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RESEARCH ARTICLE

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Genetic variants in the liver kinase B1-AMP-activated protein kinase pathway genes and pancreatic cancer risk

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Abstract

The liver kinase B1-AMP-activated protein kinase (LKB1-AMPK) pathway has been identified as a new target for cancer therapy, because it controls the glucose and lipid metabolism in response to alterations in nutrients and intracellular energy levels. In the present study, we aimed to identify genetic variants of the LKB1-AMPK pathway genes and their associations with pancreatic cancer (PanC) risk using 15 418 participants of European ancestry from two previously published PanC genome-wide association studies. We found that six novel tagging single-nucleotide polymorphisms (SNPs) (i.e, *MAP2* rs35075084 T > deletion, *PRKAG2* rs2727572 C > T and rs34852782 A > deletion, *TP53* rs9895829 A > G, and *RPTOR* rs62068300 G > A and rs3751936 G > C) were significantly associated with an increased PanC risk. The multivariate logistic regression model incorporating the number of unfavorable genotypes (NUGs) with adjustment for age and sex showed that carriers with five to six NUGs had an increased PanC risk (odds ratio = 1.24, 95% confidence interval = 1.16-1.32 and $P < 0.0001$), compared to those with zero to four NUGs. Subsequent expression quantitative trait loci (eQTL) analysis further revealed that these SNPs were associated with significantly altered mRNA expression levels either in 373 normal lymphoblastoid cell lines (*TP53* SNP rs9895829, $P < 0.05$) or in whole blood cells of 369 normal donors from the genotype-tissue expression project (GTEx) database [*RPTOR* SNP rs60268947 and rs28434589, both in high linkage disequilibrium ($r^2 > 0.9$) with *RPTOR* rs62068300, $P < 0.001$]. Collectively, our findings suggest that these novel SNPs in the LKB1-AMPK pathway genes may modify susceptibility to PanC, possibly by influencing gene expression.

KEYWORDS
genome-wide association study, pancreatic cancer risk, single-nucleotide polymorphism (SNP)

1 | INTRODUCTION

Pancreatic cancer (PanC) is one of the most lethal human malignancies, with an overall 5-year survival rate lower than 10% and a median survival of 6 months, and PanC is also the fourth leading cause of cancer-related death and is expected to become the second within the next decade in the United States.[1,2] The exact cause of PanC is not yet well understood, though several risk

Abbreviations: CI, confidence interval; FPRP, false positive report probability; GWAS, genome-wide association studies; NUGs, number of unfavorable genotypes; OR, odds ratio; PanC, pancreatic cancer; SNP, single-nucleotide polymorphism.

factors have been identified, including smoking, morbid obesity, having a family history of PanC or pancreatitis, and having certain hereditary conditions.[3,4] More importantly, patients with PanC rarely exhibit symptoms at the early stages, until the disease reaches an advanced stage, which is one of the main reasons for the observed, generally poor survival rates.[4,5] Therefore, prevention and early diagnosis at a curable stage are desperately needed to reduce PanC mortality.

Since Otto Warburg first proposed a connection between cellular metabolism and tumorigenesis nearly 100 years ago, pointing out a new direction for cancer research,[6,7] numerous studies have been reported on molecular mechanisms that link the signaling pathways that control the metabolism to cell growth, in which the metabolism reprogramming was found to be necessary for cancer initiation and progression, a hallmark of cancer.[8,9] PanC is characterized by a severely hypoxic and nutrient-deprived microenvironment, with some specific metabolically adaptive mechanisms, such as the Warburg effect, glutamine addiction and autophagy, that all contribute to PanC development and progression, in addition to both oncogenes/tumor suppressors and tumor microenvironment.[10,11] Therefore, targeting any of specific metabolic adaptions becomes an emerging strategy for PanC diagnosis and treatment.[12,13]

Liver kinase B1 (LKB1), also known as STK11, directly activates the AMP-activated protein kinase (AMPK), which is responsible for nutrient sensing and metabolism reprogramming, and LKB1 is inactivated by mutations found in PanC, and the loss of LKB1 is thought to drive tumorigenesis.[14–16]

AMPK is a master regulator of metabolic homeostasis by sensing cellular energy status, the AMP:ATP ratio. When there is an increase in the cellular AMP:ATP ratio, which reflects a decrease in energy supply, AMPK is phosphorylated and activated, promoting catabolic processes and inhibiting anabolic processes to increase the energy level.[17]

Recent studies found that meisoindigo can preferentially kill cancer stem cells by interfering cell metabolism via inhibition of LKB1 and activation of AMPK in PanC.[18] Similar results have also been drawn from metformin, which influences PanC progression by activating the LKB1-AMPK pathway, including inhibition of cell division, promotion of apoptosis, and autophagy, down-regulation of circulating insulin, and activation of the immune system.[19]

Therefore, we hypothesize that genetic variants of the LKB1-AMPK pathway genes are associated with PanC risk. To test such a hypothesis, we conducted a comprehensive meta-analysis of genetic variants in the LKB1-AMPK pathway genes using two previously published genome-wide association study (GWAS) datasets from the PanScan (i.e., the Pancreatic Cancer Cohort Consortium) and Pancreatic Cancer Case-Control Association Study. We also focused our analysis on the identified single-nucleotide polymorphisms (SNPs) that may change the mRNA expression levels of the genes and thus are likely have functional consequences.

2 | METHODS

2.1 | Study population and GWAS data

The analysis used two previously published PanC GWASs: Pancreatic Cancer Cohort Consortium (PanScan, phs000206.v5.p3) and the Pancreatic Cancer Case-Control Association Study (dbGaP#:phs000648.v1.p1), which included 15 418 participants (8474 cases and 6944 controls). The PanScan GWAS has three phases, including PanScan I, II and III (1760 cases and 1780 controls in PanScan I; 1457 cases and 1666 controls in PanScan II; and 1538 cases and 0 controls in PanScan III).[20–23] We then merged the PanScan II and PanScan III into one dataset, PanScan II/III, because PanScan III had only cases available in dbGaP.[24] Another GWAS dataset was from the Pancreatic Cancer Case-Control Consortium (PanC4) that consisted of 3719 cases and 3498 controls from the United States, Europe and Australia (**Figure S1**). All the participants in these GWASs were of European ancestry, and a written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations for each of the participating institutions, and the present study followed the protocols approved by Duke University Health System Institutional Review Board.

