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Original Article

Genetic variants in the Hedgehog signaling pathway genes are associated with gastric cancer risk in a Chinese Han population

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Abstract

The Hedgehog signaling pathway participates in the occurrence and progression of cancers including gastric cancer. We conducted this study to evaluate whether genetic variants in the Hedgehog signaling pathway genes would affect gastric cancer risk. Multi-marker Analysis of GenoMic Annotation (MAGMA) was used to investigate the aggregated genetic effects of single nucleotide polymorphisms (SNPs) assigned to candidate genes. The relationship between SNPs and gastric cancer risk was estimated by multivariate logistic regression analyses. Gene expression was calculated using databases obtained from The Cancer Genome Atlas (TCGA) and The Gene Expression Omnibus (GEO). Kaplan - Meier plotter was used to evaluate the association between gene expression with gastric cancer survival. Tumor Immune Estimation Resource 2.0 (TIMER 2.0) was applied to determine the correlation between selected gene expression and the immune cell infiltration degree. We identified that the G allele of rs2990912 in *KIF27* was associated with higher gastric cancer risk, especially in the young and male subgroups. The expression of *KIF27* in gastric cancer tissues was higher than that in normal tissues, leading to poor survival in gastric cancer patients. Besides, *KIF27* expression was related to immune cell infiltration and positively correlated with PD-L1 expression. Our findings highlight the key role of genetic variation in the

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Hedgehog signaling pathway genes in gastric cancer susceptibility, which may provide important insights into the diagnosis, prognosis, and treatment of gastric cancer.

Keywords: gastric cancer, Hedgehog signaling pathway, genetic susceptibility, molecular epidemiology

Introduction

Gastric cancer is the fifth most common cancer globally, with more than 1 000 000 new cases are diagnosed each year and is responsible for more than 8% of all oncological deaths in 2018[1]. The incidence of gastric cancer is the highest in East Asia, especially in China, followed by Central and Eastern Europe[1]. Gastric cancer ranks the second in incidence and mortality in China[2]. Although there is a steady decreasing trend in the incidence and mortality rates of gastric cancer because of the change of dietary habits, decrease in *Helicobacter pylori* infection, and reduction in the smoking prevalence, gastric cancer is still a huge threat to human health due to its nonspecific early stage disease symptomatology and the lack of effective screening methods[3]. More than 80% of patients with gastric cancer are diagnosed as advanced with poor clinical outcomes and the 5-year survival rate is less than 20%[4]. Therefore, studies on the mechanism of gastric cancer occurrence and development may improve the early diagnosis and prognosis of gastric cancer.

Genome-wide association studies (GWASs) have evaluated a large number of single-nucleotide polymorphisms (SNPs) related to complex diseases, including gastric cancer[5–6]. The standard approach of GWAS in single-variant level misses an amount of gastric cancer risk-related SNPs with a moderate effect because of the strict threshold at $P<5\times10^{-8}$. Gene-based and pathway-based analyses have been applied in GWAS to identify SNPs with moderate effects but significantly related to disease by adopting a looser threshold[7–9].

The Hedgehog signaling pathway is an evolutionary conservative pathway that transmits signals from the cell membrane. Its members have been considered to participate in embryonic development, adult tissue homeostasis, tissue repair, and carcinogenesis[10–11]. The Hedgehog signaling pathway mainly consists of the Hedgehog (HH) ligands, which include Sonic hedgehog (SHH), Indian hedgehog (IHH) and Desert hedgehog (DHH), Smoothened (SMO), Patched 1 (PTCH1), Patched 2 (PTCH2), Suppressor of Fused (SUFU), Kinesin family member 7 (KIF7), Kinesin family member 27 (KIF27), GLI family zinc finger 1 (GLI1), GLI family zinc finger 2 (GLI2), GLI family zinc finger 3 (GLI3), and GLI family zinc finger 4 (GLI4)[12]. This pathway exerts its biological function by affecting the balance between GLI activator form (GLIA) and GLI repressor form (GLIR). The Hedgehog signaling pathway is associated with self-renewal, survival, proliferation, and invasion of tumor cells and related to angiogenesis, fibrosis, immune evasion, and neuropathic pain in the tumor microenvironment[13]. GLI2 overexpression induces loss of gastrin and antral hyperplasia[14]. A lower Hedgehog-interacting protein (HHIP) level is positively correlated with gastric cancer metastasis. HHIP can mediate the progression and metastasis of gastric cancer by regulating its promoter methylation level in a feedback manner[15]. miRNA-150 targets Sufu, a tumor suppressor, and subsequently promotes cell proliferation, migration, and epithelial-mesenchymal transition *via* dual activation of Hedgehog and Wnt/β-catenin signaling pathway[16]. GLI mediates mTOR-induced programmed death-ligand 1 (PD-L1) expression in gastric cancer[17]. Apatinib, an oral small molecule tyrosine kinase inhibitor used to treat advanced gastric cancer, inhibits gastric cancer stem cells by blocking the SHH pathway[18]. Several studies have reported that genetic variants in the Hedgehog signaling pathway genes can influence the risk of various cancers such as bladder cancer[19–20], but most of them only concentrate on a few genes, and the existing evidence for the influence of SNPs in the Hedgehog signaling pathway genes on gastric cancer susceptibility in Chinese population is limited.

