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Prognostic significance of GRINA in Colorectal cancer

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Abstract

Background: Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate-Associated Protein 1 (GRINA), also named TMBIM3, has been deemed to have oncogenic activities in gastric cancer. However, the role and molecular mechanisms of GRINA in colorectal cancer (CRC) remain largely unknown. We evaluated its prognostic value in CRC by an integrated bioinformatics analysis and experimental verification.

Methods: Patients with CRC were derived from TCGA and GTEx databases. We first confirmed the expression profiles of GRINA in human cancers and compared GRINA expression between CRC and normal colorectal tissues by Wilcoxon rank sum test and Wilcoxon signed rank test. χ^2 test and logistic regression analysis was used for correlation analysis, and Wilcoxon rank sum test or Kruskal-Wallis rank sum was performed to evaluate the connection between GRINA expression and clinical features. Furthermore, Kaplan-Meier's analysis and Cox regression analysis were used to assess the correlation between GRINA expression and clinical outcomes. Colony formation and wound-healing assays were performed to observe the impact of GRINA on cell proliferation and motility. GO annotation, PPI network and GSEA were utilized to explore the underlying function of GRINA. Finally, we analyzed the correlation of GRINA in CRC with immune infiltration levels by ssGSEA.

Results: We found that GRINA expression was upregulated in diverse human cancer types and especially in CRC. Higher GRINA expression was closely related to unfavorable clinicopathologic features and clinical outcomes. GRINA overexpression promotes CRC proliferation and migration in vitro. Functional enrichment analyses suggested that GRINA is involved in the most significant terms including anion transmembrane transport, cell proliferation related pathways, metabolic pathways and DNA damage and repair related pathways. GRINA expression was most positively correlated with NK CD56dim cells and

negatively associated with Th2 cells through analyzing GRINA expression and immune infiltration.

Conclusion: GRINA may be a potential prognostic biomarker and therapeutic target in CRC. Moreover, GRINA might accelerate CRC development and EMT by regulating the crosstalk of WNT and MYC signaling which contribute to the unfavorable survival rate and adverse immune infiltration in CRC patients.

Introduction

Colorectal cancer (CRC) is the third leading cause of morbidity and mortality in both sexes worldwide^{1,2}, causing a heavy economic and national burden. In 2020, there will be an estimated 104,610 newly diagnosed cases and more than fifty thousand cancer-related death, respectively². Notably, in adults, the incidence rate is increasing rapidly and accounts for approximately 10% per year^{2,3}. CRC includes major subtypes of colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ). Current therapeutic strategy of CRC recommended by NCCN guidelines mainly includes curative surgical resection for early stage, systemic chemoradiotherapy and targeting therapy for advanced stage or tumor recurrence⁴. However, the distant metastasis, relapse of disease and chemotherapy resistance are often occurred, leading to treatment failure⁵. In addition, due to the high heterogeneity, complicated pathogenesis and insidious lesion site of CRC, it is difficult to diagnose in the early stage. Despite continuous improvements in surgery, progressed imaging techniques and new drugs development, inefficient early diagnosis rate may result in limited five-year survival rate of CRC⁶. Hence, it is urgent to discover more effective biomarkers for early diagnosis, therapeutic strategies and prognostic assessment.

Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate-Associated Protein 1 (GRINA), located on chromosome 8q24.3, is a member of N-methyl D-aspartate (NMDA) receptors. NMDA receptors are involved in a variety of nervous system diseases such as cerebral ischemia, major depressive disorder (MDD) and schizophrenia (SCZ), which control the pivotal process of neuronal cell death or neurotransmitters release⁷. Recently, some investigators have revealed that NMDA receptors are also intimately linked to a tumor progression of multiple cancer types. In CRC, blocking NMDA receptors can inhibit aerobic glycolysis to weaken the viability and migration of colon cancer cells⁸. The VEGF expression and migration ability of pancreatic cancer cells are curbed through inhibition of NMDA receptor *in vivo* and *in vitro*⁹. Blocking NMDA receptor promotes tumor cell apoptosis in breast cancer or small cell lung cancer^{10,11}, and NMDA receptor is also considered to be a potential therapeutic target for ovarian cancer¹². GRINA, also known as TMBIM3, belongs to the Transmembrane BAX Inhibitor Motif Containing (TMBIM) family which are a group of cell death regulators¹³. Recent studies have shown that GRINA can inhibit apoptosis of gastric cancer cells and modulate aerobic glycolysis to facilitate tumor progression in gastric cancer¹⁴. However, the role of GRINA in CRC has not been developed.