2.2 | Gene selection, genotyping and imputation

The keywords "LKB1-AMPK" was searched in Molecular Signatures Database (MSigDB; http://www.broadinstitute.org/gsea/msigdb/index.jsp), and all the 58 related genes located on autosomal chromosomes were selected from REACTOME and PID (details presented in **Table S1**). The GWAS genotyping was performed using Illumina HumanHap550v3.0, Human 610-Quadv1_B, HumanOmniExpress-12v1.0 and HumanOmniExpressExome-8v1.0.[25–27] The SNPs located both in these genes and their ± 500-kb flanking regions were extracted for further imputation by the IMPUTE2 software, using the 1000 Genomes (Phase 1, Release 3) Project as the reference dataset.[28,29]

For quality control, the imputed SNPs with an information/accuracy score > 0.4 were qualified for further analysis (with details presented in Table 1 and **Figure S2**). As a result, there were 14 557 SNPs, 15 866 SNPs, and 14 263 SNPs within 5.0 kb up- and downstreams of the 58 LKB1-AMPK pathway genes from populations of the PanScan I, PanScan II/III, and panC4 studies, respectively. The final meta-analysis for all three datasets contained 12 777 SNPs that met the inclusion criteria: a missing rate < 5%, MAF > 1% and Hardy-Weinberg equilibrium (HWE) test P value > 1×10^{-5}.

2.3 | Association analysis

Unconditional multivariable logistic regression analysis including sex, age, and top five principal components was performed using PLINK (version 1.90), assuming an additive genetic model, with assessment of genomic data to control for potential population stratification. The

TABLE 1 Associations between 16 SNPs in the AMPK-LKB1 pathway genes and pancreatic cancer risk with an FPRP ≤ 0.02.

SNP rs ID#	Locus	Gene	Allelea	IS1b	IS2b	IS3b	EAF1c	EAF2c	EAF3c	OR (95% CI)d	*Pe*	FPRP
rs35075084	2q34	*MAP2*	CT > C	0.540	0.602	0.545	0.019	0.023	0.023	0.76 (0.64-0.90)	0.0010	0.020
rs2727572	7q36.1	*PRKAG2*	C > T	0.982	0.979	0.982	0.473	0.454	0.445	1.08 (1.03-1.13)	0.0011	0.019
rs12668489	7q36.1	*PRKAG2*	C > T	0.985	0.982	0.986	0.473	0.456	0.448	1.08 (1.03-1.13)	0.0010	0.018
rs2538046	7q36.1	*PRKAG2*	G > A	0.995	0.993	0.991	0.505	0.486	0.474	1.09 (1.04-1.14)	0.0003	0.005
rs34852782	7q36.1	*PRKAG2*	TA > T	0.886	0.860	0.791	0.283	0.272	0.275	1.10 (1.05-1.16)	0.0002	0.003
rs17884306	17p13.1	*TP53*	C > T	0.979	0.973	0.971	0.060	0.058	0.057	0.83 (0.75-0.93)	0.0004	0.007
rs17879377	17p13.1	*TP53*	C > T	0.972	0.969	0.966	0.051	0.053	0.050	0.84 (0.74-0.94)	0.0011	0.020
rs9891744	17p13.1	*TP53*	C > T	0.980	0.975	0.976	0.060	0.059	0.058	0.82 (0.73-0.91)	0.0001	0.002
rs75732100	17p13.1	*TP53*	C > T	0.985	0.980	0.981	0.060	0.058	0.058	0.82 (0.74-0.92)	0.0002	0.004
rs35850753	17p13.1	*TP53*	C > T	0.963	0.948	0.952	0.021	0.022	0.023	0.74 (0.62-0.88)	0.0004	0.008
rs9895829	17p13.1	*TP53*	A > G	0.991	0.987	0.991	0.061	0.058	0.058	0.82 (0.74-0.92)	0.0002	0.003
rs17883323	17p13.1	*TP53*	G > T	0.989	0.987	0.991	0.061	0.058	0.057	0.83 (0.74-0.92)	0.0002	0.004
rs8079544	17p13.1	*TP53*	G > T	1.000	1.000	1.000	0.060	0.058	0.058	0.82 (0.74-0.92)	0.0002	0.003
rs62068300	17q25.3	*RPTOR*	G > A	0.973	0.974	0.978	0.308	0.320	0.330	0.92 (0.87-0.97)	0.0008	0.015
rs17848685	17q25.3	*RPTOR*	C > G	0.729	0.701	0.822	0.213	0.228	0.234	0.91 (0.86-0.97)	0.0011	0.019
rs3751936	17q25.3	*RPTOR*	G > C	0.897	0.883	0.906	0.264	0.268	0.258	0.91 (0.86-0.97)	0.0008	0.014

Abbreviations: CI, confidence interval; EAF, effect allele frequency; FPRR, false positive report probability; IS, imputation info score; OR, odds ratio; SNP, single-nucleotide polymorphism.

[a]Referring to "common allele/effect allele."

[b]IS1 was IS in PanScan I controls; IS2 was IS in PanScan II/III controls; IS3 was IS in PanC4 controls.

[c]EAF1 was EAF in PanScan I controls; EAF2 was EAF in PanScan II/III controls; EAF3 was EAF in PanC4 controls.

[d]Fixed effects models were used when no heterogeneity was found between studies (Q test $P > 0.10$ and $I^2 < 50.0\%$); otherwise, random effects models were used.

