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Whole genome sequencing identifies rare germline variants enriched in cancer related genes in first degree relatives of familial pancreatic cancer patients

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Abstract
First-degree relatives (FDRs) of familial pancreatic cancer (FPC) patients have increased risk of developing pancreatic ductal adenocarcinoma (PDAC). Investigating and understanding the genetic basis for PDAC susceptibility in FPC predisposed families may contribute toward future risk-assessment and management of high-risk individuals. Using a Danish cohort of 27 FPC families, we performed whole-genome sequencing of 61 FDRs of FPC patients focusing on rare genetic variants that may contribute to familial aggregation of PDAC. Statistical analysis was performed using the gnomAD database as external controls. Through analysis of heterozygous premature truncating variants (PTV), we identified cancer-related genes and cancer-driver genes harboring multiple germline mutations. Association analysis detected 20 significant genes with false discovery rate, q < 0.05 including: PALD1, LRP1B, COL4A2, CYLC2, ZFYVE9, BRD3, AHDC1, etc. Functional annotation showed that the significant genes were enriched by gene clusters encoding for extracellular matrix and associated proteins. PTV genes were over-represented by functions related to transport of small molecules, innate immune system, ion channel transport, and stimuli-sensing channels. In conclusion, FDRs of FPC patients carry rare germline variants related to cancer pathogenesis that may contribute to increased susceptibility to PDAC. The identified variants may potentially be useful for risk prediction of high-risk individuals in predisposed families.

KEYWORDS
cancer genes, familial pancreatic cancer, first-degree relatives, rare germline variants, whole genome sequencing
1 | INTRODUCTION

The global incidence of pancreatic cancer is 5.5 per 100,000 for men and 4.0 per 100,000 for women. Among the cases, 5%–10% are estimated to be familial pancreatic cancer (FPC). FPC is an inherited malignancy with familial clustering, defined by presentation of at least one pair of first-degree relatives (FDRs) with pancreatic ductal adenocarcinoma (PDAC), in the absence of a known hereditary cancer syndrome. Familial aggregation of FPC confers increased risk of PDAC among relatives. It is estimated that in FPC predisposed families, individuals with 2 FDRs with PDAC have a 6.4-fold higher risk of developing PDAC, while individuals with at least 3 FDRs with PDAC have a 32-fold higher risk of disease. These reports characterize predisposed FDRs as high-risk individuals (HRIs). International consensus guidelines recommend yearly screening of HRIs including FDRs in FPC predisposed families – with genetic testing for PDAC predisposition genes being a potential tool for future risk assessment and stratification of HRIs.

We have recently analyzed familial correlation of FPC in a Danish nationwide family cohort and estimated a high heritability of 51% in FDRs to FPC patients. The high genetic predisposition for FPC calls for efforts to identify genetic variations underlying the pathogenesis of the disease. In the literature, multiple genome-wide association studies (GWASs) have been performed and addressed common single nucleotide polymorphisms (SNPs) associated with sporadic pancreatic cancer (SPC). However, only a few studies have focused on FPC, perhaps due to limited sample availability, as FPC represents a rare sub-group of pancreatic cancer, estimated to be accountable for around 10% of all PDAC cases.

Despite great efforts, previous genetic association studies have only detected a limited number of susceptibility loci for PDAC. GWAS is based on a “common disease, common variant” hypothesis with common variants referring to allelic variants present in more than 1%–5% of the population. The common variants confer relatively small increments in the risk of disease. Instead of focusing on common SNPs, a better choice is to analyze rare single nucleotide variants (SNVs) using next generation sequencing (NGS) and statistical models for joint analysis of rare variants.

Studies using NGS techniques have identified prominent gene mutations in BRCA1, BRCA2, CDKN2A, PALB2, and ATM to be associated with FPC. Nonetheless, only about 12% of all PDAC cases carry any of these mutations – meaning that the germline-component of >80% of all FPC cases remains unknown. A recent whole genome sequencing (WGS) analysis has demonstrated that the genetic architecture of FPC is highly heterogeneous, and the currently identified genetic variants account for a limited genetic component underlying the disease susceptibility. The genetic heterogeneity of FPC means that susceptibility variants could be private to certain individuals or families – a situation that imposes challenges in identifying the relevant genetic variants.

The high degree of genetic similarity between FPC patients and their FDRs, 50% on average, suggests that the latter are valuable samples for conducting genetic association studies. We have performed the first Nordic WGS study on FDRs of FPC patients from a nationwide cohort in the Danish population. Analysis and characterization of rare germline variants in FDRs of FPC patients could help reveal the molecular basis underlying the high genetic susceptibility of FPC.

2 | MATERIALS AND METHODS

2.1 | Sample collection

A nationwide cohort of 27 Danish families with susceptibility to FPC is currently included in a screening program for PDAC at the Department of Medical Gastroenterology, Odense University Hospital, Denmark. Each family was diagnosed with genetic predisposition to FPC at the department of clinical genetics in their home region, prior to inclusion in the screening program. In concordance with previous definitions at our institution and international consensus criteria, familial predisposition for FPC was defined as presence of either: (1) Two FDRs with PDAC, with at least one of the cases debuting at age < 50 year; or (2) at least three FDRs with PDAC.