Thereby, present research aimed to evaluate the prognostic value of GRINA expression in CRC with publicly available RNA-seq data retrieved from The Cancer Genome Atlas (TCGA) database portal (<https://cancergenome.nih.gov/>) and Genotype-Tissue Expression (GTEx) project (<https://www.gtexportal.org/home/datasets>). Our findings indicated that GRINA is expressed at high levels and correlated with poorer clinical outcomes in CRC. The potential

molecular mechanisms of GRINA were elucidated using GO annotation, PPI network and GSEA analyses. Furthermore, we explored the molecular pathogenesis may be involved by analyzing the GRINA expression with the immune infiltration level in CRC. Taken together, our data implicated that GRINA could serve as an essential prognostic biomarker and therapeutic target for CRC by bioinformatics analysis.

Materials and methods

RNA sequencing information from TCGA and bioinformatics analysis

TCGA data was initially utilized to analyze the association between differential expression and clinical prognosis. Those RNA-seq data without clinical information were excluded. Finally, a large cohort of COAD and READ dataset was analyzed by patients (n = 619, Workflow Type: level 3 HTSeq-FPKM) with corresponding clinical features were extracted from TCGA (<https://portal.gdc.cancer.gov/>). Subsequently, level 3 HTSeq-FPKM data were transferred into TPM (transcripts per million reads) for followed data analysis. Some clinical information in 619 cases was unavailable or unknown, which was taken as missing values. Median value served as cut-off point for dividing high and low expression groups of GRINA in tumor samples. Our research complied with the publication guidelines supported by TCGA.

Statistical analysis

All statistical analyses were assessed and exhibited using R (v.3.6.2). To compare the GRINA expression between CRC and normal colorectal tissues, Wilcoxon rank sum test was used in two independent samples and Wilcoxon signed rank test was performed in paired samples. For the association between GRINA expression and clinical pathologic characteristics, Wilcoxon rank sum test, Kruskal-Wallis test and logistic regression were selected to calculate continuous variables, while Pearson chi-square test (χ^2) statistic or Fisher's exact test to analyze categorical variables across groups. Survival curves of CRC patients in TCGA were structured using Kaplan-Meier method and determined with log-rank statistical analysis. Univariate and multivariate Cox regression models were constructed to assess the influence of GRINA expression on overall survival (OS) along with other clinical features (stage, CEA level, history of colon polyps, colon polyps present, neoplasm type, age, gender, weight, height, TP53 status, KRAS status and BRAF status). Results of Cox regression models were displayed as the hazard ratio (HR) with 95% confidence interval (CI). Spearman correlation was taken to analyze the correlation between GRINA and the infiltration of immune cells. Wilcoxon rank sum test was applied to evaluate the immune cells infiltration levels between different groups (high and low) of GRINA. All hypothesis tests were two-tailed with a significance cut-off at 0.05.

Establishment and evaluation of the Nomogram for CRC prognostic model

Based on the independent prognostic factors identified by multivariate analysis, the nomogram for CRC prognostic model was established using R packages rms and followed by a calibration curve to assess the prognostic accuracy. A risk score (RS) was calculated for each CRC patient by the risk assessment system. CRC patients in the TCGA cohort were dichotomized into high and low risk groups judging from the Median value of RS. Two-tailed tests with *P*-values less than 0.05 were considered statistically significant.

Colony formation assay

Cells were plated onto six-well culture plates, and incubated in a 10% FBS - containing medium for about 10 days. The colonies were fixed in methanol for 10 minutes and stained by crystal violet for 20 minutes, then observed under a microscope and photographed.

