Development of a novel lipid metabolism-related model predicting the prognosis of gastric cancer and exploration the role of NPR3 in gastric cancer metastasis

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Abstract

Aim

To establish a novel lipid metabolism-related (LMR) prognostic model for gastric cancer (GC) and explore the potential mechanism of natriuretic peptide receptor-3 (NPR3) in the process of GC metastasis.

Method

LMR genes were identified from the Gene Set Enrichment Analysis (GSEA) and mRNA expression profile were download from The Cancer Genome Atlas (TCGA) database. We used the R package “limma” to obtain the LMR differentially expressed genes (DEGs) between GC and adjacent tissues. Consensus clustering was then performed based on the expression of LMR DEGs using the R package “ConsensusClusterPlus”. We adopted the weighted correlation network analysis (WGCNA) to obtain the best module related to metabolic subtypes. A prognostic model based on 6 LMR genes (FBLN7, NPY1R, VTN, NPR3, EPHB3 and AUH) was established through least absolute shrinkage and selection operator (LASSO) penalized Cox regression analysis based on progression-free interval (PFI). In addition, we verified the NPR3 expression in several GC cell lines by quantitative Real-time PCR and Western Blotting, and explored the effect of NPR3 on GC cell migration using the wound healing assay and transwell test. We performed immunohistochemistry (IHC), H&E and collagen staining on 42 GC tissues to clarify the clinical significance of NPR3 in gastric cancer.

Results

2 LMR subtypes (C1 and C2) were confirmed using consensus clustering of 153 LMR-DEGs. Compared with C1, C2 was associated with a worse prognosis, especially in terms of PFI (HR: 1.64, 95%CI: 1.15–2.33, P < 0.001). Using WGCNA and univariate cox regression, 558 genes were screened out to build and optimize the model. Finally, a novel predictive formula system based on 6 genes (FBLN7, NPY1R, VTN, NPR3, EPHB3 and AUH) were constructed and the time-dependent area under the receiver operating characteristic curve (time-ROC, 1/3/5 years) was 0.79/0.77/0.71 and 0.73/0.68/0.64 in the training set (N = 214) and validation set (N = 141), respectively. In addition, we found that NPR3 over-expression could promote the migration of GC cells. And its expression was higher in tumor tissues than in paracancerous tissues and patients with high expression of NPR3 were more likely have the vascular invasion (OR: 5.056, 95%CI: 1.159–22.060, p = 0.031) and higher stage (OR: 5.100, 95%CI: 1.336–19.470, p = 0.017).

Conclusion

We established a novel LMR prognostic model predicting the prognosis of gastric cancer, and found that NPR3 can promote the tumor migration and vascular invasion of gastric cancer.
Introduction

The global incidence of gastric cancer (GC) ranks fifth among cancers and the mortality rate ranks third, which has brought a serious burden on the economy and public health to threaten the people health and safety all over the world [1]. Though endoscopic submucosal dissection (ESD) or surgical resection of lesions is the most widely used for the treatment at present, the prognosis of GC patients still remains grim. In addition, GC is a heterogeneous disease, individuals vary widely in response to the new therapies such as adjuvant chemotherapy and immunotherapy [2]. Therefore, effective monitoring and prediction tools for GC prognosis are urgently needed.

As one of the three major nutrients, lipids is crucial in cell membrane formation and cellular related signal transduction. Lipid metabolism process has attracted wide attention for their participation in carcinogenic signal. Due to specific needs of catabolism and anabolism, fast proliferating tumor cells have metabolic reprogramming at the lipid level. Increasing de-novo lipogenesis and exogenous uptake of lipids can promote fatty acid catabolism and early growth of cancer cells to form tumor focus. Previous studies have shown that some LMR proteins, such as fatty acid transporter (CD36) and fatty acid transport protein family (SLC27) were highly expressed in cancer and were associated with poor prognosis in GC, breast cancer, prostate cancer and other cancers, and statins combined with targeted drugs can improve chemotherapy resistance [3-4]. Some key rate-limiting enzymes and transcriptional regulators related to fat and cholesterol production, such as fatty acid synthase (FASN) and sterol regulatory element binding proteins (SREBP), were reported to be involved in signal transduction and interaction between tumor cells and a variety of non-tumor cells including adipocytes, immune cells and fibrous cells in tumor microenvironment (TME). Lipid metabolism plays an important role in tumor proliferation, metastasis and chemotherapy resistance [5].

