagues, six different populations of different ethnicities including a total of 14 666 cases and 221 897 controls.

Our results identified *CDKN2B-AS1*-rs1412832-T, situated on chromosome 9p21.3, to be associated with PDAC risk with a level of significance lower than the threshold imposed by multiple testing. The T allele was associated with increased risk of developing PDAC. The association was significant in five out of the six populations used and was borderline in the last one, with an effect very similar in all populations, resulting in no heterogeneity ($I^2 = 21.3\%$). Considering the meta-analysis of all the subjects together the association was below the genome-wide significance threshold ($P = 5.25 \times 10^{-9}$).

The 9p21.3-rs1412832 lies in a long noncoding RNA (lncRNA) gene called *CDKN2B-AS1/ANRIL*. This lncRNA modulates the expression of several genes situated in its proximity, among which *CDKN2B* (p16), that is frequently mutated in several cancer types, including familial and sporadic pancreatic cancer, supporting our theory that common genetic variability in high-penetrance mutation genes may influence pancreatic cancer development. According to GTEx, the T allele of 9p21.3-rs1412832 increases the expression of *CDKN2B-AS1/ANRIL* in fibroblasts. Considering that this antisense downregulates *CDKN2B* it is plausible to hypothesise that the T allele, that is associated with increased PDAC risk downregulates *CDKN2B*. This is particularly interesting given that fibroblasts are a key component of pancreatic cancer tumour microenvironment and are involved in the crosstalk between cancer cells and infiltrating immunocytes.[25] Considering that *CDKN2B-AS1/ANRIL* regulates many targets it is not surprising that the genetic variability at this locus is associated with a plethora of human traits. The list of the 102 associations reported in GWAS Catalogue includes risk of breast cancer, colorectal cancer, various upper respiratory cancers, glioma and diabetes. To support the

association between 9p21.3 and pancreatic cancer there are two studies conducted within the PANDoRA consortium that report the association of two SNPs at this locus with pancreatic cancer risk. One, rs3217992 is associated with PDAC risk,[20] the other, rs2518719 (r^2 = .075) with neuroendocrine pancreatic cancer (PNET) risk.[14] The first SNP (rs3217992) is situated in the same gene as rs1412832 (74 319 bps apart) but shares a very weak LD both in individuals of European ethnicity (r^2 = .26; 1000 genomes, Europeans) and in Asians (r^2 = .03; 1000 genomes, East Asians), while the second SNP (rs2518719) is a missense variant of the *CDKN2A* gene and is completely independent from rs1412832 in Caucasians (r^2 = .075) and monomorphic in Asians. Therefore, the LD values indicate that all these SNPs represent independent signals across a region of 107 115 kb.

All this evidence strongly suggests a causative association between one or more polymorphic variants in the 9p21.3 region and pancreatic cancer risk; however, it remains unclear which variant or variants are responsible for the associations identified. Considering that rs1412832 is situated in a lncRNA that has multiple targets makes it an intriguing candidate; however, fine-mapping approaches, using high coverage sequencing, followed by functional studies, will be needed to better understand the biologic mechanisms that link the complex regulation of this locus, its genetic variability and so many complex human phenotypes, including pancreatic cancer. However, considering the complex genomic context of the 9p21.3 locus, even with Next Generation Sequencing techniques it would be difficult to define which of the polymorphisms is responsible for the associations. It is also plausible that the increase in PDAC risk may result from the interplay or additive effect of many low penetrance variants in the region. Considering the importance of this locus in so many human phenotypes several studies have attempted to better characterise the mechanistic link between the genetic variability and the traits but, at present, it still is not clear. One of the more popular hypotheses is, however, the deregulation of p16 gene product.[26]

A clear strength of our study, apart from the large sample size permitting to identify even small effects, is that we observed an association in independent populations of different ethnicity. The majority of GWAS loci have been identified in Caucasians and interethnic validation of risk SNPs is certainly warranted, although varying LD blocks in different populations makes this task difficult.[27] Genetic diversity among different ethnicities is clearly a hot topic in genetics and finding a susceptibility marker that is valid across Europeans and Asian is important for a better understanding the disease aetiology.

In addition, the statistically significant association across multiple ethnicities dramatically decreases the chances of this been a spurious finding.

Another association worth mentioning is between polymorphisms in the *CHEK2* locus and PDAC risk. One of the polymorphisms in that region was already reported as a susceptibility locus by a GWAS and therefore was not genotyped in PANDoRA since the consortium was part of the replication in the original article. Interestingly though, all the *CHEK2* SNPs significant in the discovery phase showed a statistically significant association in JaPAN but not in FinnGen, highlighting once again the importance of validating susceptibility loci across multiple ethnicities.

Our study also highlights the importance of secondary analysis of GWAS data, using large replication populations to validate the findings, as a tool for discovering new risk loci in complex human disease. In fact, rs1412832 has not shown a genome-wide significant association in any of the datasets and therefore it was not followed up in the replication set of any of the published GWAS, representing a clear false negative. However, combining all the datasets together we observed a robust statistical association that was consistent in all populations, also considering the agreement with the literature. Therefore, GWAS results represent just the tip of the iceberg and more studies like the one performed here are needed to uncover more of the still missing heritability and the genetic architecture of PDAC. Furthermore, fine mapping approaches of large studies and consortia will be extremely useful in understanding the mechanistic link discovered in association studies.

In conclusion, our results suggest that a common variant in a regulatory region close to a gene that frequently presents high penetrance mutations is also associated with PDAC risk, through a very robust genome wide statistical association.

AUTHOR CONTRIBUTIONS

Daniele Campa: Conceived and designed the study; wrote the first draft of the article. **Matteo Giaccherini:** Performed the lab work; analysed the data; wrote the first draft of the article. **Riccardo Farinella:** Performed the lab work. **Manuel Gentiluomo:** Analysed the data. All the authors contributed with the interpretation of the data. All authors contributed to the writing and approve of the final version of the article. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

DATA AVAILABILITY STATEMENT

For the publicly available data used in our study (PanScan, PanC4, JaPAN, FinnGen) data sources and handling of the data are described in the Materials and Methods. The PANDoRA primary data for this work will be made available to researchers who submit a reasonable request to the corresponding author, conditional to approval by the PANDoRA Steering Committee and the Ethics Commission of the Medical Faculty of the University of Heidelberg. Data will be stripped from all information allowing identification of study participants.

ETHICS STATEMENT

Each participating study obtained approval from the responsible institutional review board (IRB) and IRB certification permitting data sharing in accordance with the NIH Policy for sharing of Data Obtained in NIH-Supported or NIH-Conducted Genome Wide Association Studies. The PANDoRA study protocol was approved by the Ethics Commission of the Medical Faculty of the University of Heidelberg. In accordance with the Declaration of Helsinki, written informed consent was obtained from each participant. The FinnGen study was approved by the ethical Review Board of the Hospital District of Helsinki and Uusimaa. FinnGen participants provided written, informed consent. For JaPAN, written informed consent was obtained from all study participants, and the study protocol was approved by the Ethical Review Board of Aichi Medical University, the Institutional Ethics Committee of Aichi Cancer Center, the Human Genome and Gene Analysis Research Ethics Committee of Nagoya University, and the ethics committees of all participating hospitals.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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