[e]Obtained from the meta-analysis of the three PanC studies.

principal components were computed by GCTA (genome-wide complex trait analysis) software and the top five principal components with *P* value less than 0.001 were selected from the logistic regression analysis from all the three studies (**Table S2**), and an odds ratio (OR) and its 95% confidence interval (CI) were estimated for each SNP with PLINK.[30] A meta-analysis was further performed by using the results of 12 777 SNPs based on β estimates and standard errors with Stata software (v 12, State College, Texas, US). Cochran's Q statistics and I^2 were used to assess the heterogeneity.[31] If the Cochran's Q-test *P* value > 0.100 and the heterogeneity statistic $I^2 < 50\%$, a fixed-effects model was applied. Otherwise, a random-effects model was used.

False positive report probability (FPRP) is the probability of no true association between a genetic variant and disease risk, given a statistically significant finding. We chose FPRP to correct for multiple testing, because more than 90% of SNPs (12 379 out of 12 777 under investigation) included in the present study were imputed. The FPRP approach with a prior probability of 0.01 and a hazards ratio (HR) of 2.0 was assigned for an association with genotypes and alleles of each SNP to reduce the probability of false-positive findings. The association between each SNP and PanC risk was evaluated with an additive genetic model, in which a cut-off FPRP value ≤ 0.02 was considered as a significant association. The multivariable stepwise logistic regression model was also used to identify independent SNPs. The number of unfavorable genotypes (NUGs) of SNPs with independent effects was calculated to assess the classification performance of the model. All individuals were further dichotomized into low-risk group (0-4 NUGs) and high-risk group (5-6 NUGs) for additional analysis. Besides, Haploview v4.2[32] was used to produce the Manhattan plot and linkage disequilibrium (LD) plot, and LocusZoom[33] was used to construct the regional association plots by using the dataset from the 1000 Genomes Project. Linear regression was used to analyze the correlations between SNPs and corresponding mRNA expression levels. All statistical analyses were performed with SAS software (version 9.4; SAS Institute, Cary, NC), if not specified otherwise

2.4 | *In silico* functional predication and validation

To predict potential functions of the significant SNPs, we used two *in silico* tools: RegulomeDB (http://regulomedb.org/)[34] and HaploReg (http://www.broadinstitute.org/mammals/haploreg/haploreg.php).[35] We performed the expression quantitative trait loci (eQTL) analysis to estimate the associations between SNPs and mRNA expression levels of the corresponding genes by using the mRNA expression data from the lymphoblastoid cell lines of 373 Europeans available in the 1000 Genomes Project[36] and 127 tumor tissues in The Cancer Genome Atlas (TCGA)[37] as well as the eQTL results from the

genotype-tissue expression project (GTEx) database for the whole blood (n = 369) and normal pancreatic tissues (n = 220) (http://www.gtexportal.org/home/).[38] In addition, we also compared the mRNA expression levels of targeted gene between tumor and adjacent normal tissues available in the Oncomine™ database (https://www.oncomine.org/).[39]

3 | RESULTS

3.1 | Single locus analysis

The research workflow of the present study design is shown in Figure 1. We first estimated the associations between selected SNPs [with a minor allele frequency (MAF) ≥ 0.01] and PanC risk with the unconditional logistic regression analysis for each of the three populations of European ancestry with 14 557 SNPs, 15 866 SNPs, and 14 263 SNPs for PanScan I, PanScan II/III, and PanC4, respectively; the single locus analysis revealed that these three study populations had 623, 1713, and 911 SNPs with a nominal $P < 0.05$, respectively (**Figure S3**). Then, we included a total of 12 777 SNPs in a meta-analysis of the three populations and found that 589 SNPs remained to be associated with PanC risk at $P < 0.05$ in an additive genetic model, of which 16 SNPs on *MAP2*, *PRKAG2*, *TP53*, and *RPTOR* passed multiple testing corrections with an FPRP ≤ 0.02 (**Figure S4A;** Table 1).

Although seven SNPs of *TP53* (i.e., rs17884306, rs17879377, rs9891744, rs75732100, rs9895829, rs17883323, and rs8079544) located at 17p13 have been previously reported by the AURORA pathway-based analyses,Query Deleted,[40] the *TP53* rs35850753 and other eight SNPs (i.e., *MAP2* rs35075084, *PRKAG2* rs2727572, rs12668489, rs2538046 and rs34852782 and *RPTOR* rs62068300, rs17848685 and rs3751936) located at 2q34, 7q36.1, and 17q25.3, respectively, are novel findings, for which we performed additional *in silico* analysis for their functional relevance. The results of these SNPs in each of GWAS datasets and the final meta-analysis are summarized in Table 2. All these SNPs showed a low to moderate heterogeneity among the three GWAS datasets (all Q-test $P > 0.100$ and $I^2 < 50.0$).

3.2 | LD analysis and stepwise analysis

For the LD analysis, three (i.e., rs2727572, rs12668489 and rs2538046) of the four *PRKAG2* SNPs shared a high LD ($r^2 \geq 0.80$, **Figure S4B** and **Figure S5A**); seven (i.e., rs17884306, rs17879377, rs9891744, rs75732100, rs9895829, rs17883323, and rs8079544) of the eight *TP53* SNPs shared a high LD ($r^2 \geq 0.80$, **Figure S4D** and