In this study, we used a comprehensive strategy to screen the effective SNPs in key genes of Hedgehog signaling pathway using bioinformatics tools and functional analysis, and explored the possible mechanisms of the risk SNPs and corresponding host genes involved in gastric oncogenesis.

Materials and methods

Study subjects and genotyping

Gastric cancer GWAS data (phs000361. v1. p1)

was downloaded from the database of Genotype and Phenotype. The Illumina 660W Quad chip was used for genotyping. The nongenotyped variants were imputed on the basis of the 1000 Genomes Project using IMPUTE for the gastric cancer GWAS[21]. A total of 3725 individuals (1625 gastric cancer cases and 2100 controls) were included in this study. This population was derived from 4987 samples of the case-control and case-only components of the Shanxi Upper Gastrointestinal Cancer Genetics Project (Shanxi). The population characteristics were described previously[22] and characteristic information was summarized in ***Supplementary Table 1*** (available online).

Steps for selecting genes and SNPs

We selected 31 key Hedgehog signaling pathway genes from Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/) and the previously published studies. Gene position was obtained from UCSC Genome Browser (***Supplementary Table 2***, available online, https://genome.ucsc.edu/). A total of 17 962 SNPs in the 31 reference gene regions were genotyped. ***Fig. 1*** shows SNPs screening process. Firstly, we determined 3164 eligible SNPs in 27 genes after the quality control using the criteria as: minor allele frequency (MAF) >0.05, P>0.05 for Hardy-Weinberg equilibrium (HWE), and call rate >95%. Then, we evaluated the aggregated single-variant effect into each candidate gene on gastric cancer risk using Multi-marker Analysis of GenoMic Annotation (MAGMA)[23]. MAGMA is a tool for gene-based and gene-set pathway analysis with GWAS data. The Z-value of MAGMA reflects the strength of the association between each gene and the phenotype, and a higher value corresponds to a stronger association. Finally, we predicted the function of SNPs located at risk genes using Regulome DB (https://regulomedb.org/regulome-search/), SNP info Web Server (https://manticore.niehs.nih.gov/), and HaploReg (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php), and selected the tagged SNPs using pair-wise linkage disequilibrium (LD) analysis based on the GWAS data ($r^2 \geqslant 0.8$). SNPs with a score higher than 5 in Regulome DB were removed.

Expression analysis

Gene expression data, genotype data, cancer pathological feature, and *H. pylori* infection information of patients with gastric cancer were downloaded from The Cancer Genome Atlas (TCGA) stomach adenocarcinoma (STAD) datasets. Gene expression information obtained from the Gene Expression Omnibus (GEO) database (GSE66229, GSE13911, and GSE37023) was used to analyze the expression pattern of genes in gastric tumor and normal tissues. The GTEx dataset (https://www.gtexportal.org/home/) was used to analyze the expression quantitative trait loci (eQTL) for candidate SNPs on genes. The influence of gene expression on