In physiological condition, natriuretic peptide receptor 3 (NPR3) can promote the degradation of natriuretic peptide hormone (NP) through binding and internalization. Ross J et al. pointed out that NPR3 in adipocytes can reduce insulin-stimulated glucose uptake and de-novo lipogenesis, resulting in obesity, insulin resistance and even hepatitis [6]. NPR3 has been reported to have opposite effects in different tumor types. In osteosarcoma and hepatic carcinoma, NPR3 has been reported to be able to block the PI3K/AKT pathway to arrest the process of cell cycle and induce apoptosis [7]-[8]. However, some studies have shown that up-regulation of NPR3 can promote proliferation and inhibit apoptosis in colorectal cancer cells, while knockdown of NPR3 in renal clear cell carcinoma promotes metastasis [9-10]. The role of NPR3 in gastric cancer remains unclear, which prompts us to explore the role of NPR3 in prognostic prediction and metastasis of gastric cancer.

In this study, we constructed a lipid metabolism-related (LMR) risk prediction model based on 6 genes (FBLN7, NPY1R, VTN, NPR3, EPHB3 and AUH) of GC through least absolute shrinkage and selection operator (LASSO) penalized Cox regression analysis. The time-dependent receiver operating characteristic (time-ROC) curve revealed that the model demonstrated better predictive performance in
terms of progression-free interval (PFI). Further, we found that NPR3 was highly expressed in GC cells, and verified the role of NPR3 in promoting tumor cell invasion in gastric cancer.

Materials and method

Acquisition of LMR genes and GC mRNA expression profile

Gene Set Enrichment Analysis (GSEA) was conducted to gain LMR genes on the website (https://www.gsea-msigdb.org/gsea/index.jsp). And we used following 6 most common pathways as the key words: metabolism of lipid, sphingolipid metabolism, fatty acid metabolism, glycerophospholipid metabolism, peroxisome proliferator-activated receptor alpha and transcriptional regulation of white adipocyte differentiation. The mRNA expression profile (FPKM) of GC (tumor tissues = 375, adjacent tissues = 32) was obtained from TCGA (https://portal.gdc.cancer.gov/), then we converted ENSG_ID to Gene Symbol via GENCODE data (https://www.gencodegenes.org/human/), eliminated the genes and samples whose missing values were more than 50%, and further used the impute.knn function of R package “impute” to complete the missing values, the nearest neighbor Kappa index was 10, and finally carried out standardization processing based on log2 (X + 1).

LMR differentially expressed genes (DEGs) and Consensus clustering

We analyzed the LMR-DEGs between GC and adjacent tissues using R package “limma” (version 3.40.6) with the cut-off criteria set as P < 0.05, and fold change (FC) ≥ 1.5 [11]. Using the R package “org.HS.eg.db” (version 3.1.0), we performed the Gene ontology (GO) functional annotation and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis [12] to clarify the biological process, subcellular localization, molecular function and involved pathway between the DEGs and total LMR-genes. Basing on the LMR-DEGs level, we employed Consensus-ClusterPlus to identify various metabolic subtypes in 375 GC patients [13], utilizing agglomerative “Partitioning Around Medoids” (PAM) clustering with a 1-Pearson correlation distances and resampling 80% of the samples for 10 repetitions. The optimal number of clusters was determined using the empirical cumulative distribution function plot. We obtained clinical data from UCSC Xena (https://xenabrowser.net/), and drew the Kaplan-Meier curve of each subtype, indicators including overall survival (OS), PFI and disease specific survival (DSS).

Weighted correlation network analysis (WGCNA)

After analyzing the pathway and prognosis differences in metabolic subtypes, we used R passage WGCNA to screening LMR-module [14]. Firstly, the Pearson’s correlation matrices and average linkage method were both performed for all pair-wise genes, then a weighted adjacency matrix was constructed. A soft-thresholding parameter, $\beta$, was set to 5 with $R^2 = 0.86$ in our study to highlight strong correlations between Genes while penalizing weaker correlations. The adjacency was transformed into a topological overlap matrix (TOM), which could measure the network connectivity of a gene defined as the sum of its adjacency with all other genes for network gene ration, and the corresponding dissimilarity (1-TOM) was calculated. Genes with comparable expression profiles were grouped into gene modules using average
linkage hierarchical clustering based on the TOM-derived dissimilarity measure, with a minimum size of 60 for the genes dendrogram. With setting the sensitivity to 3 and module merge threshold to 0.25, we finally obtained several modules and identified the best module correlated to metabolic subtypes. To establish a predictive model rather than explore mechanism network, we further screened predictive genes of PFI (P < 0.01) via univariate cox regression rather than analyse the hub genes.