Wound-healing assay

Cells were cultured in six-well plates and cultured until 24 h. Then the monolayers were disrupted with a plastic pipette tip. Photographs were taken when cells migrated into the wounded area and three fields of each point were recorded.

Identifying Differentially Expressed Genes (DEGs) of GRINA in CRC

Gene expression profiles quantified by HTSeq-Counts were first compared between GRINA high and low groups in CRC, and DESeq2 package¹⁵ was further applied to identify DEGs. The volcano plot and heatmap drawn by R, presented meaningful DEGs in CRC between GRINA high and low expression groups. Log fold change (logFC) > 2.0 and adjusted $P < 0.05$ were set as the cut-off criterion for DEGs.

GO functional annotation of DEGs and Gene set enrichment analysis (GSEA) pathway analyses

To better investigate and visualize biological functions behind DEGs, Gene ontology (GO) annotation was utilized to annotate biological processes (BPs), cellular components (CCs) and molecular functions (MFs) of genes. signaling pathways. GSEA was performed to analyze the relevant signaling pathways by considering Hallmark gene sets in the Molecular Signatures Database (MSigDB) as the reference gene sets. R package clusterProfiler (3.6.0)¹⁶ was carried out for GO and GSEA to analyze functional annotation and pathways. FDR q-value < 0.25, adjusted p -value < 0.05 and NES > 1 were deemed statistically significant.

Protein-protein interaction (PPI) network of DEGs construction

Metascape (<http://metascape.org>)¹⁷ which is a free and comprehensive tool for gene annotation was selected to perform PPI enrichment analysis from the following databases: BioGrid, InWeb IM, and OmniPath. Further, Molecular Complex Detection (MCODE) algorithm was applied to identify densely connected network regions.

Immune infiltration analysis by single-sample Gene Set Enrichment Analysis (ssGSEA)

To elucidate the correlation of GRINA expression in CRC with immune infiltration levels, 24 types of tumor infiltrating immune cells were inferred from CRC gene expression profiles, including NK cells, B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells et al. GSVA package (<http://bioconductor.org/packages/stats/bioc/GSVA/>) in R¹⁸ was performed in ssGSEA to quantify immune infiltration of CRC.

Results

GRINA expression profiles in human cancers

To predict the GRINA expression patterns in human cancers, we downloaded the RNA-seq data of multiple malignancies from TCGA and GTEx databases which were uniformly processed into TPM using the Toil workflow¹⁹ on UCSC XENA Browser (<https://xenabrowser.net/datapages/>). We first analyzed the transcriptional levels of GRINA across 33 types of cancers from TCGA and normal tissues (TCGA + GTEx) using Wilcoxon rank sum test. As displayed in Fig. 1A, GRINA expression was markedly upregulated in majority of cancer types, such as urothelial bladder cancer (BLCA), breast cancer (BRCA), cervical squamous cell carcinoma (CESC), cholangiocarcinoma (CHOL),

COAD, esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck cancer (HNSC), kidney renal clear cell carcinoma (KIRC), papillary renal cell carcinoma (KIRP), acute myeloid leukemia (LAML), brain lower grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), READ, skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumor (TGCT), uterine corpus endometrial carcinoma (UCEC) and uterine carcinosarcoma (UCS). In the comparison of pan-cancer cohort and normal tissues based on TCGA, GRINA expression was significantly increased in BLCA, BRCA, CHOL, COAD, ESCA, HNSC, KIRC, KIRP, LIHC, READ, STAD, thyroid carcinoma (THCA) and UCEC (Fig. 1B).