FDRs to the FPC cases in each family are offered inclusion in the screening program after reaching a certain age (i.e. 5 years younger than the earliest age of onset of PDAC in the family); but no later than at 50 years of age. The screening program for PDAC of FDRs includes yearly imaging of the pancreas (with endoscopic ultrasonography, and fine needle biopsy if relevant), along with PDAC blood markers (i.e. Cancer antigen 19–9, CA19–9) – with the possibility to individualize the program for each individual.

Individuals in the screening program, comprising of FDRs to FPC patients currently without presentation of PDAC, received informed consent to participate in the WGS study. Sixty-one FDRs were included in the study, and a sample of 10 mL full blood was collected from each individual for sequencing analysis. The cohort profiles of the FPC predisposed families included in the screening program are described in detail in a previous study.

2.2 | Ethics

Data and sample collection from relevant individuals were conducted with the approvals from the Danish National Committee on Health Research Ethics (NVK) (project number: 1604008) and the Danish Data Protection Agency (project number: 18/54160).

2.3 | Sequencing analysis

A total of 61 FDRs from FPC patients were whole genome sequenced using DNA extracted from peripheral blood. In brief, 20 μg of genomic DNA per sample was sequenced using the TruSeq DNA PCR free kit (Illumina, Inc). Sequencing was performed on a NovaSeq 6000 (Illumina, Inc). Sequence reads were analyzed and aligned to the human reference genome (hg19) using Illumina DRAGEN software.
Variants were annotated using VarSeq (Golden Helix, Inc.) with (i) functional consequence in RefSeq gene transcripts, (ii) zygosity, (iii) minor allele frequency (MAF) determined using publicly available variant databases (gnomAD) and (iv) presence in ClinVar.

2.4 Filtering and interpretation of variants

We applied filtering using VarSeq (https://www.goldenhelix.com/products/VarSeq/), version 2.2.1 (Golden Helix, Inc.) for downstream filtering. All variants were first filtered with a minimum of 10× coverage, nonsynonymous, and presented in the exome region or splice sites, which represented a range of 60.4%–95.6% of targeted bases. Filtered variants were then processed twice, one for each parameter. The first parameter which covers the possibility of a compound heterozygous, autosomal recessive, multifactorial inheritance, or de novo, was set to a population frequency of ≤0.01 (gnomAD and ExAC). The second parameter, which covers the dominant inheritance of single nucleotide polymorphisms (SNPs) and small insertions and deletions (INDELs), was set to a frequency of ≤0.0001. We removed sequence variants belonging to (1) pseudogenes using annotations provided by EnsDb.Hsapiens.v86 package in Bioconductor (DOI: 10.18129/B9.bioc.EnsDb.Hsapiens.v86); and (2) segmental duplication (humanparalogy.gs.washington.edu). Multi-mapped reads and artefacts were also removed from subsequent analysis.

Variants were then classified into (1) a group of functional variants including frameshift variants, inframe deletion, inframe insertion, initiator codon variants, splice acceptor variants, splice donor variants, stop gained variants and missense variants; and (2) a group of synonymous variants including splice region variants, stop retained variants, and 5′ UTR premature start codon gain variants.

VarSeq (https://www.goldenhelix.com/products/VarSeq/) was used for functional prediction of nonsynonymous variants. Clinical significance (benign, likely benign, pathogenic, likely pathogenic, uncertain significance, etc.) of variants was assessed based on ClinVar submitted records as recommended by ACMG/AMP, and on evaluation by local clinicians and biologists using an inhouse assessment catalog. Variants assessed as benign or likely benign were filtered out from the nonsynonymous group. Likewise, variants assessed as pathogenic or likely pathogenic were removed from the synonymous group.

Functional interpretation of SNVs was provided by dbNSFP (database for nonsynonymous SNPs' functional predictions), a database developed for functional prediction and annotation of all potential non-synonymous SNVs in the human genome.20 The dbNSFP via VarSeq contains variant effect classifications from six functional prediction algorithms. Pathogenicity prediction was provided by the PHRED-like score, a scaled score based on CADD (Combined Annotation-Dependent Depletion) scores 1.4.21 CADD is a tool for scoring the deleteriousness of SNVs as well as insertion/deletions variants in the human genome, with CADD score (C-Score) for a given variant assessed based on diverse genomic features derived from surrounding sequence context, gene model annotations, evolutionary constraint, epigenetic measurements and functional predictions. The PHRED-like C-Score is defined as $-10 \log \left( \frac{\text{rank}}{\text{total}} \right)$, by ranking C-Score of a variant relative to all possible 8.6 billion substitutions in the human reference genome.

2.5 The genome aggregation database

The Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org) is an open source database developed for aggregating and harmonizing both exome and genome sequencing data. It is the world's largest public collection of human genetic variations and a popular resource for basic research and clinical variant interpretation. The version 2 dataset (GRCh37/hg19) spans 125 748 exome sequences and 15 708 whole-genome sequences from unrelated individuals sequenced by various genetic studies. We make use of the WGS data of gnomAD as an external control for statistical analysis.