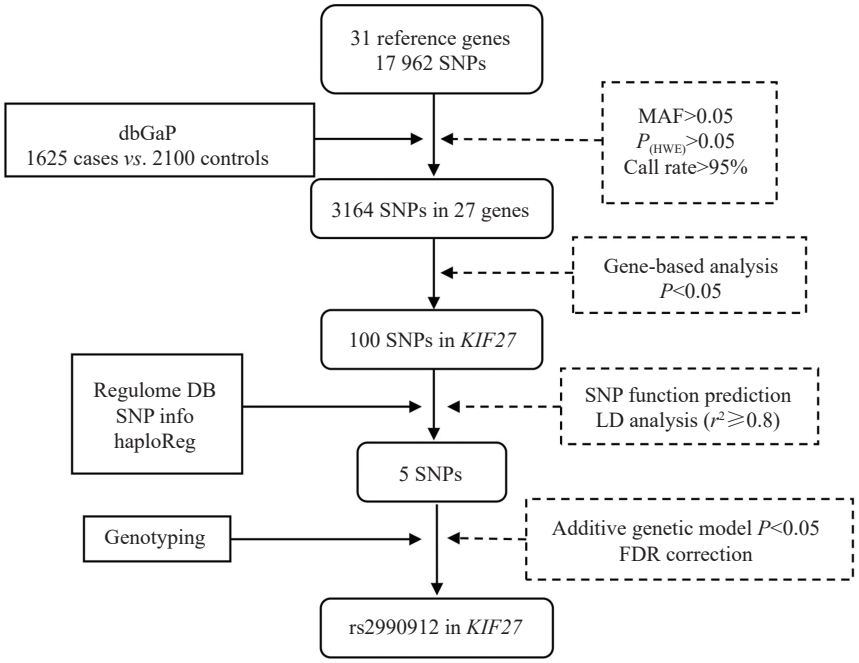

Fig. 1 **Flow diagram.** Flowchart for selecting SNPs in the Hedgehog signaling pathway. MAF: minor allele frequency; HWE: Hardy-Weinberg equilibrium; LD: linkage disequilibrium; FDR: false discovery rate.

gastric cancer patient's survival was measured by Kaplan-Meier plotter. Correlation analysis was conducted by the Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/).

Immune infiltration analysis

Tumor Immune Estimation Resource 2.0 (TIMER2.0) was used to explore the correlation between candidate gene expression and immune cell infiltration degree. This tool is useful for systematic analysis of the immune infiltration across pan cancers, providing the abundance of immune infiltrates estimated by a variety of immune deconvolution methods according to the expression profiles of TCGA tumors. TIMER, xCell, MCP-counter, CIBERSORT, EPIC, and quantTIseq were used to estimate the level of immune infiltration. The "Gene" module was used to analyze the correlation between gene expression and the level of immune infiltration in gastric cancer. The analysis results were visualized by scatter plots. By selecting the "Purity Adjustment" option, partial Spearman correlation was used for correlation analysis, and the Rho value was performed to indicate the degree of correlation. The "Gene_Corr" module was used to explore the correlation between the selected genes and immune markers in gastric cancer.

Statistical analysis

The distributions of variables in gastric cancer cases and controls were assessed using a Student's *t*-test. A Chi-square test was performed to analyze HWE in controls. Odds ratios (ORs) and their 95% confidence intervals (CIs) were examined using multivariate logistic regression analyses to evaluate genetic variants of SNPs on gastric cancer risk. The covariates of age and sex were adjusted. False discovery rate (FDR) correction was performed to adjust *P* values. The two-sided Mann-Whitney test was utilized to compare the significance of different gene expression between gastric tumor and normal tissues. R (version 3.5.1) and PLINK (version 1.90) were used to perform all statistical analyses.

Results

Gene-set analysis and SNP selection

The mechanism of 31 genes involved in the Hedgehog signaling pathway is presented in ***Supplementary Fig. 1*** (available online). Steps for selecting SNPs are shown in ***Fig. 1***. In terms of gene-set analysis using MAGMA for genes in the Hedgehog signaling pathway, we identified that *KIF27* harboring 100 SNPs (Z=1.88, P=0.03; ***Fig. 2*** and ***Supplementary Table 3***, available online) showed the strongest relationship with the gastric cancer susceptibility. After function prediction based on Regulome DB, SNP info Web Server, HaploReg, and LD analysis, only 5 SNPs were kept for further investigation (***Supplementary Table 4***, available online).

In single-variant genetic association analysis, rs2990912 and rs2065516 were detected to be significantly correlated with gastric cancer susceptibility in the additive model. After FDR correction, only rs2990912 in *KIF27* was related to gastric cancer susceptibility (P=2.77×10^{-2}; ***Table 1***).