Next, we explored the GRINA expression status in CRC, as Fig. 1C shown, GRINA mRNA expression was elevated in all TCGA CRC types ($n = 619$) compared to TCGA and GTEx normal ($n = 667$) ($p < 0.001$). Based on TCGA, GRINA was still overexpressed in 619 CRC tissues contrasted with 51 normal colorectal tissues ($p < 0.001$) as seen in Fig. 1D. Meanwhile, Fig. 1E also exhibited the identical result that GRINA expression in CRC tissues ($n = 50$) was higher than that in paired adjacent colorectal tissues ($n = 50$) ($p < 0.001$). Additionally, we distinguished CRC tissues from adjacent nontumor tissues to assess the efficacy of GRINA expression by receiver operating characteristic (ROC) curve. As expected, GRINA expression suggested an effectively discrimination ability with an area under the ROC curve of 0.96 (Fig. 1F). The THPA database demonstrated that the cytoplasmic membrane localized GRINA was highly expressed in CRC tissue compared with normal tissue using Immunohistochemistry (IHC) analysis (Fig. 1G). Taken together, we could observe that GRINA expression was often overexpressed in diverse human cancer types and especially in CRC.

Association between GRINA expression and clinicopathologic features in CRC

Initially, RNA-seq data with clinical information of CRC samples were extracted from TCGA. A total of 619 CRC patients with clinicopathological features were eligible for further analysis after excluding those data without available clinical records. The correlation analysis using Pearson chi-square test between GRINA expression and clinicopathological variables in CRC indicated that higher GRINA expression was significantly correlated with N stage ($P = 0.011$), M stage ($P = 0.003$), pathologic stage ($P = 0.001$), neoplasm type ($P = 0.017$), BRAF status ($P < 0.001$), KRAS status ($P = 0.039$) and TP53 status ($P < 0.001$), but not with T stage, gender, CEA level, history of colon polyps, colon polyps present, neoplasm type, age, weight, and height (Table 1). All the variables used in chi-square test are also recruited into logistic regression analysis (Table 2). Likewise, the logistic model identified an increased risk of GRINA expression level among patients with N stage (OR = 1.59 (1.16-2.20) for N1 & N2 vs. N0, $P = 0.004$), M stage (OR = 2.10 (1.31-3.44) for M1 vs. M0, $P = 0.003$), pathologic stage (OR = 1.74 (1.26-2.42) for Stage III & Stage IV vs. Stage I & Stage II, $P < 0.001$), neoplasm type (OR = 0.64 (0.44-0.91) for colon adenocarcinoma vs. rectum adenocarcinoma, $P = 0.014$), BRAF status (OR = 0.34 (0.19-0.60) for Mut vs. WT, $P < 0.001$), KRAS status (OR = 0.68 (0.48-0.97) for Mut vs. WT, $P = 0.032$) and TP53 status (OR = 3.40 (2.36-4.95) for Mut vs. WT, $P < 0.001$).

Furthermore, Wilcoxon rank sum test or Kruskal-Wallis rank sum was conducted to evaluate the connection between GRINA expression and clinical characteristics. As demonstrated in Fig. 2A-G, our results revealed that the upregulated expression level of GRINA was positively associated with advanced N stage ($p = 0.045$), M stage ($p = 0.010$), pathologic stage ($p = 0.003$), rectum adenocarcinoma neoplasm type ($P < 0.001$), BRAF WT status ($P < 0.001$), KRAS WT status ($P = 0.019$) and TP53 Mut status ($P < 0.001$). Our data above implied that GRINA expression was closely related to unfavorable clinicopathologic features.

High GRINA expression in CRC correlates with poor clinical outcomes

Aim to better clarify the clinical significance of GRINA expression involved in CRC patients, as described by Kaplan-Meier's analysis, patients with higher GRINA expression level strongly associated with a shorter overall survival (OS) ($p = 0.022$; Fig. 3A) and disease-specific survival (DSS) ($p = 0.006$; Fig. 3B) in CRC.

Besides, univariate and multivariate Cox regression analyses were applied to identify the risk factors associated with OS and DSS (Table 3). In univariate analysis, T stage (T3 & T4 vs. T1 & T2), N stage (N1 & N2 vs. N0), M stage (M1 vs. M0), Pathologic stage (Stage III & Stage IV vs. Stage I & Stage II), CEA level (> 5 vs. ≤ 5) and a high level of GRINA were significantly associated with a worse OS and DSS, while OS was also affected by age (> 65 vs. ≤ 65). Multivariate Cox regression analysis was subsequently visualized by including variables which were statically meaningful in univariate analysis. M stage was an independent prognostic factor for both OS ($P = 0.035$) and DSS ($P = 0.001$). GRINA expression pattern remained an independent prognostic factor for OS of CRC patients ($P = 0.020$). Furthermore, Pathologic stage ($P = 0.025$) and age ($P < 0.001$) were risk factors for OS.

