HERITABILITY AND GENETIC ARCHITECTURE

OF PANCREATIC CANCER

by
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ABSTRACT

Pancreatic cancer is currently the third leading cause of cancer deaths in the US. Identified common susceptibility loci for pancreatic cancer only account for a small fraction of phenotypic variations, highlighting the "missing heritability" problem. Array heritabilities were underestimated because of the inadequate coverage on rare variants. The goal of this study was to generate a more comprehensive and less biased estimate of heritability for pancreatic cancer and to infer the genetic architecture of pancreatic cancer through genomic partitioning of the estimated heritability.

In the Pancreatic Cancer Case-Control Consortium (PanC4) study of 3,568 cases and 3,363 controls, the heritability estimated from the imputed variants was 21.2% (s.e. = 4.8%). Genomic partitioning found more than one-third of the estimated heritability attributable to rare variants with minor allele frequency (MAF) < 0.01. Across the functional group of intronic, intergenic, coding and regulatory variants, intronic variants account for most estimated heritability. Identified GWAS loci explained 4.1% of the total phenotypic variations for pancreatic cancer. These findings suggest that the yet-to-be-identified rare and common variants are both key contributors to the "missing heritability" of pancreatic cancer, and it's important to include both coding and regulatory regions in the search of disease-causing variants for pancreatic cancer.

In the combined sample of 569 familial pancreatic cancer (FPC) cases and 754 controls from Alzheimer's Disease Neuroimaging Initiative (ADNI) study, whole-genome sequencing (WGS) variants explained 38.8% (s.e. = 15.7%) of total phenotypic variance for pancreatic cancer. This higher estimate of heritability for pancreatic cancer is likely a result of a more

completed characterization of genetic variation across the genome in the sequencing data as well as a stronger genetic background in the FPC cases.

In addition, variants in 11 established FPC genes only explained 0.4% of total phenotypic variance for pancreatic cancer in PanC4 study, which reflected the limitation of current statistical approaches in capturing the contribution of rare variants to the heritability of a trait.

Findings from this study provided valuable insights into the genetic architecture of pancreatic cancer which would help understand the disease etiology and to aid in disease screening, diagnosis, prognosis and therapy.

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CHAPTER ONE

INTRODUCTION

Over the past 20 years, pancreatic cancer remains one of the deadliest cancers and a major public health challenge. This chapter provides the rationale of studying heritability for pancreatic cancer and how the results of the current study may guide the future strategies for gene discovery efforts. First, the key statistics of pancreatic cancer, including the prevalence, incidence, and survival rate are presented to emphasize the critical issues associated with the disease. Second, the risk factors and their impact on pancreatic cancer are reviewed. Then the limitations of the past heritability analysis on pancreatic cancer are discussed, followed by a description of the missing heritability problem in the context of pancreatic cancer. The chapter concludes with the purpose and goals of current study.

Epidemiology of Pancreatic Cancer

The pancreas is an organ located in the abdomen, behind the stomach. The pancreas has two main types of cells. The exocrine cells make up the majority of the pancreas and release enzymes for food digestion. The endocrine cells are hormone-producing cells that regulate blood sugar. These two types of cells in the pancreas form different types of tumors that present distinct characteristics and require different treatments. The vast majority of pancreatic tumors occur in the exocrine tissues. Pancreatic ductal adenocarcinoma (PDAC) is the most common type that represents > 90% of all pancreatic cancers[1].

Pancreatic cancer is a rare disease. The lifetime risk of developing cancer in the pancreas is about 1.5% by age 80 for both men and women[2]. Like all cancers, pancreatic cancer is more prevalent in aged populations. The incidence of pancreatic cancer is sharply elevated after age 55, and the median age at diagnosis is 70 years in the United States. On January 1, 2014, the

estimated prevalence of pancreatic cancer in the United States was 0.0165%[2]. Despite its low incidence, pancreatic cancer has the highest case-fatality rate of all common cancer types. It has surpassed breast cancer becoming the third-leading cause of cancer deaths in the US, responsible for an estimated 44,330 deaths in 2018. Both the incidence and mortality of pancreatic cancer have been increasing steadily over the past decade. By 2030, pancreatic cancer is predicted to become the second most common cause of cancer mortality, after lung cancer [3]. In contrast to dramatic declines in cancer-related deaths for other malignancies, the 5-year survival rate for pancreatic cancer remains low (currently 8%). The poor prognosis of pancreatic cancer is primarily attributed to its silent symptoms at early stage, the lack of reliable, accurate and non-invasive detection methods, and the lack of effective treatments for non-localized disease. More than half (52%) of patients are diagnosed at a distant stage, for which 5-year survival is less than 3%. Conventional treatments, such as chemotherapy, radiation or a combination of these modalities, have had little impact on the course of disease in late-stage cancer patients.

Risk Factors for Pancreatic Cancer

The rapid progression and short survival of patients with pancreatic cancer make research on this cancer particularly challenging. Patients are often too ill to participate in research studies, or to provide necessary information and/or bio-specimens required to answer various research questions. Studies in pancreatic cancer often have small sample sizes and hence insufficient power to draw conclusive inferences. Over the past two decades, great efforts had been made to establish longitudinal cohorts of pancreatic cancer. Large-scale collaborations of these cohorts (i.e. consortia) have provided unprecedented opportunities in revealing the causes and risk factors for pancreatic cancer.

Family History

Family history is a long-recognized risk factor for pancreatic cancer and an important predictor of disease risk. Studies have suggested that approximately 5-10% of pancreatic cancer patients report a close relative with pancreatic cancer[4,5]. Most epidemiological studies have demonstrated a 2- to 3-fold increase in risk of pancreatic cancer among individuals with affected first-degree relatives (FDRs)[6–15]. Some studies have shown even higher risk. A Swedish study reported a standardized incidence ratio (SIR) for pancreatic cancer of 1.73 (95%CI: 1.13-2.54) in offspring with at least one parent who presented with PDAC[16]. In a prospective study from the National Familial Pancreas Tumor Registry (NFPTR), the SIRs for pancreatic cancer in comparison to the SEER (Surveillance, Epidemiology, and End Results) rates were 6.4 (95% CI: 1.8-16.4) and 32.0 (95% CI: 10.2-74.7) in individuals with two and three FDRs with pancreatic cancer, respectively[17].

Cigarette Smoking

Cigarette smoking is the best-characterized risk factor for pancreatic cancer. Risk of pancreatic cancer for current smokers is about twice that for never smokers[11,18–32]. A dose-response relationship was observed with smoking intensity (i.e. number of cigarette per day) [18,19,21,22,24,33–35] and duration (i.e. number of years)[21,22,33]. A higher amount of cigarette smoking over time (i.e. pack-years) is also associated with an elevated risk of pancreatic cancer[21,24,33,36]. In several studies, smoking cessation lowered the risk of pancreatic cancer among former smokers to be comparable to never smokers after 5-10 years[19,21,22]. Given that near half of the general population being current or former smokers, it was estimated that about 11-32% of all pancreatic cancer cases could be attributable to cigarette smoking[37].

Chronic Pancreatitis

There is an increasing body of evidence suggesting chronic pancreatitis as a risk factor for pancreatic cancer. The relationship between chronic pancreatitis (CP) and pancreatic cancer was complicated. Prospective cohort studies in CP patients found up to 60% pancreatic cancer occurences within the first two years of CP diagnosis[38–42]. There was a substantially higher risk of pancreatic cancer diagnosis in the first two years, and the risk decreased with time[18,38,39,43,44]. It was suggested that a proportion of these pancreatic cancer cases may be initially misdiagnosed as CP, and hence represent a potential diagnostic delay of up to 2 years[45]. Given the rapid progression and low survival rate of pancreatic cancer, misdiagnosis would become less likely with longer periods of observation. Therefore, a lag period of two years was often considered in studies evaluating the association between CP and cancer of the pancreas. Such cohort studies had reported a 6- to 27-fold increase in risk of pancreatic cancer among CP patients compared to the general population[38–44,46–48]. In the most recent meta-analysis of 9 cohort studies and 4 case-control studies, the relative risk of pancreatic cancer in CP patients was 16.2 (95%CI: 12.6-20.7), 7.9 (95%CI: 4.3-14.7) and 3.53 (95%CI: 1.7-7.4) with a 2-year, 5-year, and >=9-year lag period, respectively[49]. Although the risk declined over time, it remained significantly elevated almost 10 years following a diagnosis of CP. In addition, increased risk of pancreatic cancer was also observed in non-smokers and non-alcoholic CP patients[25,42–44]. It was estimated that about 1-5% of CP patients would develop pancreatic cancer at 10 years after CP diagnosis[41,42,46,48,50].

Diabetes Mellitus

A bi-directional relationship has been noted between diabetes mellitus (DM) and pancreatic cancer. The risk of pancreatic cancer was correlated negatively with the duration of DM, and the highest risk was found among DM patients diagnosed within less than one year [51,52]. It was hypothesized that recent-onset diabetes could be an early manifestation of pancreatic

cancer. Early symptoms of pancreatic cancer may also favor the diagnosis of diabetes (or vice versa), thus partly explaining the excess risk in patients with a recent diagnosis of diabetes. Meta-analysis has reported an overall 2-fold increased risk of pancreatic cancer in patients with long-standing DM (duration of DM > 2 years), and a significant 50% excess risk of pancreatic cancer was still evident among patients who had diabetes for 10 or more years [51,53,54]. This association is independent of alcohol consumption, BMI, and smoking status [51,52]. Although most published studies made no distinction between type-I and type-II DM, the majority of the patients were diagnosed with type-II DM. Studies in patients with type-I DM were often small and inconclusive. In a meta-analysis of 39 pancreatic cancer studies, the risk of pancreatic cancer was similarly elevated in patients with type-I DM (RR=2.00, 95%CI: 1.37-3.01)[55]. This finding was later supported by a population-based cohort study in Taiwan involving 14,619 patients with type-I DM, where a significantly increased risk was observed in both men (SIR = 2.61, 95%CI: 1.35-4.56) and women (SIR = 4.10, 95%CI: 2.35-6.66)[56]. People with prediabetes may also have a higher risk of pancreatic cancer. There is a linear dose-response relationship between fasting blood glucose concentration and the risk of pancreatic cancer across prediabetes and diabetes, with every 0.56 mmol/L (10 mg/dL) increase in fasting blood glucose associated with a 14% increase in the risk of pancreatic cancer[57].

Obesity

Association between obesity and pancreatic cancer is often weak and less conclusive, partly due to the concurrence of obesity and diabetes. Population-based cohort studies found 1.3-2.0 fold increase in risk of pancreatic cancer among obese individuals (BMI >= 30 kg/m^2 or BMI >= 35 kg/m^2) [58–65], after adjusting for history of diabetes. Being obese is also associated with worse clinical outcome in patients with pancreatic cancer [66–69]. However, in several studies,

the weak association between obesity and pancreatic cancer was only observed in men[70–72] or women[73,74]. Higher risk of pancreatic cancer is also more likely to be observed in people who were obese in early adulthood/adolescence or in those with substantial increment in BMI [63,71,75,76]. Findings from many of these cohort studies were not conclusive due to limited power resulting from the low incidence of the disease and the competing risk of deaths among obese individuals [35,64,65,77].

Genetic Factors

Pancreatic cancer is more prevalent in families with several hereditary syndromes, including hereditary breast and ovarian cancer (HBOC), Peutz-Jeghers syndrome (PJS), familial atypical multiple mole melanoma (FAMMM), hereditary pancreatitis (HP) and Lynch syndrome (LS). These genetic syndromes are reported to be associated with a substantially higher risk of pancreatic cancer. High-penetrance germline mutations of their predisposing genes are responsible for the occurrence of the disease in 8-30% familial pancreatic cancer (FPC) cases[78–81]. These mutations have also been detected in sporadic cases with and without a family history of the disease[82–84]. Because of the high prevalence of these genes (*BRCA1/2*, *CDKN2A*, *PRSS1*, *STK11*, *PALB2*, *ATM*, *MLH1*, *MSH2*, *MSH6*, and *PMS2*) in FPC families, they are considered the established FPC genes. An in-depth review of the risk of pancreatic cancer in these families and the predisposing genes of these genetic syndromes can be found in Chapter Two.

Genome-wide association studies (GWAS) allow for the unbiased evaluation of common genomic variants associated with pancreatic cancer. Six pancreatic cancer GWAS have been conducted by the Pancreatic Cancer Cohort Consortium (PanScan) and the Pancreatic Cancer Case Control Consortium (PanC4) in populations of European ancestry. A total of 21 pancreatic cancer susceptibility loci located in 18 genomic regions have been discovered in European

populations. The two GWAS in Asian populations reported an additional five loci associated with pancreatic cancer. Details of these susceptibility loci can be found in Chapter Two.

Family-based Heritability and Missing Heritability Problem

A central question in the study of the inheritance of human disease is whether the observed variation of a particular trait is due to environmental factors or genetic factors – the nature versus nurture debates. Heritability is formally defined as the proportion of phenotypic variance of a trait in a given population at a given time that is attributable to variation in genetic factors. The broad-sense heritability (H^2) measures the combined effects of all loci, including possible allelic interactions within loci (dominance) and between loci (epistasis). The narrow-sense heritability (h^2) quantifies the contribution of additive genetic effects which is the sum of the average effects of parents' genes that give rise to the mean genotypic value of their progeny (Supplementary Notes). Historically, narrow-sense heritability is considered a more useful parameter to predict the selection response in the breeding of plants and livestock animals. In the study of human traits, it is generally believed that the resemblance of a trait between relatives is mostly due to the additive genetic effects[85]. Therefore, current heritability studies have been primarily focused on the narrow-sense heritability.

Heritability is a population-specific parameter since both the genetic variance and environmental variance can vary across different populations. Differences in allele frequencies, the effect sizes and the mode of gene actions lead to changes in genetic variance of a trait. The shift of environmental exposures can also change heritability. In practice, heritability of the same trait is often quite similar across populations and species[85].

Family-based Heritability

Traditionally, heritability was estimated in family-based studies, as simple functions of the regression of offspring phenotypes on parental phenotypes, the correlation of full or half-sibs, and the difference in the correlation of monozygotic (MZ) and dizygotic (DZ) twin pairs. For example, in the classic twin studies, Falconer quantifies the heritability of a trait as twice the difference between the DZ resemblance (covariance or correlation) and the MZ resemblance[86].

The rarity and fast progression of pancreatic cancer have made heritability studies of the disease particularly difficult. The often-quoted estimate of heritability for pancreatic cancer was reported by a population-based twin study. Lichtenstein and his colleagues collected data on 44,788 pairs of twins listed in the Swedish, Danish, and Finnish twin register, aiming to assess the relative importance of heritable and environmental factors in causing cancer at 28 anatomical sites [87]. The heritability for pancreatic cancer (h^2_{fam}) was estimated to be 0.36 (95% CI: 0 – 0.53). The low precision of the heritability estimate is likely due to the small number of twin pairs affected by the disease. Despite the large sample size, there were only 3 pairs of concordant MZ twins and 1 pair of concordant DZ twins in men and women combined. Similarly, the most recent twin study with 80,309 monozygotic and 123,382 dizygotic twin individuals from population-based twin registers of Denmark, Finland, Norway and Sweden failed to estimate the heritability for pancreatic cancer due to having less than five pairs of concordant twin pairs diagnosed with the disease[88]. The limited statistical power of family-based studies has prohibited the further exploration of the genetic contribution to the risk of pancreatic cancer.

A general concern with family-based heritability is the potential inflation due to either the common environmental factors shared by relatives or the non-additive genetic effects. A crucial assumption underlying the twin study design is that the common environmental variation is equal among MZ and DZ twins [89,90]. The validity of this assumption is controversial since even

monochorionic and dichorionic MZ twins show differences in the magnitude of shared environmental effects for some traits[91,92]. Violation of equal-environment assumption is particularly alarming in twin studies of behavioral phenotypes[93,94]. In addition, the design of twin studies does not allow the estimate of non-additive genetic variance which would also lead to an overestimation of the narrow-sense heritability[89,90]. Heritability estimated from other types of family-based studies depends on the appropriate partitioning of observed variation into unobserved genetic and environmental factors. Recent studies have reported an overestimation of family-based heritability from statistical models that did not account for the shared environmental factors[95]. This inflation in family-based heritability was observed in a number of well-studied quantitative traits and binary diseases, including several common cancer phenotypes (e.g. lung cancer, prostate cancer and breast cancer)[95–98]. Moreover, family-based studies require large random population-based sampling of relative pairs, regardless of their disease status. Incomplete ascertainment will lead to a biased estimate of heritability (ascertainment bias). A previous study suggested that when the rate of diagnosis among relatives of probands was higher than the rate of diagnosis in population, the estimate of heritability from family-based studies were likely inflated[99].

Missing Heritability Problem

Pancreatic cancer is a genetically highly heterogeneous disease. Despite the large sample sizes of current GWAS studies, the identified common susceptibility loci together explain < 5% of the total phenotypic variation for pancreatic cancer [100–102]. Comparing to the family-based estimate of heritability (h^2_{fam}= 36%), it appears that a large proportion of heritability is unexplained, highlighting the so-called “missing heritability” problem.

There have been extensive debates on the source of missing heritability in complex diseases or traits. It is reasonable to assume that due to the differences in genetic architecture (number, type, effect size and frequency of susceptibility variants) across traits, missing heritability may take a different form for different diseases[103]. Many explanations for missing heritability have been suggested; each has different implications for the design of the next round gene discovery efforts [104]. Although inflated family-based heritability may partially explain the missing heritability for many complex traits or diseases, such inflation is likely to be trait-specific and remains debatable for pancreatic cancer. The fact that known GWAS loci only account for a small fraction of variance explained by all common variants implies that much of the missing heritability could be due to variants whose effects are too small to reach genome-wide significance. Thus, larger sample sizes are required to increase the power of GWAS studies for identification of yet-to-be-detected common variants. Rare variants are neither directly genotyped nor well tagged by the genotyping arrays. If the missing heritability is largely due to rare variants, extending coverage on these variants through whole-exome or whole-genome sequencing (WGS) in a large sample is more important for continued progress in gene discoveries. An excess of significant GWAS associations in non-coding regions underscores the importance of including both coding and regulatory regions in the search for disease-associated variants [105,106]. Understanding the genetic architecture of the disease is the key to find missing heritability and to guide the future strategies in discovering the disease-causing genes.

Population-based Heritability and GREML Approach

The Single-Component GREML Approach

To address the missing heritability problem, alternative approaches have been developed to estimate heritability in populations of unrelated individuals. GWAS relies on a very stringent

P value (e.g. 5×10^{-8}) to reduce the occurrence of false-positive results, but it may cause many real associations to be missed. Instead of focusing on the statistical significance of individual causal variants, these approaches are designed to capture the cumulative effects of all causal variants simultaneously.

One of the most widely applied methods in heritability analysis is GREML, which stands for the genomic-relatedness-based restricted maximum-likelihood (REML) approach. This approach assumes the effect sizes of causal variants are independent of the linkage disequilibrium (LD) and are inversely proportionate to their minor allele frequency (MAF), which implies that the per-variant contribution to heritability is constant across MAF spectrum[107]. Comparing to the family-based studies, this approach utilizes the very distant relationships between unrelated individuals to estimate heritability explained by causal variants passed down from their remote common ancestors. Therefore, estimates of heritability using GREML are less biased by shared environmental factors.

The simplest form of GREML approach is the single-component GREML (GREML-SC), where the disease status as cases (=1) or controls (=0) is modeled as a function of one or more fixed effects (u) including the intercept and covariates, a random genetic effect (g), and a residual effect (e) in a mixed linear model:

$$y = u + g + e \tag{1}$$

The total phenotypic variance is the sum of the variance due to random genetic effects ($G\sigma_g^2$) and the variance due to residual effects ($I\sigma_e^2$):

$$var(y) = G\sigma_g^2 + I\sigma_e^2 \tag{2}$$

Assuming all the causal variants are known, a genetic relationship matrix (GRM) G estimated from all causal variants can be obtained to determine the genetic similarities between

pairwise individuals. Given G, the variance due to random genetic effects can be estimated using the REML approach and the proportion of phenotypic variance explained by all causal variants is defined as

$$h^2 = \sigma_g^2/(\sigma_g^2 + \sigma_e^2) \quad (3)$$

For binary phenotypes, the estimates of variance need to be transformed from the observed 0-1 scale to the unobserved continuous 'liability' scale using a probit transformation[108], and a population prevalence of the disease (K) need to be specified to correct for the oversampling of cases in the case-control study.

Array-based Heritability

Heritability estimated from equation (3) can be considered as the overall heritability given all causal variants were known. However, in practice, we know little about the causal variants for a trait or disease. Instead, this approach has been widely applied in GWAS to estimate the proportion of phenotypic variation explained by the additive effects of all genotyped single-nucleotide polymorphisms (SNPs), termed array heritability. In this case, a different GRM (A) is constructed based on the genetic similarities estimated from all genotyped SNPs.

$$var(y) = A\sigma_g^2 + I\sigma_e^2 \quad (4)$$

The heritability is then defined as $h^2_{array} = \sigma_g^2/(\sigma_g^2 + \sigma_e^2)$, which estimates the array heritability, not the overall heritability. Like in GWAS, how much of the phenotypic variation can be captured by genotyped SNPs depends on the LD between the causal variants and the genotyped SNPs. Because GWAS arrays target primarily SNPs with MAF > 1%, the genetic variance due to rare variants (MAF < 1%) is not well captured in array heritability estimates. As a result, the array heritability is always an underestimation of the overall heritability.

In a collection of large case-control GWAS from the National Cancer Institute's Cancer Genomic Research Laboratory, the array heritability of pancreatic cancer was estimated to be 0.098 (95% CI: 0.037 – 0.160)[102]. Using the GWAS data from the Pancreatic Cancer Cohort Consortium (PanScan), about 0.18 (95%CI: 0.06 – 0.30) of the total phenotypic variation of pancreatic cancer was explained by common genotyped single-nucleotide polymorphisms (SNPs) [100]. In the Pancreatic Cancer Case-Control Consortium (PanC4) samples of Caucasian ancestry, 0.164 (95% CI: 0.104 – 0.224) of the total phenotypic variation was due to genome-wide common variants[101]. When combining the PanC4 data with PanScan data, the array heritability was estimated to be 0.131 (95%CI: 0.099 – 0.163)[101]. The variability of array heritability in these studies likely reflects the differences in study populations (though all study populations listed above are of European ancestry) and genotyping arrays.

Motivations of This Study

The genomics era has brought new tools and resources to study the relationship between phenotype and genotype. The development of novel statistical methods offers new research avenues to estimate heritability in unpedigreed populations using different types of genetic data. The previous estimates of array heritability have direct implications on the common causal variants in the susceptibility of pancreatic cancer, but provide no information regarding the rare causal variants. In addition, recent studies have found bias in array heritability estimated using GREML-SC, due to the uneven tagging of causal variants. To obtain a more comprehensive and less biased estimate of heritability for pancreatic cancer, this study is motivated by two factors: (1) the availability of imputation and whole-genome sequencing (WGS) data, and (2) the improvements on heritability estimates using the multi-component GREML approach.

Imputation and Whole-genome Sequencing Data

Comparing to genotyping arrays, imputation and WGS data both provide a more completed characterization of genetic variation across the genome, particularly on variants at the lower end of MAF spectrum. With the extended coverage on rare and low-frequency variants, the LD between causal variants and the analyzed variants would be greatly improved using imputation and WGS data. Simulation studies have demonstrated that heritability estimated from imputed variants was able to capture almost all variation at common causal variants and a proportion of variation at rare causal variants[109]. If rare variants do contribute to the susceptibility of pancreatic cancer, we would expect to see an increase in heritability estimate using imputation data ($h^2_{imputed}$) in relative to array data. With sufficient sample sizes, WGS data in theory has the potential to capture all causal variants and hence generate the closest estimate of overall heritability, which could further approach to the family-based heritability for pancreatic cancer.

The Multi-Component GREML Approach

Despite its wide applications in GWAS studies, heritability estimated from GREML-SC is known to be biased, due to the average LD between causal variants and analyzed variants being higher or lower than the average LD between analyzed variants[110]. Even with whole-genome sequencing data, the GREML-SC estimate of heritability would be biased if the causal variants had a different MAF spectrum or LD property than the variants used in the analysis[109]. Such bias could be ameliorated by the multi-component GREML approach with additional stratification on MAF and/or LD.

The multi-component GREML approach takes a general form as,

$$y = u + \sum_t^T g_t + e \quad (5)$$

where more than one genetic components (g_t) are included in a mixed linear model. The total phenotypic variance now becomes the sum of variance attributable to each genetic component as well as the residual effects,

$$var(y) = \sum_t^T A_t \sigma_t^2 + I\sigma_e^2 \quad (6)$$

and the heritability for a trait can be calculated as

$$h^2 = \sum_t^T \sigma_t^2 / (\sum_t^T \sigma_t^2 + \sigma_e^2) \quad (7)$$

The multi-component GREML approach has two variations: GREML-MS and GREML-LDMS. In a MAF-stratified GREML (GREML-MS) analysis, the number of genetic components corresponds to the number of MAF groups defined at specific thresholds. This approach can adjust for the difference in LD caused by a difference in the MAF spectrum but is unable to correct for the region-specific LD heterogeneity across the genome. Empirical data suggested that GREML-MS was able to minimize most bias observed in GREML-SC estimates[111]. If the causal variants tend to be enriched in genomic regions with higher or lower LD than average (e.g. DNase I-hypersensitive sites), the GREML-MS estimates of heritability will be biased[109]. The LD- and MAF-stratified GREML (GREML-LDMS), which uses a sliding window approach to fit the region-specific LD heterogeneity across the genome, is demonstrated to produce more valid estimates of heritability across different simulation scenarios of causal variant distributions[109,110]. Because GREML-LDMS fits a large number of components, a much larger sample size is required to obtain acceptable standard errors (e.g. < 0.05).

By partitioning variants into narrower MAF and LD ranges in multi-component models, the LD between causal variants and analyzed variants get closer to the LD between analyzed variants, and therefore the variance estimates associated with each partitioned GRM are likely to be approximately unbiased. The multi-component GREML approach not only provides less

biased estimates of heritability, but also allow the investigation of the genetic architecture of complex traits through the estimation of heritability components from different variant sets.

Goals of this Study

Pancreatic cancer represents a major public health challenge. The limited progress in prevention, early detection, and treatment of pancreatic cancer underscore the urgent need for continued research efforts to illuminate the biology and etiology of the disease. Inherited genetic variation plays an important role in both the familial and sporadic occurrences of pancreatic cancer, and may be used to characterize populations who will benefit from early detections. Heritability (narrow-sense heritability) measures the contribution of additive genetic effects to the susceptibility of a disease. In the past, family-based heritability analysis for pancreatic cancer was limited because of the rarity of the disease. Such studies often lack the sufficient statistical power to produce meaningful estimates. In addition, heritability estimated from family-based studies may be inflated due to shared environmental factors within families. The newly developed statistical approaches allow the assessment of heritability in unrelated individuals, which teases apart the contribution of environmental factors shared among relative. However, heritability derived from GWAS studies are generally underestimated since rare variants are not well captured by genotyping arrays.

Finding the missing heritability of pancreatic cancer requires an accurate estimate of heritability and a better understanding of the genetic variation underlying the disease. The goal of this study is to comprehensively evaluate the genetic contribution to the susceptibility of pancreatic cancer using both whole-genome sequencing data and imputed data. These data provide a near-complete characterization of the genetic variation across the genome, minimizing the bias in heritability estimation due to inadequate coverage of causal variants, particularly

those at the lower end of MAF spectrum. The application of the multi-component GREML approach in this study took into account the unknown MAF distributions and LD properties of causal variants, generating a less biased estimate of heritability for pancreatic cancer. Using heritability measures, we explored the genetic architecture of pancreatic cancer through partitioning the estimated heritability by MAF, chromosomes and functional annotations. This analysis was aimed to answer critical questions about the role of rare and common variants in the susceptibility of pancreatic cancer, as well as the importance of coding and regulatory variants in pancreatic cancer risk. In addition to the reported GWAS loci for pancreatic cancer, we also quantified the contributions of established FPC genes, which further helped determine the source of missing heritability.

Supplementary Notes

Observed phenotypes (P) of a trait can be partitioned into a simple statistical model representing the contribution of the unobserved genetic (G) and environmental factors (E).

$$Phenotype(P) = Genotype(G) + Environment(E) \quad (1)$$

The variance of the observed phenotype (σ_P^2) can be expressed as a sum of unobserved underlying variance due to genetic (σ_G^2) and environmental factors (σ_E^2).

$$\sigma_P^2 = \sigma_G^2 + \sigma_E^2 \quad (2)$$

The broad-sense heritability (H^2) is defined as the proportion of phenotypic variance that can be attributed to the variance of genetic factors.

$$H^2 = \frac{\sigma_G^2}{\sigma_P^2} \quad (3)$$

The genetic variance can be further partitioned into the variance due to additive (σ_A^2), dominance (σ_D^2) and epistatic genetic effects (σ_I^2).

$$\sigma_G^2 = \sigma_A^2 + \sigma_D^2 + \sigma_I^2 \quad (4)$$

The narrow-sense heritability (h^2) is defined as the proportion of phenotypic variance that can be attributed to the additive genetic effects.

$$h^2 = \frac{\sigma_A^2}{\sigma_P^2} \quad (3)$$

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CHAPTER TWO

INHERITED PANCREATIC CANCER

Pancreatic cancers arise through a series of genetic events both inherited and acquired. Inherited genetic changes, both high penetrance and low penetrance, are an important component of pancreatic cancer risk, and may be used to characterize populations who will benefit from early detection. Furthermore, pancreatic cancer patients with inherited mutations may be particularly sensitive to certain targeted agents, providing an opportunity to personalized treatment. Family history of pancreatic cancer is one of the strongest risk factors for the disease, and is associated with an increased risk of cancers at other sites, including but not limited to breast, ovarian and colorectal cancer. The goal of this chapter is to discuss the importance of family history of pancreatic cancer, and the known genes that account for a portion of the familial clustering of pancreatic cancer.

Family History and Risk of Pancreatic Cancer

Family history is a long-recognized risk factor for pancreatic cancer and an important predictor of disease risk. Studies have suggested that approximately 5-10% of pancreatic cancer patients report a close relative with pancreatic cancer[1,21,2]. Most epidemiological studies have demonstrated a 2- to 3-fold increase in risk of pancreatic cancer among individual with affected first-degree relatives (FDRs)[3–12]. However, some studies have shown even higher risk. A Swedish study reported a standardized incidence ratio (SIR) for pancreatic cancer of 1.73 (95%CI: 1.13-2.54) in offspring with at least one parent affected with pancreatic ductal adenocarcinoma (PDAC)[13]. In a prospective study from the National Familial Pancreas Tumour Registry (NFPTR), the SIRs for pancreatic cancer in comparison to the SEER (Surveillance,

Epidemiology, and End Results) rates were 6.4 (95% CI: 1.8-16.4) and 32.0 (95% CI: 10.2-74.7) in individuals with two and three FDRs with pancreatic cancer[14].