**Construction and optimization of predictive model**

According to the proportion of 3:2, we divided GC patients into the training set (N = 214) and verification set (N = 141) by SPSS 23.0 's randomized generator, and used t or Chi-square test to verify the balance of clinical characteristics. Then we performed the LASSO regression using the “glmnet” package [15] to construct a original model to predict PFI in the training set, and the efficacy was evaluated by time-ROC curve even in the verification set. If the overall efficiency or the stability in internal verification was poor, multivariate cox regression (step back wald method) and clinical characteristics were used to increase efficiency, portability and internal stability. In different data sets, time-ROC, KM and decision curve analysis (DCA) curve were used to evaluate the efficiency, stability and clinical benefit of the final model.

**Single sample gene set enrichment analysis (ssGSEA) to identify the changeable pathways in GC subtypes**

We divided GC patients into high and low groups based on the final model score and NPR3 expression, and performed ssGSEA (version 3.0) to find enrichment pathways in different subgroups. Then we set the minimum gene set to 5, the maximum gene set to 5000, and resample a thousand times. Pathways with P < 0.05, false discovery rate (FDR) < 0.25, and normalized enrichment score (NES) > 1.5 or < -1.5 were considered to be related. Before Vitro experiment, we retrieved the online data of NPR3 from The Human Protein Atlas (HPA, https://www.proteinatlas.org/) and Gene Set Cancer analysis (GSCA, http://bioinfo.life.hust.edu.cn/GSCA/#/) to determine the subsquent verification method.

**Cell culture and transfection**

Cell line GES-1 were obtained from the cell bank of SAIBAIKANG biotechnology company (Shanghai, China), while other 5 human GC cell lines were obtained from the Chinese Academy of Sciences (SNU-1, AGS, NCI-N87, Beijing; HGC27, Kunming; MKN45, Shanghai). All cells were cultured in RPMI-1640 medium (Biological Industries, Cromwell, CT, United States) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Cromwell, CT, United States), and maintained in a 37°C incubator with a 5% CO2. We used the cloning vector (pcDNA3.1-NPR3, insert size: 1638bp, Tsingke, Nanjing) to overexpress NPR3, while empty vector (pcDNA3.1-vector, Tsingke, Nanjing) was used as the negative control. The AGS cells with 60-80% confluence in 6 wells dishes were transfected with 2ug recombinant plasmid and 5ul Hieff Trans® Liposomal Transfection Reagent (Yeasen, Shanghai, China) without FBS. After 6 hours, Changing the medium containing 10% FBS and continue to culture to 48h.

**RNA extraction and RT-qPCR assay**
Total RNA was extracted from human cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer’s instructions. The reverse transcription reaction (RT) was performed with Reverse Transcription kit (Vazyme, Nanjing, China). The RT-qPCR reactions were performed with a SYBR Green PCR Kit (Vazyme, Nanjing, China), measured in Quadruplicate and performed on an Applied Biosystems 7900HT sequence detection system (Applied Biosystems). β-actin was used as an internal control for mRNA. The relative expression levels of the target genes were calculated using the comparative 2−ΔΔCt method. All primers used in this study were listed in Supplementary Table S1, and repeated three times.

**Western blotting**

Samples were homogenized in RIPA buffer, sonicated, and prepared for Western blotting. Protein were separated by electrophoresis on 10% SDS-PAGE gels before semi-drytransfer to the PVDF membrane. NPR3 and β-actin were detected using antibodies purchased from Abcam (EPR12716, Berlin, Germany) and Proteintech. All results were repeated three times.