Based on the four independent prognostic factors identified by multivariate analysis, a nomogram for 1-, 3-, 5-year OS was built. In the nomogram, a higher total number of points predict a worse prognosis. As we have seen in Fig. 3C, the contribution to the OS trend from the age was the maximum, and GRINA expression affected the minimum. The C-index for the nomogram was 0.779 (95% CI: 0.748-0.810). Then a calibration curve was generated to assess the prognostic accuracy (Fig. 3D). The bias-corrected line in the calibration plot was found to be close to the gray line which represents the linear best fit. Collectively, our findings verified that GRINA may serve as a dominating independent prognostic factor for CRC patients.

The prognostic value of GRINA expression profiles for OS in subgroup analysis

As indicated in Fig.4A, further subgroup analyses were carried out to investigate the prognostic value of GRINA expression in patients with CRC who were stratified according to the different diagnostic parameters (T, N stages and Pathologic stage) based on TCGA. HR based GRINA expression signature was proven to be a potential marker for predicting OS in different subgroups, including T3 & T4 (HR = 1.546 (1.069-2.238), $p = 0.021$), N1 Stage (HR = 2.349 (1.111-4.968), $p = 0.025$), II & Stage III & Stage IV (HR = 1.588 (1.090-2.313), $p = 0.016$). Moreover, Kaplan-Meier survival curves for OS were plotted using survminer R package. High expression levels of GRINA presented a worse OS in T3 & T4, N1 Stage and II & Stage III & Stage IV subgroups (Fig.4B-D).

GRINA facilitates CRC proliferation and migration in vitro

To elucidate whether GRINA functions as a tumor promotor, we carried out colony formation and wound-healing assays to see whether it affects cancer cell growth. As shown in Fig.5A,

colony formation ability was promoted by upregulated GRINA in HCT116. Then we undertook wound-healing assay to screen the impact of GRINA on CRC cells migratory properties. GRINA overexpression led to a more rapid healing of scratch wounds (Fig. 5B). Taken together, GRINA was proved to possess strong tumor promotive activity in CRC cells.

GO annotation and PPI network construction of GRINA related DEGs in CRC

Gene expression profiles of CRC in TCGA were first divided into two cohorts by defining the quartile as cut-off point. Setting $|\log FC| > 2.0$ and adjusted $P < 0.05$ as the cut-off criterion, DESeq2 package was further carried out to identify DEGs based on HTSeq-Counts profiles from TCGA. Altogether, 641 DEGs were identified statically significant between GRINA-high and GRINA-low groups, including 110 up-regulated and 531 down-regulated genes. These aberrant DEGs were presented by volcano plot (Fig. 6A). Heatmap drawn by R further showed the top 10 meaningful co-expressed differentially genes between GRINA-high and GRINA-low groups (Fig. 6B).

To better understand the potential functions of GRINA connected DEGs in CRC, GO annotation was employed with clusterProfiler in R. The regulation of GRINA interactive genes mainly participate in one BP, three CCs and top four MFs (Fig. 6C), mainly including anion transmembrane transport, lamellar body, apical plasma membrane, apical part of cell, sulfotransferase activity, vitamin binding, organic acid transmembrane transporter activity, carboxylic acid transmembrane transporter activity.

We further conducted a Metascape PPI enrichment analysis to further probe the relationship between GRINA and CRC. The PPI network of DEGs and two most significant MCODE components extracted from the PPI network are implicated in Fig 6D, E. A subsequent process and pathway enrichment analyses were applied to each MCODE component independently, biological functions were mainly observed in MCODE_1: sulfur compound metabolic process, glycoprotein metabolic process; MCODE_2: G alpha (i) signaling events, GPCR ligand binding, Class C/3 (Metabotropic glutamate/pheromone receptors).