2.6 Statistical analysis

Proxy external controls association test (ProxECAT): ProxECAT22 is a statistical method specifically developed for analysis of WGS data using existing large databases as external controls (here gnomAD). Different from the conventional case–control design that focuses on genetic variants predicted as functional and compares their frequencies between groups, ProxECAT makes use of non-functional variants as a proxy for how well variants within a genetic region are sequenced and called within a sample. It compares the ratio between variant frequency and proxy frequency (λ*, λproxy) in cases with that in the external controls to adjust for group differences in sequencing technology, in processing (i.e. processing of DNA samples), and in read depth for creating the internal and external datasets, with the null hypothesis:

$$\frac{i(g^*, \text{case})}{\lambda(\text{proxy, case})} = \frac{i(g^*, \text{control})}{\lambda(\text{proxy, control})}$$

Where $g^*$ represents the gene of interest and $\lambda$ is the rate of variants per N cases or controls. As the maximum likelihood estimates have a closed form under Poisson distribution, statistical significance of estimates can be inferred by a likelihood ratio test.22 In summary, ProxECAT is a gene-based burden test that includes non-functional variants to enable the use of existing databases as external controls in statistical testing. The model has been integrated in a R package, ProxECAT, to assist implementation of the method (https://github.com/hendriau/ProxECAT).

2.7 Over-representation analysis

Over-representation analysis (ORA) is used to assess if the overlap of identified significant genes with genes from a functional cluster (biological pathway, a compiled list of cancer related genes) is significantly
different from being random by calculating the probability from a hypergeometric distribution:

\[
p(X \geq k) = 1 - \sum_{r=0}^{k} \binom{m}{r} \binom{N-m}{n-r} / \binom{N}{n}
\]

where \( N \) is the number of all genes in the genome, \( m \) is the number of genes in a functional cluster, \( n \) is the number of genes identified as significant, \( k \) is the number of overlapping genes under testing. The R function \( \text{phyper}() \) was used for calculating the hypergeometric probability.

ORA has been implemented in a web tool for biological pathway analysis, the gene-set enrichment analysis (GSEA), to test if genes in one biological pathway is over-represented in a list of identified significant genes. GSEA was performed on canonical pathways at https://www.gsea-msigdb.org/gsea/index.jsp.

**Figure 1** Workflow of whole genome sequencing analysis [Colour figure can be viewed at wileyonlinelibrary.com]

**Figure 2** Premature truncating variant (PTV) analysis results illustrated with a histogram for the genes distributed by the number of PTVs they carry (a) and with a scatter plot for genes ordered by the number of hosting PTVs (b). The red colored dots are genes with 4 or more PTVs [Colour figure can be viewed at wileyonlinelibrary.com]
The analytical pipeline from sequencing analysis, filtering, statistical testing, functional annotations to the final report is illustrated in Figure 1.

3 | RESULTS

3.1 | Sequencing analysis

We sequenced a total of 61 unaffected FDRs of FPC patients (sex: 25 female/36 male; median age: 59 years [37–84]) (Table S1), with an average output (median) of 15 161 SNVs per samples (range: 14678-15 630), after applying in-house filtering pipelines using VarSeq. In total, 60 778 SNVs were detected. Among them, 2397 SNVs were detected in all samples; and 16 533 SNVs were detected only once in 61 samples (referred to as private mutations, accounting for 27.2% of all detected SNVs). In Figure S1, we show the frequency of number of SNVs by number of times of detection in the 61 samples. As described in the Methods section, we further filtered all variants to remove SNVs from pseudogenes, pseudogene homology, segmental duplicates, and multi-mapped variants, leaving 46 033 unique SNVs for subsequent analysis.

3.2 | Analysis of premature truncating variants

PTVs represent a type of variants within a gene that create an early stop codon, leading to a shortened or truncated protein and resulting in serious functional consequences. Following Roberts et al. (2016),16 we filtered SNVs using the following criteria (i) nonsense variants, splice-site variants (splice donor variant, splice acceptor variant), and frameshift INDELs (frameshift variants); (ii) heterozygous in the germline; (iii) minor allele frequency (MAF) < 0.01 from gnomAD and (iv) present in only one individual, i.e. “private” or shared by FDRs in a family, i.e. “familial”, obtaining a total of 492 heterozygous PTVs in 448 genes. We then counted the number of PTVs in each gene.