**Wound healing assay and transwell assay**

AGS cells were seeded into six-well plates and grown to 90-100% confluence. The cell layer was scraping with a 1000μL sterile pipette tip and washed with three times with PBS to remove the floating and detached cells, then cultured without FBS medium and images were acquired under a microscope at two time points (0, 24 h). Cell migration abilities were evaluated by transwell chambers (Corning Life Sciences, Bedford, MA, United States). Briefly, a total of 3 × 10⁴ AGS cells suspended in 200ul media without FBS were seeded in the upper chambers. Then, 500 μL of culture medium containing 20% FBS was added to the lower chambers. After incubation at 37°C for 24 h, the cells in the lower chambers were fixed with 4% paraformaldehyde for 30 min, stained with crystal violet (Beyotime, Shanghai, China) for 30 min. Finally, two random fields were microscopically examined. All results were repeated three times.

**Immunohistochemistry (IHC), H&E and sirius red staining**

A total of 42 pathologically confirmed GC tissues were obtained from Nanjing Drum Tower Hospital, and the expression of NPR3 (Rabbit, 26706-1-AP, proteintech) was detected by IHC staining. Staining of NPR3 was scored by the two pathologists blind to the clinical data by applying a semi-quantitative immunoreactivity score (IRS), which determined by the product of staining intensity and staining area under imageJ. In our study, IRS of 0–1 and IRS of 2–4 were classified as low and high expression of NPR3, respectively. In addition, we stained these tissues by the Sirius Red Kit (PHYGENE,PH1098) to determine the degree of fibrosis.

**Statistical analysis methods**

Generate graphics using GraphPad Prsim 8 software. In addition to R analysis, the data were analyzed using SPSS (version 22.0, Chicago, IL, USA), with statistical tests including chi square test, log rank test,
and student t-test. All tests were conducted bilaterally, with P<0.05 indicating a statistically significant difference.

**Results**

**LMR-DEGs screening**

We totally obtained 776 LMR genes from GSEA (740 with a unique match and detectable expression in TCGA-STAD data), as shown in **Table S2-3**, and 153 DEGs between GC and adjacent tissues were confirmed, of which 89 were up-regulated and 64 were down-regulated. The volcano map and heat map of top 50 genes were shown in **Figure 1A-B**. Next, we compared the pathways and molecular functions of 153 DEGs and 776 LMR genes by GO and KEGG. It was found that AMP-activated protein kinase (AMPK) pathway, glycerolipid metabolism pathway and peroxisome pathway appeared in the top 10 pathways of DEGs, while sphingolipid metabolism and its signaling pathway, peroxisome proliferators-activated receptors (PPAR) pathway increased in proportion. GO suggested that fatty acid catabolic, monocarboxylic acid metabolic and regulation of lipid metabolic process newly appeared in DEGs. The above results confirmed the rationality of self-defined screening of DEGs, as shown in **Figure 1C-F**.

**Metabolic subtypes clustering and model predictors screening**

Using the 153 DEGs, clustering analysis divided 375 GC patients into two metabolic subtypes, as shown in **Figure 2A-E**. Kaplan-Meier curve showed that C2 was worse than C1 in every prognosis indicators: OS (HR: 1.43, 95%CI: 1.03-1.98, P = 0.03, N = 354), DSS (HR: 1.68, 95%CI: 1.11-2.56, P = 0.01, N = 353), and PFI (HR: 1.64, 95%CI: 1.15-2.33, P = 5.9e-3, N = 355), as shown in **Figure S1A-C**. WGCNA divided 57226 mRNA into 41 modules, and “blue” module (5549 mRNA) had the strongest correlation with the PAM metabolic subtypes (r: 0.63, p = 7.5e-43). In addition, this module also weakly correlated with age, T stage and differentiation grade. By univariate COX regression analysis, we further selected 558 mRNA (P < 0.01) to establish the original model of PFI (according to the P value), as shown in **Figure 3A-D**.