Familial clustering was considered the first evidence supporting the genetic predisposition to pancreatic cancer. Reports of multiple siblings in one generation and individuals in three consecutive generations affected by pancreatic cancer are strong evidence of a hereditary form of the disease following Mendelian inheritance patterns[15–19]. This was later supported by segregation analyses, which favored a major gene model that was predicted to follow an autosomal dominant pattern of a rare allele[20]. As demonstrated in the observational epidemiologic studies, individuals with a family history of pancreatic cancer are at an increased risk of developing the disease themselves. In addition, a population-based twin study in Europe has estimated the heritability for pancreatic cancer to be 36% (95% CI: 0-53%)[21]. As more evidence of the genetic basis of pancreatic cancer has emerged, an operational definition of familial pancreatic cancer (FPC) was proposed to facilitate investigations of the inherited components of the disease. FPC is defined as kindreds with at least a pair of FDRs diagnosed with PDAC. Comparing to the general population, FPC kindred members have a 7- to 9-fold increased risk of pancreatic cancer[14,22]. Risk is even higher among members of FPC kindreds with a young-onset case (< 50 years; SIR = 9.31, 95% CI: 3.42-20.28) than those without (SIR = 6.34, 95% CI: 4.02-9.51)[22].

Both prospective and retrospective studies have found increased risks of other cancers in relatives of pancreatic cancer patients, particularly breast cancer[23,24], melanoma[24], ovarian[25], and colorectal cancer[24,26]. Study from NFPTR reported an increased risk of dying from cancer of the breast (weighted standardized mortality ratio (wSMR) = 1.66, 95%CI: 1.15-2.34), ovarian (wSMR=2.05, 95%CI: 1.10-3.49), bile duct (wSMR = 2.89, 95%CI: 1.04-6.39) and bladder

(wSMR = 1.90, 95%CI: 1.00-3.30) in FDRs of FPC probands[27]. Elevated mortality of colon cancer (wSMR = 2.31, 95%CI: 1.30-3.81) and prostate cancer (wSMR = 2.31, 95%CI: 1.14-4.20) were also observed among the relatives of young-onset (< 50 years old) pancreatic cancer probands[27]. These findings suggest a shared genetic etiology between pancreatic cancer and several other cancers, and the potential benefits of surveillance at-risk relatives for cancers with established screening guidelines.

Pancreatic Cancer Genes

Pancreatic cancer is more prevalent in families with several hereditary syndromes, for which the predisposing genes have been identified, including *BRCA1* and *BRCA2* associated with hereditary breast and ovarian cancer (HBOC), *STK11* associated with Peutz-Jeghers syndrome (PJS), *CDKN2A/p16* associated with familial atypical multiple mole melanoma (FAMMM), mismatch repair (MMR) genes associated with Lynch syndrome, and *PRSS1* associated with hereditary pancreatitis (HP). These genetic syndromes are reported to be associated with a substantially higher risk of pancreatic cancer. The recent discovery of germline mutations in *PALB2* and *ATM* genes in FPC kindreds has extended the list of established high- and moderate-risk pancreatic cancer genes (Table 1).

BRCA1/2

BRCA1 and *BRCA2* gene are well-known high-penetrant predisposing genes for hereditary breast and/or ovarian cancer (HBOC). These genes are involved in the DNA damage response and DNA double-strand breaks repair. Pancreatic cancer is the third most common cancer associated with *BRCA1*/2 mutations, though the penetrance at age 70 is much lower[28–31]. The prevalence of *BRCA2* mutations in pancreatic cancer patients is 1.4-8.2% for patients unselected for family history[32–36], about 6-10% among FPC patients[37–42], and up to 17.2% in

families with 3 or more pancreatic cancers[38,41,43]. Comparing to the general population, the risk of pancreatic cancer is about 2-6 fold in *BRCA2* carriers[28,44] and 2-5 fold in *BRCA1* carriers [29,30,44]. Several studies had reported a higher risk of pancreatic cancer in *BRCA2* carriers than in *BRCA1* carriers[28,31,44–46]. *BRCA1/2* mutation carriers are at particularly high risk (SIR = 4-10) for early onset pancreatic cancer[28–30,44]. FDRs of *BRCA1/2* carriers, regardless of their carrier status, have a significantly higher risk of pancreatic cancer than the general population[44,46].

PALB2

PALB2 gene is a tumor suppressor that interacts closely with both *BRCA1* and *BRCA2* during double-strand DNA repair. Mutations of *PALB2* had previously been associated with familial breast cancer[47]. Jones *et al.* first reported the discovery of truncating mutations of *PALB2* gene in four FPC probands from the NFPTR [48]. Since then, pathogenic mutations of *PALB2* gene have been found in 0.4-4% FPC families, majority of which were families with history of both pancreatic cancer and breast/ovarian cancer[49–55].

ATM

ATM is a breast cancer susceptibility gene that coordinates the DNA double-strand breaks repair. Deleterious mutations of *ATM* gene were first reported by Roberts and his colleagues in two FPC families with at least three members affected by pancreatic cancer[56]. In the subsequent analysis, four additional *ATM* mutations were found in 166 FPC patients compared to none in 190 spouse controls[56]. To date, *ATM* mutations are found in 1-5% patients with pancreatic cancer[35,36,42,57–59].

STK11

Peutz-Jeghers syndrome (PJS) is caused by germline mutations in the *STK11* gene[60–62]. PJS patients are at very high risk of developing cancer during their lifetimes, particularly

gastrointestinal cancer and gynecological cancer. The cumulative risk of developing any gastrointestinal cancer is 38-66% at age 70[63]. Compared to the general population, PJS patients have a 76-140 fold elevated risk for pancreatic cancer[64–66]. The cumulative risk of developing pancreatic cancer at age 70 in PJS patients is 11-55%[64–67].

CDKN2A

CDKN2A is a tumor suppressor gene that is considered a major cause of familial melanoma. In melanoma-prone families of European ancestry, pancreatic cancer is the second most common type of cancers associated with *CDKN2A* mutations. Longitudinal studies in these families have found a 15- to 80-fold increased risk of pancreatic cancer in carriers of *CDKN2A* mutations comparing to the general population[68–73]. The risk of developing pancreatic cancer is also higher in FDRs of carriers than in FDRs of non-carriers (RR = 7.4 [95%CI: 2.3-18.7])[74].

Mismatch repair genes

Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch Syndrome, accounts for 2-5% of all colorectal cancer. It is caused by inactivating mutations of DNA mismatch repair (MMR) genes: *MLH1*, *MSH2*, *MSH6* and *PMS2*. While several studies found no increase in risk of pancreatic cancer in Lynch Syndrome patients[75–78], others reported an approximately 7- to 10-fold elevated risk of developing pancreatic cancer in carriers of MMR gene mutations[79–82]. The relative risk of pancreatic cancer is higher at younger age[79,80]. The cumulative risk of developing pancreatic cancer at age 70 among mutation carriers was estimated to be 3.68% (95% CI: 1.45% - 5.88%) [80].

PRSS1

Hereditary pancreatitis (HP) is autosomal-dominant disorder characterized by recurrent episodes of acute pancreatitis in childhood and frequent progression to chronic pancreatitis.

Germline mutations in *PRSS1* are responsible for the majority of HP cases. Comparing to the general population, the risk of pancreatic cancer is about 69-fold higher in HP patients, and the median age of cancer onset was at least 15 years earlier [83–86]. About 20-50% HP patients would develop pancreatic cancer at age 70[83–85,87]. The risk is even higher among smokers with HP who tend to develop pancreatic cancer 20 years before non-smokers[85,88].

Germline Mutations in Sporadic Cases

Inherited genetic alterations are not restricted to patients with FDRs affected by pancreatic cancer. Mutations of pancreatic cancer genes are also found in non-FPC patients with or without family history. Three germline mutations in *BRCA2* was found in 41 (7.3%) sporadic pancreatic cancer patients without family history[32]. Of the 14 *BRCA* mutation carriers identified from 306 consecutive incident PDAC patients, only two had a family history of PDAC[33]. Salo-Mullen *et al.* reported a 7.4% prevalence of *BRCA* mutations in 27 PDAC patients of Ashkenazi Jewish ancestry without a family history of breast, ovarian or pancreatic cancer[34]. Studies have reported 0-3% sporadic or unselected pancreatic cancer patients carrying *PALB2* mutations, leading to an aggregated prevalence of 0.75%[55]. In an unselected group of 854 pancreatic cancer patients, twelve of them had germline *BRCA2* mutations, ten with *ATM*, three with *BRCA1*, and two with *PALB2*, the majority of whom had no FDR affected by the disease[36]. Larger scale studies are currently underway to evaluate the mutation prevalence in apparently sporadic pancreatic cancer and expanding genetic testing beyond the current guidelines.

Targeted Therapy

Understanding genetic predisposition of pancreatic cancer has important implication for the development and translation of targeted therapies. Tumors with mutations in *BRCA1/2*, *PALB2*, and *ATM* are highly sensitive to DNA-damaging related treatments such as crosslinking

agents[89–91] and poly(ADP-ribose) polymerase inhibitors (PARPi)[92–95]. Preclinical studies have demonstrated improved sensitivity to chemotherapeutic agents and ionizing radiation in pancreatic cancer cells treated by these agents[96–98]. The clinical benefits of using crosslinking agents and PARPi in patients with pancreatic cancer are currently being investigated. Preliminary results have shown promising efficacy of these agents, particularly in patients with *BRCA2*-associated pancreatic cancer[99–102].

Common Low-Risk Susceptibility Loci

Genome-wide association studies (GWAS) allow for the unbiased evaluation of common genomic variants associated with pancreatic cancer. To identify common susceptibility variants, six pancreatic cancer GWAS have been conducted by the Pancreatic Cancer Cohort Consortium (PanScan) and the Pancreatic Cancer Case Control Consortium (PanC4) in populations of European ancestry, including PanScan I in 2009[103], PanScan II in 2010[104], PanScan III in 2014[105], PanC4 in 2015[106], an imputation analysis of PanScan I-III[107] and a recent meta-analysis of PanScan I-III and PanC4. A total of 21 pancreatic cancer susceptibility loci located in 18 genomic regions have been discovered in European populations (Table 2).

PanScan I

PanScan I was a two-stage GWAS including 1,896 patients with incidence pancreatic cancer and 1,939 controls in the discovery stage, as well as 2,457 cases and 2,654 controls in the replication stage. The most significant variant (rs505922) on chromosome 9q34.2 was mapped to the first intron of *ABO* blood group gene [103]. The association of ABO loci with pancreatic cancer has been robustly replicated in studies of European[105,106,108,109] and Asian populations[110–112]. These findings are consistent with the observation that individuals with blood group O had a lower risk of pancreatic cancer than those with groups A or B. About 17% to 19.5% of all

pancreatic cancers in populations of European descent was attributable to the inheritance of a non-O blood group[113,114].

PanScan II

From 3,851 pancreatic cancer cases and 3,934 controls, PanScan II identified three novel genomic regions on chromosome 13q22.1 (a large non-genic region), chromosome 1q32.1 (*NR5A2*) and chromosome 5p15.33 (*TERT-CLPTM1L*) to be significantly associated with pancreatic cancer[104]. The locus on chromosome 13q22.1 (rs9543325) was mapped to a large gene desert flanked by the *KLF5* and *KLF12* genes. Both genes encode a transcription factor involved in cell transformation, proliferation, and carcinogenesis. Several studies have reported the overexpression of the *KLF5* gene in pancreatic cancer [115–118]. The *KLF2* gene, on the other hand, was found to be downregulated in PDAC tumor tissues, and its expression may suppress the malignant transformation of PDAC cancer cells through its regulation of beta-catenin/TCF signaling[119].

Two variants on chromosome 1q32.1 are associated with pancreatic cancer independently. The first significant variant (rs3790844) identified in PanScan II is located in the first intron of the *NR5A2* gene[104]. Imputation analysis of PanScan I-III detected the second variant in the upstream of *NR5A2* (rs2816938). *NR5A2* encodes the nuclear receptor subfamily 5 group A member 2, a transcription factor that activates or inhibits transcription of specific target genes. Overexpression of NR5A2 was observed in resected PDAC tumor tissues and was associated with reduced survival time in PDAC patients[120]. Heterozygous Nr5a2 mice exhibit increased rates of pancreatic acinar to ductal metaplasia and impaired recovery after chemically induced acute pancreatitis[121,122]. Loss of Nr5a2 accelerates the development of oncogenesis

driven by Kras[121,122]. These findings suggest a tumor suppressor role of *NR5A2* that protects pancreas from KRAS driven pre-neoplastic changes.

Four independent pancreatic cancer risk loci have now been identified in the multi-cancer *TERT-CLPTM1L* region on chromosome 5p15.33. The first pancreatic cancer risk locus identified in PanScan II is located in the intron 13 of *CLPTM1L* (rs401681). PanScan III reported a second independent risk locus on chromosome 5p15.33, tagged by a synonymous variant within the second exon of *TERT* (rs2736098) [105]. A third independent risk locus located in the first intron of *TERT* gene (rs2853677) was discovered through a candidate gene analysis in 5,550 pancreatic cancer cases and 7,585 control subjects from PANDoRA (PANcreatic Disease ReseArch) consortium and PanScan [123]. Recently, imputation of PanScan I-III and replication in PANDoRA and PanC4 found a fourth risk locus for pancreatic cancer in this genomic region (rs35226131), which is located about 200bps upstream of the transcriptional start site of *TERT* [107]. The chromosome 5p15.33 region contains two plausible candidate genes: *TERT*, which encodes the catalytic subunit of telomerase reverse transcriptase and *CLPTM1L*, which encodes the cleft lip and palate-associated transmembrane 1 like protein. TERT is a component of the protein and RNA complex that maintains telomere ends. Mutations in TERT promoter region were frequent in multiple tumor types and were correlated with increased TERT expression and telomerase activation[124]. Common variants in the *TERT* region were associated with leukocyte telomere length in patients with breast and ovarian cancer[125]. A recent study had reported an association between the minor allele of rs401681 and shorter telomere length in pancreatic cancer patients, which was consistent with the observation that telomere shortening occurs as an early event in pancreatic tumorigenesis[126–128]. Overexpression of *CLPTM1L* gene are observed in lung and pancreatic cancer tissues[129–131]. CLPTM1L protects tumor cells from genotoxic

apoptosis and is required for Ras-induced oncogenic transformation[129,130,132]. It's also found that overexpression of CLPTM1 may lead to an abrogation of normal cytokinesis and promote cell proliferation in pancreatic cancer cells[131].

PanScan III

The PanScan III study population combined 1,582 newly genotyped pancreatic cancer cases and 5,203 control subjects with PanScan I cohort and sought replication in 2,576 cases and 6,662 controls from the PANDoRA consortium. Four new risk loci for pancreatic cancer was identified on chromosome 7q23.2 (*LINC-PINT*), 16q23.1 (*BCAR1*), 13q12.2 (*PDX1*) and 22q12.1 (*ZNRF3*). The signal on 7q32.3 was marked by an intronic variant (rs6971499) in *LINC-PINT*, a long intergenic p53-induced non-protein coding RNA located between *MKLN1* (Muskelin 1) and *KLF14* (Kruppel-like factor 14). Muskelin is an intracellular protein that mediates cell adhesive and cytoskeletal responses to the extracellular matrix[133]. KLF14 is a member of the Kruppel-like family of transcription factors that may act as a suppressor of KRAS-mediated cell growth through regulation of the cyclin A promoter[134]. Loss of KLF14 may also trigger centrosome amplification, aneuploidy and spontaneous tumorigenesis[135]. KLF14 has also been associated with several metabolic phenotypes including type-2 diabetes mellitus (T2DM), a known risk factor for pancreatic cancer[136–139].

A synonymous variant residing in the last exon of *BCAR1* was noted on 16q23.1 (rs7190458). Breast cancer anti-estrogen resistance 1 (BCAR1), also known as p130Cas is a member of the Cas (Crk-associated substrate) family of adaptor proteins with important regulatory roles in migration, cell cycle control and apoptosis[140]. Altered expression and activity of p130Cas is known to promote metastasis and drug resistance in multiple cancers[140,141]. In addition, two chymotrypsinogen gene, *CTRB1* and *CTRB2* are also located closely to the

detected signal. As important members of a family of serine proteases secreted by the pancreas into the gastrointestinal tract[142], these two genes are plausible target for susceptibility variants at this locus. The detected signal for pancreatic cancer is also in proximity of a susceptibility locus (rs7202877) for type-I and type-II diabetes[143,144] that was found to impair beta-cell function[145] and influences expression of CTRB1/2 in pancreas tissues[146].

The top ranked variant on chromosome 13q12.2 (rs9581943) is located in the promoter region of the *PDX1* (pancreatic and duodenal homeobox1 protein 1) gene. Pathway analysis of GWAS data identified *PDX1*, along with *NR5A2*, *HNF1A*, and *HNF4G*, as important genes for pancreatic development[147]. The protein encoded by *PDX1* is a transcriptional activator of several genes. It is essential in the early development of pancreas[148], and plays a major role in beta-cell function and glucose-dependent regulation of insulin gene expression. Heterozygous mutations in *PDX1* resulted in impaired glucose tolerance and symptoms of diabetes as seen in maturity-onset diabetes of the young type 4 (MODY4) and late-onset T2DM[149–151].

The signal on chromosome 22q2.1 (rs16986825) maps to an intron in *ZNRF3* (zinc and ring finger 3), which encodes a cell surface transmembrane E3 uniquitin protein ligase that is a negative regulator of the WNT signaling pathway[152]. Additionally, a low-penetrance breast cancer gene, *CHEK2,* is also located in proximity to the detected signal. This gene encodes a cell-cycle checkpoint kinase that cooperates with p53, BRCA1 and ATM and regulates cell division in response to DNA damage[153]. Germline mutations and variants of *CHEK2* had been implicated in susceptibility to several cancer types[154–158], including familial pancreatic cancer[41,42,159].

PanC4

PanC4 was conducted on 9,925 pancreatic cancer cases and 11,569 controls, pooling 4,164 newly genotyped cases and 3,792 controls from nine studies in the PanC4 consortium with PanSan I and II cohorts in the gene discovery stage, and analyzed an independent set of 2,497 cases and 4,611 controls from the PANDoRA consortium in the replication stage. Not only this study replicated all previously identified risk loci for pancreatic cancer in European populations, three novel associated signals were also detected on chromosome 17q25.1 (*LINC00673*), 7p13 (*SUGCT*) and 3q29 (*TP63*)[106]. Significant association was also found on chromosome 2q13.3 (*ETAA1*), a region with prior suggestive evidence in the Han Chinese[160].

The top variant on 17q25.1 (rs11655237) maps to *LINC00673* (long inter-genic non-protein coding RNA 673). This association was also observed in a Han Chinese population (rs11655237, OR = 1.26, $P = 3.95\times10^{-14}$)[161]. Through its epigenetic regulation of gene expression, *LINKC00673* may function as an oncogene in several types of cancers. Overexpression of LINC00673 promotes tumor proliferation, invasion and metastasis in non-small-cell lung cancer [162–164] and tongue squamous cell carcinoma[165], and was correlated with poor prognosis in breast cancer[166]. In contrast, expression of LINC00673 was significantly lower in PDAC cancer cells than in normal cells and tissues and overexpression of LINC00673 in the PDAC cell line substantially reduced the rate of cell proliferation. It was found that the single-nucleotide change at rs11655237 creates a miR-1231 binding site, which diminishes the effect of LINC00673 in an allele-specific manner and thus confer susceptibility to pancreatic tumorigenesis [161].

PanC4 reported a significant association on 7p13 with an intronic variant (rs17688601) of the *SUGCT* (succinyl-CoA:glutarate-CoA transferase) gene. Mutations of this gene cause a benign form of glutaric aciduria (glutaric aciduria type III), a rare metabolic abnormality

characterized by persistent isolated accumulation or excretion of glutaric acid [167]. The role of this gene in pancreatic cancer risk is unclear.

Two strongly correlated intronic variants of *TP63* (tumor protein p63) were found to be associated with pancreatic cancer in PanC4 (top variants rs9864771). Protein encoded by *TP63* (p51/p63) is a p53 homologue with pleiotropic functions including cell proliferation, survival, apoptosis, differentiation, senescence, and aging. Frequent overexpression of p63 was observed in resected PDAC tissues[168]. It was suggested that different isoforms of p63 have opposite effects. While TAp63 induces cell death and cell cycle arrest with tumor suppressor features[169], DNp63 as the predominant isoform in pancreatic cancer cell lines, promotes pancreatic cancer growth, motility and invasion[170,171]. As a tumor suppressor, p63 had reduced anti-oncogenetic effects compared with p53 in human cancer cells[172]. However, loss of p63 can cooperate with loss of p53, leading to higher tumor burden and metastasis as seen in genetic mice models[168,171]. It is hypothesized that it is the ratio of TAp63 and DNp63 that determines the biological outcome and chemo-sensitivity.

Imputation Analysis of PanScan I-III

The imputation analysis of the GWAS data in 5,107 cases and 8,845 controls from PanScan I-III had uncovered three new pancreatic cancer signals on chromosome 1q32.1 (*NR5A2*), 8q24.21 (*MYC*), and 5p15.33 (*CLPTM1L-TERT*), all of which are independent from previously reported susceptibility variants[107].

The detected variants on 8q24.21 (rs10094872) is a novel risk loci for pancreatic cancer, independent from the previously reported loci with suggestive evidence in PanScan III (rs1561927). These two variants are both located in the 2 Mb region known to contain multiple susceptibility loci that influence risk of bladder, breast, prostate, colorectal, lung, ovarian,

pancreatic, and renal cancer[173–177]. *MYC* (MYC proto-oncogene, bHLH transcription factor) is the gene located in the closest proximity to the detected variant. Oncogene MYC is a transcription factor that has been implicated in the pathogenesis of one-third of all human malignancies, and may play an important role in KRAS-driven neoplastic transformation in the pancreas[178]. MYC overexpression occurs in up to 42% of advanced PDAC[179,180]. Activation of MYC in adult mice has led to the development of ductal adenocarcinomas with metastasis to the liver[181]. Although evidence have suggested regulatory roles of the 8q24.21 risk loci in the expression of MYC, functional analysis are warranted to allow a deeper understanding of the underlying mechanism[178].

Meta-analysis of PanScan I-III and PanC4

A recent meta-analysis of 9,040 pancreatic cancer patients and 12,496 controls of European ancestry from PanScan and PanC4 has identified a significant association on chromosome 7p12 (*TNS3*). Replications of promising signals in the PANDoRA consortium yield another four genome-wide significant loci on chromosome 1p36.33 (*NOC2L*), 8q21.11 (*HNF4G*), 17q12 (*HNF1B*) and 18q21.32 (*GRP*).

The variant at 1p36.33 (rs13303010) maps to the first intron of the *NOC2L* gene, which encodes the nucleolar complex protein 2 homology, also known as novel INHAT repressor (NIR). NIR is an inhibitor of histone acetyltransferases (HAT) that can bind to p53 at promoters and inhibit p53-mediated gene transactivation by blocking histone acetylation[182]. This protein can also bind to TAp63, a tumor suppressor protein closely related to p53, resulting in the inhibition of TAp63-dependent transactivation of p21[183]. Notably, an intronic variant of the *TP63* gene had previously been identified as a susceptibility locus for pancreatic cancer[106]. Increased expression of *NOC2L* was found in pancreatic cancer cell lines as compared with

histologically normal pancreatic cancer samples[184]. This gene is also among the seventeen genes differentially methylated in breast tumor samples[185].

The locus at 7p12 was marked by an intronic variant (rs73328514) in *TNS3*. This gene encodes Tensin 3, a member of the Tensin family of intracellular proteins that is known as a negative regulator of cell migration and adhesion [186–188] and may play an important role in tumorigenesis and cancer metastasis[186,189,187].

An intronic variant of *HNF4G* on chromosome 8q21.11(rs2941471) was found to be associated with pancreatic cancer. The *HNF4G* gene encodes hepatocyte nuclear factor 4 gamma, a member of nuclear receptor (NRs) that regulate gene expression in a spatiotemporal manner. *HNF4G* is one of the orphan NRs whose endogenous ligands and biological functional are unknown. In addition to this meta-analysis, two pathway analysis of GWAS data had each identified *HNF4G* as a susceptibility factor for pancreatic cancer[147,190]. Common variants of this gene were found to be associated with breast cancer[191]. This gene is also involved in the growth and invasion of bladder cancer[192,193]. In addition, a variant of *HNF4G* correlated with rs2941471 has been significantly associated with variations in serum urate concentrations (rs2941484, r^2 = 0.56, in 1000 Genomes EUR)[194], consistent with a previous report of elevated urate levels as an early event of pancreatic cancer[195].

The signal at 17q12 was located in the fourth intron of *HNF1B*, encoded hepatocyte nuclear factor 1-beta, another member of NRs that is known to regulate the early development of pancreas and cell differentiation[196]. Mutations of this gene cause a specific type of maturity-onset diabetes of the young (MODY5), also known as renal cysts and diabetes syndrome (MIM [137920]). Common variants of this gene are associated with type-2 diabetes mellitus and related phenotypes[197–199], and hence may also contribute to the susceptibility of pancreatic cancer.

Genetic and epigenetic changes of this gene are also associated with risk of endometrial, ovarian and prostate cancer[200–204].

At 18q21.32, the detected association was marked by rs1517037, about ~10kb upstream of the *GRP* gene. This gene encodes a member of the bombesin-like family of gastrin-releasing peptides that stimulate exocrine pancreatic secretion and release various gastrointestinal peptides[205,206]. Studies have suggested a potential role of *GRP* in pancreatic tumor growth[207].

GWAS in Asian Populations

Two GWAS have been conducted in populations of Asian descent (Table 3). The Japanese pancreatic cancer study of 991 cases and 5,209 controls found suggestive associations on chromosome 6q25.3 (*FOXQ1*), 12p11.21 (*BICD1*) and 7q36.2 (*DPP6*) [208]. The second GWAS in a Chinese population of 3,584 pancreatic cancer cases and 4,868 controls (ChinaPC) identified five susceptibility loci on chromosome 21q21.3 (*BACH1*), 21q22.3 (*TFF1*), 10q26.11 (*PRLHR*), 22q13.32 (*FAM19A5*), and 5p13.1 (*DAB2*)[160]. The most significant association identified in ChinaPC was for rs372883, a variant located in the 3' untranslated region (3'UTR) of *BACH1* (BTB domain and CNC homolog 1) gene on chromosome 21q21.3. BACH1 is a transcription factor that belongs to the cap 'n' collar type of basic region leucine zipper factor family (CNC-bZip). Recent studies have demonstrated a critical role of BACH1 in cell migration and metastasis through its regulation of metastasis-related gene expression in breast, colon and prostate cancer [209–211]. The second significant association was detected on chromosome 21q22.3 (rs1547374). This region harbors the trefoil family protein 1 (*TFF1*) gene that encodes secretory proteins expression in gastrointestinal mucosa. Upregulated expression of TFF1 in precursor lesions of PDAC, including pancreatic intraepithelial neoplasia (PanIN), intraductal papillary-mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MSNs), suggests its potential

involvement at the early stage of pancreatic carcinogenesis [212–214]. Recent studies found that reduced expression of TFF1 in the invasion front of human PDAC was associated with lymph node metastasis and poor survival in patients with PDAC[215]. In pancreatic cancers, expression of TFF1 promotes tumorigenesis by suppressing oncogene-induced senescence [216] and is correlated with increase metastasis[217]. An intronic variant (rs2255280) in *DAB2* (clathrin adaptor protein) gene region on 5p13.1 was among the identified susceptibility loci in ChinaPC. Frequent loss expression of DAB2 in human malignant cancer cells suggests its potential role as a tumor suppressor[218]. Overexpression of DAB2 inhibits cell growth, migration and invasion, and was correlated with poor survival in cancer patients[219–222]. Significant association on 10q26.11 was observed for a regulatory variant of *PRLHR* (prolactin releasing hormone receptor) gene (rs12413624). Polymorphisms of this gene was associated with colorectal cancer[223]. A intronic variant of *FAM19A5* (family with sequence similarity 19 member A5) gene on 22q13.32 was also associated with an increased risk of pancreatic cancer (rs5768709). This gene encodes a TAFA protein expressed predominately in brain and may function as brain-specific chemokines or neurokines[224]. Prior to the ChinaPC study, there is no implication of *PRLHR* or *FAM19A5* in the risk of pancreatic cancer and thus their susceptibility role is currently unknown.

Current Status of Screening High-Risk Populations

Screening and early detection of pancreatic cancer offer the best chance of reducing the high mortality rates of this disease. The goal of screening asymptomatic individuals is to identify pancreatic cancer at early stage or, ideally to identify high-grade precancerous lesions that can be resected to prevent the development of cancer. Because of the low incidence of pancreatic cancer in the general population, population level screening will demand a highly specific screening assay. Selective screening of individuals at increased risk for pancreatic cancer is considered

worthwhile. The International Cancer of the Pancreas Screening (CAPS) Consortium recommends screening on FDRs of FPC patients, patients with PJS, and carriers of *CDKN2A/p16*, *BRCA2*, and carriers of MMR gene mutations with ≥1 affected FDR[225] . FDRs of FPC patients represent a group of high-risk individuals that are relatively easy to identify in clinical settings. Of all identified risk factors for pancreatic cancer, PJS confers the greatest risk for the disease, making PJS patients good candidates for pancreatic cancer screening. Among the established pancreatic cancer genes, germline *BRCA2* mutations followed by *ATM* account for the highest percentage of inherited pancreatic cancer[36,42,58,40,34]. It is recommended that *BRCA2* mutation carriers with ≥1 affected FDR and those with two or more affected family members should be considered for screening, particularly Ashkenazi Jewish individuals. In addition, given the substantially higher risk of pancreatic cancer in patients with *CDKN2A/p16* and mismatch repair (MMR) gene mutations, screening is also recommended to those mutation carriers with >1 affected FDRs.

Endoscopic ultrasonography (EUS) and/or MRI/magnetic resonance cholangiopancreatography (MRCP) are recommended by CAPS as initial screening tools. However, the CAPS Consortium could not reach consensus on ages to initiate or stop surveillance, the interval for follow-up imaging, nor on the long-term management of initial abnormal results. Screening and early detection strategies should be accompanied by effective treatment or preventive strategies if they are to produce a significant survival benefit. Given morbidity and mortality associated with pancreatic surgery, there is little consensus about when surgery is required for a pancreatic lesion in asymptomatic high-risk individuals. Multidisciplinary assessment is however recommended to make individualized decision of the necessity of surgical intervention. The lack of consensus on many aspects of pancreatic cancer

screening underscores the need for more research to fill the knowledge gap and to make evidence-based decisions.

Summary and Future Directions

Pancreatic cancer is a rare and deadly disease with the highest case-fatality rate of any major cancer. Due to the lack of effective means for prevention, diagnosis and treatment, pancreatic cancer remains a major public health challenge. Family history, cigarette smoking, chronic pancreatitis, and diabetes are well-established risk factors for pancreatic cancer. Pancreatic cancer is fundamentally a genetic disease caused by both inherited and acquired genetic mutations. Family-based heritability analysis reported 36% of pancreatic cancer was due to genetics. Familial pancreatic cancer kindreds and patients affected by certain genetic syndromes, for example HP, PJS, HBOC, FAMMM, and HNPCC, are at particularly high risk of pancreatic cancer. About 15-20% of FPC are caused by germline mutations in one of the established pancreatic cancer gene (*BRCA2, ATM, PALB2, PRSS1*, S*TK11*, *BRCA1*, *CDKN2A*, *MLH1*, *MSH2*, *MSH6*, and *PMS2*). The genetic basis of susceptibility underlying the majority of FPC cases, however, remains unexplained. To date, GWAS of pancreatic cancer have discovered 16 low-risk susceptibility loci in European populations and 5 in Asian populations, many of which had strong biological plausibility. Together, these GWAS loci explained < 5% of pancreatic cancer. Screening, surveillance and management guidelines for genetically high-risk individuals are currently evolving. DNA-damaging related agents are promising in treating pancreatic cancer caused by mutations in *BRCA1*, *BRCA2*, *PALB2 or ATM* genes. Identification of disease-causing genes can aid in the characterization of individuals at highest genetically defined risk in which effective prevention approach can be developed.