| Gene names | Chr | Position 1st PTV | PTVs | Private PTVs | Familial PTVs |
|------------|-----|-----------------|------|--------------|---------------|
| CCDC40     | 17  | 78 013 765      | 6    | 6            | 0             |
| MUC19      | 12  | 40 791 701      | 5    | 3            | 2             |
| CRIPAK     | 4   | 1 388 414       | 4    | 3            | 1             |
| ATXN2      | 12  | 112 036 779     | 3    | 2            | 1             |
| HMCN2      | 9   | 133 061 568     | 3    | 3            | 0             |
| KLHL35     | 11  | 75 141 449      | 3    | 3            | 0             |
| SAMQ1      | 19  | 14 200 862      | 3    | 2            | 1             |
| ZNF667     | 19  | 56 952 566      | 3    | 2            | 1             |
| ANO7       | 2   | 242 128 114     | 2    | 2            | 0             |
| ATP8B3     | 19  | 1 795 984       | 2    | 1            | 1             |
| BTN2A1     | 6   | 26 466 202      | 2    | 1            | 1             |
| C8orf44    | 8   | 67 590 023      | 2    | 2            | 0             |
| CCDC7      | 10  | 32 856 799      | 2    | 0            | 2             |
| CELSR1     | 22  | 46 832 075      | 2    | 0            | 2             |
| COL6A5     | 3   | 130 159 330     | 2    | 1            | 1             |
| COL6A6     | 3   | 130 284 459     | 2    | 2            | 0             |
| DNAH14     | 1   | 225 231 637     | 2    | 2            | 0             |
| DRD4       | 11  | 640 071         | 2    | 2            | 0             |
| GFY        | 19  | 49 931 883      | 2    | 0            | 2             |
| HOMEZ      | 14  | 23 744 823      | 2    | 0            | 2             |
| HSD3B1     | 1   | 120 056 818     | 2    | 0            | 2             |
| IQGAP3     | 1   | 156 509 221     | 2    | 2            | 0             |
| KRT77      | 12  | 53 086 340      | 2    | 2            | 0             |
| PKM        | 15  | 72 523 315      | 2    | 0            | 2             |
| PRB3       | 12  | 11 420 755      | 2    | 2            | 0             |
| RBM33      | 7   | 155 532 530     | 2    | 2            | 0             |
| RETSAT     | 2   | 85 576 627      | 2    | 1            | 1             |
| TEX15      | 8   | 30 703 647      | 2    | 2            | 0             |
| ULK4       | 3   | 41 756 779      | 2    | 0            | 2             |
| ZNF880     | 19  | 52 888 070      | 2    | 2            | 0             |
A full list of genes with at least one PTV is shown in Table S2. Figure 2(A) is a histogram for the genes distributed by the number of PTVs they carry. The majority of genes have only 1 PTV. There are 22 genes with 2 PTVs and 8 with ≥3 PTVs (Table 1). Figure 2(B) plots the 448 genes ordered by the number of hosted PTVs (red labelled dots for genes with more than 4 PTVs). In Table 1 and Table S2, it is clear that the number of heterogenous PTVs harboured by the top genes are contributed mainly by private mutations, although there are also genes solely with PTVs shared by FDRs in a family.

Detailed information of each of the PTVs is presented in Table S3 with one row for each sample carrying the mutation. A “familial” PTV is carried by samples from the same family, while “private” PTVs can only be found in one sample (row). As can be seen in Table S3, many of the genes are enriched by PTVs with a high PHRED score and a high voting of functional prediction (dbNSFP Functional Prediction Voting), indicating high significance in functional implications. The trend of positive correlation (Spearman correlation coefficient 0.26, p < 1.87e-06) between PHRED score and dbNSFP functional prediction is clearly shown in Figure S2.

### 3.3 Rare variants association analysis

A total of 12,297 variants with MAF < 0.01 are available for association analysis. These variants are from 7,229 genes, among them 531 genes have at least one functional nonsynonymous mutation and one synonymous mutation which were tested using ProxECAT. The Q-Q plot in Figure 3 displays significant genes with p-values deviating from the random distribution. The Manhattan plot in Figure 4 shows the genes along chromosomal locations. There are 20 significant genes with FDR < 0.05 (p < 1.5e-03) (Table 2), 84 genes with p < 0.05 (Table S4). The top 6 genes (p < 1e-05, FDR < 1e-03) include PALD1, LRP1B, COL4A2, CYLC2, ZFYVE9, BRD3. PALD1 (paladin) on chromosome 10 is highly significant (p < 1.53e-33) as it stands out from the other genes in Figures 3-4.

There were 7 SNVs observed in PALD1, 6 missense and 1 splice region variants. Only 1 missense mutation (10:72294183, Ref/Alt: C/T) and the splice region variant met MAF < 0.01. Sixteen SNVs were observed for LRP1B (low-density lipoprotein receptor-related protein 1B, chromosome 2) after filtering, among them 6 had MAF < 0.01 with 5 missense mutations and 1 splice region variant. We observed 12 SNVs in COL4A2 (collagen type IV alpha2 chain), 4 of them had MAF < 0.01. After filtering, 1 missense variant (13:111155578, Ref/Alt: G/A) and 1 splice region variant remained. For CYLC2 (cycin 2), there were 6 SNVs and 3 of them had MAF < 0.01, with 1 missense variant (9:105767091, Ref/Alt: C/A) and 2 splice region variants. The ZFYVE9 (zinc finger FYVE-type containing 9) gene has 3 SNVs among which 2 SNVs had MAF < 0.01, with 1 splice acceptor variant (1:52729440, Ref/Alt: -/CA) and 1 splice region variant. There were 5 SNVs for BRD3 (bromodomain containing 3) gene, only 2 of them had MAF < 0.01 with 1 missense mutation (9:136899924, Ref/Alt: T/C) and 1 synonymous splice region variant.
Table 2 also has another BRD gene, BRD4 with 2 missense mutations (19:15350625, Ref/Alt: C/T; 19:15350625, Ref/Alt: C/T) and 1 splice region variant.

Detailed information of each rare nonsynonymous variant in the significant genes in Table 2 can be found in Table S5 showing all genes with p < 0.05. In general, these variants have high PHRED scores (>20), although low scores are also observed in some of the variants. Notably, the vast majority of the nonsynonymous variants are missense mutations.