Association between SNP rs2990912 of *KIF27* and the risk of gastric cancer

We investigated the significance of the association between SNP rs2990912 with gastric cancer risk using additive, dominant, codominant, and recessive genetic effect models. The frequencies of AA, AG and GG were 83.4%, 16.0%, and 0.6% in gastric cancer cases and 86.6%, 13.1%, and 0.3% in controls, respectively. More individuals carried G allele were observed in cases than in controls compared with the AA genotype in the dominant model (OR, 1.29; 95% CI, 1.07–1.55; P=7.39×10^{-3}, ***Table 2***), indicating that individuals who carried rs2990912 G allele had a 1.29-fold increased risk of gastric cancer.

Further stratification analyses were performed according to age and gender. The results showed that individuals carried G allele in rs2990912 had a prominently increased gastric cancer risk compared with subjects carried A allele in the subgroups of younger individuals with an age less than 60 years (OR, 1.43; 95% CI, 1.10 –1.86; P=7.56×10^{-3}) and males (OR, 1.27; 95% CI, 1.03 –1.56; P=7.39×10^{-3}, ***Table 3***). We further conducted Kaplan-Meier survival analysis using rs2990912 genotype and clinical data obtained from TCGA to evaluate whether rs2990912 played a role in gastric cancer patients' survival. As shown in ***Supplementary Fig. 2*** (available online), rs2990912 did not change the survival of gastric cancer patients.

Gene expression in gastric tumor and normal tissues

We evaluated the expression pattern of *KIF27* in gastric tumor and normal tissues using TCGA and GEO datasets, and found that *KIF27* expression in tumors was significantly higher than that in normal

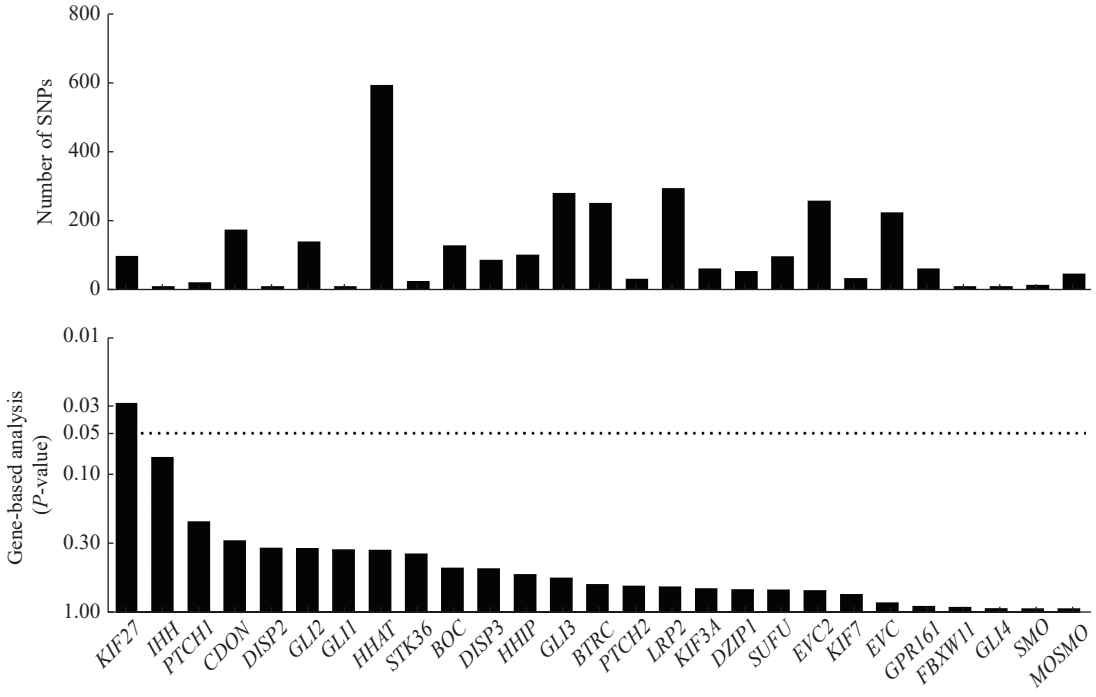

Fig. 2 **Gene-set analysis.** Gene-set analysis using MAGMA for genes in the Hedgehog signaling pathway. The top panel shows the SNP numbers located in genes; and the bottom panel shows the *P*-value of genes. SNPs: single nucleotide polymorphisms; MAGMA: Multi-marker Analysis of GenoMic Annotation.