GSEA pathway enrichment analyses of GRINA in CRC based on MSigDB

As many pathways contribute to tumor initiation and maintenance, the poor clinical outcome associated with high GRINA expression may be involved in promoting CRC by activating a number of signaling pathways. GSEA was performed to identify signaling pathways that are differentially activated in CRC by comparing data sets that had low and high expression of GRINA. GSEA revealed significant differences (FDR q-value < 0.25 , adjusted p -value < 0.05 and NES > 1) in enrichment of gene sets collected from MSigDB (h.all.v7.0.symbols.gmt). Only the 20 most significantly upregulated enriched signaling pathways based on NES were listed in supplementary Table 1 and visualized in Fig. 7A-I. GSEA - based pathway analyses suggested that cell proliferation, metabolic, DNA damage and repair related pathways were commonly enriched in GRINA high expression phenotype. Of concern was that (1) cell proliferation related pathways: WNT_BETA_CATENIN pathway, MYC pathway, epithelial mesenchymal transition (EMT), KRAS pathway, IL-2_STAT5 pathway; (2) metabolic pathways: bile acid and xenobiotic metabolism; (3) DNA damage and repair related pathways: reactive oxygen species (ROS), DNA repair. Above all, our results indicated that GRINA may play a potential role in promoting CRC tumorigenesis and progression.

The correlation between GRINA and immune infiltration in CRC

To illustrate the association between GRINA expression and immune infiltration quantified by ssGSEA in CRC, spearman correlation was applied for analysis. GRINA expression was

positively correlated with the abundances of immunocytes (NK CD56dim cells, Treg, NK cells, etc), and negatively associated with Th2 cells, T helper cells, Tcm, etc (Fig. 8A, p -value < 0.01). As assumed in Fig. 8B-G, NK CD56dim cells ($R = 0.307$, $p < 0.001$), Treg ($R = 0.283$, $p < 0.001$), NK cells ($R = 0.193$, $p < 0.001$) presented a positive connection with GRINA expression, whereas Th2 cells ($R = -0.387$, $p < 0.001$), T helper cells ($R = -0.322$, $p < 0.001$), Tcm ($R = -0.285$, $p < 0.001$) was significantly negatively correlated with GRINA expression. Additionally, we compared the immune infiltration levels of NK CD56dim cells, Treg, NK cells, Th2 cells, T helper cells and Tcm between the high and low GRINA expression groups, and the findings were consistent with the above data (Fig. 7H-M).

Discussion

The morbidity and mortality of colorectal carcinoma (CRC) are staying at a high level across the world²⁰. About 60% of CRC patients are diagnosed at a relatively late stage with an average 5-year survival rate of about 13% which seriously threatened the physical and mental health of human beings²¹. In addition, the rising incidence of CRC among young people (before age 50) is an emerging trend^{22,23}. Occurring through three main pathways including adenoma-carcinoma sequence, serrated pathway and inflammatory pathway, CRC can be divided into various subtypes according to tumor anatomical location or overall molecular mutation. Therefore, CRC is a heterogeneous disease in etiology, which increases the difficulty of early detection and effective prevention. With the improvement of molecular biology techniques in recent years, the mining of TCGA database to screen molecular biomarkers affecting the development of CRC has provided a promising insight for early diagnosis before the onset of symptoms and treatment strategy for CRC patients²⁴.

GRINA/TMBIM3 is one of such candidate genes with limited exploration in CRC. All members of the TMBIM family have inhibitory activities in different apoptotic setting, and its underlying mechanisms include death receptor regulation, calcium homeostasis regulation, endoplasmic reticulum stress signaling, autophagy, reactive oxygen species (ROS) production and so on¹³. Study shows that GRINA protein deficiency initiates cold-induced ER stress and cell death by activating an intrinsic apoptotic pathway²⁵. GRINA also been recorded to have oncogenic activities, a pro-survival and anti-apoptotic role in gastric cancer cells by modulating glycolysis metabolism¹⁴. To date, no researches have focused on the role of GRINA in CRC and its contribution to clinical prognosis, biological functions and molecular pathogenesis. Our study provides the preliminary proof for a momentous relationship between GRINA and CRC by integrated bioinformatics analysis.