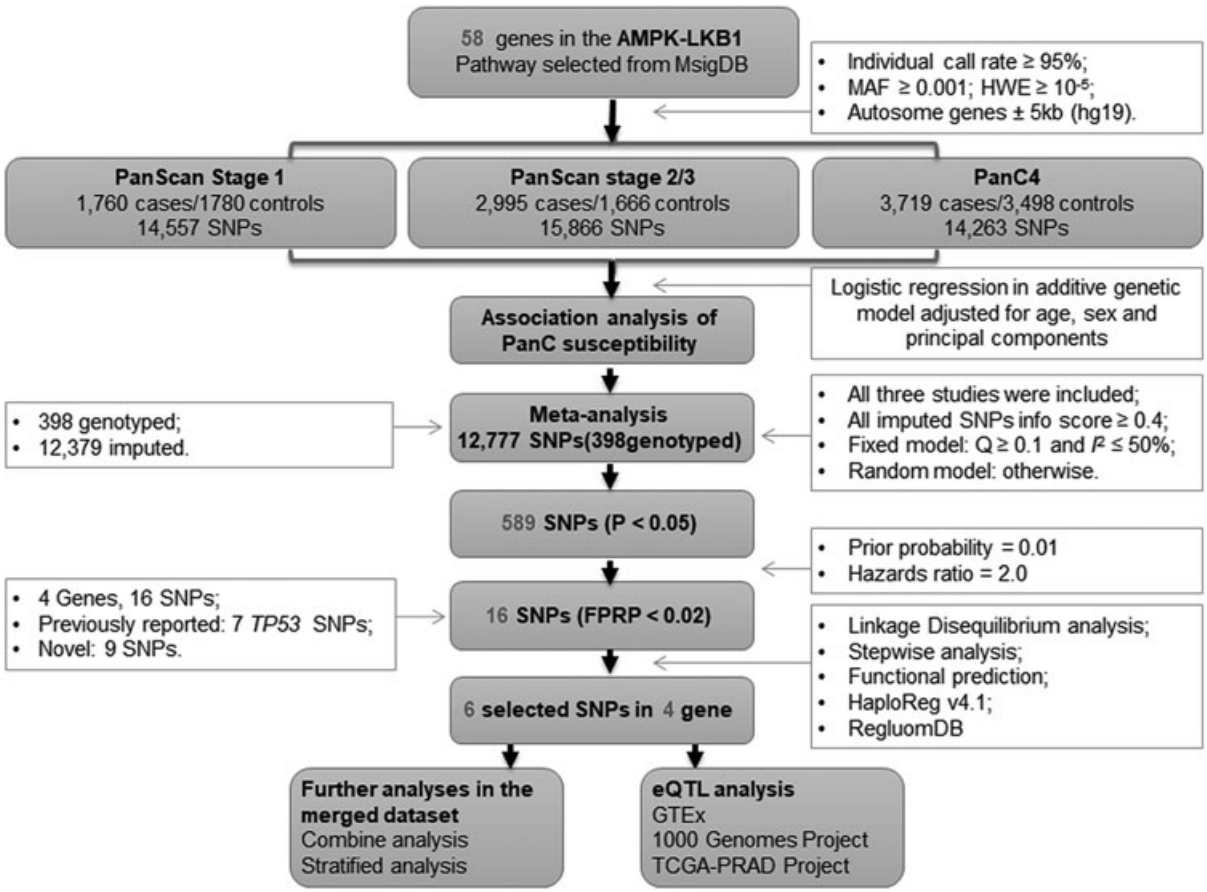

FIGURE 1 The workflow of the analysis. eQTL, expression quantitative trait loci; FPRP, false positive report probability; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; PanC, pancreatic cancer; SNP, single-nucleotide polymorphism

TABLE 2 The results of these 16 SNPs in each of GWAS datasets and the final meta-analysis.

				PanScan_stage 1		PanScan_stage 2/3		PanC4		All GWASs combined		Heterogeneity	
SNP rs ID#	Allele	Position	Gene	OR (95% CI)[a]	*P*[a]	OR (95% CI)[a]	*P*[a]	OR (95% CI)[a]	*P*[a]	OR (95% CI)	*P*	*Q*	I^2
rs35075084	CT > C	210473015	*MAP2*	0.78 (0.54-1.13)	0.188	0.88 (0.66-1.18)	0.400	0.68 (0.54-0.86)	0.001	0.76 (0.64-0.90)	0.0010	0.398	0
rs2727572	C > T	151298246	*PRKAG2*	1.03 (0.94-1.13)	0.509	1.08 (1.00-1.18)	0.064	1.10 (1.03-1.18)	0.005	1.08 (1.03-1.13)	0.0011	0.541	0
rs34852782	TA > T	151566171	*PRKAG2*	1.08 (0.97-1.20)	0.144	1.11 (1.01-1.22)	0.036	1.11 (1.03-1.20)	0.005	1.10 (1.05-1.16)	0.0002	0.902	0
rs12668489	C > T	151301725	*PRKAG2*	1.04 (0.95-1.15)	0.362	1.08 (0.99-1.17)	0.087	1.10 (1.03-1.17)	0.005	1.08 (1.03-1.13)	0.0010	0.675	0
rs2538046	G > A	151314162	*PRKAG2*	0.94 (0.85-1.03)	0.161	1.07 (0.98-1.16)	0.132	1.11 (1.04-1.19)	0.002	1.09 (1.04-1.14)	0.0003	0.715	0
rs17884306	C > T	7572101	*TP53*	0.81 (0.65-0.99)	0.044	0.82 (0.68-0.99)	0.039	0.85 (0.73-0.99)	0.032	0.83 (0.75-0.93)	0.0004	0.912	0
rs17879377	C > T	7574721	*TP53*	0.88 (0.70-1.10)	0.256	0.78 (0.64-0.95)	0.015	0.85 (0.73-1.00)	0.044	0.84 (0.74-0.94)	0.0011	0.706	0
rs9891744	C > T	7574864	*TP53*	0.79 (0.64-0.98)	0.032	0.81 (0.67-0.98)	0.028	0.83 (0.72-0.97)	0.016	0.82 (0.73-0.91)	0.0001	0.817	0
rs75732100	C > T	7576348	*TP53*	0.80 (0.65-0.99)	0.037	0.83 (0.69-1.00)	0.046	0.84 (0.72-0.97)	0.018	0.82 (0.74-0.92)	0.0002	0.947	0
rs35850753	C > T	7578671	*TP53*	0.94 (0.67-1.31)	0.712	0.60 (0.43-0.83)	0.002	0.73 (0.58-0.93)	0.010	0.74 (0.62-0.88)	0.0004	0.168	43.97
rs9895829	A > G	7578679	*TP53*	0.79 (0.64-0.98)	0.029	0.83 (0.69-1.00)	0.046	0.84 (0.72-0.97)	0.020	0.82 (0.74-0.92)	0.0002	0.913	0
rs17883323	G > T	7579619	*TP53*	0.79 (0.64-0.98)	0.029	0.82 (0.68-0.99)	0.042	0.84 (0.73-0.98)	0.024	0.83 (0.74-0.92)	0.0002	0.896	0
rs8079544	G > T	7580052	*TP53*	0.80 (0.65-0.99)	0.036	0.82 (0.68-0.99)	0.039	0.83 (0.72-0.97)	0.017	0.82 (0.74-0.92)	0.0002	0.948	0
rs62068300	G > A	78574727	*RPTOR*	0.96 (0.87-1.07)	0.479	0.92 (0.84-1.01)	0.083	0.90 (0.84-0.96)	0.003	0.92 (0.87-0.97)	0.0008	0.537	0
rs17848685	C > G	78599562	*RPTOR*	1.00 (0.89-1.12)	0.998	0.92 (0.83-1.02)	0.099	0.87 (0.80-0.94)	0.001	0.91 (0.86-0.97)	0.0011	0.150	47.36
rs3751936	G > C	78938204	*RPTOR*	1.00 (0.90-1.11)	0.960	0.89 (0.81-0.98)	0.017	0.89 (0.82-0.96)	0.003	0.91 (0.86-0.97)	0.0008	0.178	41.98