Figures and Tables

Table 1 Pancreatic Cancer Predisposing Genes

Genes	Predisposition Syndromes	Risk of Pancreatic Cancer*
BRCA2	Hereditary breast and/or ovarian cancer	SIR = 2.20 – 5.90
BRCA1		SIR = 1.60 – 4.73
PALB2	Familial breast cancer	Increased
ATM		Increased
STK11	Peutz-Jeghers syndrome	SIR = 76.2 – 139.7
PRSS1	Hereditary Pancreatitis	SIR = 53 – 87
CDKN2A	Familial atypical multiple mole and melanoma syndrome	SIR = 14.8 – 80.8
Mismatch repair genes (*MLH1*, *MSH2*, *MHS6* and *PMS2*)	Hereditary non-polyposis colorectal cancer	No effect up to SIR = 10.68

* SIR = standardized incidence ratio

Table 2 Genetic Susceptibility Loci for Pancreatic Cancer Identified in European Populations

Chr*	Nearest Gene(s)	Top SNP†	Minor/Major Alleles	Consequence Type	Allelic OR [95% CI]‡	P-value
1p36.33	*NOC2L*	rs13303010	G/A	intron	1.26 [1.19-1.35]	8.36×10^{-14}
1q32.1	*NR5A2*	rs3790844	T/C	intron	0.77 [0.71-0.84]	2.45×10^{-10}
1q32.1	*NR5A2*	rs2816938	A/T	upstream	1.20 [1.15-1.25]	4.88×10^{-15}
2p13.3	*ETAA1*	rs1486134	G/T	downstream	1.14 [1.09-1.19]	3.36×10^{-9}
3q29	*TP63*	rs9854771	A/G	intron	0.89 [0.85-0.93]	2.35×10^{-8}
5q15.33	*TERT, CLPTM1L*	rs401681	C/T	intron	1.19 [1.11-1.27]	3.66×10^{-7}
5p15.33	*TERT, CLPTM1L*	rs2736098	T/C	synonymous	0.80 [0.76-0.85]	9.78×10^{-14}
5p15.33	*TERT, CLPTM1L*	rs35226131	T/C	upstream	0.71 [0.63-0.80]	1.70×10^{-8}
7p12	*TNS3*	rs73328514	T/A	intron	0.85 [0.80-0.90]	1.35×10^{-7}
7p13	*SUGCT*	rs17688601	A/C	intron	0.88 [0.84-0.92]	1.41×10^{-8}
7q32.3	*LINC-PINT*	rs6971499	C/T	intron	0.79 [0.74-0.84]	2.98×10^{-12}
8q21.11	*HNF4G*	rs2941471	G/A	intron	0.89 [0.85-0.93]	6.60×10^{-10}
8q24.21	*MYC*	rs10094872	T/A	intron	1.15 [1.10-1.20]	3.22×10^{-9}
9q34	*ABO*	rs505922	T/C	intron	1.20 [1.12-1.28] (het) 1.44 [1.26-1.63] (hom)	5.37×10^{-8}
13q12.2	*PDX1*	rs9581943	A/G	upstream	1.15 [1.10-1.20]	2.35×10^{-9}
13q22.1	none	rs9543325	T/C	intergenic	1.26 [1.18-1.35]	3.27×10^{-11}

16q23.1	*BCAR1, CTRB1, CTRB2*	rs7190458	A/G	synonymous	1.46 [1.30-1.65]	1.13×10^{-10}
17q12	*HNF1B*	rs4795218	A/G	intron	0.88 [0.84-0.92]	1.32×10^{-8}
17q25.1	*LINC00673*	rs11655237	T/C	non-coding transcript exon	1.26 [1.19-1.34]	1.42×10^{-14}
18q21.32	*GRP*	rs1517037	T/C	upstream	0.86 [0.80-0.91]	3.28×10^{-8}
22q12.1	*ZNRF3*	rs16986825	T/C	intron	1.18 [1.12-1.25]	1.18×10^{-8}

* Chromosomal location in NCBI genome build 37.

† SNP = single nucleotide polymorphisms

‡ OR = odds ratio; CI = confidence interval; het = heterozygotes; hom = homozygotes

Table 3 Genetic Susceptibility Loci for Pancreatic Cancer Identified in Asian Populations

Chr*	Nearest Gene(s)	Top SNP†	Minor/Major Alleles	Consequence Type	Allelic OR [95% CI]‡	P-value
5p13.1	*DAB2*	rs2255280	C/A	intron	0.81 [0.76-0.87]	4.18×10^{-10}
10q26.11	*RPLHR*	rs12413624	T/A	regulatory region	1.23 [1.16-1.31]	5.12×10^{-11}
21q21.3	*BACH1*	rs372883	C/T	3' UTR	0.79 [0.75-0.84]	2.24×10^{-13}
21q22.3	*TFF1*	rs1547374	G/A	downstream	0.79 [0.74-0.84]	3.71×10^{-13}
22q13.32	*FAM19A5*	rs5768709	G/A	intron	1.25 [1.17-1.34]	1.41×10^{-10}

* Chromosomal location in NCBI genome build 37.

† SNP = single nucleotide polymorphisms

‡ OR = odds ratio; CI = confidence interval; het = heterozygotes; hom = homozygotes

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CHAPTER THREE

HERITABILITY FOR PANCREATIC CANCER FROM IMPUTED VARIANTS

The heritability analysis was conducted in two independent case-control studies: a subset of the Pancreatic Cancer Case-Control Consortium (PanC4) study and a combined sample of familial pancreatic cancer (FPC) patients from the Familial Pancreatic Cancer Genome Sequencing Project (FPCGSP) and participants from the Alzheimer's Disease Neuroimaging Initiative (ADNI) study. This chapter presents the analysis conducted in the PanC4 study using variants imputed from 1000 Genomes Project reference panels, including the estimation of heritability explained by all imputed variants, the genomic partitioning of estimated heritability by chromosomes, minor allele frequency (MAF) categories and functional annotations, as well as the evaluation of identified GWAS loci contributing to the susceptibility of pancreatic cancer.

Study Populations

The Pancreatic Cancer Case Control Consortium (PanC4) was established to improve the understanding of the etiology of pancreatic cancer through joint, or pooled data analysis (http://panc4.org)[1] from established case-control studies of pancreatic cancer. A subset of pancreatic cancer case-control studies with available bio-samples participated in the PanC4 genome-wide association study (GWAS). Participating sites included nine hospital-based or population based case-control studies including the Central Europe study coordinated by the International Agency for Research on Cancer (IARC/Central Europe)[2], Johns Hopkins Hospital (Baltimore, MD)[3,4], Mayo Clinic (Rochester, MN)[5], MD Anderson Cancer Center (Houston, TX)[6], Memorial Sloan-Kettering Cancer Center (New

York, NY)[7], University of Toronto (Toronto ON, Canada)[8], Queensland (Queensland, Australia)[9], University of California San Francisco (San Francisco, CA)[10] and Yale University (New Haven, CT)[11]. Cases were defined as individuals with adenocarcinoma of the pancreas. Control subjects were selected from the general population or hospital catchment area as detailed in

Analysis	**Model#**	**GREML Approach***	**Mixed Linear Model†**	**List of Genetic Components**	**Calculation of h^2**
Estimates of heritability explained by imputed variants	1	GREML-SC	$y = u + g + \varepsilon$	g	$h^2_{imputed} = \sigma^2_g/(\sigma^2_g + \sigma^2_e)$
	2	GREML-MS	$y = u + \sum_{i=1}^{2} g_i + \varepsilon$	$g_{MAF<0.01}$ $g_{MAF\geq0.01}$	$h^2_{imputed} = \frac{\sum_{i=1}^{2}\sigma^2_{g_i}}{\sum_{i=1}^{2}\sigma^2_{g_i} + \sigma^2_e}$
	3	GREML-LDMS	$y = u + \sum_{i=1}^{4} g_i + \varepsilon$	$g_{MAF<0.01,LD\ below}$ $g_{MAF<0.01,LD\ above}$ $g_{MAF\geq0.01,LD\ below}$ $g_{MAF\geq0.01,LD\ above}$	$h^2_{imputed} = \frac{\sum_{i=1}^{4}\sigma^2_{g_i}}{\sum_{i=1}^{4}\sigma^2_{g_i} + \sigma^2_e}$
Genomic partitioning by chromosome	4	GREML-LDMS	$y = u + \sum_{i=1}^{88} g_i + \varepsilon$	$g_{MAF<0.01,LD\ below,chr1}$ $g_{MAF<0.01,LD\ above,chr1}$ $g_{MAF\geq0.01,LD\ below,chr1}$ $g_{MAF\geq0.01,LD\ above,chr1}$ … $g_{MAF<0.01,LD\ below,chr22}$ $g_{MAF<0.01,LD\ above,chr22}$ $g_{MAF\geq0.01,LD\ below,chr22}$ $g_{MAF\geq0.01,LD\ above,chr22}$	$h^2_{chr1} = \frac{\sum_{i=1}^{4}\sigma^2_{g_{i,chr1}}}{\sum_{i=1}^{88}\sigma^2_{g_i} + \sigma^2_e}$ … $h^2_{chr22} = \frac{\sum_{i=1}^{4}\sigma^2_{g_{i,chr22}}}{\sum_{i=1}^{88}\sigma^2_{g_i} + \sigma^2_e}$
Genomic Partitioning by MAF	5	GREML-LDMS	$y = u + \sum_{i=1}^{12} g_i + \varepsilon$	$g_{MAF<0.01,LD\ below}$ $g_{MAF<0.01,LD\ above}$ $g_{MAF\ 0.01-0.10,LD\ below}$ $g_{MAF\ 0.01-0.10,LD\ above}$	$h^2_{MAF<0.01} = \frac{\sum_{i=1}^{2}\sigma^2_{g_{i,MAF<0.01}}}{\sum_{i=1}^{12}\sigma^2_{g_i} + \sigma^2_e}$

				$g_{MAF\ 0.10-0.20,LD\ below}$ $g_{MAF\ 0.10-0.20,LD\ above}$ $g_{MAF\ 0.20-0.30,LD\ below}$ $g_{MAF\ 0.20-0.30,LD\ above}$ $g_{MAF\ 0.30-0.40,LD\ below}$ $g_{MAF\ 0.30-0.40,LD\ above}$ $g_{MAF\ 0.40-0.50,LD\ below}$ $g_{MAF\ 0.40-0.50,LD\ above}$	$h^2_{MAF\ 0.01-0.10} = \frac{\sum_{i=1}^{2} \sigma^2_{g_{i,MAF\ 0.01-0.10}}}{\sum_{i=1}^{12} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{MAF\ 0.10-0.20} = \frac{\sum_{i=1}^{2} \sigma^2_{g_{i,MAF\ 0.10-0.20}}}{\sum_{i=1}^{12} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{MAF\ 0.20-0.30} = \frac{\sum_{i=1}^{2} \sigma^2_{g_{i,MAF\ 0.20-0.30}}}{\sum_{i=1}^{12} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{MAF\ 0.30-0.40} = \frac{\sum_{i=1}^{2} \sigma^2_{g_{i,MAF\ 0.30-0.40}}}{\sum_{i=1}^{12} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{MAF\ 0.40-0.50} = \frac{\sum_{i=1}^{2} \sigma^2_{g_{i,MAF\ 0.40-0.50}}}{\sum_{i=1}^{12} \sigma^2_{g_i} + \sigma^2_e}$
Genomic partitioning by functional annotation	6	GREML-LDMS	$y = u + \sum_{i=1}^{16} g_i + \varepsilon$	$g_{MAF<0.01,LD\ below,coding}$ $g_{MAF<0.01,LD\ above,coding}$ $g_{MAF\geq0.01,LD\ below,coding}$ $g_{MAF\geq0.01,LD\ above,coding}$ $g_{MAF<0.01,LD\ below,intergenic}$ $g_{MAF<0.01,LD\ above,intergenic}$ $g_{MAF\geq0.01,LD\ below,intergenic}$ $g_{MAF\geq0.01,LD\ above,intergenic}$ $g_{MAF<0.01,LD\ below,intronic}$ $g_{MAF<0.01,LD\ above,intronic}$ $g_{MAF\geq0.01,LD\ below,intronic}$ $g_{MAF\geq0.01,LD\ above,intronic}$ $g_{MAF<0.01,LD\ below,regulatory}$ $g_{MAF<0.01,LD\ above,regulatory}$ $g_{MAF\geq0.01,LD\ below,regulatory}$ $g_{MAF\geq0.01,LD\ above,regulatory}$	$h^2_{coding} = \frac{\sum_{i=1}^{4} \sigma^2_{g_{i,coding}}}{\sum_{i=1}^{16} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{intergenic} = \frac{\sum_{i=1}^{4} \sigma^2_{g_{i,intergenic}}}{\sum_{i=1}^{16} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{intronic} = \frac{\sum_{i=1}^{4} \sigma^2_{g_{i,intronic}}}{\sum_{i=1}^{16} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{regulatory} = \frac{\sum_{i=1}^{4} \sigma^2_{g_{i,regulatory}}}{\sum_{i=1}^{16} \sigma^2_{g_i} + \sigma^2_e}$

Evaluation of GWAS loci	7	GREML-LDMS	$y = u + g_{GWAS} + \sum_{i=1}^{4} g_i + \varepsilon$	g_{GWAS} $g_{MAF<0.01,LD\ below,remaining}$ $g_{MAF<0.01,LD\ above,remaining}$ $g_{MAF\geq0.01,LD\ below,remaining}$ $g_{MAF\geq0.01,LD\ above,remaining}$	$h^2_{GWAS} = \frac{\sigma^2_{GWAS}}{\sigma^2_{GWAS} + \sum_{i=1}^{4} \sigma^2_{g_{i,remaining}} + \sigma^2_e}$

* GREML-SC = single-component GREML; GREML-MS = MAF-stratified GREML; GREML-LDMS = LD- and MAF-stratified GREML.

† The disease status in observed scale (y) is a functional of some fixed effects (μ) including intercept, age, sex, and first 20 principal components, one or more genetic components (g_i), and a residual effect (ε).

Supplementary Table 2. This study was reviewed and approved by the Institutional Review Board of the Johns Hopkins University School of Medicine and of each participating institution. Informed consent was obtained from all patients participating in this study.

Genotyping and Imputation

DNA samples from 8,052 PanC4 participants, 180 study duplicates, 176 HapMap control samples, and 26 replicates from the previous pancreatic cancer GWAS PanScan 2[12], were genotyped on the IlluminaHumanOmniExpressExome-8v1 array at the Johns Hopkins Center for Inherited Disease Research (CIDR). Genotypes were called using GenomeStudio version 2011.1, Genotyping Module 1.9.4 and GenTrain version 1.0[1]. Before imputation, individuals with excess allele sharing, duplicates, and subjects with incomplete information on age were removed. Single-nucleotide polymorphisms (SNPs) with the following characteristics were excluded: positional duplicates, more than two discordant calls in study duplicates, technical failures, or missing call rate > 2%, more than one Mendelian error in HapMap control trios, Hardy-Weinberg equilibrium p-value < 10^{-6}, sex difference in allele frequency > 0.2 for autosomes/XY in samples of European ancestry, monomorphic SNPs and SNP on sex chromosomes. Overall 7,956 samples and 821,150 SNPs passed the applied quality control filters and were imputed using IMPUTE v2[13] with the 1000 Genomes (Phase 3, v3)[14] as the reference panel.

Quality Controls on PanC4 After Imputation

Additional QC filters were applied on the imputed PanC4 data to minimize the impact of poorly imputed variants in the heritability analysis (Figure 1). After imputation, there were 7,956 samples and 81,671,345 autosomal variants in the dataset. A total of 372 known non-European samples were removed. The imputation generated three probabilities for genotype AA, AB and BB at each site of a given sample. To facilitate the heritability analysis, PLINK[15] was used to

convert the probabilities to hard genotypes. For genotype calls with high certainty (i.e. one of the probabilities was significantly higher than the other two), PLINK considers the genotype with the highest probability as the hard genotype. For genotype calls with low certainty (defined as the difference between the highest two probabilities is less than 0.1), PLINK sets the genotype to missing. After being converted to hard genotype calls, about 1.9% variants with missingness greater than 5% were excluded. As recommended for heritability analysis, variants that failed HWE exact test at $p < 10^{-6}$ and those with minor allele count less than 5 (equivalent to a minor allele frequency of 0.03%) were excluded to minimize the noise caused by extremely rare variants. More than 60% of the variants were excluded due to being monomorphic after imputation. Because heritability analysis in case-control studies is particularly sensitive to hidden relatedness in the study population, a relatedness cutoff of 0.025 was applied to further exclude 653 distantly related individuals. The genetic relationship was determined using 99,138 common (MAF > 0.05) and independent (pairwise $r^2 > 0.20$) variants directly genotyped in our dataset. The final dataset contains 6,931 samples and 16,112,030 sites, including 68,512 multi-allelic sites. After splitting these multi-allelic variants into multiple records, there were 16,184,129 variants in the final dataset. Minor allele frequencies (MAF) of these variants were calculated in PLINK. Annotation of the variants was obtained from ANNOVAR[16]. The functional predictions were derived from the NCBI Reference Sequence Database.

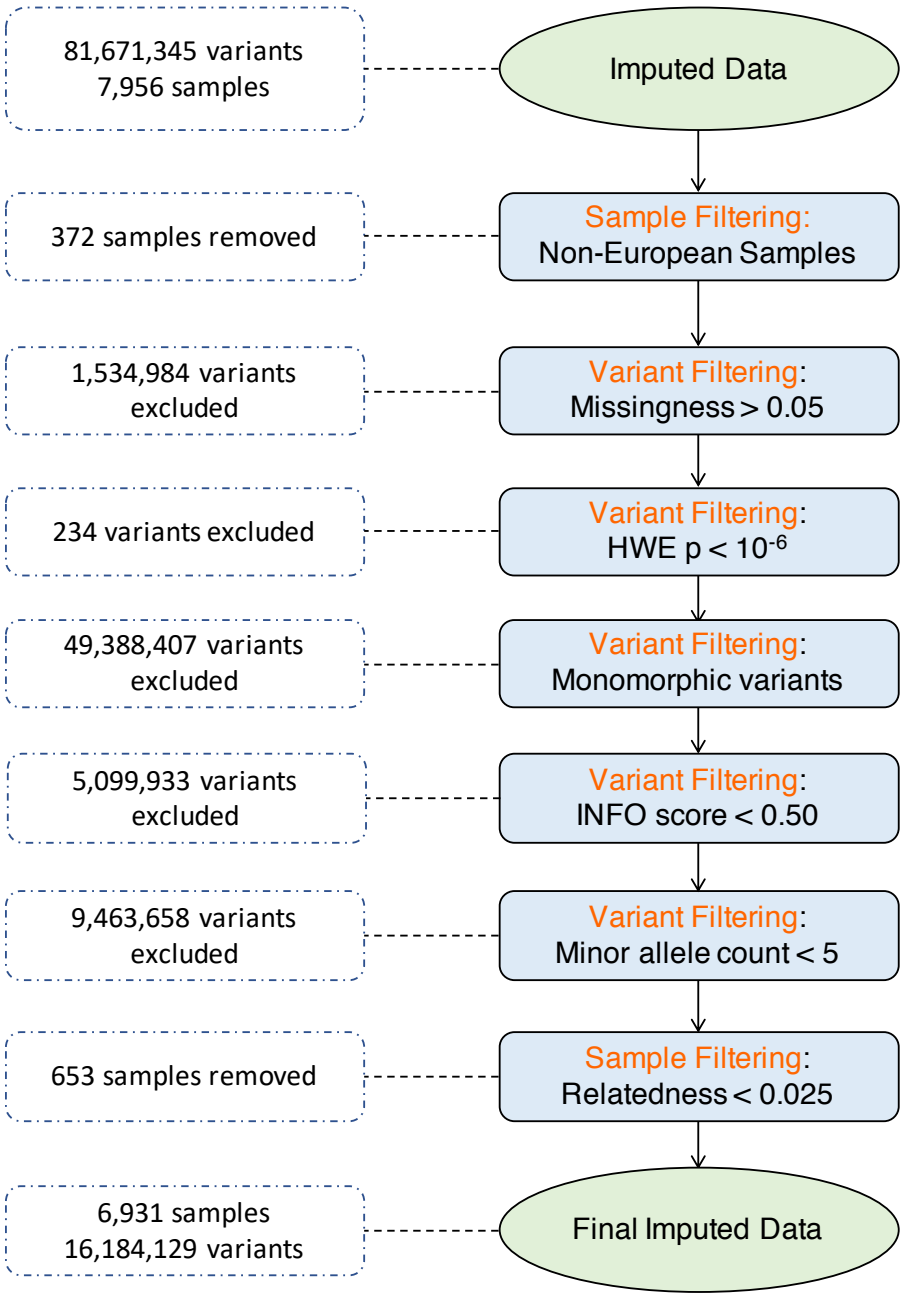


Figure 1 Overview of quality control procedures on PanC4 imputed data. Circles with a solid line represent WGS data at different quality control stage. Boxes with a solid line represent filters applied in an order directed by arrows. Boxed with dashed line detail changes in WGS data resulting from the implementation of each filter.

Statistical Analysis

This study aimed to assess the contribution of the additive genetic effects in the susceptibility of pancreatic cancer. The recently developed restricted maximum likelihood (REML) approach was used to obtain comprehensive estimates of heritability for pancreatic cancer. First, the narrow sense heritability due to additive genetic effects was estimated from all imputed variants across the genome using three forms of REML approach: GREML-SC (single-component GREML), GREML-MS (MAF-stratified GREML) and GREML-LDMS (LD- and MAF-stratified GREML)[17]. The validity of estimated heritability from these GREML approaches depends on the MAF distribution and LD properties of the causal variants, which is unknown to us. By applying all three forms of GREML approach to our dataset, we were able to evaluate the validity of the estimated heritability by comparing the results from different GREML approaches and select the model that generates a less biased estimate for subsequent analysis. Potential bias in the heritability estimation due to residual population stratification and/or cryptic relatedness were assessed to ensure the validity of the results. Next, the estimated heritability was partitioned by MAF, chromosomes, and functional annotation, to infer the genetic architecture of pancreatic cancer. The GREML-LDMS approach was applied to estimate the variance explained by each subset of variants conditioning on the rest of the genome. These joint analyses would account for the correlations between variants in different categories. Lastly, the contributions of identified GWAS loci to the disease heritability was quantified. All the models and corresponding calculation of heritability in this analysis are outlined in Supplementary Table 1.

Estimates of Heritability Explained by Imputed Variants

The heritability explained by imputed variants ($h^2_{imputed}$) was estimated using three forms of REML approach: GREML-SC, GREML-MS, and GREML-LDMS. A detailed

description of these forms of GREML approach can be found in Chapter One. In the GREML-SC analysis, the variance due to additive genetic effects of the imputed variants was estimated by fitting one single genetic relationship matrix (GRM) calculated from all autosomal variants in a mixed linear model (Model #1). The proportion of variance explained by all autosomal variants (i.e. heritability) was then defined as $\boldsymbol{h^2_{imputed}} = \boldsymbol{\sigma^2_g}/(\boldsymbol{\sigma^2_g} + \boldsymbol{\sigma^2_e})$.

In the GREML-MS analysis, all autosomal variants were stratified into two MAF groups: 0.0003 – 0.01 and 0.01 - 0.50. The predictor GRMs were calculated from variants in each MAF category and were fit jointly in a mixed linear model (Model #2). The overall heritability was then defined as $\boldsymbol{h^2_{imputed}} = \sum_{\boldsymbol{i=1}}^{\boldsymbol{2}} \boldsymbol{\sigma^2_{g_i}} /(\sum_{\boldsymbol{i=1}}^{\boldsymbol{2}} \boldsymbol{\sigma^2_{g_i}} + \boldsymbol{\sigma^2_e})$.

LD stratification was applied in the GREML-LDMS analysis to further account for the region-specific heterogeneity in LD. In brief, individual variants were first allocated to a large number of overlapping segments with an average length of 100 kb. The mean LD r^2 score for each segment was derived from the LD r^2 score of individual variants in the segment. Based on the segment LD scores, all variants were stratified into two groups (i.e. above or below the mean segment LD score). After the LD stratification, the variants in each LD group were further binned into two MAF categories (0.003-0.01 and 0.01-0.50) as described in the GREML-MS analysis. The predictor GRMs were calculated from each LD-MS group and fitted jointly in a mixed linear model (Model #3). The proportion of variance explained by all autosomal variants was calculated as $\boldsymbol{h^2_{imputed}} = \sum_{\boldsymbol{i=1}}^{\boldsymbol{4}} \boldsymbol{\sigma^2_{g_i}} /(\sum_{\boldsymbol{i=1}}^{\boldsymbol{4}} \boldsymbol{\sigma^2_{g_i}} + \boldsymbol{\sigma^2_e})$.

For a binary trait like pancreatic cancer, the estimates of variance need to be transformed from the observed 0-1 scale to the unobserved continuous 'liability' scale using a probit transformation[18]. To correct for the oversampling of cases in the case-control study, a population prevalence of the disease (K) was specified. Based on the 2009-2011 SEER report, the lifetime

risk of being diagnosed with cancer of the pancreas by age 85 was K = 0.0149 for US Whites[19]. Unless specified, the default average information algorithm was applied to run REML iterations in all heritability analysis. All models in the analysis were adjusted for age, sex and the first 20 PCs which were calculated using 99,138 common (MAF > 0.05) and independent (pairwise r^2 > 0.20) variants directly genotyped in our dataset. The incorporation of the first 20 PCs in the model is recommended to control for potential population substructure in the study sample, as the presence of population stratification would bias the estimate of heritability[17]. The variance in liability scale was reported along with its standard errors. Results from GREML-SC, GREML-MS, and GREML-LDMS were compared and the model that generates a less biased estimate of heritability was selected for the subsequent analysis.

Heritability analysis is particularly sensitive to population substructure. Any residual relatedness or population stratification in the study population could inflate the estimates of heritability. The presence of residual population stratification or relatedness can lead to correlations between chromosomes, which can be detected in a separate analysis by fitting only one chromosome at a time in a mixed linear model. In this separate analysis, the variance explained by any given chromosome ($h^2_{c_sep}$) would also capture some of the variance caused by other chromosomes. The joint analysis would protect against such inter-chromosomal correlations because the estimate of variance explained by each chromosome (($h^2_{c_joint}$) is conditional on the other autosomes in the model. To quantify the effect of remaining population structure, the difference between $h^2_{c_sep}$ and $h^2_{c_joint}$ was regressed on chromosome length (i.e. the number of variants).

$$\boldsymbol{h^2_{c_sep} - h^2_{c_joint} = b_0 + b_1 \times L_c + e} \quad \textbf{(4)}$$

The intercept of this regression (b_0) indicates inflation due to cryptic relationship (irrespective of chromosomal length L_c) and b_1 indicates inflation due to population stratification (correlates with L_c). Because the difference between $h^2_{c_sep}$ and $h^2_{c_joint}$ represents the overall effect of all the other 21 autosomes on a given chromosome, the proportion of variance attributed to the cryptic relationship and population stratification across the whole genome is approximately equal to $b_0 \times \frac{22}{21} + b_1 \times \frac{sum(L_c)}{21}$. This analysis was conducted based on results from the GREML-SC approach.

Partitioning by Chromosome, MAF and Functional Annotation

Genomic partitioning of estimated heritability was performed using GREML-LDMS approach, where all autosomal variants were stratified into 4 LD-MS groups as described previously (MAF < 0.01 vs. MAF ≥0.01 and below vs. above median LD score). Additional stratification was applied according to the type of partitioning analysis (by MAF, chromosome or functional annotation). Variance explained by each subset of variants was estimated in joint analysis conditioning on the remaining variants in the genome.

To determine the variance captured by each autosomal chromosome, the variants in 4 LD-MS groups were further allocated to 22 autosomal chromosomes, resulting in 88 LD-MS-chromosome strata. The GRMs from each LD-MS-chromosome stratum were calculated and fitted jointly in a mixed linear model (Model #4). In this analysis, the Fisher scoring approach was applied for variance estimation. The variance captured by each chromosome was aggregated from variance due to 4 LD-MS groups allocated to each chromosome. Standard errors of the summed variance were calculated from the sample variance/covariance matrix using the delta method. Linear regression was performed to assess the correlations between variance explained

by individual chromosome and the length of the chromosome. The length of a chromosome was represented by the total number of variants in the chromosome.

To improve the resolution in MAF distribution of causal variants, variants were binned into six MAF categories: 0.0003 – 0.01, 0.01 – 0.10, 0.10 – 0.20, 0.20 – 0.30, 0.30 – 0.40 and 0.40 – 0.50. Variance explained by each MAF category was estimated from a mixed linear model with 12 genetic components (Model #5). GRM calculated from each component was fitted jointly in the model conditioning on all other components. The variance captured by each MAF category was aggregated from variance due to 2 LD strata within the MAF category. Standard errors of the summed variance were calculated from the sample variance/covariance matrix using the delta method.

All imputed variants were initially annotated into six functional groups according to ANNOVAR annotations: coding, intergenic, intronic, ncRNA, UTR, and upstream/downstream. Due to the limited number of variants in some of these groups, exonic and splicing variants were grouped together as coding variants whereas variants coded as ncRNA, UTR or upstream/downstream were combined as regulatory variants. Therefore, all variants were mapped into four functional groups: coding, intergenic, intronic and regulatory. Variants in each of the four functional groups were further stratified into 2 MAF categories and 2 LD groups. In the joint analysis of all functional groups, the variance explained by each functional category was summed from variance due to 4 LD-MS strata within the functional category (Model #6). Standard errors of the summed variance were calculated from the sample variance/covariance matrix using the delta method.

Contributions of Identified GWAS Loci

The common low-risk susceptibility loci for pancreatic cancer have been reported in six genome-wide association studies (GWAS) in European populations and two GWAS in Asian populations (Chapter Two). A total of 26 GWAS loci reached the genome-wide significance were included in this analysis. The index SNP of these 26 GWAS loci were first identified in our dataset. If not available, the variants with the strongest LD (pairwise r^2) to the index SNP were included to capture the GWAS signals. Then all variants within ±250 kb of the index SNP were grouped together with the index SNP as a single genetic component. The remaining variants across the genome were stratified into 2 MAF categories and 2 LD groups as described previously. The variance explained by the GWAS loci was estimated by fitting 5 GRMs jointly in a mixed linear model (Model #7).

Results

The final sample consists of 3,568 pancreatic cancer cases and 3,363 controls (Tables

Table 4). Case and control samples were similar in sex ratio and age distributions. All subjects in this case-control cohort were aged 40 years or older.

A shows the distribution of minor allele frequencies (MAF) in the final dataset containing 16,184,129 variants. The majority of the variants are rare and low-frequency variants with a MAF < 5%. The remaining variants are evenly distributed across the MAF categories. The majority of the variants in the final dataset are intergenic (52.8%) or intronic (37.4%). About 1% of the sequenced variants are located in the coding regions (

B).