Table 1 **Association between two significant SNPs in the Hedgehog signaling pathway and gastric cancer risk**

Chr	SNP	Gene	Allele[a]	MAF (case)	MAF (control)	OR (95% CI)[b]	*P*[b]	*P*[c]
9	rs2990912	*KIF27*	A/G	0.09	0.07	1.29(1.08–1.54)	5.53×10^{-3}	2.77×10^{-2}
9	rs2065516	*KIF27*	C/T	0.44	0.47	0.90(0.82–0.99)	2.84×10^{-2}	7.10×10^{-2}

[a]Reference allele/effect allele; [b]*P*-values for additive model adjusted for sex and age in logistic regression model; [c]*P*-values after false discovery rate correction. Chr: chromosome; MAF: minor allele frequency; OR: odds radio; CI: confidence interval.

Table 2 **Association between rs2990912 in KIF27 and gastric cancer risk**

Genotypes	Cases		Controls		OR (95% CI)[a]	*P*[a]
	N	%	*N*	%		
AA	1324	83.4	1761	86.6	1.00	
AG	253	16.0	266	13.1	1.27 (1.06–1.54)	1.16×10^{-2}
GG	9	0.6	6	0.3	1.95 (0.69–5.51)	2.10×10^{-1}
Additive model					1.29 (1.08–1.54)	5.53×10^{-3}
Dominant model (AG+GG *vs.* AA)					1.29 (1.07–1.55)	7.39×10^{-3}
Recessive model (GG *vs.* AG+AA)					1.88 (0.66–5.32)	2.35×10^{-1}

[a]ORs and *P*-values were adjusted for age and sex in logistic regression model. OR: odds radio; CI: confidence interval.

tissues (***Fig. 3***). We observed the expression of *KIF27* in the pathological characteristics of gastric cancer using clinical data obtained from TCGA and found that *KIF27* expression was significant different in tumor stage Ⅱ and Ⅲ (***Supplementary Fig. 3***, available online)**.**

Additionally, we assessed the impact of SNPs on the *KIF27* expression in different tumor grades and stages using data obtained from TCGA database and performed the eQTL analysis using the GTEx database. As shown in ***Supplementary Fig. 4*** (available online), rs2990912 genotype did not affect

Table 3 **Stratified analysis for rs2990912 and gastric cancer risk in additive model**

Variables	rs2990912 (cases/controls)						OR (95% CI)[a]	*P*[a]
	AA		AG		GG			
	N	%	*N*	%	*N*	%		
Age (years)								
<60	591/840	83.0/87.1	115/123	16.2/12.8	6/1	0.8/0.1	1.43 (1.10–1.86)	7.56×10^{-3}
≥60	733/921	83.9/86.1	138/143	15.8/13.4	3/5	0.3/0.5	1.17 (0.92–1.49)	1.94×10^{-1}
Sex								
Male	1029/1197	83.8/86.7	191/180	15.6/13.0	8/4	0.6/0.3	1.27 (1.03–1.56)	7.39×10^{-3}
Female	295/564	82.4/86.5	62/86	17.3/13.2	1/2	0.3/0.3	1.34 (0.95–1.89)	9.32×10^{-2}

[a]ORs and *P*-values for additive model adjusted for sex and age in logistic regression model. OR: odds radio; CI: confidence interval.