Similar to Figure S2, the PHRED scores show an obvious correlation with dbNSFP Functional Prediction Voting (Figure S3), suggesting that high PHRED scores are associated with high functionality of the variants. However, the degree of correlation is much higher than that for the PTVs (Spearman correlation coefficient 0.78 vs 0.26) with even higher statistical significance (p-value 3.07e-07 vs 1.87e-06). Notably, the vast majority of the nonsynonymous variants are missense mutations.

3.4 Over-represented gene clusters

The 448 genes harbouring at least one PTV were submitted to GSEA for over-representation analysis of canonical pathways using the hypergeometric test. Five canonical pathways are significantly over-represented (FDR < 0.05) among the 2868 pathways from the GSEA databases encompassing 40 071 genes in universe (Table 3). The gene set “transport of small molecules” consisting of 728 genes has 26 genes overlapping with the list of 448 PTV genes resulting in a FDR < 2.44e-03. The small gene set “butyrophilin (BTN) family interactions” has only 12 genes. Four of them can be found in the PTV genes with FDR < 1.29e-02. Gene set “ion channel transport” is formed by 183 genes. Eleven of them can be found in the list of PTV genes leading to a significant over-representation with FDR < 1.29e-02. “Innate immune system” is a large gene set containing 1117 genes. Among them, 30 overlapped with the PTV genes (FDR < 2.35e-02). There are 8 PTV genes in overlap with the 106 genes in “Stimuli-sensing channels” resulting in a FDR < 2.25e-02.

Next, we submitted the 20 significant genes tested by ProxECAT in Table 2 to GSEA. One pathway concerning the extracellular matrix (ECM) was significantly over-represented (Table 3). Among the 275 genes in the pathway, 4 genes (COL4A2, BMPER, COL4A4) are represented in the list of significant genes in Table 2, displaying a significant overlap with a hypergeometric p value of 9.42e-06 (FDR < 2.75e-02). The pathway is an ensemble of genes encoding core extracellular matrix including ECM glycoproteins, collagens, and proteoglycans.

3.5 Enrichment analysis of cancer driver genes

We finally tested overlap of cancer driver genes in the list of genes hosting heterogenous PTVs and in the list of significant genes

| Gene name | Chrom | Start position (basepair) | Number of functional variants | Number of synonymous variants | ch2 | p-value | FDR |
|-----------|-------|--------------------------|-------------------------------|-------------------------------|-----|---------|-----|
| PALD1     | 10    | 72 288 965               | 1                             | 1                             | 145.67 | 1.53e-33 | 8.85e-31 |
| LRP1B     | 2     | 141 115 595              | 5                             | 1                             | 46.18 | 1.08e-11 | 3.11e-09 |
| COL4A2    | 13    | 111 082 772              | 1                             | 1                             | 23.32 | 1.37e-06 | 2.64e-04 |
| CYLC2     | 9     | 105 767 091              | 1                             | 2                             | 22.33 | 2.30e-06 | 3.32e-04 |
| ZFYVE9    | 1     | 52 729 426               | 1                             | 1                             | 19.81 | 8.55e-06 | 9.60e-04 |
| BRD3      | 9     | 136 899 924              | 1                             | 1                             | 19.52 | 9.96e-06 | 9.60e-04 |
| AHDC1     | 1     | 27 875 385               | 2                             | 1                             | 16.18 | 5.76e-05 | 4.75e-03 |
| MCM5      | 22    | 35 796 605               | 1                             | 1                             | 15.23 | 9.50e-05 | 6.87e-03 |
| KCNAB2    | 1     | 6 086 512                | 1                             | 1                             | 14.91 | 1.13e-04 | 7.24e-03 |
| DMBT1     | 10    | 124 339 274              | 6                             | 1                             | 14.71 | 1.26e-04 | 7.26e-03 |
| MYRFL     | 12    | 70 329 887               | 1                             | 1                             | 13.95 | 1.88e-04 | 9.88e-03 |
| SGSM3     | 22    | 40 797 647               | 2                             | 1                             | 13.45 | 2.45e-04 | 1.18e-02 |
| IGF2BP3   | 7     | 23 358 880               | 1                             | 1                             | 12.11 | 5.03e-04 | 2.24e-02 |
| HN1       | 17    | 73 132 258               | 1                             | 1                             | 11.93 | 5.53e-04 | 2.24e-02 |
| BRD4      | 19    | 15 350 200               | 2                             | 1                             | 11.83 | 5.82e-04 | 2.42e-02 |
| RIF1      | 2     | 152 300 231              | 1                             | 1                             | 11.41 | 7.30e-04 | 2.64e-02 |
| SGK2      | 20    | 42 196 593               | 1                             | 1                             | 11.12 | 8.56e-04 | 2.91e-02 |
| LAMB4     | 7     | 107 720 041              | 1                             | 1                             | 10.75 | 1.05e-03 | 3.36e-02 |
| NYAP1     | 7     | 100 082 846              | 3                             | 1                             | 10.26 | 1.36e-03 | 4.14e-02 |
| BMPER     | 7     | 34 010 036               | 1                             | 1                             | 10.08 | 1.50e-03 | 4.33e-02 |
tested using ProxECAT, respectively. To do that, the number of overlapping genes between the detected gene lists and a collection of 460 cancer driver genes identified by Dietlein et al (2020)\textsuperscript{23} was counted and tested using hypergeometric test. Among the 84 genes in Table S4, one gene, the KIT Proto-Oncogene (\textit{KIT}), overlapped with the cancer driver gene list. Although only one overlap, the hypergeometric test reported a \( p < 4.2\times 10^{-3} \), suggesting significant enrichment of cancer driver genes. The missense variant in \textit{KIT} gene (4:55595566, Ref/Alt: C/T) has a PHRED score of 32 and 6 out of 6 votes predicted as damaging.