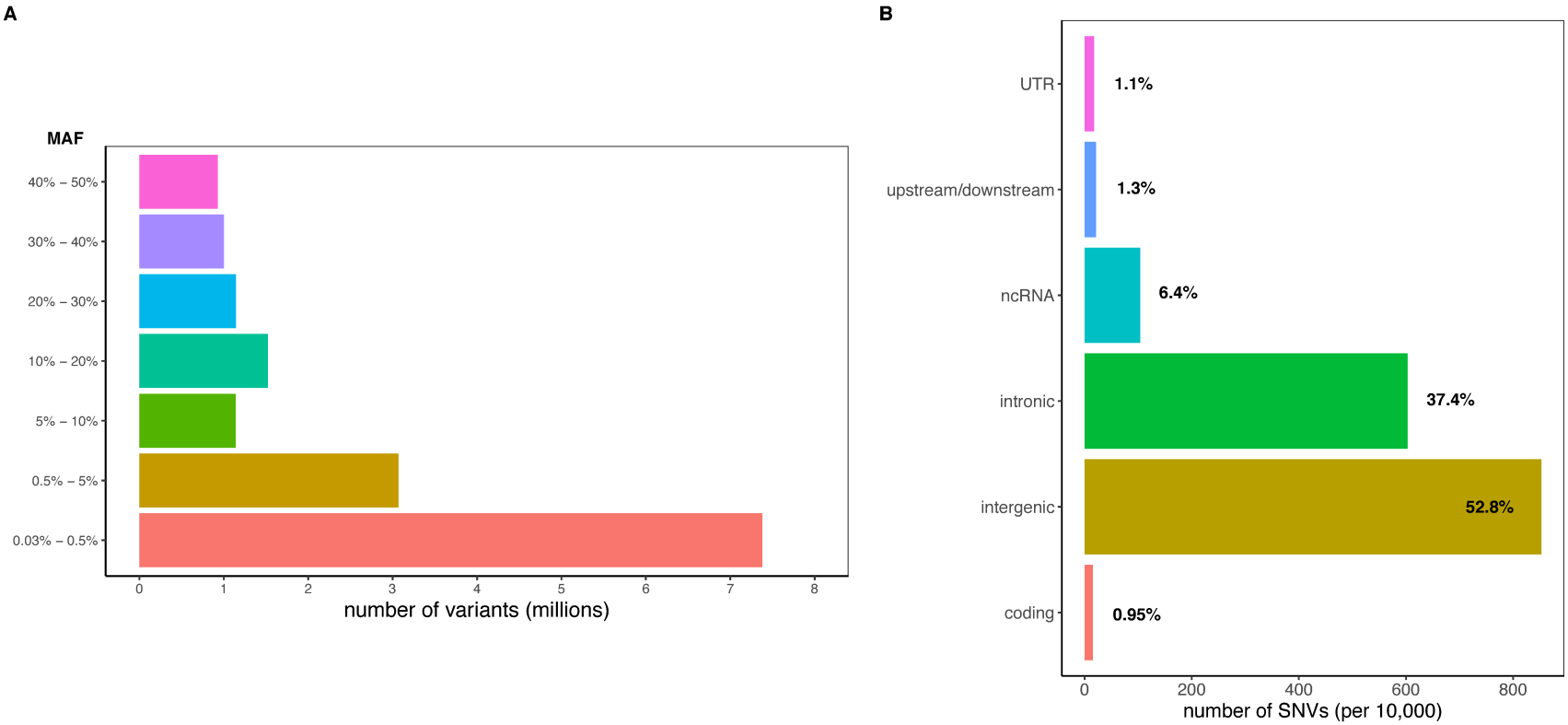


Figure 2 Minor allele frequency (A) and functional annotation (B) distribution of PanC4 imputed variants.

Estimates of Heritability Explained by Imputed Variants

The heritability for pancreatic cancer was calculated in three models applying different forms of GREML approach. The heritability for pancreatic cancer explained by all imputed variants ($h^2_{imputed}$) was estimated to be 16.0% (s.e. = 4.6%) in the single-component model (Model #1). When stratified at MAF of 0.01 (Model #2), the estimate of heritability from GREML-MS was 12.9% (s.e. = 3.9%), lower than the estimate from GREML-SC model. When further stratified by LD (Model #3), the heritability for pancreatic cancer explained by all imputed variants was estimated to be 21.2% (s.e. = 4.8%). Given the substantial differences in the estimates of heritability from these three models, the GREML-LDMS estimate was considered the less biased. Unless specified, all subsequent analysis was conducted based on the GREML-LDMS model.

By comparing the estimated variance explained by each chromosome in a separate ($h^2_{c_sep}$) analysis to a joint ($h^2_{c_joint}$) analysis, the proportion of variance attributed to the residual population stratification and/or cryptic relatedness in our dataset was estimated to be 0.31%, which is unlikely to spuriously inflate the estimate of overall heritability.

Genomic Partitioning of Genetic Variation

Genomic partitioning of the estimated heritability can provide valuable insights on the underlying genetic architecture of the disease. Partitioning by chromosome was conducted in a GREML-LDMS model (Model #4) and the estimated variance associated with each chromosome is shown in Figure 3. Chromosome 9 account for the largest proportion of genetic variation for pancreatic cancer (h^2 = 2.3%, s.e. = 1.6%), followed by chromosome 7 (h^2 = 2.1%, s.e. = 1.8%). Chromosome 8 (h^2 = 1.8%, s.e. = 1.7%), chromosome 16 (h^2 = 1.8%, s.e. = 1.4%), chromosome 5 (h^2 = 1.5%, s.e. = 1.9%), chromosome 2 (h^2 = 1.5%, s.e. = 2.0%), and chromosome 1 (h^2 =

1.5%, s.e. = 2.0%) also explained a fair amount of phenotypic variance for pancreatic cancer. On all these abovementioned chromosomes, common susceptibility loci for pancreatic cancer have been identified in GWAS studies. Regression of the variance explained by individual chromosome on the length of the chromosome found no correlations as shown in Figure 4.

The GREML-LDMS model with 2 MAF categories and 2 LD groups (Model #3) suggest that imputed variants with MAF ≤ 0.01 (48.4% of all imputed variants) explains 5.2% (s.e. = 3.6%) of the phenotypic variance of pancreatic cancer, while common variants (MAF > 0.01) account for about 16.0% (s.e. = 3.5%) of the phenotypic variance (

	Affected Individuals (N = 3,568)	**Unaffected Individuals (N = 3,363)**
Female, N (%)	1,510 (42.3%)	1,491 (44.3%)
Age, N (%)		
40-49	335 (9.4%)	358 (10.6%)
50-59	768 (21.5%)	849 (25.2%)
60-69	1275 (35.7%)	1,140 (33.9%)
70-79	900 (25.2%)	786 (23.4%)
>80	290 (8.1%)	230 (6.8%)

Table 5). When the imputed variants were stratified into 6 MAF categories and 2 LD groups (Model #5), the overall heritability for pancreatic cancer was estimated to be 19.9% (s.e. = 5.0%), which is quite similar to the result from the previous model with 2 MAF categories (Model #3). Partitioning of the estimated heritability by six MAF categories also found a substantial amount of genetic variation at rare variants for pancreatic cancer, with $h^2 = 6.9\%$ (s.e. = 3.8%) for variants with MAF ≤ 0.01, all of which is attributed to variants with lower than average LD scores (Figure 5). Variants with 0.01 < MAF ≤ 0.10 explain a comparable amount of variance for pancreatic cancer ($h^2 = 6.2\%$, s.e. = 3.1%) but mostly from variants with above average LD scores (Figure 5).

In the genomic partitioning by functional groups, intronic and intergenic variants, which contain 37.3% and 52.7% of all imputed variants, account for 12.4% (s.e. = 6.8%) and 6.0% (s.e. = 6.8%) of phenotypic variance for pancreatic cancer, respectively (

	Above mean LD		**Below mean LD**		**Row sum**	**s.e.** *	**No. Variants (%)**
	Est	**s.e.** †	**Est**	**s.e.** †			
MAF ≤ 0.01	0.0523	0.037	0	0.051	0.052	0.036	8,354,405 (51.6%)
MAF > 0.01	0.0142	0.017	0.1460	0.032	0.160	0.035	7,829,724 (48.4%)
Column sum	0.067	0.040	0.146	0.058			
Total sum	**0.212**	**0.048**					
No. Variants (%)	8,092,536 (50%)		8,091,593 (50%)				

* s.e. for row sum and column sum was calculated from variance/covariance matrix.

Table 6). Coding variants, including exonic and splicing variants, despite being the smallest functional group, explained 1.0% (s.e. = 3.9%) of phenotypic variance for pancreatic cancer. The remaining 1.8% variance (s.e. = 3.5%) was attributed to variants in regulatory regions (e.g. UTR, ncRNA and upstream/downstream).

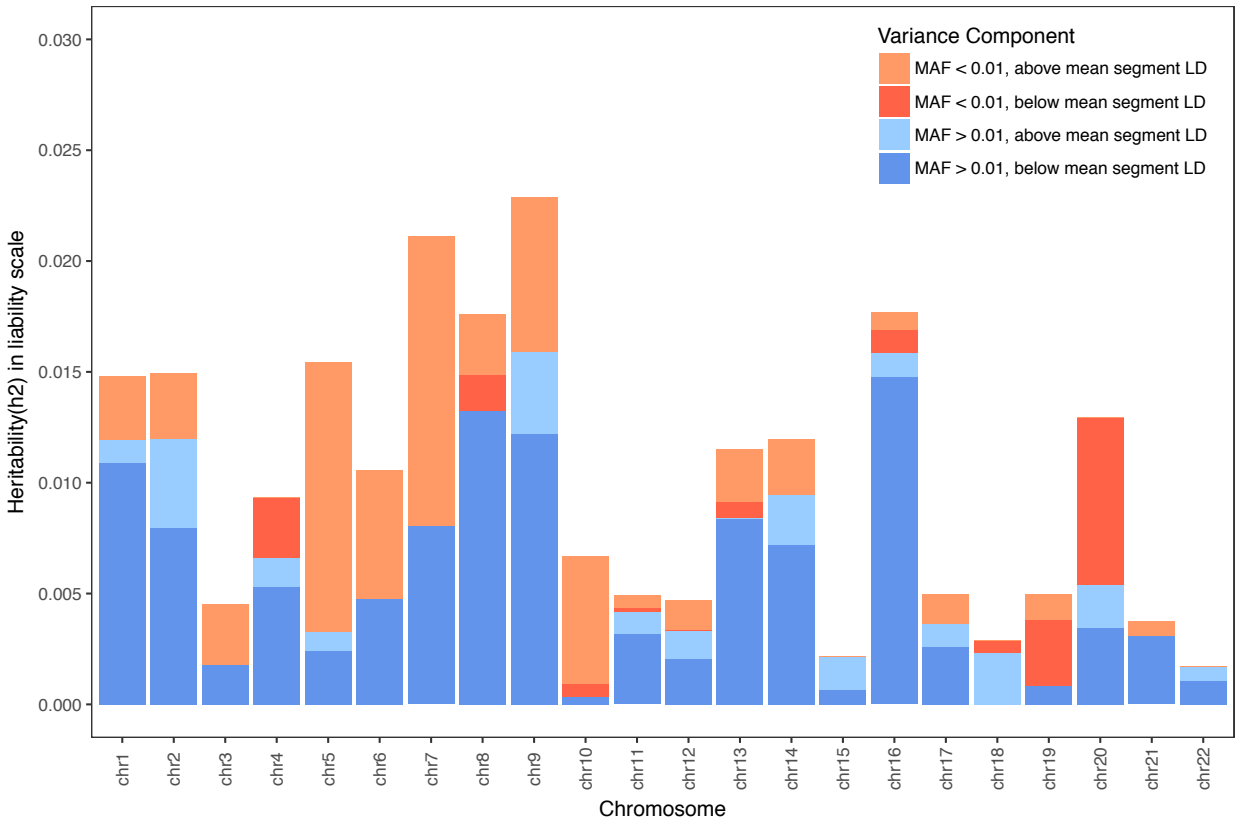


Figure 3 Estimated variance explained by variants on each autosomal chromosome. Variants on each chromosome were stratified into 2 MAF categories and 2 LD groups. The estimated variance associated with individual chromosome was aggregated from the variance explained by four MAF-LD groups.

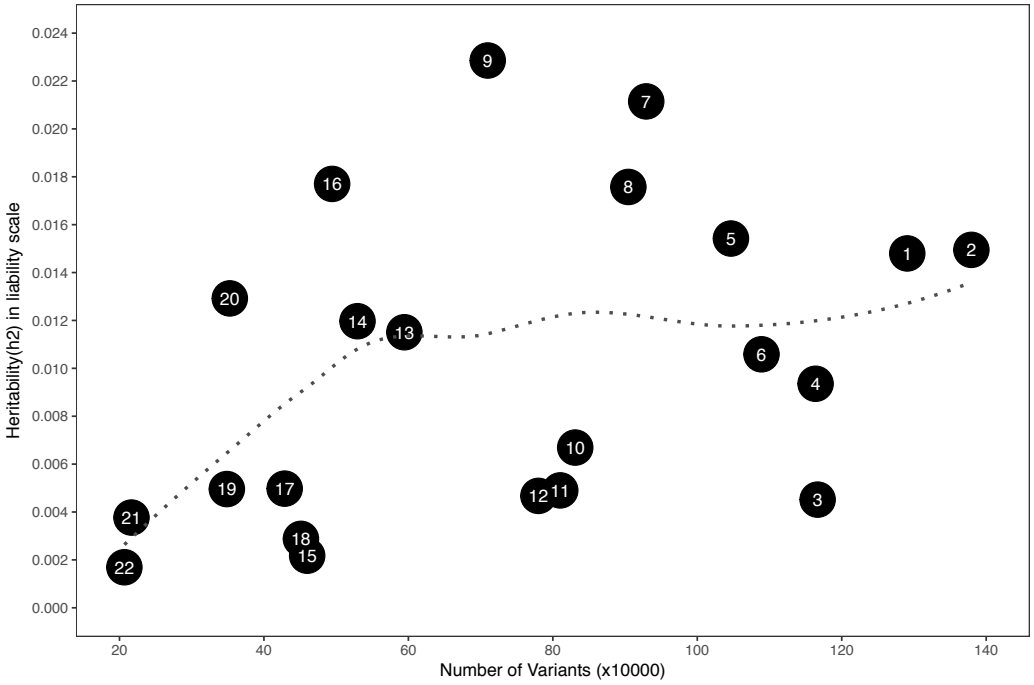


Figure 4 Correlations between the estimated variance explained by individual chromosome and the length of chromosomes. Each black circle represents a chromosome marked by the chromosome ID number. The dotted line is loess smoothed fit curve based on the data. No correlation was found between the estimated variance explained by individual chromosome and the length of chromosomes.

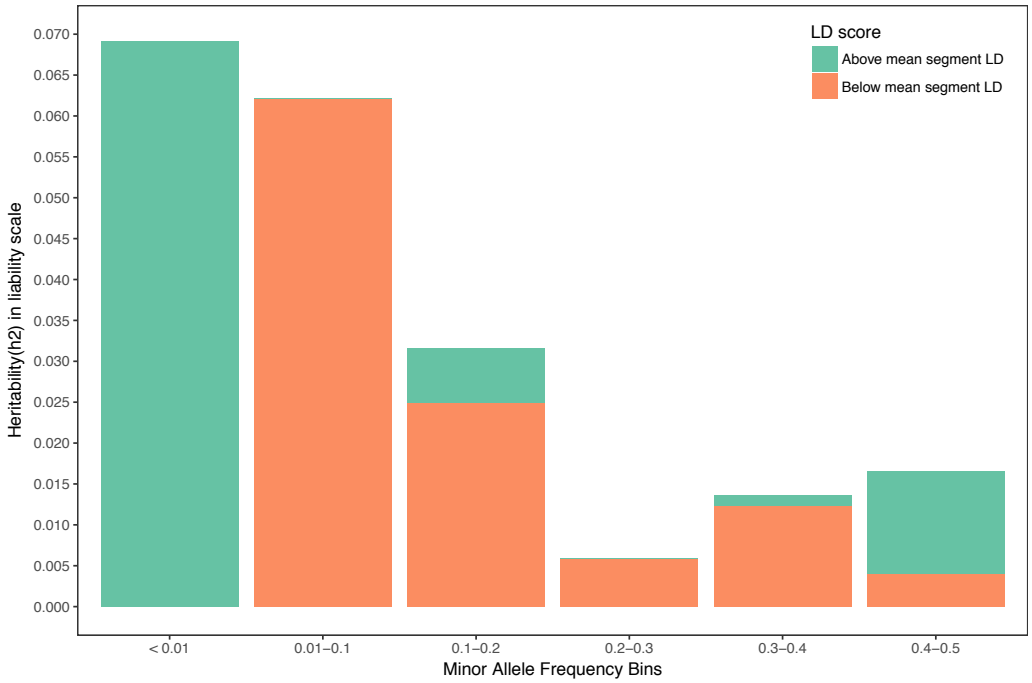


Figure 5 The estimated proportion of phenotypic variance explained by imputed variants in six MAF categories. Variants on each MAF category were further stratified into 2 LD groups (above vs. below mean segment LD score). The estimated variance attributed to each MAF category was aggregated from the variance explained by 2 LD groups.

The contribution of Identified GWAS Loci

Of the 26 common susceptibility loci reported in GWAS studies, 23 index SNPs were available in our dataset. For the three GWAS loci whose index SNP not available in our dataset, including rs2736098 on chromosome 5p13.33 (*TERT-CLPTM1L*), rs10094872 on chromosome 8q24.21 (*MYC*) and rs4795218 on chromosome 17q12 (*HNF1B*), variants in strong LD (pairwise r^2) with these index SNP were included in the analysis (Supplementary Table 3). To assess the aggregate contribution of these 26 GWAS loci to the estimated heritability for pancreatic cancer, a total of 72,225 variants located within ±250 kb of the index SNP was analyzed. Together these variants explained 4.1% (s.e. = 0.8%) of phenotypic variance for pancreatic cancer.

Discussion

Our study represents the first systematic investigation of the genetic architecture of pancreatic cancer. From the variants imputed to the 1000 Genomes Project reference panels, the heritability for pancreatic cancer explained by imputed variants ($h^2_{imputed}$) was estimated to be 21.2% (s.e. = 4.8%). This estimate is considerably lower than the estimates from traditional twin study[20](i.e. h^2_{fam} = 36%) but substantially higher than the array heritability estimates (h^2_{array}) which ranged from 9.8% to 18% [1,21,22]. Previously in the same PanC4 sample, the heritability for pancreatic cancer was estimated to be 16.4% (95% CI = 10.4 – 22.4%) from 620,357 directly genotyped SNPs using GREML-SC approach[1]. Comparing to this array heritability, the use of imputation data in our analysis allows $h^2_{imputed}$ to better capture the variance explained by rare and low-frequency causal variants. In addition, the application of GREML-LDMS approach stratifying variants by MAF and LD can minimize the differences in LD between causal variants and analyzed variants, and consequently reduce the bias associated with GREML-SC estimate.

Therefore, $h^2_{imputed}$ estimated in our analysis is an improved estimate of heritability than the previously reported array heritability.

However, heritability estimated from imputed variants is expected to miss some variance due to rare causal variants. This is because either the rare casual variants were not imputed using the chosen reference panels or they were excluded at quality control stage. Characterizing rare variants in imputation are challenging. It is likely that causal variants with low allele frequency or those unique to pancreatic cancer patients were not polymorphic in 1000 Genome Project reference populations and therefore were not imputed. It's also known that rare variants tend to have lower imputation accuracy than common variants. In our analysis, we applied a stringent INFO score cutoff at 0.50 to exclude variants with poor imputation quality, which affected 56.3% of all imputed variants. Since heritability analysis cannot account for the imputation uncertainty, probabilities of genotypes have to be converted to hard genotypes, which led to high missingness (> 5%) in about 1.9% of imputed variants. This conversion also caused some rare variants to become monomorphic in the data, making no contribution to the heritability analysis. Exclusion based on INFO score and missingness was likely to have a greater impact on rare variants than on common variants. Lastly, following the standard practice of GREML analysis, variants with minor allele count <5 were excluded from our analysis to avoid the noise and unstable estimations. This means that variants with extremely low frequency, such as mutations of established FPC genes, will never be included in the GREML based heritability analysis. As a result, their contribution to the susceptibility of pancreatic cancer can never be accurately reflected in heritability measures.

The underestimation of variance explained by rare variants may be partially explained the difference in h^2_{fam} and $h^2_{imputed}$. Whole-genome sequencing data in theory is capable of

capturing variance due to all causal variants, and hence generates an estimate of heritability closest to h^2_{fam}. In simulation studies, Yang and his colleagues showed that the proportion of variation at sequence variants captured by the 1000 Genomes Project imputation is almost independent of genetic architecture; and on average, about 97% of variation at common variants and about 68% of variation at rare variants could be captured by 1000 Genomes Project imputation[17]. In our GREML-LDMS analysis (Model #3), 5.2% (s.e. = 3.6%) and 16.0% (s.e. = 3.5%) of phenotypic variance for pancreatic cancer can be attributable to rare and common variants, respectively. Extrapolated from the proportion of variation at common and rare variants captured by 1000 Genomes Project imputation, the adjusted estimate of heritability for pancreatic cancer from $h^2 = \frac{h^2_{rare}}{0.68} + \frac{h^2_{common}}{0.97}$ would be 24.1%.

Even with the adjustment, the estimated heritability is still considerably lower than the family-based heritability (h^2_{fam}) of 36%. There are two possible explanations for the remaining missing heritability. First, the estimate of heritability from twin studies may be inflated owing to the shared environmental factors. Differences in shared environmental factors in MZ twins and DZ twins if not properly modeled may lead to an over-estimation of heritability. Although evidence of such inflation has been reported for a number of quantitative and binary traits[23], it is likely to be trait-specific and remains debatable for pancreatic cancer. Secondly, even with the adjustment, the estimated heritability for pancreatic cancer is still likely to be an underestimation since GREML estimates can never capture variance due to extremely rare mutations. Mutations of established FPC genes have previously been detected in 3-10% of sporadic pancreatic cancer patients[24–26]. Given that cases in the PanC4 study were unselected for their family history, the combined prevalence of these mutations in the PanC4 study was expected to be similar to that in published studies. In the most recent study, some of the established FPC genes were found to

have large effects on the risk of pancreatic cancer in sporadic cases, with an odds ratio ranging from 2.58 to 12.33[26]. Given the prevalence and effect sizes, variants of these established FPC genes were expected to explain a measurable fraction of phenotypic variation for pancreatic cancer. However, due to its low frequencies, these rare mutations likely were not imputed using 1000 Genome Projected reference panels or had been removed at quality controls. Without these rare pathological mutations, variants of the established FPC genes only explain a scanty amount of phenotypic variance for pancreatic cancer (0.4%) in our study. Using whole-genome sequencing data in a large enough cohort would largely lessen the underestimate of these very rare variants. However, even with WGS data, the standard practice is to exclude variants with small minor allele count (MAC, e.g. MAC < 3 or < 5), in order to minimize the noise in the analysis. As a result, the GREML estimate of heritability will always be an underestimation of the overall heritability, and the magnitude of underestimation depends on the underlying genetic architecture of the trait. For cancer phenotypes known to be affected largely by rare mutations, the bias can be substantial.

To infer the underlying genetic architecture of pancreatic cancer, the estimate of overall heritability was partitioned by MAF, chromosome, and functional annotations. Partitioning by chromosome ranked chromosome 9, 7, 8, 16, 5, 2 and 1 as top contributors to the heritability of pancreatic cancer. This is consistent with the GWAS findings as on all these chromosomes common susceptibility loci have been discovered (Chapter Two). Our results also suggest that variants in genomic regions with lower LD tend to explain a larger proportion of variance than those in regions with higher LD (14.6% vs. 6.7%, Table 1). Rare variants in lower than average LD regions appear to make little or no contribution to the estimated heritability for pancreatic cancer in our analysis (Table 5, Figure 5), which could be a result of under-representation of

such variants in the imputation data. As discussed above, since heritability estimated from imputed variants captures almost all variation at common variants but only a proportion of variation at rare variants, results from partitioning by chromosome are likely driven by common causal variants, some of which had been identified through GWAS studies.

Results from partitioning by MAF showed a non-negligible proportion of phenotypic variance explained by variants with MAF ≤ 0.01. More than 1/3 of the estimated heritability is attributable to rare variants. As suggested in simulation studies, this is likely an underestimate since not all variation at rare variants is captured by 1000 Genomes Project imputation, particularly those extremely rare variants that are known to confer risk in FPC cases. Our results demonstrate an important role of rare variants in the susceptibility of pancreatic cancer, which emphasize the necessity of WGS studies. High-coverage sequencing studies with a near-complete characterization of genetic variation would allow examination of variants with extremely low frequency or unique to specific populations. However, obtaining a sufficiently large sample to permit such sequencing analysis remains a significant challenge.

On the other hand, common variants still account for a large proportion of estimated heritability in our study. Previous GWAS studies in the PanC4 study reported that 3.0% (95% CI = 2.0 – 3.9%) of the total phenotypic variation was accounted for by established association regions[1]. In our analysis, known GWAS loci explained 4.1% of phenotypic variance for pancreatic cancer, leaving > 10% of the phenotypic variance unexplained. The increment in genetic variance due to known GWAS loci in our study is likely resulting from the inclusion of the five novel loci discovered in a recent meta-analysis of four pancreatic cancer consortium studies[27]. The large proportion of unexplained heritability highlights the importance of continuously searching for common susceptibility loci for pancreatic cancer. SNP-array based

genotyping followed by imputation will remain a more cost-effective strategy than WGS studies. However, larger sample sizes will be required to increase the power of current GWAS for identification of yet-to-be detected causal common and low-frequency variants. As imputation reference panel of large sample size (e.g. Haplotype Reference Consortium, HRC) becoming available, the imputation accuracy is expected to be greatly improved for low frequency and rare variants, hence increase the power of detecting associations on these variants[28,29]. An alternative approach to improve the imputation quality on rare variants is to use a population-specific reference panel, which would provide better enrichment of population-specific variants that are likely to be monomorphic in commonly used reference populations[30–32]. This could be particularly useful for a low-prevalence disease like pancreatic cancer, known to be largely affected by rare variants. Currently, a pancreatic cancer specific haplotype reference panel is under construction from high-coverage WGS (50x) of 638 pancreatic cancer cases of European ancestry. For future imputation and meta-analysis, there is great potential to gain power using this disease-specific reference panel, in combination with 1000 Genomes Project or HRC panels, for rare variants in particular.

Across four functional groups, intronic variants account for most phenotypic variance of pancreatic cancer (12.4%). Interestingly, 12 out of 21 GWAS loci identified in the European population are mapped to intronic variants. However, it is unclear whether these variants are of direct functional significance, as opposed to simply being in linkage disequilibrium with another functional SNP in the vicinity. The coding variants, comprising about 1% of imputed variants, account for 1% of the phenotypic variance of pancreatic cancer. This is likely an underestimate since a proportion of rare or extremely rare coding variants were not imputed or removed by quality controls. Comparing to the other three functional groups, the proportion of variants with a

MAF < 0.005 is much higher among coding variants (Supplementary Figure 1). It is possible that the poor imputation accuracy on rare and extremely rare variants has a greater impact on coding variants than variants in other three functional groups.

Conclusion

In a subset of the PanC4 sample of 3,568 pancreatic cancer cases and 3,363 controls, the heritability for pancreatic cancer is estimated to be 21.2% from imputed variants. This estimate is less biased than reported array-based heritability but remains an underestimation of overall heritability due to limited coverage on rare and extremely rare variants. It is known that rare mutations are presented in 8-30% familial cases and in 3-10% of sporadic cases. Current statistical approaches, such as GREML used in this study, is incapable of capturing variance due to these rare mutations. Genomic partitioning by chromosome ranks several autosomal chromosomes as top contributors to the estimated heritability. Since these results are largely driven by common variants, they provide very little guidance on physical locations of causal variants. Rare variants with MAF < 0.01 explain about 1/3 of the estimated heritability, suggesting an important role of these variants in the susceptibility of pancreatic cancer. Whole-genome sequencing that determines the genetic variation underlying the full spectrum of pancreatic cancer will be necessary for rare causal variant discovery. However, establishing a sufficiently large cohort for sequencing analysis is very costly. Combining existing sequencing cohorts is an interesting strategy that is worth being further investigated for its performance in identifying rare causal variants. In our study, known GWAS loci only account for 4.1% of the phenotypic variance for pancreatic cancer, significantly less than the amount of variance attributable to common variants (16.0%). Imputation and meta-analysis have proven fruitful in the search for common and low-frequency susceptibility loci for pancreatic cancer, and remain

the most cost-effective strategy in future gene discovery research for the disease. Increasing the sample size and improving the imputation accuracy would be the key to ensure progress in such studies. In addition to using a larger reference panel like Haplotype Reference Consortium, a disease-specific reference panel for pancreatic cancer would further improve the imputation on variants with extremely low frequency or unique to the disease populations.

Tables

Table 4 Characteristics of Analyzed PanC4 Cohort

	Affected Individuals (N = 3,568)	**Unaffected Individuals (N = 3,363)**
Female, N (%)	1,510 (42.3%)	1,491 (44.3%)
Age, N (%)		
40-49	335 (9.4%)	358 (10.6%)
50-59	768 (21.5%)	849 (25.2%)
60-69	1275 (35.7%)	1,140 (33.9%)
70-79	900 (25.2%)	786 (23.4%)
>80	290 (8.1%)	230 (6.8%)

Table 5 Estimates of Variance Explained by Imputed Variants from GREML-LDMS Analysis

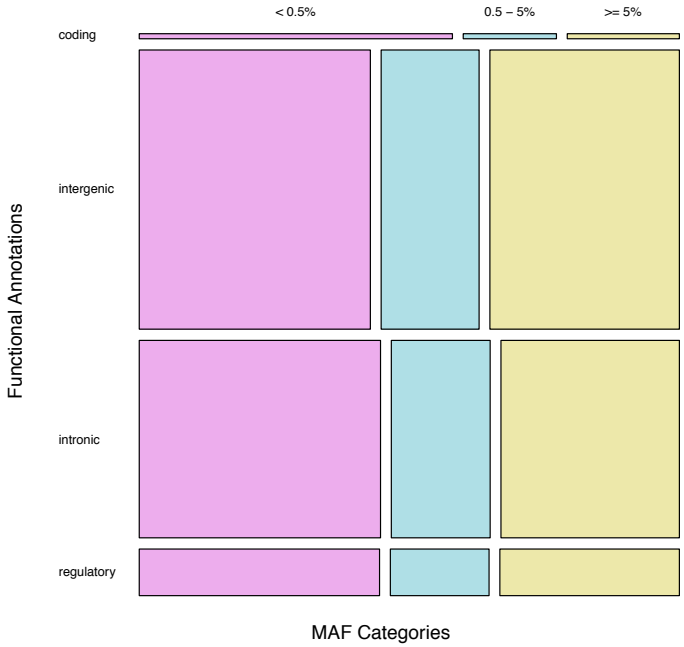
	Above mean LD		Below mean LD		Row sum	s.e. *	No. Variants (%)
	Est	s.e. †	Est	s.e. †			
MAF ≤ 0.01	0.0523	0.037	0	0.051	0.052	0.036	8,354,405 (51.6%)
MAF > 0.01	0.0142	0.017	0.1460	0.032	0.160	0.035	7,829,724 (48.4%)
Column sum	0.067	0.040	0.146	0.058			
Total sum	**0.212**	**0.048**					
No. Variants (%)	8,092,536 (50%)		8,091,593 (50%)				

* s.e. for row sum and column sum was calculated from variance/covariance matrix.