**Development of the new 6 genes predictive model**

We identified λ = 0.1 in LASSO regression and there were 12 genes still retained their coefficients, predictive formula: P = AP007216.2 * 0.047 - AUH * 0.022 + BHLHE41 * 0.016 - EPHB3 * 0.092 + FBLN7 * 0.217 + GDF6 * 0.181 + NPY1R * 0.133 + VTN * 0.012 + CLDN11 * 0.239 - MMP12 * 0.226 + NPR3 * 0.008 + PAK3 * 0.002, as shown in **Figure 4A**. The time-ROC of training set and validation set were 0.78/0.80/0.78 and 0.58/0.60/0.46, respectively, as shown in **Figure 4B-C**. Considering the excessive number of genes and poor stability of time-ROC, multivariate cox regression was adopted to reduce collinearity to optimize the model. The results of step by step forward and backward Wald method were consistent, and finally we gained a 6 genes model as following: P = 0.537 * FBLN7 + 0.370 * NPY1R + 0.191 * VTN + 0.254 * NPR3 - 0.253 * EPHB3 - 0.585 * AUH. The time-ROC of training set and validation set were 0.79/0.77/0.71 and 0.73/0.68/0.64, respectively, as shown in **Figure 4D-E**. To note, the addition of independent risk factors such as gender and clinical stage did not significantly improve the efficiency.
(time-ROC of the training set and validation set was 0.79/0.75/0.68 and 0.73/0.73/0.73, respectively, with cut-off value: 0.524), as shown in Table S5, Figure S2A-D. Therefore, we determined the six-gene model, verified the best cut-off value (-1.205) in the training set and the verification set. KM curve showed that this value could well distinguish patients with poor prognosis even in subgroup analysis, DCA curve obviously showed its clinical benefit, as shown in Figure 4F-I, while subgroup analysis were shown in Figure 5A-H.

**Enrichment pathway of the new 6 genes (new6) model in ssGSEA**

For high group of the new6 (> -1.205), totally 14/186 tumor-related pathways up-regulated, including calcium signaling pathway, vascular smooth muscle contraction, glycosphingolipid biosynthesis ganglioside series, regulation of actin cytoskeleton, focal adhesion, gap junction, hedgehog signaling pathway, cell adhesion molecules (CAMs), mitogen-activated protein kinases (MAPK) signaling pathway, basal cell carcinoma, leukocyte transendothelial migration, transforming growth factor beta (TGF-β) signaling pathway, peroxisome proliferators-activated receptors (PPAR) signaling pathway, and melanoma. For low group of the new6, there were totally 8/186 cancer-suppressing pathway up-regulated, including nucleotide excision repair, base excision repair, cell cycle, DNA replication, basal transcription factors, P53 signaling pathway, mismatch repair and non-homologous end joining, which further explained the possible reasons for the better prognosis of the low score group from the mechanism, shown in Figure 5I-J.

**Expression of NPR3 and potential mechanism exploration**

Among the 6 genes in our model, the role of FBLN7, NPY1R, VTN and EPHB3 in GC have been described in previous studies [16-19], while the role of NPR3 in tumor cells remains controversial and expected to be clarified. HPA showed that NPR3-mRNA only expressed in gastric mucus-secreting cells and fibroblasts. In 354 GC patients, patients with high expression of NPR3 had a worse prognosis (cut-off value: 0.58, p = 0.0006), as shown in Figure S3A-D. While GSCA online analysis also suggested that GC patients with a high NPR3-mRNA expression level have a higher risk of death (OS, PFI, DSS), even though its average expression in the tumorous was lower than the normal. In addition, pathway prediction analysis showed that NPR3 might inhibit the process of apoptosis, cell-cycle, and activate epithelial–mesenchymal transition (EMT). Combined with the above situation, we detected the mRNA expression of NPR3 in 6 cell lines (GES-1, AGS, HGC27, SNU-1, NCI-N87 and MKN45), and RT-PCR showed that the NPR3 mRNA level in HGC27 was higher than that in GES-1 (a normal human gastric mucosal epithelial cell line), while the expression of NPR3 in other cells was lower than that of GES-1, especially in AGS cells. At the protein level, NPR3 expression was higher in NCI-N87 phase than in other GC cells and GES-1, as shown in Figure 6A. We then overexpressed NPR3 in AGS cells, as shown in Figure 6B. The wound healing test and transwell experiment suggested that NPR3 over-expression could promote the migration of AGS, as shown in Figure 6C-D.