Likewise, among the 448 PTV genes in Table S2, 12 overlaps were found, \textit{TCHH}, \textit{TMPRSS15}, \textit{CHIT1}, \textit{ZNFB3}, \textit{AIM2}, \textit{SPATA31E1}, \textit{PRDM2}, \textit{DIS3}, \textit{ATP11A}, \textit{CCDC66}, \textit{NFKBIE}, \textit{TNFRSF10C}, resulting in a hypergeometric \( p < 2.2\times 10^{-16} \), an extremely highly significant enrichment of cancer driver genes.

\section{DISCUSSION}

FDRs of FPC patients are at increased risk of developing PDAC and other cancers due to high degree of genetic relatedness and aggregation of risk genes within family members. Through performing the first WGS on FDRs of FPC patients in a nationwide cohort, we have found enrichment of rare genetic mutations in genes with significant implication in cancer pathogenesis.

\subsection{The relevance of rare premature-truncating variants}

By shortening the protein-coding sequence of genes, PTVs can lead to altered function of the hosting genes including gain or loss of gene function through for example nonsense mediated mRNA decay.\textsuperscript{24} Identifying PTV associations to human diseases is a useful way to detect drug targets and to gain disease insights. Using the same approach as Roberts et al (2016),\textsuperscript{16} we were able to examine PTV genes in FDRs and prioritize them for further assessment.

On top of Table 1 is CCDC40 (coiled-coil domain containing protein) harbouring 6 heterogenous PTVs, all of which are “private” mutations. One study showed that the gene is associated with primary immunodeficiency diseases,\textsuperscript{25} which may be related to PDAC development.\textsuperscript{26} The MUC (mucin) genes encode a family of high molecular weight, heavily glycosylated proteins that form a protective coat around cancer cells. They are critical in the pathogenesis of pancreatic cancer and are associated with resistance to cytotoxic drugs, cancer invasiveness, metastases, and cell proliferation.\textsuperscript{27} Expression of MUC genes has been shown to be associated with precursor lesions (pancreatic intraepithelial neoplasia, PanIN) in pancreatic cancer and overall survival.\textsuperscript{28} Among the other genes in Table 1, somatic mutations in DNAH (dynein axonemal heavy chain) were reportedly associated with gastric cancer survival and treatment response.\textsuperscript{29} A SNP in ANO7 was found to be associated with risk of aggressive prostate cancer with elevated expression of the gene correlating with disease severity and outcome.\textsuperscript{30}

Besides the observed PTVs, we also found pathogenic variants in known FPC susceptibility genes\textsuperscript{16} that met our PTV definition but with allelle frequency missing from the Ensembl Variation database. Among the 12 known FPC predisposition genes, we observed two pathogenic variants in \textit{BRCA2} gene in two families (one in each family), one frameshift variant in \textit{ATM} gene in one family, one frameshift variant in \textit{CDKN2A} gene in one family, and one stop gained variant in \textit{MSH6} in one family. These pathogenic variants together with the above well-defined PTVs can serve as candidates for segregation

\begin{table}
\centering
\begin{tabular}{ |l|c|c|c|c|c|c| }
\hline
\textbf{Gene set name} & \textbf{# Genes in Gene Set (K)} & \textbf{Description} & \textbf{# Genes in overlap (k)} & \textbf{k/K} & \textbf{p-value} & \textbf{FDR} \\
\hline
\textbf{PTV genes:} & & & & & & \\
\textbf{REACTOME} & 728 & Transport of small molecules & 26 & 0.04 & 8.36e-07 & 2.44e-03 \\
\textbf{Butyrophilin (BTN) family interactions} & 12 & Butyrophilin (BTN) family interactions & 4 & 0.33 & 8.96e-06 & 1.29e-02 \\
\textbf{Ion channel transport} & 183 & Ion channel transport & 11 & 0.06 & 1.33e-05 & 1.29e-02 \\
\textbf{Innate immune system} & 1117 & Innate immune system & 30 & 0.03 & 3.43e-05 & 2.35e-02 \\
\textbf{Stimuli-sensing channels} & 106 & Stimuli-sensing channels & 8 & 0.08 & 4.03e-05 & 2.35e-02 \\
\hline
\textbf{ProxECAT genes:} & & & & & & \\
\textbf{NABA} & 275 & Ensemble of genes encoding core extracellular matrix including ECM glycoproteins, collagens and proteoglycans & 4 & 0.01 & 9.42e-06 & 2.75e-02 \\
\hline
\end{tabular}
\caption{Functional pathways over-represented by all PTV genes and ProxECAT significant genes}
\end{table}
analysis in the FPC families, when similar sequencing data are available on the probands (i.e. FPC patients in the respective families). Such analysis will help with identifying novel causal variants and further characterize the functional profiles of known variants in FPC.