In our research, using publicly available data, we first revealed GRINA expression is obviously upregulated in majority of cancer types and the increased expression of GRINA was observed in CRC during the unfavorable clinicopathologic features. The prognostic analysis from more than 600 clinical patients further confirmed the prognostic value of GRINA in CRC. GRINA expression pattern was considered to be an independent prognostic factor for OS of CRC patients from Cox regression analyses which followed a nomogram for predicting 1-, 3-, 5-year OS. Finally, we found that higher GRINA expression presented a worse OS in T3 & T4, N1 Stage and II & Stage III & Stage IV subgroups.

For in-depth exploration of molecular mechanism of GRINA, GO annotation and GSEA pathway were performed. GRINA and its associated genes were primarily involved in anion

transmembrane transport, and were structural components of apical part of cell and apical plasma membrane, they also participated in organic acid and carboxylic acid transmembrane transporter activity. Moreover, GSEA - based pathway enrichments indicated that cell proliferation related pathways were most commonly associated with high expression phenotype of GRINA. These findings are consistent with the molecular pathways implicated in CRC carcinogenesis. WNT pathway has been deemed to affect immune infiltration by enhancing T cell transmigration and regulating immune suppressor cells^{26,27}. And we have known that β -catenin, a key component of the canonical WNT signaling pathway, the translocation of transcription factor β -catenin from the cytoplasm to the nucleus led to transcription of c-myc, and mesenchymal markers ((N-cadherin, vimentin), hence induced EMT and accelerated tumor progression²⁸. The MYC oncogene is a transcription factor that is one of the most commonly activated oncogenes in the pathogenesis of many types of human cancer including CRC^{29,30}. Recently, Dhanasekaran and colleagues proved that MYC could remodel the tumor immune microenvironment cooperating with Twist1, they can elicit a cytokinome to reprogram the crosstalk between cancer cells and macrophages and drives tumor aggression³¹. Thus, crosstalk between WNT signaling and MYC signaling may be significant in CRC biology and immune microenvironment.

To further prove our hypothesis, we explored the molecular pathogenesis may be involved by analyzing the GRINA expression with the immune infiltration level in CRC. In our study, we found that GRINA expression was positively correlated with the abundances of immunocytes NK CD56dim cells, Treg and NK cells. NK CD56dim is a subtype of NK cells, compared with NK CD56bright cells which primarily produce large amounts of cytokines, the normal NK CD56dim cells have the potential to kill tumor cells^{32,33}. Importantly, Krijgsman, etc. found that the phenotype of circulating NK CD56dim cells are altered and the amounts of cells were significantly increased in CRC patients contrasted with the normal group³⁴, which implied that the alterable phenotype of NK CD56dim cells might contribute to CRC. Moreover, increasing Treg cells suppress the immune system and circulating Treg was significantly upregulated in CRC and other tumor patients compared with healthy donors³⁴⁻³⁷, which suggests tumor escape in CRC by increasing Treg may support tumor progression in different kinds of mechanisms^{38,39}. Combining with our data imply that GRINA maybe mediated by NK CD56dim cells and Treg to regulate immune reaction, and thus enhance tumor development.

We also found that Th2 cells, T helper cells, Tcm was significantly negatively associated with GRINA expression. Consistent with our study, Tcm has long been demonstrated superior in vivo expansion, persistence, and antitumor capacities⁴⁰. Th2 cells, a crucial subtype of CD4+ T helper cells, have evolved to play crucial roles in choreographing adaptive immune responses⁴¹. However, the role of Th2 cells in tumors is still obscure. Researchers showed that Th2 cells couldn't impact patient's prognosis in CRC and they have different influences on different tumors^{39,42}.