Table 6 Estimates of Variance Explained by Imputed Variants in Four Functional Groups

		Above mean LD		Below mean LD		Row sum	s.e.	No. Variants (%)	Sub-category sum	s.e.
		Est	s.e.	Est	s.e.					
Coding	MAF ≤ 0.01	0.0016	0.022	0.0036	0.029	0.005	0.036	103,133 (0.6%)	0.010	0.039
	MAF > 0.01	0.0050	0.009	0	0.014	0.005	0.017	50,753 (0.3%)		
Intergenic	MAF ≤ 0.01	0.0334	0.036	0	0.059	0.033	0.063	4,305,707 (26.6%)	0.060	0.068
	MAF > 0.01	0	0.015	0.0267	0.026	0.027	0.030	4,220,706 (26.1%)		
Intronic	MAF ≤ 0.01	0.0416	0.037	0	0.055	0.042	0.061	3,173,417 (19.6%)	0.124	0.066
	MAF > 0.01	0.0086	0.014	0.0735	0.025	0.082	0.028	2,856,226 (17.6%)		
Regulatory	MAF ≤ 0.01	0	0.024	0	0.036	0	0.043	746,866 (4.6%)	0.018	0.045
	MAF > 0.01	0.0002	0.009	0.0181	0.014	0.018	0.016	676,634 (4.2%)		

Supplementary Figures and Tables



Supplementary Figure 1 MAF distribution of variants in four functional groups. The heights of the boxes are proportional to the percentage of all variants being annotated as coding, intergenic, intronic or regulatory variants. The widths of the boxes are proportional to the percentage of variants in each functional group fallen into each MAF categories.

Supplementary Table 1 Models and Calculations of h^2 in Heritability Analysis

Analysis	Model#	GREML Approach*	Mixed Linear Model†	List of Genetic Components	Calculation of h^2
Estimates of heritability explained by imputed variants	1	GREML-SC	$y = u + g + \varepsilon$	g	$h^2_{imputed} = \sigma^2_g/(\sigma^2_g + \sigma^2_e)$
	2	GREML-MS	$y = u + \sum_{i=1}^{2} g_i + \varepsilon$	$g_{MAF<0.01}$ $g_{MAF\geq 0.01}$	$h^2_{imputed} = \frac{\sum_{i=1}^{2}\sigma^2_{g_i}}{\sum_{i=1}^{2}\sigma^2_{g_i} + \sigma^2_e}$
	3	GREML-LDMS	$y = u + \sum_{i=1}^{4} g_i + \varepsilon$	$g_{MAF<0.01,LD\ below}$ $g_{MAF<0.01,LD\ above}$ $g_{MAF\geq 0.01,LD\ below}$ $g_{MAF\geq 0.01,LD\ above}$	$h^2_{imputed} = \frac{\sum_{i=1}^{4}\sigma^2_{g_i}}{\sum_{i=1}^{4}\sigma^2_{g_i} + \sigma^2_e}$
Genomic partitioning by chromosome	4	GREML-LDMS	$y = u + \sum_{i=1}^{88} g_i + \varepsilon$	$g_{MAF<0.01,LD\ below,chr1}$ $g_{MAF<0.01,LD\ above,chr1}$ $g_{MAF\geq 0.01,LD\ below,chr1}$ $g_{MAF\geq 0.01,LD\ above,chr1}$ … $g_{MAF<0.01,LD\ below,chr22}$ $g_{MAF<0.01,LD\ above,chr22}$ $g_{MAF\geq 0.01,LD\ below,chr22}$ $g_{MAF\geq 0.01,LD\ above,chr22}$	$h^2_{chr1} = \frac{\sum_{i=1}^{4}\sigma^2_{g_{i,chr1}}}{\sum_{i=1}^{88}\sigma^2_{g_i} + \sigma^2_e}$ … $h^2_{chr22} = \frac{\sum_{i=1}^{4}\sigma^2_{g_{i,chr22}}}{\sum_{i=1}^{88}\sigma^2_{g_i} + \sigma^2_e}$
Genomic Partitioning by MAF	5	GREML-LDMS	$y = u + \sum_{i=1}^{12} g_i + \varepsilon$	$g_{MAF<0.01,LD\ below}$ $g_{MAF<0.01,LD\ above}$ $g_{MAF\ 0.01-0.10,LD\ below}$ $g_{MAF\ 0.01-0.10,LD\ above}$ $g_{MAF\ 0.10-0.20,LD\ below}$ $g_{MAF\ 0.10-0.20,LD\ above}$ $g_{MAF\ 0.20-0.30,LD\ below}$ $g_{MAF\ 0.20-0.30,LD\ above}$	$h^2_{MAF<0.01} = \frac{\sum_{i=1}^{2}\sigma^2_{g_{i,MAF<0.01}}}{\sum_{i=1}^{12}\sigma^2_{g_i} + \sigma^2_e}$ $h^2_{MAF\ 0.01-0.10} = \frac{\sum_{i=1}^{2}\sigma^2_{g_{i,MAF\ 0.01-0.10}}}{\sum_{i=1}^{12}\sigma^2_{g_i} + \sigma^2_e}$ $h^2_{MAF\ 0.10-0.20} = \frac{\sum_{i=1}^{2}\sigma^2_{g_{i,MAF\ 0.10-0.20}}}{\sum_{i=1}^{12}\sigma^2_{g_i} + \sigma^2_e}$

				$g_{MAF\ 0.30-0.40,LD\ below}$ $g_{MAF\ 0.30-0.40,LD\ above}$ $g_{MAF\ 0.40-0.50,LD\ below}$ $g_{MAF\ 0.40-0.50,LD\ above}$	$h^2_{MAF\ 0.20-0.30} = \frac{\sum_{i=1}^{2} \sigma^2_{g_{i,MAF\ 0.20-0.30}}}{\sum_{i=1}^{12} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{MAF\ 0.30-0.40} = \frac{\sum_{i=1}^{2} \sigma^2_{g_{i,MAF\ 0.30-0.40}}}{\sum_{i=1}^{12} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{MAF\ 0.40-0.50} = \frac{\sum_{i=1}^{2} \sigma^2_{g_{i,MAF\ 0.40-0.50}}}{\sum_{i=1}^{12} \sigma^2_{g_i} + \sigma^2_e}$
Genomic partitioning by functional annotation	6	GREML-LDMS	$y = u + \sum_{i=1}^{16} g_i + \varepsilon$	$g_{MAF<0.01,LD\ below,coding}$ $g_{MAF<0.01,LD\ above,coding}$ $g_{MAF\geq0.01,LD\ below,coding}$ $g_{MAF\geq0.01,LD\ above,coding}$ $g_{MAF<0.01,LD\ below,intergenic}$ $g_{MAF<0.01,LD\ above,intergenic}$ $g_{MAF\geq0.01,LD\ below,intergenic}$ $g_{MAF\geq0.01,LD\ above,intergenic}$ $g_{MAF<0.01,LD\ below,intronic}$ $g_{MAF<0.01,LD\ above,intronic}$ $g_{MAF\geq0.01,LD\ below,intronic}$ $g_{MAF\geq0.01,LD\ above,intronic}$ $g_{MAF<0.01,LD\ below,regulatory}$ $g_{MAF<0.01,LD\ above,regulatory}$ $g_{MAF\geq0.01,LD\ below,regulatory}$ $g_{MAF\geq0.01,LD\ above,regulatory}$	$h^2_{coding} = \frac{\sum_{i=1}^{4} \sigma^2_{g_{i,coding}}}{\sum_{i=1}^{16} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{intergenic} = \frac{\sum_{i=1}^{4} \sigma^2_{g_{i,intergenic}}}{\sum_{i=1}^{16} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{intronic} = \frac{\sum_{i=1}^{4} \sigma^2_{g_{i,intronic}}}{\sum_{i=1}^{16} \sigma^2_{g_i} + \sigma^2_e}$ $h^2_{regulatory} = \frac{\sum_{i=1}^{4} \sigma^2_{g_{i,regulatory}}}{\sum_{i=1}^{16} \sigma^2_{g_i} + \sigma^2_e}$
Evaluation of GWAS loci	7	GREML-LDMS	$y = u + g_{GWAS} + \sum_{i=1}^{4} g_i + \varepsilon$	g_{GWAS} $g_{MAF<0.01,LD\ below,remaining}$ $g_{MAF<0.01,LD\ above,remaining}$ $g_{MAF\geq0.01,LD\ below,remaining}$ $g_{MAF\geq0.01,LD\ above,remaining}$	$h^2_{GWAS} = \frac{\sigma^2_{GWAS}}{\sigma^2_{GWAS} + \sum_{i=1}^{4} \sigma^2_{g_{i,remaining}} + \sigma^2_e}$

* GREML-SC = single-component GREML; GREML-MS = MAF-stratified GREML; GREML-LDMS = LD- and MAF-stratified GREML.

† The disease status in observed scale (y) is a functional of some fixed effects (μ) including intercept, age, sex, and first 20 principal components, one or more genetic components (g_i), and a residual effect (ε).

Supplementary Table 2 Description of the Studies Included in PanC4 Case-Control Consortium

Study	Cases	Controls	Accrual Years	Source of Cases	Source of Controls	Control Matching	Age of Diagnosis for Cases (SD) [†]	Age of Controls (SD) [*]	Male (%)	Cauc. (%) [†]
IARC	448	456	2006-2010	Academic hospitals	General practitioners	Age; sex; region	63.84 (11.16)	61.88 (11.88)	57	100
Johns Hopkins Hospital	315	81	2007-2011	Clinic	Spouse in-law	None (in-law)	64.29 (11.53)	63.45 (14.73)	51	93
Mayo Clinic	1104	1027	2000-2010	Clinic	Primary care patients	Age; sex; race; residence	65.92 (11.09)	63.34 (10.58)	56	93
MD Anderson	616	509	1997-2007	Hospital	Friends and spouse of non-pancreatic cancer patients	Age; race; sex	62.6 (9.69)	59.2 (10.6)	59	100
Memorial Sloan Kettering	317	139	2000-2008	Clinic	Patient spouses and patient visitors	None	64.01 (10.39)	61.68 (10.98)	64	87
Toronto	402	401	2003-2012	Population-based cancer registry	Family medicine clinic database	Age; sex; ethnicity	64.92 (11.03)	62.95 (11.73)	50	85
Queensland	559	604	2007-2011	Population-based hospitals & cancer registry	Electoral rolls	Age; sex	66.64 (11.26)	67.51 (10.91)	60	96
UCSF	253	248	2006-2010	Two UCSF clinics	Three UCSF clinics	Frequency matched by age and sex	62.52 (10.35)	60.40 (10.96)	54	82
YALE	156	366	2005-2009	Population-based hospitals & cancer registry	Enhanced RDD	Frequency matched by age and sex	67.01 (10.43)	65.15 (10.6)	59	93
Total	4170	3831					64.76	63.09	58	95

[*] SD: standard deviation

[†] Percent self-reported as Caucasians

Supplementary Table 3 Availability of the Index SNP of Pancreatic Cancer GWAS Loci in the Data

CHR*	Nearest Gene(s)	Index_SNP†	Position	Population	Availability	Alternative	Pairwise_r^2
1p36.33	*NOC2L*	rs13303010	894573	European	yes	NA	NA
1q32.1	*NR5A2*	rs3790844	200007432	European	yes	NA	NA
1q32.1	*NR5A2*	rs2816938	199985368	European	yes	NA	NA
2p13.3	*ETAA1*	rs1486134	67639769	European	yes	NA	NA
3q29	*TP63*	rs9854771	189508471	European	yes	NA	NA
5q15.33	*TERT, CLPTM1L*	rs401681	1322087	European	yes	NA	NA
5p15.33	*TERT, CLPTM1L*	rs2736098	1294086	European	no	rs548166886	1.000
5p15.33	*TERT, CLPTM1L*	rs35226131	1295373	European	yes	NA	NA
7p12	*TNS3*	rs73328514	47488569	European	yes	NA	NA
7p13	*SUGCT*	rs17688601	40866663	European	yes	NA	NA
7q32.3	*LINC-PINT*	rs6971499	130680521	European	yes	NA	NA
8q21.11	*HNF4G*	rs2941471	76470404	European	yes	NA	NA
8q24.21	*MYC*	rs10094872	128719884	European	no	rs9642880	0.598
9q34	*ABO*	rs505922	136149229	European	yes	NA	NA
13q12.2	*PDX1*	rs9581943	28493997	European	yes	NA	NA
13q22.1	none	rs9543325	73916628	European	yes	NA	NA
16q23.1	*BCAR1, CTRB1, CTRB2*	rs7190458	75263661	European	yes	NA	NA
17q12	*HNF1B*	rs4795218	36078510	European	no	rs3110645	1.000

17q25.1	*LINC00673*	rs11655237	70400166	European	yes	NA	NA
18q21.32	*GRP*	rs1517037	56878274	European	yes	NA	NA
22q12.1	*ZNRF3*	rs16986825	29300306	European	yes	NA	NA
5p13.1	*DAB2*	rs2255280	39394989	Asian	yes	NA	NA
10q26.11	*RPLHR*	rs12413624	120278944	Asian	yes	NA	NA
21q21.3	*BACH1*	rs372883	30717737	Asian	yes	NA	NA
21q22.3	*TFF1*	rs1547374	43778895	Asian	yes	NA	NA
22q13.32	*FAM19A5*	rs5768709	48929569	Asian	yes	NA	NA

* Chromosomal location in NCBI genome build 37.

† SNP = single nucleotide polymorphisms; Index SNP = variant reported to be associated with pancreatic cancer

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CHAPTER FOUR

HERITABILITY FOR PANCREATIC CANCER FROM WGS VARIANTS

The heritability analysis was conducted in two independent case-control studies: a subset of the Pancreatic Cancer Case-Control Consortium (PanC4) study and a combined sample of Familial Pancreatic Cancer (FPC) patients from the Familial Pancreatic Cancer Genome Sequencing Project (FPC-GSP) and participants from the Alzheimer's Disease Neuroimaging Initiative (ADNI) study. This chapter presents the analysis conducted in the FPC-ADNI study, including the estimate of heritability from WGS variants in FPC-ADNI study, as well as the comparison of heritability estimates between FPC-ADNI and PanC4 study using the same set of genotyped variants.

Study Populations and Genetic Data

Familial Pancreatic Cancer (FPC) Patients

FPC-GSP is a multi-institutional study, encompassing 10 familial pancreatic cancer (FPC) registries across North American, with the goal of identifying additional pancreatic cancer susceptibility genes through whole-genome sequencing (WGS) analysis (https://www.familialpancreaticcancer.org/). Patients with FPC were enrolled into the National Familial Pancreatic Tumor Registry (NFPTR) at Johns Hopkins or one of the FPC registries participating at the following sites: Dana-Farber Cancer Institute (Boston, MA), Karmanos Cancer Institute (Detroit, MI), Mayo Clinic (Rochester, MN), McGill University Health Centre (Montreal, Quebec, Canada), Memorial Sloan Kettering Cancer Center (New York, NY), Mount Sinai Hospital (Toronto Ontario, Canada), University of Michigan (Ann Arbor, MI), University of Pennsylvania (Philadelphia, PA), and University of Pittsburgh (Pittsburgh, PA). FPC families are defined as kindreds with at least two first-degree relatives (FDRs) diagnosed with pancreatic

ductal adenocarcinoma (PDAC). Of 593 FPC kindreds, a total of 658 patients were selected for sequencing as they were either untested or tested negative for any previously known deleterious mutation in an establish FPC gene[1]. Diagnosis of pancreatic cancer was self-reported. When possible, all cancer diagnoses were pathologically confirmed.

Germline DNA samples were obtained from blood (n=454), Epstein-Barr virus transformed peripheral blood lymphocytes (lymphoblastoid cell line, LCL) (n=158), or non-tumor tissue (n=26). These samples were whole-genome sequenced using Illumina HiSeq 2000 at 30x depth of coverage and genotyped with the HumanOmni2.5-8v1 SNP array (Illumina, Inc., San Diego, CA) by Personal Genome Diagnostics. Whole genome sequencing generated 200 base pair (2×100 bp reads) per fragment in the final library. Sequence reads were analyzed using ELAND v2 (Illumina) for alignment to the human reference genome hg19/GRCh37 and CASAVA v1.7 for variant calling[2].

Alzheimer's Disease Neuroimaging Initiative (ADNI) Subjects

ADNI is an ongoing, longitudinal, multicenter study launched in 2004. The overarching goals of ADNI project were to characterize clinical, genetic, imaging and biochemical biomarkers of Alzheimer's Disease (AD) over the course of disease progression, and to establish standardized methods for imaging/biomarker collection and analysis for ultimate use in clinical trials [3]. The ADNI project has three phases, ADNI1, ADNI GO and ADNI2, with distinct research objectives. The full ADNI cohort recruited 822 individuals aged 55-90 years from 59 participating sites across the United States and Canada. The cohort consists of subjects with normal cognition, with early or late mild cognitive impairment (MCI) or with AD. Individuals with cancer were not eligible to participate in ADNI [3].

As genetic factors play an important role in Alzheimer's disease, a key aim of the ADNI is to combine genetics with imaging and clinical data to help investigate mechanisms of the disease. Of 822 ADNI participants, 818 with a sufficient amount of DNA sample were genotyped and sequenced. DNA samples of these individuals were derived from either peripheral blood (n=731) or immortalized LCL (n=87)[4]. These samples were genotyped on HumanOmni2.5 SNP array and sequenced on non-CLIA WGS platform at Illumina at 30X depth (Illumina, Inc., San Diego, CA). The WGS data were aligned and called using CASAVA on reference genome hg19/GRCh37 per Illumina's protocol in the spring of 2013. After basic quality control checks, the released dataset contains 809 ADNI samples.

Quality Controls on FPC-ADNI Study

A series of quality control (QC) procedures were implemented on WGS data to minimize the impacts of low-quality variants, mishandling of DNA samples, cryptic relatedness, population substructures and batch effects on heritability estimates. Insertion/deletion variants were not included in our analysis. **Error! Reference source not found.** presents an overview of these QC procedures. Prior to QC, the FPC WGS data consisted of 658 samples sequenced on 56,330,793 single-nucleotide variants (SNVs) with a transition to transversion ratio (Ti/Tv) of 1.47. The ADNI WGS data contained 809 samples sequenced on 59,444,512 SNVs with a Ti/Tv of 1.52. Before merging the FPC WGS and ADNI WGS data, genotypes with genotyping quality score (GQ) < 20 or read depth (DP) < 10 were set to missing. Variants in annotated super-duplicated regions were excluded. Samples were dropped if they had non-reference discordance rate (NRD) > 5% or mismatched sex. In addition, twenty FPC samples were removed due to being duplicates or with a non-FPC diagnosis. After merging the WGS data of FPC and ADNI cohort, we further removed non-European samples as well as closely related individuals (first-

and second-degree relatives). In the end, variants with the following characteristics were further excluded: monomorphic variants, missingness > 10%, Hardy-Weinberg Equilibrium exact test with $p < 10^{-}6$, and minor allele count < 3. The final dataset contains 1,323 samples on 14,055,912 single-nucleotide variants (SNVs). The Ti/Tv ratio in the final dataset was 2.07, which was considered adequate for WGS data.

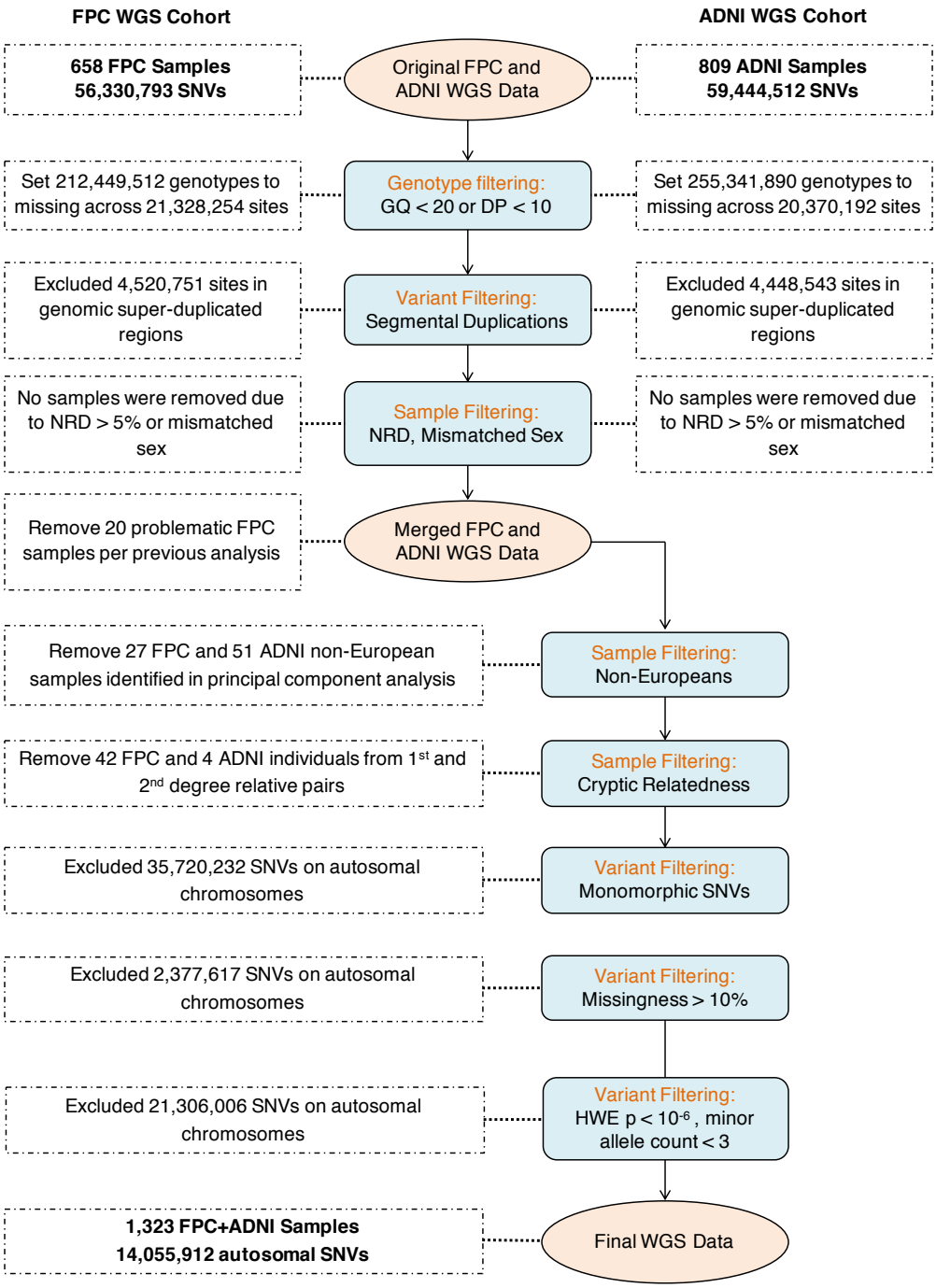


Figure 6 Overview of quality control procedures on FPC-ADNI WGS data. Circles with the solid line represent WGS data at different quality control stage. Boxes with the solid line represent filters applied in an order directed by arrows. Boxed with dashed line detail changes in WGS data resulting from the implementation of each filter. The bolded text emphasizes the number of samples and variants in WGS data at the beginning and the end of quality controls.

Genotype Filtering: GQ < 20 and DP < 10

Filtering on GQ scores was recommended for WGS analysis. CASAVA variant caller uses a Bayesian approach to update a prior genotype distribution based on the observed sequencing reads to produce a final predicted genotype distribution. This final genotype distribution is summarized as a set of quality scores (GQ). Briefly, at each site the probability of all possible genotypes was calculated based on the relative distribution of reads of different bases (i.e. A, T, C, G). Then a prior distribution based on a reference sequence was applied to adjust the genotype probabilities and to determine the GQ. Higher GQ values indicate higher confidence in the called genotypes. Filtering on GQ scores is highly recommended, but the thresholds are often arbitrary and vary by study.

Another filtering metric often used in sequencing data is read depth (DP) values. The DP values generated by CASAVA represent the total number of reads mapped to the site that passed read filtering (i.e. low-quality reads are dropped). It is not the number of reads used to call the most likely genotype, but the total number of reads used to determine the probability of all possible genotypes. No minimum DP threshold was enforced to make genotype calls. However, genotype calls with read depth greater than 3 times the chromosomal mean were removed to minimize potentially spurious calls that occur near the centromeres and other high-copy number regions. Like GQ values, DP thresholds are often arbitrary and vary by study.

GQ < 20 and DP < 10 were often applied in sequencing studies to exclude low-quality calls. To evaluate the efficiency of these two filters in our dataset, the true-variant rate and error rate were calculated in 11 pairs of duplicated FPC samples[5]. The true-variant rate is the proportion of concordant SNVs that remained in the dataset after applying the filter, whereas the error rate is the proportion of discordant SNVs that remained after applying the filter. The true-

variant rate and error rate were each averaged across 11 pairs of duplicated samples. At GQ < 20, 97.0% true variants and 2.5% errors were retained in the dataset. While at DP < 10, the true-variant rate and error rate was 96.9% and 8.7%. When both filters were applied, 96.1% true variants were retained, and the error rate was reduced to 1.3%. A higher cutoff value at GQ or DP may further lower the error rate at a high expense of removing a large number of true variant calls. Because the number of concordant genotype calls was considerably larger than the number of discordant genotype calls, the cutoff of GQ < 20 and DP < 10 would maximize the true variant calls at an acceptable error rate. Genotypes with GQ < 20 or DP < 10 were therefore set to missing, which affects about 38% and 33% variants in FPC WGS and ADNI WGS data, respectively.

Variant Filtering: Segmental Duplications

Previous studies have suggested that WGS has limited capacity to resolve the structure of duplicated regions within genomes, particularly those > 15 kb in length and show > 97% sequence identity[6]. Assembly of sequence reads from these duplicated regions is difficult and more prone to errors. Over the years a list of segmental duplications in the human genome that are > 1kb in length and > 90% identity has been constructed. The coordinates of these segmental duplications in GRCh37 reference human genome were obtained from the Segmental Duplication Database (http://humanparalogy.gs.washington.edu). SNVs located in these super-duplicated regions were excluded from our analysis, lowering the total number of SNVs by 4,520,751 (8.0%) in FPC WGS data and by 4,448,543 (7.5%) in ADNI WGS data (Figure 1).

Samples Filtering: Non-reference Discordance Rate (NRD)

A high non-reference discordance rate (NRD) is an indicator of problematic samples. For each sample in ADNI (N = 809) or FPC (N = 637) data, we calculated the NRD as the proportion

of non-reference genotypes differed between the WGS data and Omni2.5M array data. We limited this analysis to autosomal variants shared by the WGS data and Omni2.5M array data, excluding small insertion/deletions, SNVs with call rate < 0.95 and those with a MAF < 5% in Omni2.5M data. Concordant and discordant genotypes were identified using a PERL module in VCFtools (*vcf-compare*)[7]. Because sequencing errors in WGS data would affect the NRD calculation, we performed the analysis in the genome after removing the genotypes with GQ < 20 or DP < 10 and excluding variants in the super-duplicated regions.

Figure 7 showed the NRD per sample in the FPC WGS data and ADNI WGS data, respectively. The NRD rates in FPC WGS data ranged from 1.04% to 1.77% with a mean of 1.07% (Figure 7A). Sample FPC0713 with an NRD of 1.77% was distributed further away from the cluster of most samples but was considered acceptable (< 5%). The distribution of NRD rates in the ADNI WGS data was quite similar to that in the FPC WGS cohort, with a slightly higher mean of 1.11% and a slightly narrower range between 1.06% and 1.46% (Figure 7B). One sample 12AD48485 in the ADNI WGS data was separated from the main cluster but was considered acceptable as well.

All samples in FPC WGS and ADNI WGS data had NRD < 2%. Therefore, no sample in either cohort was removed due to an NRD > 5%.

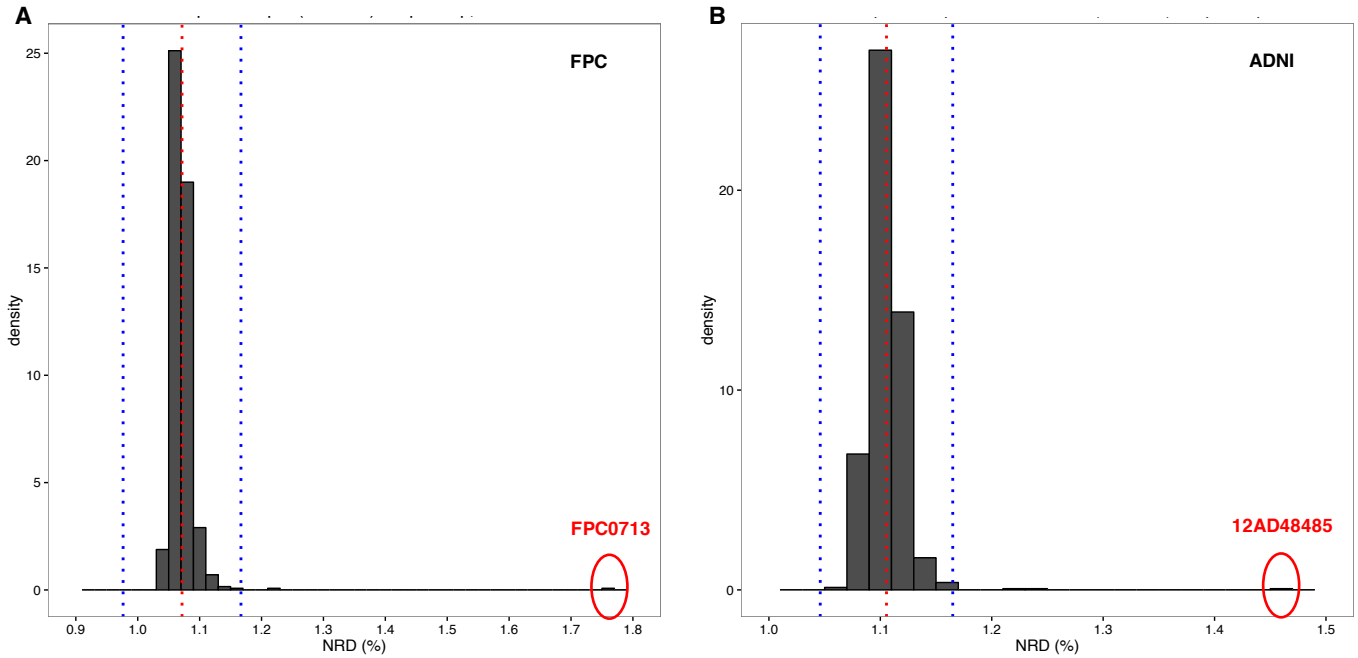


Figure 7 Distributions of non-reference discordance rate (NRD) per sample in (A) FPC WGS and (B) ADNI WGS data. The red dotted lines represent the mean NRD average across all samples in each data. The blue dotted lines represent the NRD percentage ± 3 standard deviations from the mean. Samples circled and marked with their ID in each data had an NRD significantly higher than the remaining samples.