4.2 The relevance of significant genes by association test

The gene showing the highest statistical significance in association analysis using ProxECAT is PALD1 (Table 2). By using machine learning, Deeb et al (2015) found PALD1 as one of their four most predictive proteins for classifying diffuse large B-cell lymphoma patients. In another study, knockdown of the expression of PALD1 was found to enhance the angiogenesis of immortalized human endothelial cells to promote cancer development. By screening for base-specific mutations, Tuupanen et al (2014) found a hotspot mutation in PALD1 for colorectal cancer.

Mutational and transcriptional changes in some of the significant genes in Table 2 have previously been shown to be associated with PDAC. Brar et al (2019) found LRPIB (low-density lipoprotein receptor-related protein IB) mutations are more frequent in metastatic lesions than in primary pancreatic tissue suggesting that mutation in this tumor suppressor gene may promote PDAC metastasis. Two genes in Table 2, BRD3 and BRD4, belong to the bromodomain and extraterminal (BET) family of proteins, which is one of the most prominent transcriptional vulnerabilities in human cancer – serving as potential therapeutic targets in cancer treatment. It has been shown that BET bromodomain inhibitors can block growth of pancreatic cancer cells. A recent study showed that AHDC1 gene is upregulated by competing endogenous RNA (ceRNA) interactions between IncRNAs and miRNAs to promote the progression of cervical cancer.

Overexpression of MCM (mini-chromosome maintenance) genes was significantly associated with PDAC progression and prognosis, and expression of MCMs could serve as prognostic and therapeutic biomarkers for PDAC. The missense mutation found in MCM5 in this study could function as cis-regulatory mutation affecting expression of the gene (as an expression quantitative trait locus, eQTL) and contribute to the potential risk of PDAC. DMBT1 (deleted in malignant brain tumors 1) is a tumor suppressor gene. Secretion of COOH-terminal fragment of DMBT1 has been revealed from PDAC cell lines, while the gene was also found to be differentially expressed in PDAC. The presently detected missense mutations in the gene may cause dysregulation of its tumor suppressor activity, thereby promoting the development of PDAC. Likewise, increased expression of IGF2BP3 (insulin-like growth factor 2 mRNA-binding protein 3) was found to promote invasiveness and metastasis of PDAC, while dysregulation of SGK2 (serum/glucocorticoid regulated kinase 2) affected treatment response in PDAC. The Rif1 gene has emerged as a conserved regulator of chromosome maintenance, acting to control DNA replication and repair. The gene is found highly upregulated in pancreatic cancer cell lines and is considered as a potential biomarker for diagnosis and therapeutic treatment of pancreatic cancer.

In summary, we identified multiple genes that have previously been shown to be associated with cancer development, progression and metastasis – with some of the genes being directly linked with PDAC pathogenesis. The expression of our detected significant genes could have been affected by the germline mutations through, for example, DNA-transcription factors resulting in altered expression of the corresponding protein or by increased affinity for binding of the micro-RNAs. Given the high importance of gene expression in PDAC, it will be tempting to elucidate the underlying regulatory mechanisms involved.

Notably, the rare variants in the detected significant genes are predominantly missense variants (Table S5). The effects of missense mutations in cancer predisposition genes have been long discussed and it is suggested that efforts to identify new susceptibility genes should not ignore missense variations considering their important roles in cancer susceptibility. Large regions of BRCA1 and BRCA2 carry missense variations, although those occurring in the cold-spot regions have been recently shown to be unlikely pathogenic. It has been found that the majority of pathogenic variants in the breast cancer gene TP53 are missense variants, while the missense variants in two other breast cancer genes, ATM and CHEK2 are probably equally or even more important than PTVs in terms of their frequencies.

As indicated in Figure S3, the PHRED score is significantly correlated with predicted functionality, thus suggesting strong functional relevance of the rare missense variants from our association test. For all rare nonsynonymous variants (mostly missense) in genes tested with \( p < 0.05 \) (Table S5), the median PHRED score is 15.1, while variants in genes tested with FDR < 0.05 (\( p < 1.5 \times 10^{-3} \)) have a median PHRED score of 23. This again indicates that the missense variants in highly significant genes are more functionally relevant than the variants in less significant ones.

4.3 The relevance of enriched pathways

In Table 3, the only pathway enriched by the 20 significant genes from the ProxECAT association analysis is the core matrisome pathway comprising ECM glycoproteins, collagens and proteoglycans. In a recent network-based analysis of gene expression data on FPC and sporadic pancreatic cancer, increased activity in extracellular structure and ECM organization was found. It is interesting that significant association of ECM pathways was found by two different omics approaches (WGS and transcriptomics) suggesting that the detected rare variants could be involved in the regulation of genes of the ECM pathways. Among the 4 overlapping genes in the pathway, LAMB4 (subunit of the laminin gene family) is one of the most widely expressed ECM proteins and exerts many important functions in multiple organs. COL4A2, DMBT1, BMPER are also highly involved in ECM pathways as COL4A2 constitutes one of the most abundant components of nearly all basement membranes – which is a thin, pliable sheet-like type of ECM, that provides cell and tissue support. DMBT1 encodes for an ECM protein responsible for epithelial to mesenchymal transition and differentiation.
endothelial regulator) has been shown to be highly expressed in multiple malignant tumors (lung, colon, and cervix).