In conclusion, the current study provides multiple layers of evidence for the magnitude of GRINA in the occurrence of CRC. Our study is the first report indicating GRINA is a potential molecular marker for prognosis of CRC, and the crosstalk between WNT signaling and MYC signaling may be the underlying mechanisms for EMT induction and tumor progression. In addition, our results suggest that GRINA may play a novel

immunomodulatory role in tumor immunity. We made a hypothesis that GRINA may influence tumor immunology and promote CRC progression and EMT by regulating the crosstalk of WNT and MYC signaling. Our study was performed based on computational data from TCGA and GTEx, and we confirmed the promotive function of GRINA in CRC, but lacking strong verification for the potential molecular mechanism of GRINA. In the further, it still requires subsequent functional validation and randomized clinical trials to prove the biologic impact of GRINA for CRC patients.

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Data availability All data and materials are published in the manuscript, supplementary materials on journal website or available on request.

Code availability Not Applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. GRINA expression levels in different types of human cancers. (A) GRINA expression patterns across 33 types of cancers from TCGA and normal tissues (TCGA + GTEx) using Wilcoxon rank sum test. (B) Based on TCGA, GRINA expression levels were contrasted between pan-cancer cohort and normal tissues. (C) GRINA mRNA expression was compared between TCGA CRC types (n = 619) and normal tissues from TCGA + GTEx (n = 667). (D, E) Expression status of GRINA in non-paired and paired tumor and adjacent samples of CRC from TCGA. (F) A receiver operating characteristic (ROC) curve of GRINA expression was plotted for assessment of the discrimination value. (G) IHC analysis of GRINA expression in CRC and normal tissues from THPA. Representative images and detailed information are shown (Scale bars, 200 μ m). *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 2. Correlation between GRINA expression and clinicopathologic characteristics of CRC patients. (A) N stage, (B) M stage, (C) Pathologic stage, (D) Neoplasm type, (E) BRAF status, (F) KRAS status, (G) TP53 status.

Fig. 3. CRC patients with higher GRINA expression have a shorter survival period. (A, B) The Kaplan-Meier's curves of overall survival (OS) and disease-specific survival (DSS) comparing the high and low expression of GRINA in CRC. (C) Nomogram prediction of 1-, 3-, 5-year OS probability of CRC patients. (D) Calibration plot for nomogram-predicted and observed OS at 1, 3 and 5 years.

Fig. 4. The subgroup analysis of OS for evaluating the prognostic value of GRINA expression levels. (A) Forest plot for assessing the HR of GRINA for OS in subgroup analysis. (B) Kaplan-Meier analysis of OS in T stage-T3 & T4 subgroup. (C) Kaplan-Meier analysis of OS in N stage-N1 subgroup. (D) Kaplan-Meier analysis of OS in Pathologic stage- Stage II & Stage III & Stage IV subgroup.

Fig. 5. GRINA promotes proliferation and migration of CRC cells. (A) The cell proliferation was detected with colony formation assay after GRINA overexpression. (B) Wound-healing assay was monitored at 0h and 24h to detect cell migration ability of GRINA on HCT116.

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Fig. 7. GSEA (Gene set enrichment analysis) of GRINA from MSigDB hallmark gene sets. (A) Enrichment of genes in the WNT_BETA_CATENIN_SIGNALING. (B) Enrichment of genes in the MYC_TARGETS_V2. (C) Enrichment of genes in the EPITHELIAL_MESENCHYMAL_TRANSITION. (D) Enrichment of genes in the KRAS_SIGNALING_DN. (E) Enrichment of genes in the REACTIVE OXYGEN SPECIES_PATHWAY. (F) Enrichment of genes in the DNA_REPAIR. (G) Enrichment of genes in the XENOBIOTIC METABOLISM. (H) Enrichment of genes in the BILE_ACID_METABOLISM. (I) IL-2_STAT5_SIGNALING.

Fig. 8. The GRINA expression level is correlated with the immune infiltration in the tumor microenvironment. (A) Association between GRINA expression level and the relative abundances of 24 immune cells visualized by lollipop. The size of dots reveals the absolute value of Spearman R. (B-G) Spearman's correlations between GRINA expression level and the relative enrichment level of NK CD56dim cells, Treg, NK cells, Th2 cells, T helper cells and Tcm. (H-M) Immune infiltration levels of NK CD56dim cells, Treg, NK cells, Th2 cells, T helper cells and Tcm between the high and low GRINA expression groups.

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