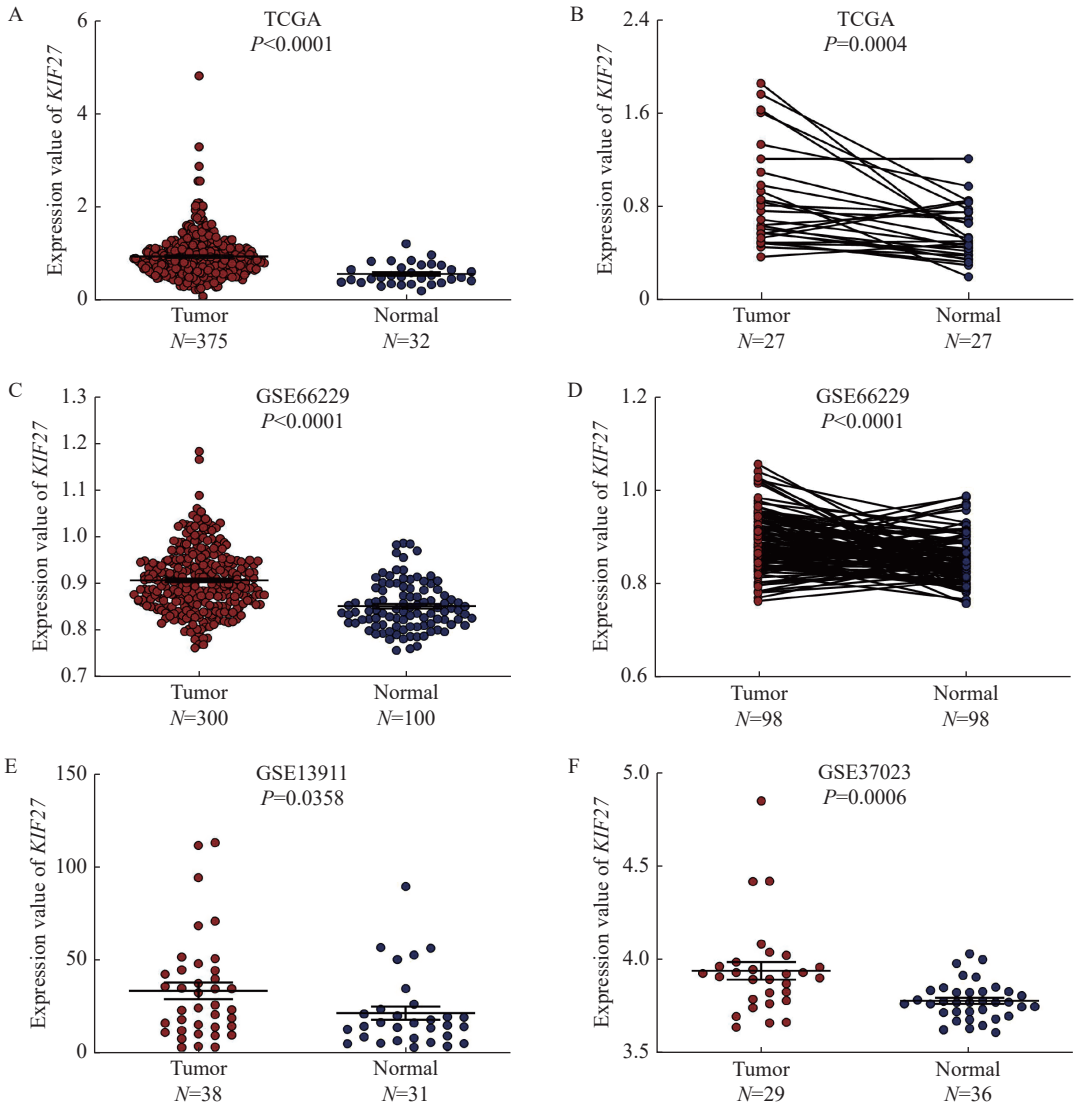

Fig. 3 **Expression of *KIF27* in cancer and normal tissues.** Differences in *KIF27* expression between normal tissues and gastric tumor tissues based on TCGA database (A and B), GSE66229 (C and D), GSE13911 (E), GSE37023 (F). *N*: number of gastric tumor or normal tissues. *P*-values were calculated using two-sided Mann-Whitney test.