One of the five pathways enriched by PTV genes, the transport of small molecules, is known to impact cancer development, metastasis, and response to treatment. The second enriched pathway is butyrophilin (BTN) family interactions. The butyrophilins are viewed as an emerging family of immune regulators. The BTN genes are functionally implicated in T cell inhibition and in the modulation of epithelial cell–T cell interactions, thus being genetically associated with inflammatory diseases. One of the BTN member genes, BTN3A2, was identified as an independent prognostic marker of triple-negative breast cancer.

Another significantly enriched pathway is related to the innate immune system. Cells of the innate immune system including: granulocytes, monocytes, macrophages, and dendritic cells, play important roles in cancer cell recognition, as well as the initiation of inflammation and antitumor immune responses. However, persistent inflammation has been shown to be a driver of tumor progression in many malignancies by promoting immune suppression and cancer metastasis, as in the case of PDAC.

Other two significantly enriched pathways in the PTV genes are the ion channel transport and stimuli-sensing channels. Both involve signaling transduction mechanisms and the capacity of cells to detect a specific stimulus depending on a characteristic combination of different ion channels. It is well known that ion channels regulate multiple cellular functions and are involved in the communication between extracellular events and intracellular signaling pathways. Altered activity of ion channels can have an impact on uncontrolled proliferation, promotion of invasion and migration of cancer. Research has indicated that certain ion channels are involved in the aberrant tumor growth and metastatic processes of PDAC. The significant enrichment of multiple pathways involved in cancer development suggests that the rare PTVs in the FDRs could impact a broad range of functional processes that jointly contribute to the increased risk of PDAC in the FPC families.

4.4 The relevance of overlapping cancer-driver genes

It is interesting that the genes from Table S2 (mainly representing PTVs of private mutations) are enriched for cancer driver genes, whose mutations give a growth advantage to cancer cells. The highly significant overlap of the 12 PTV genes with known cancer driver genes indicates that rare germline mutations could constitute a potential risk for PDAC development. Among the 12 genes, DIS3 has allele-specific expressions with decreased expression observed in carriers of pancreatic cancer risk-increasing alleles, which could therefore affect nuclear RNA processing. In another gene, TNFRSF10C (TNF Receptor Superfamily Member 10c), aberrant methylation at the promoter region has been frequently observed in pancreatic cancer cell lines suggesting that genetic variations of the gene could regulate gene activity through epigenetic mechanisms. It is interesting that the expression levels of two members of the same gene family, TNFRSF11A and TNFRSF17, have been recently shown to correlate with PDAC sub-grouping in regards to progression and therapeutic response. TMPRS515 (transmembrane serine protease 15) encodes an enzyme that converts the pancreatic proenzyme trypsinogen to trypsin, which activates other proenzymes including chymotrypsinogen and procarboxypeptidases. Strong genetic heterogeneity was found in the gene in patients with chronic pancreatitis. ZNF233 (zinc finger protein 233) has been found to be associated with pancreatic cancer in a global genomic analysis of core signaling pathways.

Overall, the observation of rare germline PTVs in cancer driver genes provides novel data in support of potentially increased risk of cancer development in FDRs in FPC predisposed families and reveal a high degree of genetic heterogeneity in FPC susceptibility.

4.5 Strengths and limitations

The identification of multiple mutations associated with pancreatic cancer and other cancer types validates the ProxECAT test as a useful tool for rare variants association studies using existing large external sequencing databases as controls. Most importantly, the identified rare variants in FDRs of FPC patients could jointly contribute to the co-aggregation of the disease within families. A limitation of the study design is that a direct association of the detected rare variants with pancreatic cancer cannot be established by such analysis, as not all FDRs may develop PDAC. Prospective follow-up data on development of PDAC among the FDRs will provide useful information to verify potential association and predictive value of the identified variants on risk of PDAC.

ProxECAT is a burden test that collapses rare variant data to estimate their enrichment within a gene region to achieve statistical power as compared to a single-variant test. By comparing difference in the ratio of functional to synonymous variants between cases and controls, ProxECAT enables the use of external controls in statistical testing. However, this also comes with a price as such a comparison can be underpowered in contrast to direct comparison of the functional variants between case and control groups. Nevertheless, the high quality of DNA samples obtained from peripheral blood and the large sample size of the external controls using the gnomAD database compensate the power issue, thus ensuring significant statistical testing by the ProxECAT analysis.

5 CONCLUSIONS

Relatives of FPC patients are at high risk of developing PDAC. Analyzing the genetic variants underlying cancer susceptibility is critical for risk assessment and early intervention. Our WGS analysis of rare variants in FDRs of FPC patients identified germline mutations and PTVs recurrent in cancer related genes and driver genes. The identified rare germline variants could contribute towards understanding the genetic basis of cancer susceptibility in relatives of FPC patients.
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CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION
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