Abbreviations: CI, confidence interval; OR, odds ratio; GWAS, genome-wide association study; SNP, single-nucleotide polymorphism

[a]Adjusted for age, sex, and top five significant principal components

Figure S5C); and three *RPTOR* SNPs (i.e., rs62068300, rs17848685 and rs3751936) showed a low LD ($r^2 \leq 0.80$, **Figure S4C** and **Figure S5B**). Having considered functional prediction and LD, we selected final eight SNPs, including *MAP2* rs35075084, two proxy *PRKAG2* SNPs (i.e., rs2727572 and rs34852782), two proxy *TP53* SNPs (i.e., rs9895829 and rs35850753), and all three SNPs of *RPTOR* (i.e., rs62068300, rs17848685 and rs3751936) for further analysis. Next, we assessed these eight representative SNPs in the presence of age, sex, and top five principal components in a multivariate stepwise logistic regression model. As a result, the genotypes of six SNPs remained significantly and independently associated with PanC risk (**Table S3**).

3.3 | Genotype effect and the joint-effect of the six significant SNPs

After stepwise analysis, we found that the genotypes of *MAP2* rs35075084 T > deletion, *TP53* rs9895829 A > G, *PRKAG2* rs2727572 C > T and rs34852782 A > deletion, and *RPTOR* rs62068300 G > A and rs3751936 G > C were significantly associated with PanC risk in both additive and dominant genetic models.

In the additive model, the association between each of these six novel SNPs and PanC risk had a linear trend as the frequency of the minor allele increased (trend test: $P = 0.0008$, $P = 0.0004$, $P = 0.0007$, $P = 0.0002$, $P = 0.0009$, and $P = 0.0009$, respectively, Table 3). Consistent with previous results of the single locus analysis, the rs2727572 T allele was associated with an increased PanC risk (OR = 1.11, 95% CI = 1.04-1.19 and $P = 0.0034$), the rs34852782 deleted allele was associated with an increased PanC risk (OR = 1.09, 95% CI = 1.02-1.16, and $P = 0.0076$), whereas the rs35075084 deleted allele, rs9895829 G, rs62068300 A, and rs3751936 C alleles were associated with a reduced risk (OR = 0.75, 95% CI = 0.64-0.89, $P = 0.0007$, OR = 0.83, 95% CI = 0.75-0.92, $P = 0.0004$, OR = 0.91, 95% CI = 0.85-0.97, $P = 0.0026$, and OR = 0.91, 95% CI = 0.86-0.97, $P = 0.0051$, respectively), compared with their corresponding wild-type allele (Table 3).

To better estimate the joint association between the six SNPs and PanC risk, we combined risk genotypes of rs35075084 TT, rs2727572 CT/TT, rs34852782 A-/—, rs9895829 AA, rs62068300 GG, and rs3751936 GG into a single genetic score as the NUGs in a dominant model. The significant association between an increased NUGs and an increased PanC risk remained ($P_{trend} < 0.0001$, Table 4). We then dichotomized all individuals into low-risk group (0-4 NUGs) or high-risk group (5-6 NUGs). As shown in Table 4, PanC risk in high-risk group was significantly greater than the low-risk group (OR = 1.24, 95% CI = 1.16-1.32, $P < 0.0001$).

Since the difference in the distribution of age existed in all the datasets, and age is a known risk factor for PanC, we performed the subgroup analysis by age group (i.e., < 60 years, 60–70 years and > 70 years) and sex to assess any potential interaction. We found that the risk associated with high-risk NUGs was more evident in the > 70 year group (OR = 1.27, 95% CI = 1.13-1.41, $P < 0.0001$) and males (OR = 1.25, 95% CI = 1.15-1.37, $P < 0.0001$); however, there was no evidence for an interaction among and between these strata ($P > 0.05$ for all, **Table S4**).

3.4 | Genotype and phenotype correlation analysis

To explore potential function of these six SNPs, we used online prediction tools and performed the eQTL analysis. We found that four SNPs are located in the intronic regions, one SNP in 5′-UTR and one SNP in 3′-UTR (**Table S5**). All SNPs are located in an enhancer region of the histone H3 mono acetyl K27, which is associated with the higher activation of transcription and defined as an active enhancer marker**.** Besides, one SNP of *TP53* and two SNPs of *RPTOR* are also located in DNase I hypersensitive sites where chromatins are sensitive to cleavage by the DNase I and lost its condensed structure, functionally related to transcriptional activity **(Figure S6)**.

We evaluated correlations between SNPs and corresponding mRNA levels in 373 normal lymphoblastoid cell lines from the 1000 Genomes Project. We found that the rs9895829 G allele was significantly correlated with decreased levels of *TP53* mRNA expression in both additive and dominent models ($P = 0.005$ and 0.039) by using the Student *t* test or linear regression analysis of the logarithm transformed expression values ($\log^2$) **(**Figure 2A and 2B**)**. No other allele was significantly correlated with increased/decreased levels of mRNA expression. However, the rs2727572 T allele of *PRKAG2*, rs62068300 A allele of *RPTOR* and rs3751936 C allele of *RPTOR* were correlated with an observable increased/decreased mRNA expression level in both additive and dominant models, though the differences did not reach the statistical level ($P = 0.399$ and 0.625, $P = 0.299$ and 0.092 and $P = 0.163$ and 0.177, respectively. **(Figure S7**). For SNP rs35075084 and rs34852782, the data for eQTL analysis were not available. Next, we used the data from 127 Europeans in the TCGA-PAAD Project to query the eQTL results and assess the correlations. However, we failed to impute the genotypes of six SNPs on the basis of the current quality control.