Sample Filtering: Mismatched Sex

Mismatched sex is another indicator of problematic samples. We performed a sex check analysis using PLINK (*--check-sex ycount*)[8], calculating the X chromosome inbreeding coefficient F to determine the sex of each sample in ADNI WGS and FPC WGS data. We used bi-allelic SNVs on the sex chromosome with MAF > 5% for this analysis, excluding variants in the pseudo-autosomal regions *(--split-x*). Linkage disequilibrium (LD) based pruning was applied to these variants at a pair-wise r^2 of 0.2.

A total of 11,638 variants on X chromosome and 148 variants on Y chromosome were used to determine sex in the FPC WGS data. There were 326 females and 312 males in this cohort according to pedigree information. Based on the empirical distribution of F estimates in FPC WGS data, we set the threshold to make female calls to 0.50. This threshold flagged two samples, FPC0145 and FPC0347, since their F estimates were > 0.80. However, all female samples in FPC WGS data had zero Y genotypes, including FPC0145 and FPC0347. The count of non-missing Y genotypes in males had a wider range than in females. After considering the sex check results from Illumina, and the non-missing Y genotype counts in our analysis, we believed that the female calls on FPC0145 and FPC0347 were justified despite their high F values.

A total of 8,501 variants on X chromosome and 55 variants on Y chromosome were used to determine sex in the ADNI WGS data. There were 446 males and 363 females in the ADNI WGS data. Estimated sex given genomic data matched the reported sex in pedigrees for all samples. In conclusion, no sample was removed due to mismatched sex in either dataset.

Merging of FPC WGS and ADNI WGS Data

We merged the FPC WGS and ADNI WGS data after applying the genotype filtering at GQ < 20 or DP < 10 and the variant filtering on segmental duplications. Evaluation of NRD rate and genetically determined sex in both FPC and ADNI samples didn't remove any sample. Before merging, we evaluated the comparability of these two datasets based on two quality metrics: site mean depth and site mean GQ.

We presented the results from chromosome 22 to illustrate the details of this analysis. We first compared the FPC WGS data and ADNI WGS data to identify shared and private SNVs using BCFtools[9]. Variants were matched by positions, reference allele and at least one alternative allele. On chromosome 22, FPC WGS data contains 598,739 SNVs and this number for ADNI WGS data was 645,982 (Figure 8). A total of 335,307 SNVs were shared by these two datasets, leaving 263,432 private SNVs in FPC WGS and 310,675 private SNVs in ADNI WGS. Majority of these SNVs were bi-allelic. The genotype filtering on GQ and DP had caused some SNVs to become monomorphic for the reference allele. After excluding the monomorphic sites, 280,917 SNVs on chromosome 22 were shared between these two datasets (Figure 8).

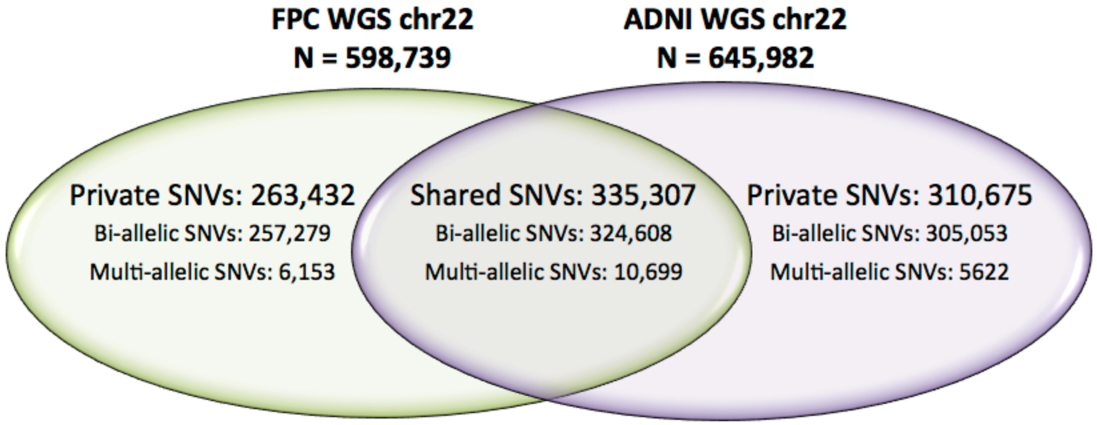
The site mean depth was defined as DP values of non-reference genotypes averaged across samples for each site. Non-reference genotypes with GQ < 20 or DP < 10 were set to missing and not included in the calculation of site mean depth. Among the 280,917 shared SNVs, the distributions of site mean depth in FPC WGS, ADNI WGS and the merged dataset were quite comparable (Figure 8). The absolute difference in site mean depth averaged across all shared SNVs was 4.83. Although the majority of the shared SNVs had an absolute difference in site mean depth ≤ 6, extremely large differences were observed in some SNVs, particularly those with low allele frequency. We plotted the difference in site mean depth of shared SNVs against the alternative allele frequency (Figure 9). The variability of difference in site mean depth was

the largest at the lower end of the alternative allele frequency (AAF). We also plotted the distributions of site mean depth by AAF groups (Figure 10). The distribution of site mean depth was more spread out in singletons and doubles (i.e. AC=1/2) than in other AAF groups. But the means and medians were very consistent across all AAF groups and were comparable in FPC WGS, ADNI WGS and the merged dataset. To evaluate the comparability of all sites on chromosome 22, not just the shared sites, we calculated the site mean depth averaged across all sites in 10-kb segments. Note that the number of SNVs located in the same 10-kb segment in FPC WGS was different from that in ADNI WGS since certain SNVs were private to the specific dataset. Then we calculated the difference in average site mean depth for each 10-kb segment between FPC WGS and ADNI WGS (Figure 11). The data suggested that the average site mean depth per 10-kb segment was consistently higher in FPC WGS than in ADNI WGS across the genomic positions on chromosome 22. But these differences were small enough to be acceptable. The same analysis demonstrated adequate comparability in all other autosomal chromosomes (data not shown).

The site mean GQ was defined as the GQ values of non-reference genotypes averaged across all samples. Genotypes excluded due to GQ < 20 or DP < 10 were not considered in the calculation. We conducted the same analysis in site mean GQ as in site mean depth, which led to the same conclusion that distributions of site mean GQ on chromosome 22 as well as other autosomal chromosomes were adequately comparable in FPC WGS, ADNI WGS and the merged dataset (data not shown).

Once the comparability of site mean depth and site mean GQ between FPC WGS and ADNI WGS was demonstrated, these two datasets were merged using BCFtools [9]. There were 20 FPC samples removed due to being duplicates or non-FPC diagnosis [10]. The merged dataset

contained 1,447 samples sequenced on 72,404,844 SNVs across all autosomal chromosomes. In the merged dataset, multi-allelic sites were split into multiple rows using BCFtools (*norm*)[9].



Distribution of Site Mean Depth (n = 280,917)

	FPC	ADNI	Abs. Difference	Merged
Min	10.0	10.0	0.0	10.0
25%	30.4	27.6	1.88	29.5
50%	37.0	33.8	3.32	35.4
75%	41.6	38.5	6.00	39.7
Max	119.0	111.0	91.0	91.2
Mean	35.5	32.7	4.83	33.9

Distribution of Site Mean GQ (n = 280,917)

	FPC	ADNI	Abs. Difference	Merged
Min	20.0	20.0	0.00	20.0
25%	119.0	110.0	6.5	102.3
50%	147.0	136.7	11.9	118.0
75%	168.0	157.8	24.3	130.5
Max	406.0	346.0	283.0	278.0
Mean	140.4	131.3	18.89	113.5

Figure 8 Comparison of FPC WGS and ADNI WGS data on chromosome 22. The green circle represents the SNVs in FPC WGS data while the purple circle represents the SNVs in ADNI WGS data. The overlap area of these two circles represents the SNVs shared by these two datasets. Distributions of site mean depth and site mean GQ were obtained from 280,917 shared SNVs that were polymorphic after merging.

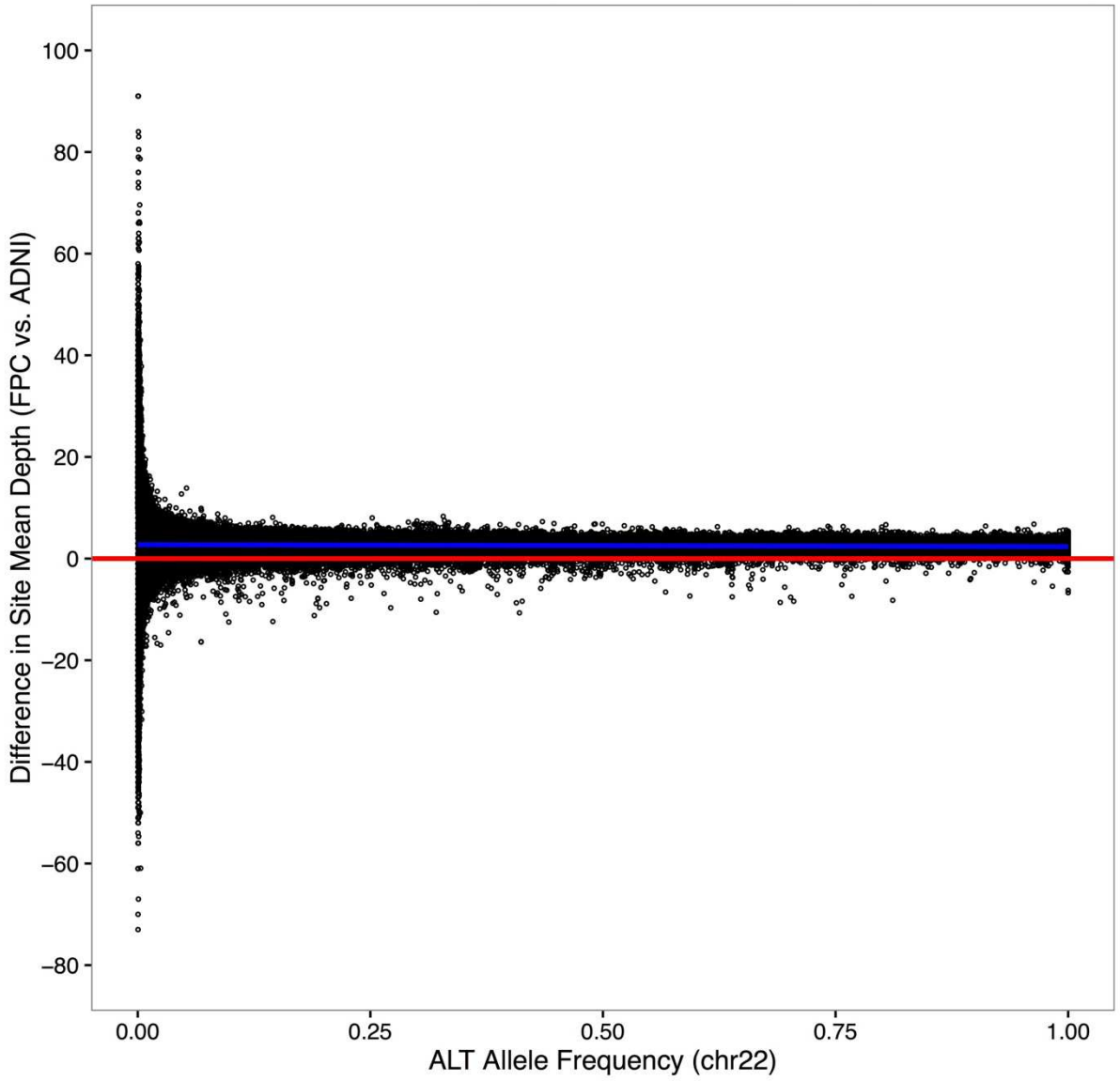


Figure 9 Differences in site mean depth by alternative allele frequencies on chromosome 22. The differences in mean depth per site were calculated among 280,917 SNVs shared FPC WGS and ADNI WGS data. The blue line indicates the difference in site mean depth (site mean depth in FPC WGS minus site mean depth in ADNI WGS) average across all sites. The red line marks the zero. The alternative allele frequency of each site was determined in the merged dataset.

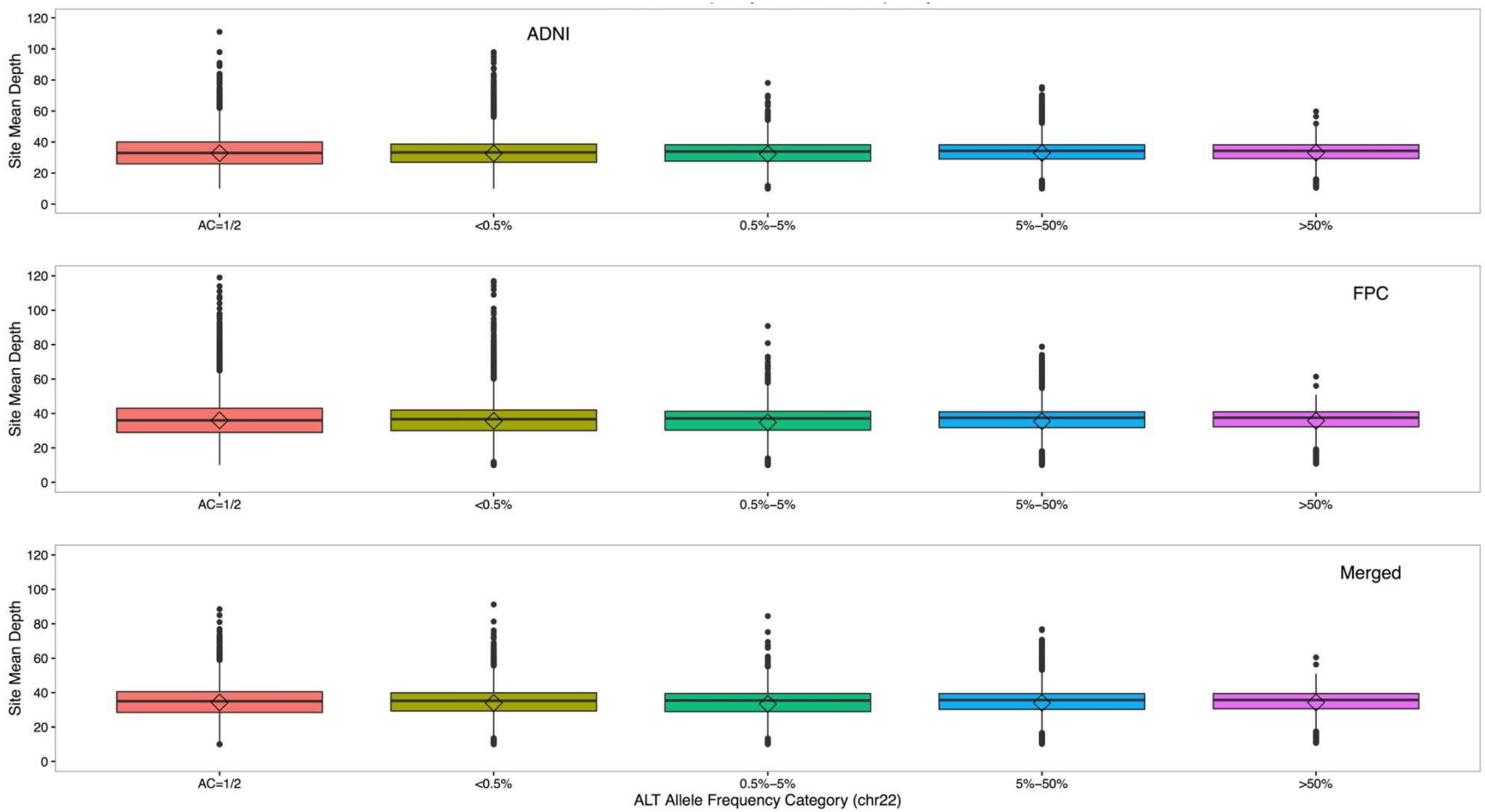


Figure 10 Site mean depth by alternative allele frequency groups on chromosome 22. The alternative allele frequencies of 280,917 SNVs shared by FPC WGS and ADNI WGS data were calculated in the merged dataset. For multi-allelic SNVs, the frequencies were summed over all alternative alleles. The diamond shape in each boxplot marks the site mean depth average across all SNVs in each alternative allele frequency group.

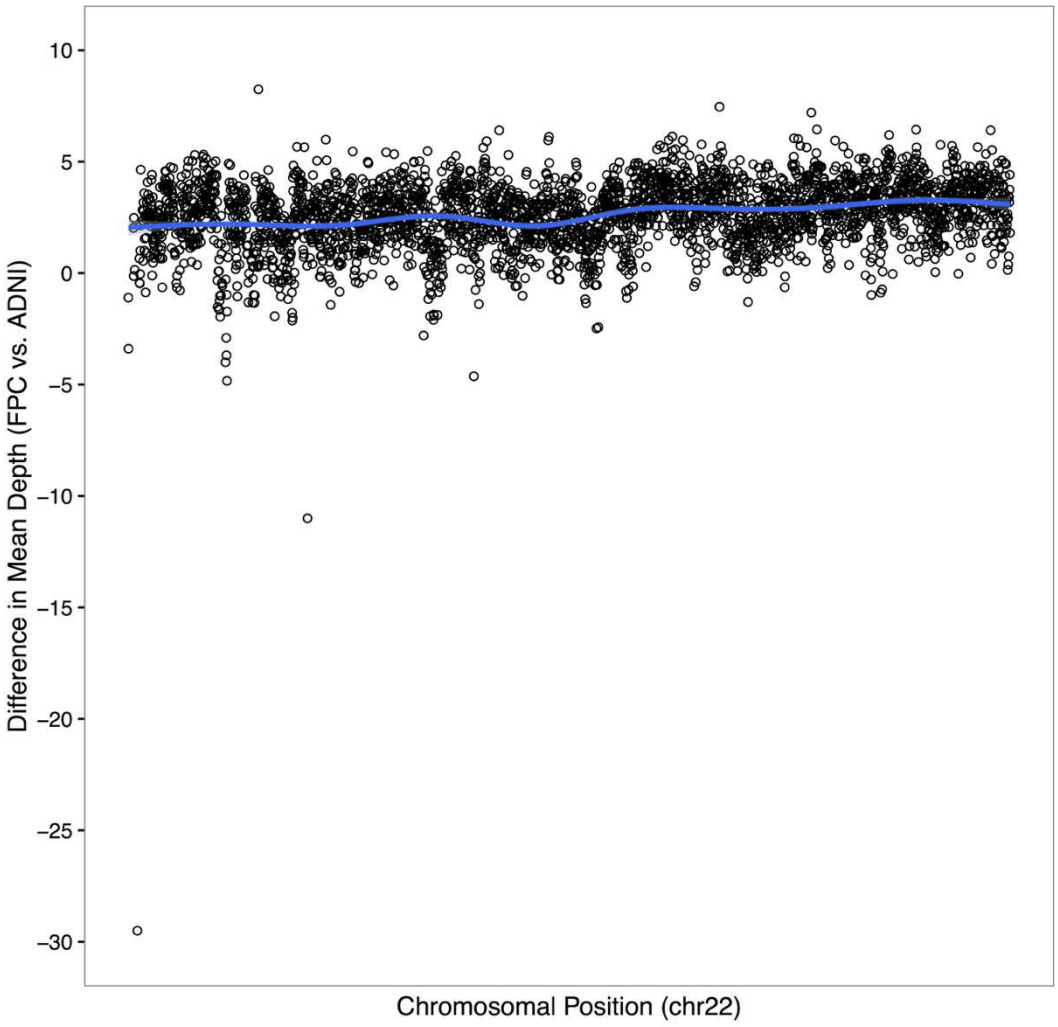


Figure 11 Differences in average site mean depth per 10-kb segment on chromosome 22. Site mean depth was averaged across all sites, shared and private, in each 10-kb segment. The differences were calculated comparing average site mean depth per 10-kb segment in ADNI WGS to that in FPC WGS. The blue line is the regressed line based on all data points.

Sample Filtering: Population Substructure

Both the FPC WGS and ADNI WGS data were self-reported to be predominately Caucasians. To identify samples of non-European ancestry, we performed principal component analysis (PCA) using reference populations from HapMap phase III. The HapMap phase III populations included two populations of European ancestry (i.e. CEU and TSI), three East Asian populations (i.e. CHD, CHB and JPT), four African populations (i.e. ASW, LWK, MKK and YRI), one South Asian population (i.e. GIH) and one Hispanic population (i.e. MEX). The genotyping data were downloaded from the International HapMap Project's FPT site (ftp://ftp.ncbi.nlm.nih.gov/hapmap/). Prior to the PCA, we excluded SNVs with any missing calls in any HapMap population, and converted the positions of the remaining SNVs in hg18/build36 to hg19/build37 using liftOver from UCSC Genome Browser[11]. Given that inferring genetic ancestry can be more challenging when family structure and cryptic relatedness is present, we only kept the founders in these HapMap populations, leaving a total of 988 samples to enter the PCA analysis. The merged FPC and ADNI WGS dataset were converted from VCF format to the binary PLINK format before the PCA analysis. During this conversion, 48,021,112 bi-allelic SNVs with at least 1 alternative allele were included.

SNVs for PCA were selected in the following steps. We first identified 336,308 common (MAF > 5%) SNVs on autosomal chromosomes shared across all HapMap populations as well as the merged dataset. Then we excluded 2,476 SNVs in high linkage disequilibrium (LD) regions and regions known to be associated with ethnicity, including the lactase regions on chromosome 2, the HLA regions on chromosome 6, and the polymorphic inversion regions on chromosome 8 and 17. LD-based pruning was conducted on the remaining SNVs at a pairwise $r^2 < 0.20$ to ensure the independence of variants. A total of 63,238 SNVs were used in PCA.

PCA was carried out using SMARTPCA in EIGENSTRAT [12]. We conducted two sets of PCA analysis on (1) the merged study population along with all HapMap phase III populations, and (2) the merged study population only. Genetic ancestry was determined based on tight clustering of study samples on the first two principal components (PCs) in the first set of analysis. In the second set of PCA analysis including only sample populations, samples with one of the first two PC values that exceeded ±1 standard deviation from the mean were considered as outliers.

The majority of the FPC and ADNI samples were clustered alongside the CEU and TSI populations, suggesting European ancestry (Figure 12A). We did, however, identify 27 FPC samples and 51 ADNI samples as outliers based on their first two PC values (Figure 12B). Among these samples, 44 were clustered closely with African populations (27 ADNI and 17 FPC) and 13 were East Asians (8 ADNI and 5 FPC). The remaining samples were inferred as Mexicans or South Asians. These 78 samples were removed from the merged dataset.

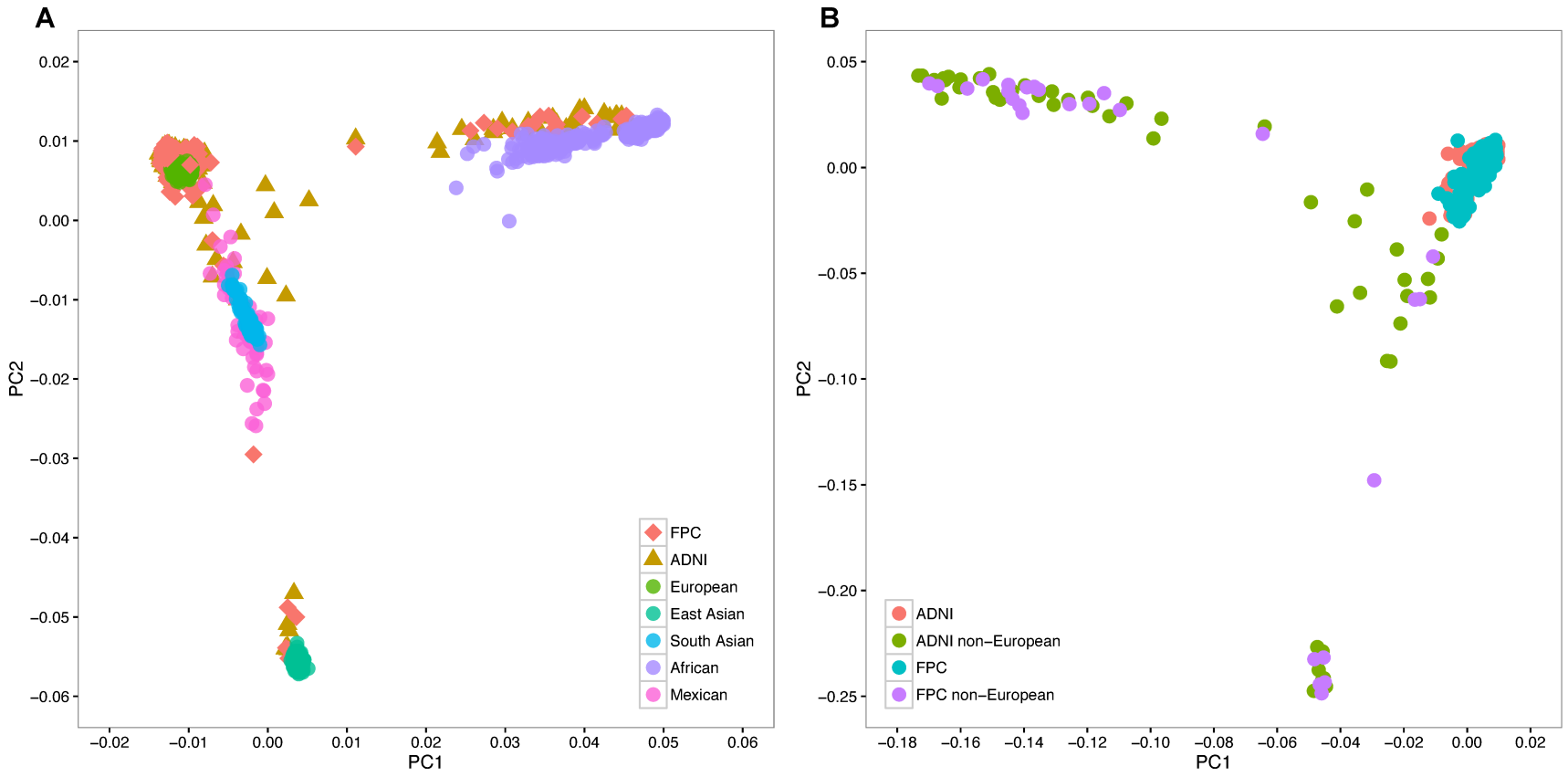


Figure 12 Principal component analysis (PCA) in the merged study population. In the first set of analysis (A), FPC samples (diamonds) and ADNI samples (triangles) were clustered alongside the HapMap phase III populations (circles). There were 44 samples clustered closely with African populations, 13 with East Asians and 21 around Mexicans or South Asians. These 78 non-European samples were marked by green and purple circles in plot (B) and were excluded from the subsequent analysis.

Sample Filtering: Relatedness

Heritability estimates in case-control studies are particularly sensitive to hidden relatedness in the study population. Including close relatives would result in the estimate of genetic variance being driven by the phenotypic correlations for these pairs, and lead to inflation possibly due to common environmental effects. To identify close relatives in the data, we first conducted an identity-by-descent (IBD) analysis separately in FPC WGS and ADNI WGS data using PLINK[8]. The genetic similarity was later assessed using GCTA[13] to determine the optimal cutoff that would eliminate the relatedness in the study population while maximizing the sample size. Prior to this analysis, 78 individuals identified as non-Europeans in the PCA analysis were excluded to minimize the bias due to population substructure, leaving 1,369 samples in the merged dataset.

First- and second-degree relatives were identified using a selected panel of SNVs in FPC WGS and ADNI WGS cohort separately. Variants were selected for IBD analysis if they were bi-allelic and independent (pairwise $r^2 < 0.10$) on autosomal chromosomes with MAF > 5%. The IBD analysis using PLINK calculated the probability of sharing 0 (Z_0), 1 (Z_1), and 2 (Z_2) alleles at each site. By comparing the estimated Z_0, Z_1, and Z_2 values to the expected, we were able to identify pairs of parent-offspring, full-siblings, half-sibling/grandparent-grandchild/avuncular, and first cousins in each cohort. In FPC WGS data, a total of 410,722 SNVs were selected for IBD analysis. We identified 3 parent-offspring pairs, 25 full-siblings, 12 first cousins and 8 pairs of other second-degree relatives (i.e. half-sibling, grandparent-grandchild or avuncular) (Figure 13A). The inferred genetic relationships of these related pairs matched the reported pedigree relationships. In ADNI WGS data, a total of 412,572 SNVs were used in the IBD analysis. Four

pairs of full-siblings were identified (Figure 13A). Pedigree information of ADNI WGS data was not available to us.

We also evaluated the relatedness using GCTA [13]. The genetic similarity for each pair of samples was calculated using variants shared by the pair. We limited this analysis to 320,379 bi-allelic SNVs with MAF > 5% on autosomal chromosomes. The relatedness value calculated from GCTA ranged from -0.025 to 0.057 (Figure 13B). We later considered two relatedness cutoffs 0.025 and 0.05 in our heritability analysis. By removing one individual from the first- and second-degree relative pairs identified in the IBD analysis, the final sample consists of 1,323 individuals.