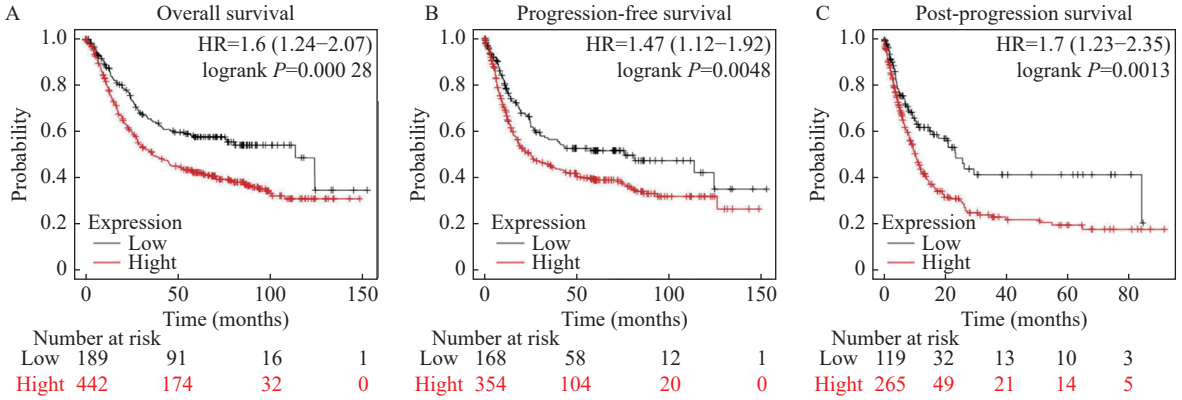

Fig. 4 **Association of *KIF27* expression with patients' survival.** Kaplan-Meier survival curve of gastric cancer patients according to the *KIF27* mRNA status by using Kaplan-Meier Plotter, an online survival analysis tool (http://kmplot.com/analysis/) of overall survival (A), progression-free survival (B), and post-progression survival (C).

KIF27 expression in each grade and stage. Although there was no eQTL of rs2990912 on *KIF27* in gastric tissues (***Supplementary Fig. 5A***, available online), we observed that rs2990912 G allele could increase *KIF27* expression in the tissues of the digestive system including liver tissues (***Supplementary Fig. 5B***, available online; P=9.60×10^{-3}) and small intestine tissues (***Supplementary Fig. 5C***, available online; P=5.30×10^{-3}). Moreover, the regulation of rs2990912 on the expression of *KIF27* in gastric tissues was consistent with that in other digestive system tissues (***Supplementary Fig. 6***, available online).

We further explored the effect of *H. pylori* infection on the expression level of *KIF27* and found no significant difference (***Supplementary Fig. 7A***, available online). In addition, *KIF27* expression did not change significantly according to the SNP genotype regardless of the presence or absence of *H. pylori* infection (***Supplementary Fig. 7B***, available online).

Effect of *KIF27* expression on gastric cancer patient survival

Kaplan-Meier plotter was applied to assess whether *KIF27* expression would affect the survival of gastric cancer patients. Individuals with higher *KIF27* expression had prominently shorter overall survival time (HR, 1.6; 95% CI, 1.24 –2.07; P=2.80×10^{-4}, ***Fig. 4A***), worse progression-free survival (HR, 1.47; 95% CI, 1.12–1.92; P=4.80×10^{-3}, ***Fig. 4B***), and poor post-progression survival (HR, 1.7; 95% CI, 1.23 –2.35; P=1.30×10^{-3}, ***Fig. 4C***). Further, in the stratified analysis on clinicopathological features, we observed that the effects of *KIF27* expression on survival were significant in the subgroups of male, stage T2, stage T4, stage N1, stage M0, Lauren classification, and differentiation (***Supplementary Table 5***, available online).

Association between *KIF27* expression and immune infiltrates

We next investigated the relationship between *KIF27* expression and immune cell infiltration degree in gastric cancer *via* the TIMER2.0 web server. The results showed that *KIF27* expression was significantly associated with the infiltration of several immune cells, including CD8$^+$ T cells (Rho=0.222, P=1.34×10^{-5}), CD4$^+$ T cells (Rho=0.398, P=8×10^{-16}), macrophages (Rho=0.144, P=5.13×10^{-3}), Tregs (Rho=0.295, P=4.93×10^{-9}), and neutrophils (Rho=0.196, P=1.19×10^{-4}, ***Supplementary Fig. 8***, available online).

The relationship between *KIF27* expression and the expression level of immune markers was further explored. As shown in ***Supplementary Fig. 9*** (available online), *KIF27* expression was related to the expression level of several immune markers, including those specific to monocytes, TAM, M1 macrophage, and M2 macrophage. In addition, the result of correlation analysis showed that *KIF27* expression was related to PD-L1 expression (Rho=0.24, P=3.2×10^{-9}, ***Supplementary Fig. 10***, available online).

Discussion

The Hedgehog signaling pathway contributes to tumorigenesis. Abnormal activation of the Hedgehog signaling pathway causes the destruction of gastric cell differentiation, loss of gastric acid secretion, and tumor transformation[24]. Dysregulation of the Hedgehog signaling leads to the invasion and