In addition, we used the data from the GTEx database and found that SNP rs60268947 and rs28434589, located in the intron of *RPTOR* and in high LD ($r^2 = 0.91$ and $r^2 = 0.96$, respectively) with SNP rs62068300, had a significant correlation with an increased level of *RPTOR* mRNA expression in whole blood cells of 369 donors from GTEx ($P = 6.5 \times 10^{-12}$ and $P = 1.5 \times 10^{-10}$, respectively, Figure 2C-D). For other SNPs, there are no significant correlations from GTEx (**Table S6**).

We also assessed the differences in mRNA expression levels of *MAP2*, *PRKAG2*, *TP53* and *RPTOR* between adjacent normal pancreatic tissues and pancreatic tumor tissues (n = 78) from the Oncomine database. We found that *MAP2* and *TP53* mRNA expression levels were statistically significantly higher ($P = 0.002$ and $P = 1.38 \times 10^{-6}$, respectively) in tumor tissues than in normal pancreatic tissues, and the mRNA expression levels of *PRKAG2* and *RPTOR* were also higher in tumor tissues than in normal pancreatic tissues, though the differences were not statistically significant ($P = 0.061$ and $P = 0.196$**;** **Figure S8**).

TABLE 3 Analysis of associations between PanC risk and the six SNPs in the dataset of PanScan and PanC4 studies.

	Group				
SNP rs ID# & genetic model	**Genotype**	**Case (%)**	**Control (%)**	**OR (95% CI)[a]**	***P*[b]**
MAP2 rs35075084 CT > C[b]					
Additive	TT	8221 (96.49)	6798 (95.60)	1.00	–
	T-	295 (3.46)	310 (4.36)	0.75 (0.64-0.89)	0.0006
	–	4 (0.05)	3 (0.04)	0.99 (0.22-4.44)	0.9871
	Trend test				0.0008
Dominant	T- + –	299 (3.51)	313 (4.40)	0.75 (0.64-0.89)	0.0007
TP53 rs9895829 A > G					
Additive	AA	7729 (90.56)	6315 (88.62)	1.00	–
	AG	788 (9.23)	790 (11.09)	0.83 (0.75-0.93)	0.0006
	GG	18 (0.21)	21 (0.29)	0.73 (0.39-1.37)	0.3285
	Trend test				0.0004
Dominant	AG + GG	806 (9.44)	811 (11.38)	0.83 (0.75-0.92)	0.0004
PRKAG2 rs2727572 C > T					
Additive	CC	2432 (28.50)	2146 (30.00)	1.00	–
	CT	4170 (48.87)	3485 (48.72)	1.09 (1.01-1.17)	0.0283
	TT	1930 (22.62)	1522 (21.28)	1.17 (1.07-1.28)	0.0008
	Trend test				0.0007
Dominant	CT + TT	6100 (71.50)	4980 (69.62)	1.11 (1.04-1.19)	0.0034
PRKAG2 s34852782 TA > Tb					
Additive	AA	4237 (49.98)	3687 (51.98)		
	A-	3492 (41.49)	2893 (40.79)	1.06 (0.99-1.13)	0.1161
	–	749 (8.83)	513 (7.23)	1.29 (1.15-1.46)	< 0.0001
	Trend test				0.0002
Dominant	A- + –	4241 (50.02)	3406 (48.02)	1.09 (1.02-1.16)	0.0076
RPTOR rs62068300 G > A					
Additive	GG	4093 (47.97)	3278 (46.00)		
	GA	3639 (42.65)	3116 (43.73)	0.92 (0.86-0.98)	0.0149
	AA	801 (9.39)	732 (10.27)	0.85 (0.76-0.95)	0.0043
	Trend test				0.0009
Dominant	GA + AA	4440 (52.03)	3848 (54.00)	0.91 (0.85-0.97)	0.0026
RPTOR rs3751936 G > C					
Additive	GG	4887 (25.28)	3886 (27.01)	1.00	–
	GC	3119 (49.55)	2710 (49.40)	0.93 (0.87-1.00)	0.0347
	CC	513 (25.17)	512 (23.59)	0.82 (0.72-0.93)	0.0025
	Trend test				0.0009
Dominant	GC + CC	3632 (74.72)	3222 (72.99)	0.91 (0.86-0.97)	0.0051

Abbreviations: CI, confidence interval; OR, odds ratio; PanC, pancreatic cancer; SNP, single-nucleotide polymorphism.
[a]Obtained from logistic regression models with adjustment for age, sex, and top five significant principal components.
[b]Base deletion.

4 | DISCUSSION

In the present study, we evaluated the associations between genetic variants in the 58 LKB1-AMPK pathway genes and PanC risk, using the two existing GWAS datasets: PanScan I, II/III from PanScan study and PanC4 from Pancreatic Cancer Case-Control Association Study.

Through the meta-analysis, we identified six novel potential susceptibility loci of *MAP2*, *PRKAG2*, *TP53* and *RPTOR* for PanC risk, located at 2q34, 7q36.1, 17p13.1 and 17q25.3, respectively. We further showed that these variants were independently or jointly associated with an increased PanC risk. Further eQTL analysis revealed that those six novel SNPs might influence the mRNA

TABLE 4 Combined risk genotypes of the six SNPs and the risk of PanC.