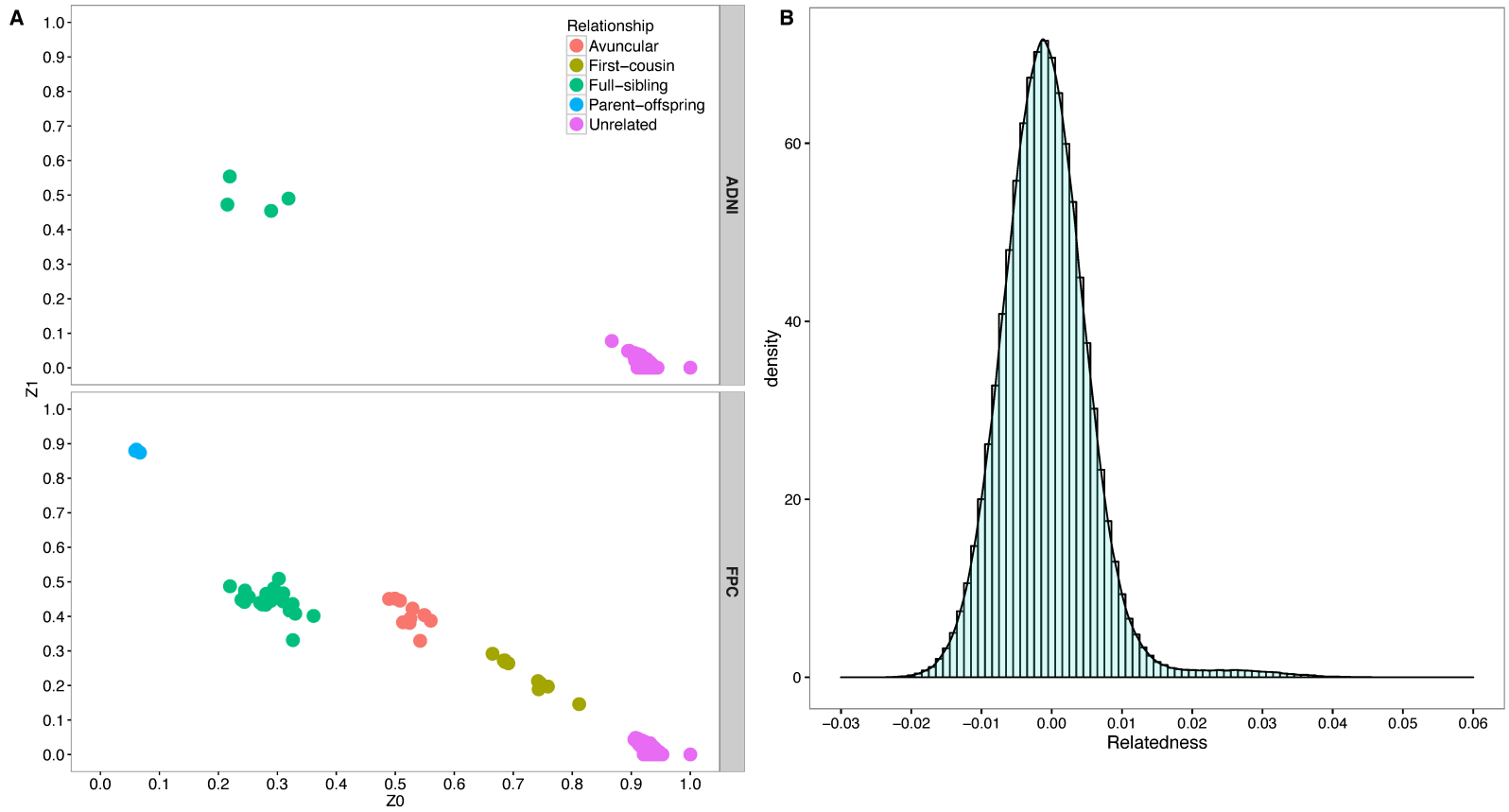
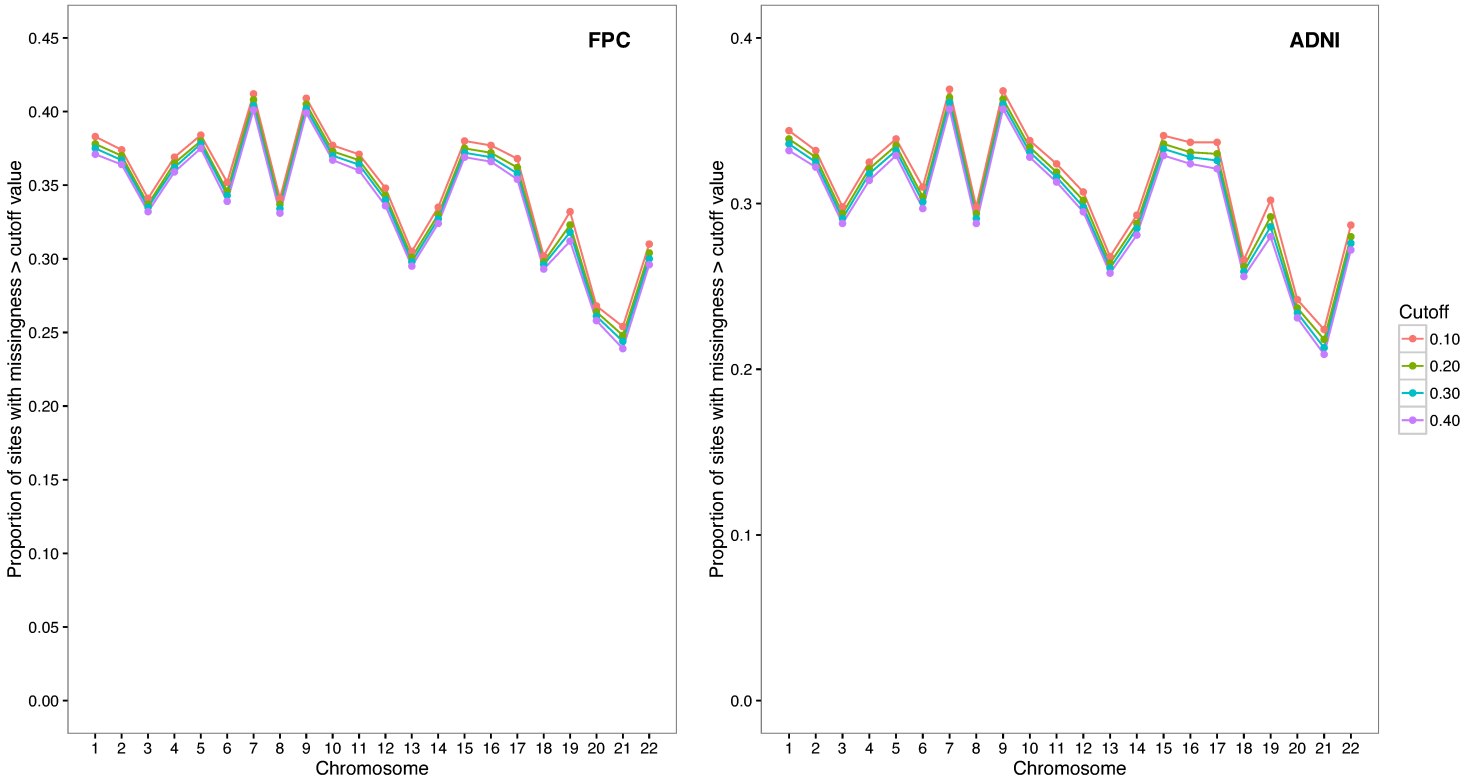


Figure 13 Z_1 versus Z_0 in identity-by-descent analysis (A) and distribution of genetic similarity values (B). Pairs of related and unrelated samples in ADNI WGS data (A above) and FPC WGS data (A below) were plotted. The genetic similarity values in (B) were estimated for samples in the merged dataset, after randomly excluding one individual from each first- and second- degree relative pairs.

Additional Site Filtering

The removal of genotypes and samples inevitably caused some variants to be homozygotes of either reference or alternative allele. About half of the SNVs (35,720,232) in the merged dataset were monomorphic and hence excluded. Missingness per site was defined as the proportion of called non-reference genotypes that were set to missing due to GQ < 20 or DP < 10. A high missingness raises concern with the poor quality of sequencing or variant calling. About 37% and 33% of variants in FPC and ADNI dataset contain missing calls, the majority of which had lost heterozygosity completely, particularly rare variants like singletons and doubletons. We assessed the proportion of variants on each chromosome with missingness greater than 10%, 20%, 30% and 40% (Figure 14). Since the differences were small across these cut-off values, a conservative value of 10% was chosen to minimize the impact of poor quality variants in the subsequent analysis, excluding an additional 2,377,617 variants. We also exclude variants that failed Hardy-Weinberg Equilibrium (HWE) exact test at $p < 10^{-6}$ (N = 51,550) and variants with minor allele count < 3 (N = 21,254,456). Variants with extremely low frequencies would cause noise and lower the precision of heritability estimates and hence were excluded from the analysis.

Figure 14 Proportion of variants with missingness greater than 10%, 20%, 30%, and 40% on each chromosome. Missingness was defined as the proportion of called genotypes that was set to missing due to GQ < 20 or DP < 10. The pattern of missingness cross chromosomes was similar in FPC and ADNI WGS dataset. A conservative cut-off value of 10% was chosen to minimize the impact of poor quality variants in the analysis.



Statistical Analysis

The analysis in this chapter is aimed to estimate the heritability for pancreatic cancer due to additive genetic effects from sequenced variants (h^2_{WGS}). The small sample size of FPC-ADNI study prohibits any stratification on MAF and/or LD. Therefore, GREML-SC approach was used to estimate the heritability. Comparisons of estimated heritability were conducted using a subset of variants shared by FPC-ADNI and PanC4 study.

Estimate of Heritability Explained by Sequenced Variants

A detailed description of the GREML approaches can be found in Chapter One. Using the GREML-SC approach, the variance due to additive genetic effects of all sequenced variants was estimated by fitting a single GRM calculated from all autosomal SNVs in a mixed linear model. The proportion of variance explained by all autosomal SNVs was defined as $\boldsymbol{h^2_{WGS}} = \boldsymbol{\sigma^2_{WGS}}/(\boldsymbol{\sigma^2_{WGS}} + \boldsymbol{\sigma^2_e})$. In this model, age, sex and first 20 principal components (PCs) were included as fix effects. The first 20 PCs were calculated using 320,379 common (MAF > 0.05) and independent (pairwise $r^2 > 0.20$) variants sequenced in the dataset. The default average information algorithm was applied to run REML iterations in all heritability analysis. A disease prevalence (K) of 0.0149 was used to correct for the ascertainment bias in the case-control cohort. Given that FPC cases constitute up to 10% of all pancreatic cancer cases and the true prevalence of FPC was unknown, the heritability was estimated with the prevalence varying from 0.1K to K. The estimates of variance were transformed from the observed 0-1 scale to the unobserved continuous 'liability' scale using a probit linear transformation[14]. The variance in liability scale was reported along with its standard errors. This analysis was conducted at relatedness cutoff of 0.05 and 0.025 to evaluate the impact of relatedness cutoffs on heritability estimates.

Comparing Estimates of Heritability in FPC-ADNI and PanC4 Study

Comparisons of estimated heritability for pancreatic cancer were performed using a subset of variants presented in both FPC-ADNI and PanC4 study. To minimize the impact of poor imputation in PanC4 data as well as the potential bias due to batch effects in FPC-ADNI data, this analysis was limited to variants that were directly genotyped in both datasets. A relatedness cutoff of 0.025 was applied to both datasets to minimize the potential bias due to residual relatedness. Heritability for pancreatic cancer was estimated with a disease prevalence (K) of 0.0149 in the PanC4 study and a disease prevalence varying from 0.1K to K in the FPC-ADNI study. Both GREML-SC and GREML-MS approach were used to estimate heritability. In the GREML-MS analysis, variants were stratified at MAF of 0.05, and the two variance components were fitted jointly in an MLM. Because of the small sample size of FPC-ADNI study, GREML-LDMS does not provide reliable estimates or convergence is not obtained. The variance in liability scale was reported along with its standard errors.

Results

The final dataset contains 1,323 samples (569 cases and 754 controls) sequenced on 14,019,172 autosomal sites, including 36,619 sites with multiple alternative alleles. After splitting the multi-allelic variants into multiple records, the final dataset consists of 14,055,912 SNVs.

A shows the distribution of MAF in the final dataset. The majority of the variants are rare and low-frequency variants with a MAF < 5%. The remaining variants are evenly distributed across the MAF categories. All variants in the final dataset were annotated by ANNOVAR[15]. The functional predictions were derived from the NCBI Reference Sequence Database. Among all variants in the final dataset, 54.2% of them were located in the intergenic regions and 37.0% were intronic. About 0.83% of the sequenced variants were coding variants (

B).

Characteristics of ADNI and FPC WGS samples at baseline are shown in Tables Table 7 and Table 8, respectively. In general, these two study populations are quite similar in terms of the age distribution and sex ratio. Individuals in both studies are primarily of European ancestry as confirmed by PCA analysis. The ADNI WGS study consists of 34.6% individuals with normal cognitive functions, 29.0% with early mild cognitive impairment and 30.4% with late cognitive impairment. The remaining 5.9% had a diagnosis of Alzheimer's' Disease (Tables

Table 7). Among the FPC cases, more than half had two relatives affected by pancreatic cancer; 30.7% had three and 13.2% had four or more relatives affected by the disease.

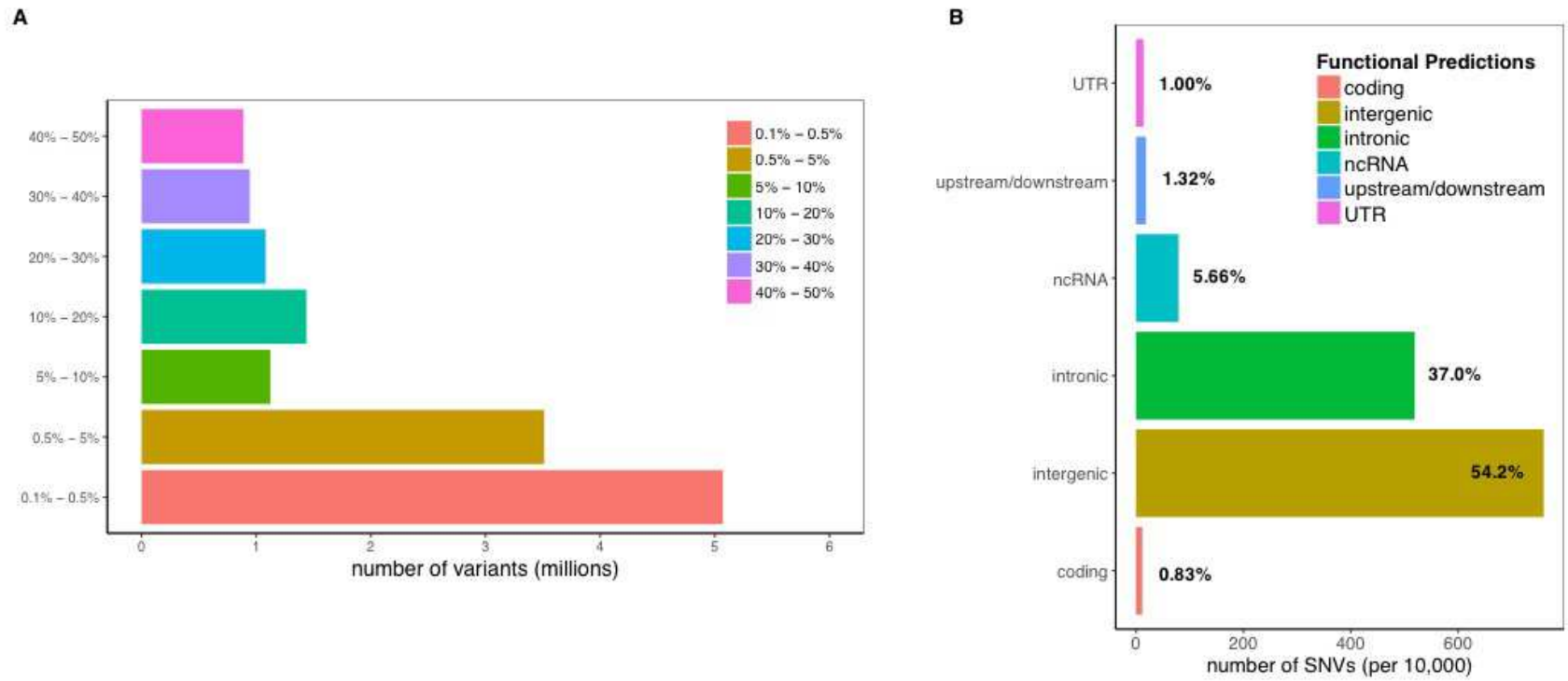


Figure 15 Minor Allele Frequency (A) and Functional Annotation (B) Distribution of WGS Variants.

Estimates of Heritability Explained by Sequenced Variants

The estimates of heritability explained by sequenced variants (h^2_{WGS}) varied by the specified population prevalence of pancreatic cancer (Table 9). Applying a relatedness cutoff further excluded distantly related individuals in our study population and hence reduced the sample size. At a relatedness of 0.025 and a sample size of 1,180 individuals, the heritability for pancreatic cancer explained by sequenced variants (h^2_{WGS}) was estimated to be 38.8% (s.e. = 15.7%), 44.0% (s.e. = 17.9%), 53.2% (s.e. = 21.6%) and 62.6% (s.e. = 25.4%) with a disease prevalence of 0.00149 (0.1K), 0.00298 (0.2K), 0.00745 (0.5K) and 0.0149 (K), respectively. Results under a relatedness cutoff of 0.05 were quite similar (Table 9).

Comparing Estimates of Heritability in FPC-ADNI and PanC4 Study

The comparison between the estimated heritability in FPC-ADNI and Panc4 study was performed using 521,282 polymorphic variants directly genotyped in both datasets. The MAF distributions of these variants are similar in FPC-ADNI and PanC4 dataset (

Supplementary Figure 2). Comparing to the full set of sequenced or imputed variants, this subset of variants has a higher proportion of common variants; over 94.4% of the 521,282 variants have a MAF > 1%. In both datasets, the relatedness cutoff of 0.025 was applied. Since one FPC case does not have array data, in total 1,179 individuals in the FPC-ADNI cohort were included in this analysis. Using the GREML-SC approach, the heritability for pancreatic cancer estimated from this subset of variants was 16.6% (s.e. = 3.3%) in the PanC4 study. In the FPC-ADNI study, the phenotypic variance for pancreatic cancer attributable to this subset of variants was 30.9% (s.e. = 11.2%) when the disease prevalence was set to 0.00149 (0.1K) and was 50.0% (s.e. = 18.1%) when the disease prevalence was 0.0149 (K). Results from the GREML-MS

analysis was similar in the PanC4 study (h^2 = 16.2%, s.e. = 3.3%) but were slightly higher in FPC-ADNI study. Specifically, when a disease prevalence of 0.00149 (0.1K) was specified, the heritability was estimated to be 32.1% (s.e. = 11.2%); and under a disease prevalence of 0.0149 (K), the estimate of heritability was 51.9% (s.e. = 18.0%) (Table 10). As expected, the estimates of heritability using this subset of variants were lower than those from the full set of imputed or sequenced variants as in Table 9 and Chapter Three.

Discussion

In this combined sample of FPC cases and ADNI participants, the heritability for pancreatic cancer from all sequenced variants was estimated to range from 38.8% (s.e. = 15.7%) to 62.6% (s.e. = 25.4%) depending on the specification of the population prevalence of pancreatic cancer (K). Misspecification of K by one factor or two could result in a 20% bias on the estimated heritability, especially when the disease is common (K >= 0.1)[14]. Unlike the PanC4 study where cases were selected regardless of the family history, affected individuals in FPC-ADNI study were all familial cases with at least two FDRs with pancreatic cancer. Since the true prevalence of FPC was unknown, four disease prevalence values, 0.0149 (K), 0.00745 (0.5K), 0.00298 (0.2K) and 0.00149 (0.1K), were evaluated to quantify the influences of K on the estimates of heritability in our study. The wide range of K specifications led to a huge difference in the estimates of heritability. Given that up to 10% of pancreatic cancer cases had a family history of the disease, the true value of heritability for pancreatic cancer is likely to be near the estimated heritability under a disease prevalence of 0.1K, where 38.8% (s.e. = 15.7%) of the phenotypic variance for pancreatic cancer was explained by all sequenced variants in the FPC-ADNI study.

However, this estimate of heritability from sequenced variants may be biased downwardly. Based on the simulation studies, estimates of heritability from the GREML-SC approach is unbiased only when the causal variants are a random subset of all sequenced variants[16]. Results from our genomic partitioning analysis in the PanC4 study (Chapter Three) suggested a distribution of causal variants on the lower end of the MAF spectrum. Moreover, the estimate of heritability in the PanC4 study has increased considerably after stratification by MAF and LD (21.2% vs. 16.0%). Given these findings in the PanC4 study, the estimate of heritability from all sequenced variants (h^2_{WGS}) using GREML-SC approach in FPC-ADNI is likely biased downwardly.

Bias in heritability estimates can also result from the exclusion of extremely rare variants. WGS data with sufficient coverage (30X) provides a near-complete characterization of genetic variations across the genome. Comparing to imputation, WGS had advantages in capturing extremely rare variants that are monomorphic in reference populations. But these extremely rare variants are often excluded from the heritability analysis to allow a reliable estimate of variance using the GREML approach. In our WGS data, more than 60% of the sequenced variants were excluded at minor allele count < 3. As a result, the genetic variance due to these variants can never be captured using current statistical analysis and the current sample size. This is particularly concerning for a cancer phenotype since rare mutations are known to play an important role in the susceptibility of most cancer types.

The estimate of heritability for pancreatic cancer from sequenced variants (h^2_{WGS})) in the FPC-ADNI study is significantly higher than the estimate from imputed variants ($h^2_{imputed}$)) in the PanC4 study (38.8% vs. 21.2%). There are three possible explanations for the difference in heritability estimates. First, the estimate of heritability in the FPC-ADNI study is inflated due to

batch effects. In the FPC-ADNI WGS data, the cases and controls were sequenced and called in different pipelines. In the presence of technical artifact, the allele frequencies between cases and controls on average will be more different than expected under the null hypothesis, and cases would appear to be more related to other cases than to controls, leading to inflated estimates of heritability. To minimize the impact of batch effects, stringent QC filters were implemented on the WGS data. In addition, the comparability of WGS data was carefully assessed before merging the FPC and ADNI datasets. Variants sequenced in the FPC and ADNI study overall showed very similar distributions of mean depth per site and mean GQ per site across the genome. The differences in these two quality metrics between the case and control samples tend to be more variable among singleton and doubleton variants, which were excluded from the heritability analysis. Although QC filters are not able to fully address the batch effects, the influence of batch effects on the estimates of heritability is likely to be small in the FPC-ADNI study. To mitigate the concern of batch effects, we also compared the estimate of heritability from 521,282 directly genotyped variants shared by the FPC-ADNI and PanC4, which also suggest a higher heritability for pancreatic cancer in the FPC-ADNI study than in the PanC4 study (32.1% vs. 16.2%). Since array data is in general less prone to technical artifact than sequencing data, the impact of batch effects on these estimates is likely to be trivial.

The second explanation for a higher estimate of heritability in the FPC-ADNI study would be the bias due to misspecification of the population prevalence of disease. Pancreatic cancer cases in FPC-ADNI study were exclusively familial cases with at least 2 FDRs affected by the disease. Currently, there is no report of the prevalence of FPC in the general population. It is estimated that up to 10% of all pancreatic cancer cases had a family history of pancreatic cancer. When limited to FPC cases, the prevalence might be even smaller. Therefore, a disease

prevalence of 0.00149, which is equivalent to 1/10 of the population prevalence of pancreatic cancer, may still be an overestimate of the true prevalence of FPC. Under a disease prevalence of 0.000745 (i.e. 1/20 of the population prevalence of pancreatic cancer), a conservative estimate of heritability from all sequenced variants in FPC-ADNI study was 34.6% (s.e. = 14.1%), which is still higher than the estimate from the PanC4 study.

Last but not least, the difference in the estimates of heritability between the FPC-ADNI and the PanC4 study may partially reflect the distinct characteristics of the study populations. As discussed above, the PanC4 study selected pancreatic cancer cases regardless of their family history, whereas all affected individuals in FPC-ADNI study were familial cases. Narrow-sense heritability is a population parameter that quantifies the relative contribution of additive genetic effects. The genetic variance depends on segregation in a population of the alleles that influence the trait, the allele frequencies, the effect sizes of the variants and the mode of gene actions[17]. Although many causal variants and genes are shared by familial and sporadic cases of pancreatic cancer, it is possible that their enrichment and Mendelian form of inheritance in high-risk families together led to a greater contribution to the susceptibility of pancreatic cancer than in general populations. On the other hand, this estimate of heritability in the FPC-ADNI study is remarkably similar to what was reported in the twin study, which was considered as the upper limit of heritability for pancreatic cancer.

Conclusion

The overall heritability for pancreatic cancer from sequenced variants was estimated to be 38.8% in the FPC-ADNI study. This estimate of heritability may be inflated due to batch effects and misspecification of disease prevalence. Though difficult to be quantified, the inflation is expected to be small. This estimate of heritability does not capture the genetic variance

attributable to extremely rare variants that are associated with the risk of pancreatic cancer. This is due to the inherent limitation of the GREML approach. The estimate of heritability from sequenced variants in the FPC-ADNI study is quite similar to the family-based heritability, but significantly higher than the estimates from imputed variants in the PanC4 study. The higher estimate of heritability in the FPC-ADNI study may suggest a greater contribution of genetic effects in familial pancreatic cancer cases than in sporadic cases. In combination with the results in the PanC4 study, the heritability for pancreatic cancer may be somewhere between 21.2% and 38.8%.

Tables

Table 7 Characteristics of ADNI WGS Samples at Baseline

Characteristics	ADNI WGS (N = 809)
Study Phase, N (%)	
ADNI1	279 (34.5%)
ADNI GO	125 (15.5%)
ADNI2	405 (50.1%)
Age at baseline (years), mean ± SD (range)	73 ± 7 (55-91)
Female gender, N (%)	363 (44.9%)
Self-report race, N (%)	
White	756 (93.4%)
Black	28 (3.5%)
Others*	25 (3.1%)
Diagnosis at baseline†, N (%)	
CN	280 (34.6%)
SMC	0 (0%)
EMCI	235 (29.0%)
LMCI	246 (30.4%)
AD	48 (5.9%)
Education (years), N (%)	
≤ 12	116 (14.3%)
>12 & ≤ 16	344 (42.5%)
> 16 & ≤ 20	349 (43.1%)

* Asian, Hawaiian/Other Pacific Islanders, American Indian/Alaskan, more than one, unknown

† CN = cognitively normal; SMC = significant memory concern; EMCI = early mild cognitive impairment; LMCI = impairment; AD = Alzheimer's Disease.

Table 8 Characteristics of FPC WGS Samples at Baseline

Characteristics	FPC WGS (N = 638)
Age group (years), N (%)	
30-49	35 (5.5%)
50-69	338 (53.0%)
70-89	251 (39.3%)
90 or above	7 (1.1%)
Unknown	7 (1.1%)
Female gender, N (%)	325 (50.9%)
No. of affected relatives, N (%)	
Two	358 (56.1%)
Three	196 (30.7%)
Four or more	84 (13.2%)

Table 9 Estimates of Heritability under Different Relatedness Cutoffs and Prevalence Values

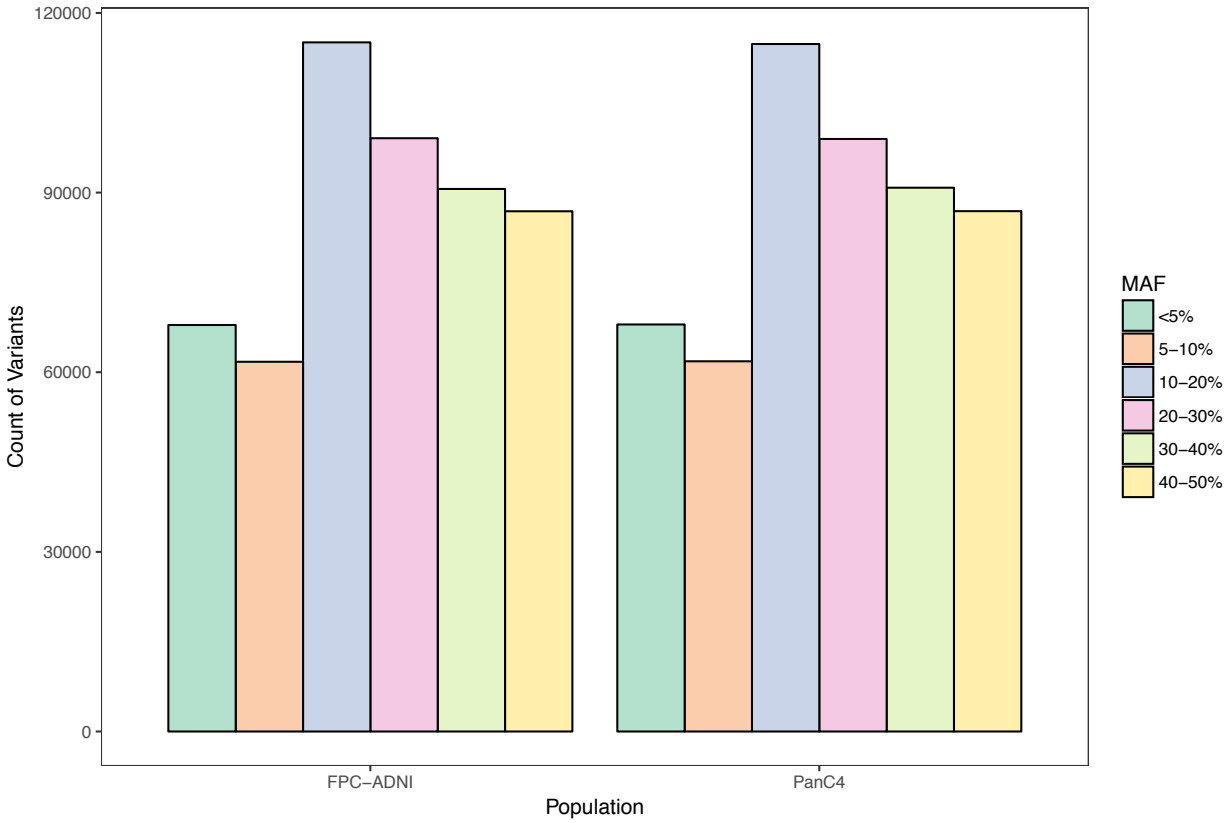
Sample Size	Prevalence	h^2	s.e.	P value
1,322 (relatedness > 0.05)	K = 0.0149	0.620	0.164	3.18×10^{-6}
	K/2 = 0.00745	0.526	0.139	3.18×10^{-6}
	K/5 = 0.00298	0.435	0.115	3.18×10^{-6}
	K/10 = 0.00149	0.384	0.102	3.18×10^{-6}
1,180 (relatedness > 0.025)	K = 0.0149	0.626	0.254	1.05×10^{-3}
	K/2 = 0.00745	0.532	0.216	1.05×10^{-3}
	K/5 = 0.00298	0.440	0.179	1.05×10^{-3}
	K/10 = 0.00149	0.388	0.157	1.05×10^{-3}

Table 10 Estimates of Heritability for Pancreatic Cancer from 521,282 Variants Shared by FPC-ADNI and PanC4 Study

Method	Cohort	Sample Size	Prevalence (K)	h^2	s.e.	P
GREML-SC	PanC4	6,931	K = 0.0149	0.166	0.033	1.70×10^{-7}
	FPC-ADNI	1,179	K = 0.0149	0.500	0.181	2.56×10^{-3}
			K/2 = 0.00745	0.424	0.154	2.56×10^{-3}
			K/5 = 0.00298	0.351	0.127	2.56×10^{-3}
			K/10 = 0.00149	0.309	0.112	2.56×10^{-3}
GREML-MS	PanC4	6,931	K = 0.0149	0.162	0.033	0.34
	FPC-ADNI	1,179	K = 0.0149	0.519	0.180	0.48
			K/2 = 0.00745	0.440	0.153	0.48
			K/5 = 0.00298	0.364	0.127	0.48
			K/10 = 0.00149	0.321	0.112	0.48

Supplementary Figures and Tables

Supplementary Figure 2 MAF Distribution of 521,282 Shared Variants in FPC-ADNI and PanC4 Study.



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CHAPTER FIVE

CONTRIBUTION OF ESTABLISHED FPC GENES IN THE HERITABILITY OF PANCREATIC CANCER

Familial Clustering and High-penetrance Mutations for Pancreatic Cancer

Inherited genetic components play an important role in the susceptibility of pancreatic cancer. It was estimated that up to 10% of pancreatic cancer patients had a family history of the disease. In recent case-control and cohort studies, 2.3 – 8.7% of pancreatic cancer patients had reported having at least one first-degree relatives (FDRs) affected by the disease, meeting the criteria of familial pancreatic cancer (FPC) (Table 11). The percentage appears to be higher in patients with early-onset pancreatic cancer (diagnosed at age ≤ 50 or < 60)[1,2]. In addition, two studies have reported a smaller proportion (< 2%) of pancreatic cancer patients having ≥ 2 FDRs with the disease [3,4]. It's important to note that obtaining an accurate family history of pancreatic cancer is very challenging as family members may be too young to have the disease when assessed, particularly for families with complex pedigree structures. Therefore, conclusive epidemiologic data on the prevalence of FPC is still lacking. Comparing to the general population, FPC kindred members have a 7- to 9-fold increased risk of pancreatic cancer[5,6]. The risk is even higher among members of FPC kindreds with a young-onset case (< 50 years; SIR = 9.31, 95% CI: 3.42-20.28) than those without (SIR = 6.34, 95% CI: 4.02-9.51)[6].