metastasis of gastric cancer and the obstruction of it reduces the proliferation of tumor cells[25]. The signaling molecules, such as *SHH*, *IHH*, *PTCH1*, and *GLI1*, are used as biomarkers in clinical oncology. The small molecule compounds targeting SMO and humanized anti-SHH antibodies are considered to be effective anti-cancer drugs for gastric cancer[12]. Previous studies have shown that genetic variants in the Hedgehog signaling pathway genes were related to the occurrence and development of diseases. The polymorphism of the *GLI1* gene SNP rs2228226 was closely related to the risk of chronic lymphocytic leukemia in the Chinese population[26]. *GLI3* genetic variants were associated with overall bladder cancer risk, and two *GLI2* SNPs and one *SHH* SNP were significantly associated with the overall survival of patients with muscle-invasive and metastatic bladder cancer[19]. The GG genotype of the *HHIP* genetic variant rs1489759 had a protective effect on chronic obstructive pulmonary disease and lung cancer[20]. Most of these studies focused on a few key genes in the Hedgehog signaling pathway, such as *GLI*, *HH*, and *HHIP*. Yet, the association between the genetic variation with gastric cancer susceptibility has rarely been reported. Although genetic variants of *GLI1* were associated with inflammatory bowel diseases susceptibility[27], we did not find a correlation between genetic variants of these reported genes and the risk of gastric cancer. Given that the Hedgehog signaling pathway plays an important role in gastrointestinal tract development, homeostasis, and malignancy, we continued to evaluate the association of the genetic variants of 31 Hedgehog signaling pathway-related genes with gastric cancer susceptibility. We found that the G allele in *KIF27* was correlated with a higher gastric cancer risk, especially in the younger and male subjects.

KIF27, kinesin family member 27, is located at human chromosome 9q22.1. *KIF27* and *KIF7* are homologs of Drosophila Cos2 in human. *KIF27* is most homologous to Cos2, sharing the common domain structure with Drosophila Cos2, while KIF7 shares only part of domain structure with Drosophila Cos2[28–30]. In Drosophila, Fu (STK36) and Cos2 regulate the cell surface accumulation and signal transduction activity of SMO by regulating SMO phosphorylation. Cos2 interacts with SMO to prevent HH-induced SMO phosphorylation, while Fu promotes SMO phosphorylation by antagonizing Cos2[31]. SMO activity plays a very important role in the downstream cascade activation by blocking GLIR and promoting GLIA[32]. Despite the conflicting reports about the role of KIF7 and KIF27 in Hedgehog signaling in vertebrates[33–34], KIF27 retains its partnership with Fu and participates in the recruitment and/or activation of proteins along the center of the cilia[35]. Evidence showed that low-level expression of KIF7 was associated with the poor prognosis of epithelial ovarian cancer and KIF7 inhibited prostate cancer development as a negative regulator[36]. It is reported that *KIF27* was involved in leukemogenesis and was a component of a minimal deleted region (MDR) in acute myeloid leukemia[37]. There has been no report about the association of *KIF27* with gastric cancer. We observed *KIF27* was significantly increased in gastric tumors compared with normal tissues. *KIF27* may be a participant in the occurrence and development of gastric cancer.

Evidence has shown that SHH is a gastric morphogen and can initiate gastritis in response to *H. pylori* infection[38]. Studies have reported that the Hedgehog signaling pathway regulates the expression of PD-L1 and may affect the anti-tumor activity of lymphocytes[39]. Furthermore, data have suggested HH/GLI inhibitor can drastically reduce PD-L1 expression and inhibit the proliferation of tumor cells in gastric cancer[40]. Given the important role of this signal in cancers, we assessed the potential impact of *KIF27* on the survival of patients with gastric cancer and found that elevated *KIF27* was related to poor gastric outcomes. Furthermore, elevated *KIF27* expression in gastric cancer was related to a higher N stage HR in OS (***Supplementary Table 5***). The results showed that *KIF27* expression was associated with immune infiltration degree in gastric cancer. We further found that *KIF27* expression was related to marker expression of different immune cell subsets. These results suggested *KIF27* may interact within the tumor microenvironment. Although our results showed that *H. pylori* infection did not affect the expression pattern of *KIF27*, we found *KIF27* was positively correlated with *PD-L1*. Thus, further functional studies are needed to determine the biological mechanisms of *KIF27* and genetic variants involved in the occurrence and development of gastric cancer.

In summary, we identified a genetic variant rs2990912 in *KIF27*, which may influence gastric cancer susceptibility in the Chinese Han population. *KIF27* and the genetic variants in it are potential

biomarkers for the assessment of gastric cancer risk and may hold important value for the diagnosis, prognosis, and therapy of gastric cancer.

Acknowledgments

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