	Group			
NUG[a]	Case (%)	Control (%)	OR (95% CI)[b]	*P*[b]
0	1 (0.01)	1 (0.01)	1.00	–
1	34 (0.40)	50 (0.71)	1.75 (0.11-29.08)	0.6963
2	459 (5.44)	467 (6.61)	1.48 (0.94-2.34)	0.0911
3	1738 (20.59)	1571 (22.25)	1.66 (1.07-2.59)	0.0253
4	3014 (35.70)	2626 (37.20)	1.75 (1.12-2.72)	0.0133
5	2450 (29.02)	1836 (26.01)	2.04 (1.31-3.18)	0.0016
6	746 (8.84)	509 (7.21)	2.23 (1.42-3.51)	0.0005
Trend test				< 0.0001
0-4	5246 (62.14)	4715 (66.78)	1.00	–
5-6	3196 (37.86)	2345 (33.22)	1.24 (1.16-1.32)	< 0.0001

Abbreviations: CI, confidence interval; OR, odds ratio; PanC: pancreatic cancer; NUG, number of unfavorable genotype; SNP, single-nucleotide polymorphism.

[a]Risk genotypes were rs35075084 TT, rs2727572 CT + TT, rs34852782 A- + —, rs9895829 AA, rs62068300 GG, and rs3751936 GG.

[b]Logistic regression analyses were adjusted for age, sex, and the top five principal components.

expression levels of corresponding genes, particularly true for the *MAP2* rs35075084 and *TP53* rs9895829.

LKB1 was initially identified as a tumor suppressor gene responsible for the familial Peutz-Jeghers syndrome and associated with an increased risk for gastrointestinal tract cancers, including PanC.[41] The AMPK, which is highly conserved in all eukaryotic cells and exists as a trimeric complex consisting of a catalytic subunit (α subunit) and two regulatory subunits (β and γ subunits), plays a role in the regulation of cellular energy homeostasis by maintaining cellular energy homeostasis in response to an increased AMP:ATP ratio and restores energy balance by inhibiting anabolic processes that consume ATP, while promoting catabolic processes that generate ATP.[12,13,17]

Together, the LKB1-AMPK pathway genes serve as a metabolic checkpoint and a central metabolic switch that governs glucose and lipid metabolisms in response to alterations in nutrients and intracellular energy levels.[42,43] Besides, LKB1-AMPK also controls for cell growth in response to environmental nutrient changes. For example, one central downstream pathway suppressed by the LKB1-AMPK pathway is the mammalian target of rapamycin (mTOR) pathway, which controls for cell growth in all eukaryotes; this signaling pathway is inhibited through the AMPK phosphorylation of tuberous sclerosis complex 2 (TSC2) and regulatorily associated protein of mTOR (RPTOR) in conditions of low intracellular ATP levels.[44–47]

Microtubule associated protein 2 (MAP2) is localized primarily in dendrites of neurons and involved in microtubule assembly.[48,49] It has been reported that MAP2 participates in the outgrowth of neuronal processes and synaptic plasticity and controls for selective axonal cargo sorting by regulating kinesin activity.[50] In addition, MAP2 is also involved in the protein kinase A-induced decrease in the invasiveness of glioma cells.[51] In the present study, we found that the *MAP2* rs34852782 deleted allele was associated with PanC risk, likely due to the resultant increase in the mRNA expression.

The protein kinase AMP-activated noncatalytic subunit γ 2 (PRKAG2), as a member of AMPK γ subunit family, mutations in which can cause inappropriate AMPK activation under resting conditions and lead to hypertrophic cardiomyopathy associated with the Wolff-Parkinson-White syndrome.[52] Previous studies revealed a nominal association of *PRKAG2* SNPs with diabetes incidence[53] and suggested that *PRKAG2* variants were involved in feed efficiency traits in beef steers.[54] The present study suggests that SNPs rs2727572 and rs34852782 located in the intron of *PRKAG2* were associated with PanC risk, and the variant-associated gene expression may be the mechanism underlying the observed association, but additional studies are needed to validate this speculation.

Tumor protein p53 (TP53) has many mechanisms of anticancer function and plays a role in apoptosis, genomic stability, and inhibition of angiogenesis.[55] *TP53* somatically mutated in 50%-80% of PanC.[2,56–58] As we mentioned before, SNPs rs9895829 of *TP53* has been previously reported by the AURORA pathway-based analyses. In our present study, we found that SNPs rs9895829 had a significant correlation with a decreased *TP53* mRNA expression level and was associated with PanC risk.

RPTOR is a crucial component of MTORC1 and negatively regulates mTOR.[59] When the intracellular energy level is low, AMPK directly phosphorylates RPTOR at Ser722 and Ser792, reducing mTOR kinase activities.[60,61] One study suggested that SNP rs11868112 of *RPTOR* exhibits a strong association with temperature variables that contribute to climate adaptations.[62] The present study found that SNPs rs62068300 and rs3751936 of *RPTOR* were associated with PanC risk. Because two SNPs (rs60268947 and rs28434589) in high LD ($r^2 > 0.90$) with rs62068300 had a significant correlation with an increased *RPTOR* mRNA expression level in whole blood cells from GTEx, and genetic variants in *RPTOR* are likely to play a role in carcinogenesis of the pancreas.

Although the present study observed associations between six novel genetic variants in the LKB1-AMPK pathway genes and PanC risk, it has also several limitations. First, we had no access to family history in the publically available GWAS datasets, which might have an impact on PanC risk. Second, since we only used the available online tools and eQTL analysis to evaluate function of a particular SNP, further functional investigations are required. Third, we are still not sure which SNP in the LKB1-AMPK pathway genes may have played a major role in or how jointly they may have an impact on PanC risk. Finally, because all selected subjects in the two GWAS studies were from Caucasian populations, the results may not be generalizable to the general populations.