Mutations of several genes have been found to be responsible for the familial clustering of pancreatic cancer. Next-generation sequencing in FPC families had identified *ATM* and *PALB2*, previously known as familial breast cancer genes, to be associated with pancreatic cancer. Increased risk of pancreatic cancer is also associated with several inherited syndromes for which the predisposing genes have been identified. The most prominent syndromes are

hereditary breast-ovarian cancer syndrome, particularly due to germ-line mutations in *BRCA1/2*, and familial atypical multiple mole melanoma syndrome, due to mutations in *CDKN2A*. In addition, *STK11* associated with Peutz-Jeghers syndrome, mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) associated with Lynch syndrome, and *PRSS1* associated with hereditary pancreatitis are among the list of genes contributing to pancreatic cancer risk. A detailed review of these genes can be found in Chapter Two.

Prevalence of High-penetrance Mutations in FPC Patients

Given the substantial risk of pancreatic cancer associated with these genes, they may be used to characterized high-risk individuals that will benefit from early detection or targeted treatment of the disease. To determine the clinical utility of these high-penetrance mutations in identifying high-risk individuals for pancreatic cancer, efforts have been made to quantify their prevalence in FPC families (Table 12). *BRCA2* is the most frequently tested gene in these studies given its long-established connection to the risk of pancreatic cancer. Mutations in *BRCA2* also account for most FPC cases, with a prevalence ranged from 2.9% to 19.2%[7–13]. The wide range of *BRCA2* mutation prevalence in these studies may partially due to the various proportion of Ashkenazi Jewish (AJ) patients in the study population. It's known that the prevalence of three *BRCA1/2* founder mutations (*BRCA1* 185delAG, *BRCA1* 5382insC, and *BRCA2* 6174delT) is much higher in Ashkenazi Jewish patients with pancreatic cancer than in non-AJ patients[14]. Following *BRCA2*, *ATM* appears to be the second most frequently mutated genes in FPC families, with a prevalence ranged from 2.4 – 8.3%[15–17,13]. Germ-line mutations of *BRCA1, CDKN2A* and *PALB2* genes were not always detected in FPC samples[11,16–19], and their prevalence was in general lower than *BRCA2* and *ATM*[12,13,16,17,20]. A recent study from the Pancreatic Cancer Genetic Epidemiology (PACGENE) Consortium assessed the prevalence of

BRCA1, *BRAC2*, *PALB2*, and *CDKN2A* mutations in 727 unrelated probands with positive family history of pancreatic cancer and reported an overall prevalence of 8% in FPC probands, which was significantly higher than that in non-FPC probands (3.5%)[12]. A follow-up study extended the testing panel to also include *ATM*, *MSH2*, and *PMS2* gene. In 302 unrelated pancreatic cancer patients from Mayo Clinic, 11.9% of the FPC patients carried at least one deleterious mutation of these seven genes while 4.3% of the non-FPC patients were carriers[13]. The overall prevalence of FPC gene mutations was 14.5% in a Japanese study with a small sample size of 54 FPC patients[21]. A substantially higher prevalence of FPC gene mutations was reported in a US study (30.4%) but this study was small and was conducted retrospectively so that not all samples were tested for the same genes[16].

Prevalence of High-penetrance Mutations in Sporadic Cases

Germ-line mutations of the established FPC genes are not limited to FPC families or patients with a positive family history of pancreatic cancer. Mutations of these genes have also been detected in apparently sporadic pancreatic cancer cases. These patients were primarily recruited in clinical settings, unselected for their family history of pancreatic cancer. Early reports of germ-line mutations in sporadic/unselected pancreatic cancer patients have been focusing on *BRCA2*[22,23] and *CDKN2A*[24,25] genes. More recently, the investigation of the prevalence of germline mutations has been extended to include all established FPC genes in studies with increased sample sizes. Hu and his colleagues evaluated the frequency of mutations in established pancreatic cancer genes among 96 unselected pancreatic cancer patients from Mayo Clinic[26]. Review of the family history of pancreatic cancer classified 7% of the patients to be FPC cases, similar to the prevalence of FPC cases observed in the general population. Germ-line mutations were detected in 9.4% of tested samples, mostly in patients with a family history

of pancreatic cancer or other related cancers (i.e. breast cancer, colorectal cancer and endometrial cancer). In the follow-up study of 3,030 patients, deleterious mutations of established FPC genes were found in 5.5% of all tested patients, including 7.9% of patients with a family history of pancreatic cancer and 5.2% patients with no family history[27]. In a similar study, Shindo and his colleagues recruited 854 pancreatic cancer patients from Johns Hopkins Hospital, the majority of whom (87.3%) had a negative family history of pancreatic cancer[28]. Among these sporadic pancreatic cancer cases, 31 mutations in known FPC susceptibility genes were identified, equivalent to an overall prevalence of 3.5%. In a recent study of 289 resected PDAC samples, 9.7% of patients carried pathogenic or likely pathogenic germline mutations of established FPC genes. The observed higher prevalence of established FPC gene mutations in this study is likely a result of a high proportion of patients (57.1%) with a personal history of another cancer[29].

In general, pancreatic cancer patients with stronger family history tend to have a higher chance of carrying mutations of known FPC genes. Mutations of these genes have also been detected in 4-10% of cases unselected for their family history[12,13,28,27,29]. Findings from these studies indicate an essential role of these high-penetrance mutations in the susceptibility of pancreatic cancer.

Contribution of Established FPC Genes in Heritability

To quantify the contribution of the established FPC genes in the susceptibility of pancreatic cancer, we estimated the variance explained by these 11 genes in a heritability analysis using imputed data in the PanC4 study. The analyzed PanC4 study consists of 3,568 pancreatic cancer patients and 3,363 controls (more details can be found in Chapter Three). These pancreatic cancer patients were selected regardless of their family history from nine hospital- or population-based case-control studies. Thus the prevalence of these FPC gene

mutations in the PanC4 study was expected to be similar to that in the general population. A multi-component analysis using GREML-LDMS approach was conducted to evaluate the contribution of the established FPC genes. A total of 9,445 variants located within ±50 kb of gene boundaries (3' UTR to 5' UTR) of the 11 FPC genes were evaluated as a single genetic component for their aggregated contribution to the estimated heritability for pancreatic cancer. The remaining variants across the genome were stratified into 2 minor-allele frequency (MAF) categories and 2 linkage disequilibrium (LD) groups as described in Chapter Three. A disease prevalence of 0.0149 was specified to transform the estimates from observed scale to liability scale and to correct for ascertainment bias in the case-control cohort. Together these variants explained 0.4% (s.e. = 0.3%) of phenotypic variance for pancreatic cancer.

This estimate appears to be much lower than what was expected given the prevalence and effect sizes of these genes in pancreatic cancer patients. Rare mutations in established FPC genes have been shown to explain a measurable fraction (4-10%) if not more of unselected pancreatic cancer. Mutations of these genes also confer a high risk of pancreatic cancer, with the per gene odds ratio ranged from 2.58 to 12.33[27]. However, the estimate of heritability using the current statistical approach cannot sufficiently capture the genetic variance attributable to these rare variants associated with the risk of pancreatic cancer.

Limitation of Current Approach in Capturing Rare Variations

Regardless of the type of data, imputation or sequencing, germ-line mutations are difficult to be characterized due to their extremely low frequencies in the general population. In imputations they are either not imputed or imputed with poor accuracy due to being monomorphic or extremely rare in the reference population. Detecting these rare mutations are possible in sequencing but requires a large sample size, which could be extremely costly for a

rare disease like pancreatic cancer. Even if these mutations are captured with sufficient accuracy in imputation or sequencing, they are often removed at the quality control stage. Including extremely low-frequency variants in the heritability analysis is currently not recommended as they would introduce noise and lead to unstable estimates. In our analysis, all variants with minor allele count less than five in the PanC4 study were excluded. Pathological mutations of FPC genes are most likely excluded in such heritability analysis. Therefore, failed to capture the variance explained by these rare mutations is expected in any GREML analysis, which would lead to a biased estimate of heritability.

The underestimation of heritability could be more evident for cancer phenotypes known to be affected by extremely rare mutations. In simulation studies, Yang and his colleagues concluded that the GREML-LDMS estimates of heritability using whole-genome sequencing data were unbiased under four different scenarios when the causal variants were (1) a random set of all variants, (2) more common than random variants, (3) less frequent than random variants, or (4) less frequent than random variants and with lower than average LD[30]. The conclusion may hold true for complex traits like human height and BMI, in which the majority of the additive genetic variance is explained by common variants[30,31]. But the genetic architecture of many cancer types may not fall into any of the four categories. The spectrum of cancer susceptibility alleles includes mutations in genes that are highly penetrant as well as low-frequency to common variants that impart a small additional risk for cancer. Given that high-penetrance mutations will most definitively be excluded from the analysis, the variance of cancer phenotypes explained by sequencing variants would be an underestimation of their true heritability. And the degree of bias may vary by cancer types, depending on the effect size and frequency of these high-penetrance mutations.

Conclusion

Heritability is a fundamental concept of genetics and its unbiased estimation is critical for understanding the genetic architecture of complex traits as well as for designing better studies in identifying disease-causing genes. Unlike traditional family-based studies, current statistical methods such as GREML approach allow the assessment of heritability in unrelated individuals, which tease apart the contribution of environmental factors shared among relatives. Although results from these approaches are sensitive to the type of data used in the analysis (array, imputation or sequencing), they can generally be improved by stringent quality controls, larger sample sizes and/or larger imputation panels. However, even when the best practices are applied, heritability is still likely underestimated, due to the limited power of these approaches in capturing genetic variation at rare mutations. The extent of such bias is likely to be trait-dependent, and more evident in cancer phenotypes known to be greatly affected by high-penetrance germ-line mutations. This limitation should be kept in mind when interpreting estimates of heritability from these approaches.

Tables

Table 11 Self-reported Family History of Pancreatic Cancer in Affected Individuals

Study (Year)	Country	Time Period	Study Design	Number of Cases*	Type of relative	Number (%) with a family history
Fernandez et al. (1994)[32]	Italy	1983-1992	Case-Control	362	First-degree	14 (3.9%)
Silverman et al. (1999)[33]	US	1986-1989	Case-Control	459	First-degree	23 (5.0%)
Inoue et al. (2003)[34]	Japan	1988-1999	Case-Control	200	Unspecified	10 (5.0%)
Hassan et al. (2007)[35]	US	2000-2006	Case-Control	802	First-degree	45 (5.6%)
Jacob et al. (2009)[36]	US (CPS-II)	1982	Prospective Cohort	7,306	First-degree	168 (2.3%)
Jacob et al. (2010)[37]	US, Europe, China (PanScan)	Varies by study	Nested Case-Control	1,183	First-degree	76 (6.4%) 6 (0.5%) (2 or more)
Austin et al. (2013)[38]	US	2005-2009	Case-Control	538	First-degree	47 (8.7%)
Elena et al. (2013)[39]	US, Europe, China (PanScan)	Varies by study	Nested Case-Control	773	First-degree	44 (5.7%)
Fehringer et al. (2014)[40]	Canada	2003-2009	Case-Control	743	First-degree	50 (7.2%)
Mizuno et al. (2014)[41]	Japan	1993-2013	Retrospective Cohort	688	First-degree	28 (4.1%)
Rosato et al. (2015)[42]	Italy	1991-2008	Case-Control	326	Unspecified	10 (3.1%)
Mocci et al. (2015)[43]	Spain	2009-2015	Case-Control	577	First-degree	36 (6.2%)
Piciucchi et al. (2015)[1]	Italy	2006-2013	Retrospective Cohort	293	First-degree	19 (6.5%) 2 (8.0%) (age ≤ 50)
Zheng et al. (2015)[44]	China	2011-2013	Case-Control	323	Unspecified	11 (3.4%)
Lakatos et al. (2016)[45]	Hungary	2012-2014	Retrospective Cohort	354	Unspecified	13 (3.6%)
Schulte et al. (2016)[46]	Australia	2007-2011	Case-Control	592	First-degree	31 (5.2%)
Takai et al. (2016)[17]	Japan	2002-2013	Retrospective Cohort	1,197	First-degree	88 (7.3%)

McWilliams et al. (2017)[47]	US, Europe, Australia, Canada (PanC4)	Varies by study	Case-Control	1,733 (age < 60)	First-degree	157 (9.1%) (age < 60)
Huang et al. (2018)[48]	US	1993-2012	Prospective Cohort	1,455	Unspecified	49 (3.4%)
Molina-Montes et al. (2018)[4]	Europe (PanGenEU)	2009-2018	Case-Control	1,431	First-degree	104 (7.3%) 28 (1.9%) (2 or more)

* Number of cases with available information on family history of pancreatic cancer.

Table 12 Prevalence of FPC Gene Germ-line Mutations in FPC Patients

Study	Country	Sample Size*	Most Commonly Tested Genes					Overall Prevalence‡
			BRCA1	*BRCA2*	*CDKN2A*	*PALB2*	*ATM*	
Bartsch et al. (2002)[18]	Germany	18	-	-	0 (0%)	-	-	-
Murphy et al. (2002)[7]	US	29	-	5 (17.2%)	-	-	-	-
Hahn et al. (2003)[8]	Europe	26	-	5 (19.2%)	-	-	-	-
Couch et al. (2007)[9]	US	146	-	8 (5.5%)	-	-	-	-
Slater et al. (2010)[20]	Europe	81	-	-	-	3 (3.7%)	-	-
Slater et al. (2010)[10]	Germany	70	-	2 (2.9%)	0 (0%)	-	-	-
Ghiorzo et al. (2012)[11]	Italy	16	0 (0%)	0 (0%)	5 (31.3%)	0 (0%)	-	
Harinck et al. (2012)[19]	Netherlands	28	-	-	-	0 (0%)	-	-
Roberts et al. (2012)[15]	US	166	-	-	-	-	4 (2.4%)	-
Zhen et al. (2015)[12]	US	515	6 (1.2%)	19 (3.7%)	13 (2.5%)	3 (0.6%)	-	41 (8.0%)
Catts et al. (2016)[49]	US	46	4 (9.5%)	7 (16.7%)	-	0 (0%)	1 (8.3%)	14 (30.4%)
Takai et al. (2016)[17]	Japan	54	0 (0%)	3 (5.6%)	-	2 (3.7%)	2 (3.7%)	8 (14.5%)
Chaffee et al. (2017)[13]	US	185	2 (1.1%)	8 (4.3%)	4 (2.2%)	1 (0.5%)	6 (3.2%)	22 (11.9%)

* The total number of FPC probands being tested for any gene. The number of FPC probands tested for each gene may be different in the same study.

† The denominator was aggregated from the number of samples tested for each specific gene across studies.

‡ Overall prevalence of all tested genes associated with pancreatic cancer, not limited to the five listed genes.

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CHAPTER SIX

CONCLUSIONS, FUTURE DIRECTIONS AND PUBLIC HEALTH SIGNIFICANCE

Summary and Conclusions

Pancreatic cancer is fundamentally a genetic disease caused by both inherited and acquired genetic alterations. Inherited genetic variation plays an important role in both the familial and non-familial (sporadic) occurrences of pancreatic cancer. Family history is a long-recognized risk factor for pancreatic cancer and an important predictor of disease risk. As previously discussed, rare mutations in 11 FPC genes confer a high lifetime risk of pancreatic cancer (Chapter Two). Large-scale genome-wide association studies (GWAS) to date have identified 26 common variants associated with a small increased risk of for pancreatic cancer in European populations and additional 5 loci in Asian populations (Chapter Two). These findings suggest that the genetic basis of pancreatic cancer consists of a wide spectrum of causal variants, ranging from extremely rare high-penetrance mutations to common low-risk susceptibility loci.

Heritability (narrow-sense heritability) is a population-based parameter that has been widely used to quantify the impact of additive genetic effect to a complex trait/disease. Prior larger scale twin studies have estimated heritability for pancreatic cancer to be 36% (95% CI: 0% - 53%)[1]. However, some studies have suggested estimates of heritability from family-based studies may be biased upwardly due to the shared environmental factors. The genomic-relatedness-based restricted maximum-likelihood (GREML) approach allows for the assessment of heritability in unrelated individuals, which could tease apart the shared environmental factors. Using this approach, the heritability for pancreatic cancer in our study was estimated to be 21.2% from imputed variants in the PanC4 study and was 38.8% from sequenced variants in the FPC-

ADNI study. These two estimates of heritability are both biased downwardly since extremely rare mutations associated with pancreatic cancer cannot be captured by the GREML approach. The higher estimate of heritability in the FPC-ADNI study could be a result of more comprehensive characterization of genetic variation in sequencing data than in imputation data, as well as the pancreatic cancer patients in the study population being exclusively FPC cases. It is possible that among FPC patients, the enrichment of high-penetrance causal variants with moderate to large effects in high-risk families together led to a greater contribution to the susceptibility of pancreatic cancer than in the general populations. Potential bias due to batch effects and/or mis-specification of disease prevalence are also possible explanations for the higher estimate of heritability in FPC-ADNI study. But their impacts are likely to be small given the stringent quality controls and the sensitivity analysis on disease prevalence. Additional analysis using the same set of variants from array data led to the same observation of a higher estimate of heritability in the FPC-ADNI study than in the PanC4 study. The estimate of heritability in the FPC-ADNI study is also very similar to the estimate from the previous twin study. These results suggest that additive genetic effects may be a greater contributor to the risk of pancreatic cancer in families with a strong genetic background, and that the true heritability for FPC could be somewhere near this estimate of heritability.

Genomic partitioning of the estimated heritability in the PanC4 study ranked chromosome 9, 7, 8, 16, 5, 2 and 1 as the top contributors to the heritability of pancreatic cancer. This is consistent with the GWAS findings as on all these chromosomes common susceptibility loci have been discovered. Across the functional group of intronic, intergenic, coding and regulatory variants, intronic variants account for most phenotypic variance of pancreatic cancer (12.4%). Interestingly, 12 out of 21 GWAS loci identified in European populations are mapped

to intronic variants. Given that the GREML approach is more powered to capture genetic variation at common variants, it was not surprising to find that these results appeared to be driven by GWAS loci. However, this does not mean that rare variants have little influence on the risk of pancreatic cancer, but rather their influence cannot be truly reflected in such analysis. For example, the contribution of coding variants is likely to be underestimated to a greater extent due to being on the lower end of the MAF spectrum than variants in other functional groups.

Results from partitioning by MAF showed that more than one-third of the estimated heritability was attributable to rare variants. Since the GREML approach can only capture a portion of variance due to rare variants, this proportion is likely to be an underestimate. Our results demonstrate an important role of rare variants in the susceptibility of pancreatic cancer, which emphasize the necessity of whole-genome sequencing (WGS) studies for the identification of variants with extremely low frequency or that are unique to specific populations. However, obtaining a sufficiently large sample to permit such sequencing analysis remain a significant challenge. On the other hand, common variants still explain a substantial amount of total phenotypic variance for pancreatic cancer (16.0%). Comparing to a previous analysis in the PanC4 study, our analysis reported a higher contribution of known GWAS loci to the susceptibility of pancreatic cancer (4.1% vs. 3.0%), likely resulting from the inclusion of the five novel loci discovered in a recent meta-analysis of four pancreatic cancer consortium studies[2]. The large proportion of still-missing heritability highlights the importance of continuing to search for common susceptibility loci for pancreatic cancer. Imputation and meta-analysis will remain a more cost-effective strategy than WGS in identifying variants with moderate to small effect sizes. However improved power must be achieved through larger sample sizes, larger or

trait-specific imputation reference panels, and a better characterization of low-frequency and rare variants.

Germ-line mutations are known to be critical in the development of pancreatic cancer. Prevalence of these high-penetrance mutations was reported to be 8-30% in FPC cases and 4-10% in sporadic cases. When evaluated in heritability analysis, variants of these 11 FPC genes together only explained 0.4% of all phenotypic variance for pancreatic cancer. These results again highlighted the inherent limitation of the GREML approach, incapable of capturing variance attributable to extremely rare mutations associated with pancreatic cancer. This limitation could yield misleading results that rare variants have a smaller influence on the susceptibility of a disease, which should be considered when interpreting results from such heritability analysis, particularly for cancer outcomes known to be affected by rare mutations.

Strengths of the Studies

Heritability for pancreatic cancer had previously been estimated from traditional family-based studies. Due to the rarity and fast progression of pancreatic cancer, these studies have limited statistical power to make conclusive inference and estimates of heritability from family-based studies may be inflated by shared environmental factors. In this study we performed a heritability analysis using the restricted maximum likelihood (REML) approach (GREML) to estimate the heritability from case-control studies of pancreatic cancer. Heritability estimated from unrelated individuals is less likely to be biased by environmental factors shared between relatives.

A number of studies have applied the GREML approach to estimate heritability for pancreatic cancer using array data. However, since variants in arrays are very selective and mostly common, these array-based heritability estimates are likely under-estimates. Our study

was conducted in two different case-control samples, each has its own strengths. The PanC4 study consists of 3,568 pancreatic cancer cases and 3,363 controls selected from nine hospital- or population-based studies, representing one of the largest case-control studies for pancreatic cancer. Samples from the PanC4 study were originally genotyped on the IlluminaHumanOmniExpressExome-8v1 array and were later imputed with 1000 Genome phase 3 populations. The imputation of array data has greatly extended its coverage to low-frequency and rare variants. In the PanC4 study, we employed three forms of GREML approach (GREML-SC, GREML-MS and GREML-LDMS) to estimate phenotypic variance explained by genetic variants. It has been demonstrated that single-component GREML (GREML-SC) estimates are biased when causal variants are not a random set of all sequence variants in terms of their MAF distribution and linkage disequilibrium (LD) structure. MAF-stratified multi-component GREML (GREML-MS) can account for the non-random MAF distribution of causal variants. Additional stratification on LD can further correct for the region-specific LD heterogeneity across the genome. The application of MAF- and LD-stratified multi-component GREML (GREML-LDMS) approach using imputation data in PanC4 study ensured our estimate of heritability be less biased than previously reported array-based estimates using the GREML-SC approach. In addition, the large sample size of the PanC4 study allows for stable estimates of variance when the number of components increases in GREML-LDMS models, and hence permits the investigation of the genetic architecture of pancreatic cancer through genomic partitioning of the estimated heritability.

The FPC-ADNI study may very likely be the only pancreatic cancer sample with WGS data. This sample was a combined sample with FPC cases from the Familial Pancreatic Cancer Genome Sequencing Project (FPC-GSP) and controls from the Alzheimer's Disease

Neuroimaging Initiative (ADNI) study, each of which is by far the largest sample of European ancestry with WGS data available for the corresponding disease. Subjects in the ADNI sample were cancer-free at baseline, and were similar in term of age distribution, sex ratio and catchment area to FPC cases, making them the ideal controls. Moreover, the cases and controls were both sequenced at Illumina using the same platform around the same period of time, allowing the merging of these WGS data to be achieved with fewer concerns about batch effects. Comparing to arrays and imputations, WGS data with sufficient coverage (30X) provides a near-complete characterization of the genetic variations across the genome, and hence minimize the bias in heritability estimation due to inadequate coverage of causal variants, particularly those at the lower end of MAF spectrum.

Limitations of the Studies

The small sample size of the FPC-ADNI study has limited our ability to evaluate the heritability for pancreatic cancer using multi-component GREML approaches. Attempts to stratify the variants into a various number of MAF categories all failed to generate a reliable estimate of variance using GCTA software. It was demonstrated that estimates of heritability from GREML-SC approach are unbiased only when the causal variants are a random subset of all sequenced variants[3]. Results from the PanC4 study suggested a distribution of causal variants for pancreatic cancer towards the lower end of the MAF spectrum. Given this, the estimate of heritability from all sequenced variants using the GREML-SC approach is likely biased downwardly.

The small size of the FPC-ADNI study also prohibits us from performing the genomic partitioning on WGS data. Our high coverage WGS data in theory is a more ideal data than imputation data to infer the genetic architecture of pancreatic cancer because of its near-complete

characterization of the genetic variation across the genome. However, any analysis involving more than one genetic component in the model failed to provide reliable estimates or convergence was not obtained.

Moreover, due to the unavailability of WGS data in an existing pancreatic cancer control population, we combined the FPC cases with subjects from ADNI study. The cases and controls were sequenced and called in slightly different pipelines. In the presence of technical artifact, the allele frequencies between cases and controls on average will be more different than expected under the null hypothesis, and cases will appear to be more related to other cases than to controls, which could lead to inflated estimates of heritability. To minimize the impact of batch effects, stringent QC filters were implemented on WGS data. In addition, the comparability of WGS data was carefully assessed before merging the FPC and ADNI dataset. Variants sequenced in the FPC and ADNI samples overall showed very similar distributions of mean depth per site and mean GQ per site across the genome. Although QC filters are not able to fully address the batch effects, the influence of batch effects on the estimates of heritability is likely to be small in the FPC-ADNI study.

Future Directions and Public Health Significance

It is estimated that pancreatic cancer will become the second leading cause of cancer death in the US by 2030. The survival rate for pancreatic cancer remains low (currently 8%) in comparison to other common cancer types, due to the lack of effective means for prevention, diagnosis and treatment. In the past few years, genetic research in pancreatic cancer has identified a number of genes and variants associated with the disease, which provide valuable insights into the complexities of its genetic architecture. Deciphering the genetic architecture is central to understanding the disease etiology and to aid in disease screening, diagnosis, prognosis

and therapy. Our study represents the first systematic investigation of the genetic architecture of pancreatic cancer.

Despite of the fruitful yields in recent GWAS and sequencing studies, the identified genes and variants for pancreatic cancer are far from explaining all heritability. Finding the missing heritability is the key step towards deciphering the genetic basis of pancreatic cancer and to help make informed decisions on the strategies and priorities of future gene discovery efforts. Our genomic partitioning analysis has found missing heritability at both rare and common variants for pancreatic cancer. Despite being underestimated, more than one-third of the estimated heritability for pancreatic cancer is attributable to rare variants, but little has been explained by known FPC genes. Since GWAS studies are not designed to detect rare associations, sequencing studies are warranted to facilitate the discovery of rare causal variants. Whole-exome sequencing can identify rare variants that directly influence gene functions but is restricted to examining the protein-coding content that comprises only 1% of the genome. In our analysis intronic variants explained the largest proportion of the estimated heritability for pancreatic cancer, emphasizing the importance of including both coding and non-coding regions in the search for disease-associated variants. WGS can measure nearly all genetic variation in the human genome and assess structural variants more accurately than WES. But owing to their cost, WGS studies have been limited by small sample sizes. Unexplained heritability at common variants was also substantial, indicating that more low-risk common susceptibility loci have yet to be identified. These variants likely have effect sizes too small to reach the genome-wide significance in current GWAS and require larger sample sizes to be detected. For low-frequency and rare variants, imputation and meta-analysis can be a more cost-effective strategy than sequencing, but the power needs to be improved with larger sample

sizes, larger imputation reference panels, or a combination with disease-specific reference panels.

Population-based heritability estimates such as those obtained in our study, provide a valuable metric for completeness of available genetic risk information, but individualized disease prevention and treatment will ultimately require identifying the variants accounting for risk in a given individual rather than on a population basis. It is our hope that results from this study provide a potentially valuable path towards the future discoveries of additional causal variants for pancreatic cancer that may permit a better characterization of individuals at the highest genetically defined risk in which effective prevention approaches can be developed and potential therapeutic targets for the disease can be explored.

Screening and early detection offer the best chance of reducing the high mortality of the disease. Selective screening of high-risk individuals is considered a cost-effective strategy. Currently, recommendation for screening is made based on individuals' family history of pancreatic cancer and their mutation status of the established FPC genes. However, based on our literature review, germ-line mutations of the established FPC genes account for about 8-30% of tested FPC cases, suggesting that pancreatic cancer is a genetically heterogeneous disease and more high-penetrance mutations are yet to be found. These high-penetrance mutations can facilitate the identification of the small proportion of the population at highest genetically defined risk for pancreatic cancer who may benefit from selective screening and early detection of the disease. Moreover, individuals with specific mutations may also benefit from targeted therapies. Tumors with mutations in *BRCA1/2*, *PALB2*, and *ATM* are highly sensitive to DNA-damaging related treatments such as crosslinking agents[4–6] and poly(ADP-ribose) polymerase inhibitors (PARPi)[7–10]. The clinical benefits of using these agents in patients with pancreatic

cancer are currently being investigated and promising results have been shown in the preliminary analysis.

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RESEARCH EXPERIENCE

Genetic Epidemiology, The Sidney Kimmel Comprehensive Cancer Center,
Baltimore, MD
2014 – 2018
Dissertation research with Dr. Alison Klein
Heritability analysis for pancreatic cancer using whole-genome sequencing data and imputation data.

Genetic Epidemiology, Johns Hopkins Bloomberg School of Public Health,
Baltimore, MD
2012-2018
Research Assistant to Dr. Priya Duggal
Exome array and exome sequencing studies on ocular disorders and infectious diseases.

Biomedical Informatics, Columbia University, New York, NY
2007-2009
Research Assistant to Dr. Chunhua Weng
Secondary use of electronic health records in public health research.

PRESENTATIONS

Poster: "Exome array analysis identified CAV1/CAV2 as a susceptibility locus for intraocular pressure" Presented at the Genomic of Common Disease annual meeting, September 2014, Potomac, MD

Poster: "Intraocular Pressure and Exome Sequencing Variation in the Beaver Dam Eye Study." (Abstract/Program #728) Presented at the 65th Annual Meeting of the American Society of Human Genetics, October 2015, Baltimore, MD.

Poster: "Whole exome sequencing and linkage analysis of patients with pulmonary non-tuberculous mycobacterial infection." Presented at the International Genetic Epidemiology Society Meeting, October 2015, Baltimore, MD

Poster: ""Transcriptomic profiling of normal pancreatic duct, precursor, and pancreatic adenocarcinoma organoids." Presented at the 2017 Genetic Research Day, February 2016, Baltimore, MD

Poster: "Whole exome sequencing and linkage analysis identified *TTK* as a candidate gene for pulmonary nontuberculous mycobacterial lung disease." (Abstract/Program #1318) Presented at the 66th Annual Meeting of the American Society of Human Genetics, October 2016, Vancouver, Canada.

Poster: "Heritability of familial pancreatic cancer using whole-genome sequencing data." Presented at the 2018 Genetic Research Day, February 2018, Baltimore MD

CONFERENCES ATTENDED

2016 The American Society of Human Genetics Annual Meeting, Vancouver, Canada
2016 The Genomic of Common Disease, Baltimore, MD
2015 The American Society of Human Genetics Annual Meeting, Baltimore, MD
2015 International Genetic Epidemiology Society Meeting, Baltimore MD
2014 The Genomics of Common Disease, Potomac, MD

TEACHING EXPERIENCE

Teaching Assistant, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Principle of Genetic Epidemiology I (1st term 2013-2014, 1st term 2014-2015, Summer Institute 2015)
Principle of Genetic Epidemiology III (3rd term 2013-2014)
Epidemiology Method 2 (2nd term 2015-2016)

HONORS AND AWARDS

The Maryland Genetics, Epidemiology, and Medicine Training Grant (MD-GEM), 2017-2018
The Maryland Genetics, Epidemiology, and Medicine Training Grant (MD-GEM), 2016-2017
The Maryland Genetics, Epidemiology, and Medicine Training Grant (MD-GEM), 2015-2016
The Maryland Genetics, Epidemiology, and Medicine Training Grant (MD-GEM), 2014-2015
The Wolfe Street Competition Award, 2015-2016