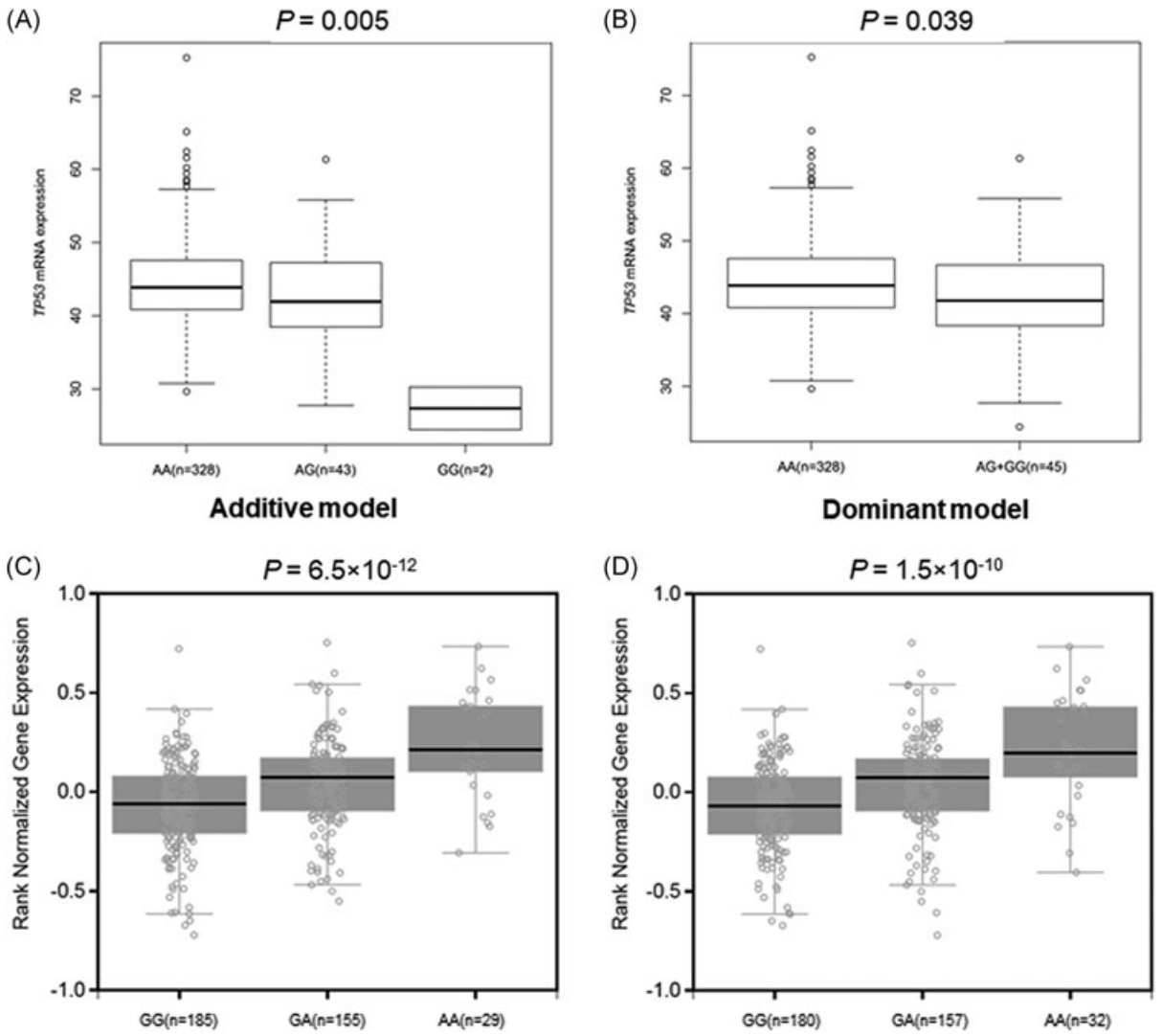

FIGURE 2 The eQTL analyses of the functional SNPs. The eQTL results of the SNP rs9895829 of *TP53* in 373 normal lymphoblastoid cell lines from the 1000 Genomes Project in (A) additive and (B) dominant model and (C,D) GTEx results of the two SNPs (rs60268947 and rs28434589) in high LD with SNP rs62068300 of *RPTOR* in whole blood cells of 369 normal participants. eQTL: expression quantitative trait loci; GTEx: genotype-tissue expression; LD: Linkage disequilibrium; SNP: single-nucleotide polymorphism

In summary, we report some significant associations between genetic variants in 58 LKB1-AMPK pathway genes and PanC risk in European populations. Specifically, six SNPs (i.e., *MAP2* rs35075084 T > deletion, *PRKAG2* rs2727572 C > T and rs34852782 A > deletion, *TP53* rs9895829 A>G, *RPTOR* rs62068300 G > A and rs3751936 G > C) were found to be significantly associated with an increased PanC risk, possibly by influencing their gene expression. More population validations and additional functional studies are needed to explore possible molecular mechanisms in the etiology of PanC.

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PanC4

The patients and controls for this study were derived from the following PANC4 studies:

Johns Hopkins National Familial Pancreas Tumor Registry, Mayo Clinic Biospecimen

Resource for Pancreas Research, Ontario Pancreas Cancer Study (OPCS), Yale University, MD Anderson Case Control Study, Queensland Pancreatic Cancer Study, University of California San Francisco Molecular Epidemiology of Pancreatic Cancer Study, International Agency of Cancer Research and Memorial Sloan Kettering Cancer Center. This study is supported by NCI R01CA154823 Genotyping services were provided by the Center for Inherited Disease Research (CIDR). CIDR is fully funded through a federal contract from the National Institutes of Health to The Johns Hopkins University, contract number HHSN2682011000111. The dbGaP accession number for this study used in this manuscript is phs000648.v1.p1.

TCGA

The results published here are in whole or part based upon data generated by The Cancer Genome Atlas pilot project established by the NCI and NHGRI. Information about TCGA and the investigators and institutions that constitute The Cancer Genome Atlas (TCGA) Research Network can be found at "http://cancergenome.nih.gov". The TCGA SNP data analyzed here are requested through dbGAP (accession#: phs000178.v1.p1).

CONFLICT OF INTERESTS

The author declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

XX, DQ, and HL collected the data, performed the data analysis and wrote the manuscript. QW conceived the idea, supervised the work and wrote the manuscript. SL, KMW, JLA, and XZ co-supervised the work and edited the manuscript. All authors edited, contributed to and approved for the final version of the manuscript.

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SUPPORTING INFORMATION

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

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