IRS suggested that NPR3 expression was higher in GC tissues than in paracancerous tissues (Z = - 5.227, P < 0.001), and high IRS (2 - 4) was a risk factor in vascular infiltration (OR: 5.056, 95%CI: 1.159-22.060, p
and tumorous stage (OR: 5.100, 95% CI: 1.336-19.470, p = 0.017), as shown in Table S6. HPA suggested NPR3 expressed in fibroblasts, so we verified the degree of fibrosis of the tissue. Sirius red staining also showed that the collagen fibers in tumor tissues were much higher than those in paracancerous tissues, as shown in Figure 7A-C. We further used ssGSEA to verify the possible mechanism of fibrosis and vascular invasion. For high-NPR3 group, totally 18/186 signaling pathways were up-regulated, including MAPK, WNT, MTOR, Hedgehog, TGF-β, calcium, adipocytokine signaling pathway, glycosphingolipid biosynthesis ganglio series, vascular smooth muscle contraction, pathway in cancer, ABC transporters, tight junctions, focal adhesion, gap junctions, regulation of actin cytoskeleton, melanoma, ECM receptor interaction and basal cell carcinoma. For low-NPR3 group, there were totally 7/186 cancer-suppressing pathways were up-regulated, including base excision repair, nucleotide excision repair, DNA replication, mismatch repair, cell cycle, drug metabolism other enzymes and non-homologous end joining, as shown in Figure 7 D-E.

Discussion

Lipid metabolism reprogramming is an important feature of gastric cancer, and existing studies mainly focus on the role of key enzymes in diagnosis, treatment and prognosis. For example, several key enzymes involved in de-novo lipogenesis of fatty acids including SREBP1C, FASN and ATP citrate lyase (ACLY) were also found to be up-regulated in gastric cancer and related to GC development, lymph node metastasis and shorter survival time [20–23]. In addition, acetyl-CoA Synthases (ACS), which can convert fatty acids into acetyl-CoA, were also found to promote the progression of GC [24–25]. Several drugs targeting these metabolic processes including proton pump inhibitors, orlistat and small molecule inhibitors of SCD1 (A939572) have been reported to show promising therapeutic effects on gastric cancer [26]. As a result, it is necessary to identify the novel lipid metabolism related prognostic biomarkers for GC.

In this study, we used LMR genes to construct a novel prognostic model for gastric cancer including 6 LMR genes (FBLN7, NPY1R, VTN, NPR3, EPHB3 and AUH) using LASSO penalized Cox regression analysis which was proved to be well behaved in both training set and verification set. Among these 6 genes, FBLN7, NPY1R, VTN and EPHB3 have previously been reported to be involved in GC tumor development [16–19]. X Bian et al. also reported that the co-family member FBLN7 was one of the independent risk factors related to GC patient prognosis [27]. Additionally, EPHB2 was reported to be associated with intestinal phenotype of gastric cancer and indicates better prognosis by suppressing gastric cancer migration [28]. Unlike most models for predicting overall survival, our model predicted progression-free interval more accurately. Based on the cut-off values of -1.205, the time-ROC in the training set and verification set were 0.79/0.77/0.71 and 0.73/0.68/0.64.

To further clarify the biological significance of this model, we performed ssGSEA to explore the enrichment pathways between high and low group. There were several pathways up-regulated in high-score group, such as glycosphingolipid biosynthesis ganglio series, regulation of actin cytoskeleton, focal adhesion, gap junction, hedgehog signaling pathway, the CAMs, MAPK signaling pathway, basal cell
cancer, leukocyte transendothelial migration, TGF-β signaling pathway, PPAR signaling pathway, melanoma and so on. Gangliosides of glycosphingolipid were reported to be involved in the process of EMT to induce cancer cells lose their epithelial features to gain mesenchymal characteristics [29]. ST3GAL5 is key enzyme for the synthesis of most complex gangliosides, Gu et al. observed that silencing ST3GAL5 could reduce the migration, invasion and anchorage-independent growth of murine breast cancer cell (4T1) in vitro as well as lung metastasis in vivo [30]. While other pathways including nucleotide excision repair, base excision repair, cell cycle, DNA replication, basal transcription factors, P53 signaling pathway, mismatch repair and non-homologous end joining (NHEJ) pathway, were elevated in the low-score group. It is well known that DNA damage repair and p53 pathway inhibit the occurrence and development of tumor cells in many kind of tumor type. While DNA double strand breaks (DSBs) are a grave threat to genome stability, NHEJ pathway could prevent cancer via efficient repair of DSBs [31]. The above results explained why the prognosis of the two groups was different. Therefore, we believed that this model has important biological significance, reflecting the enrichment of many pathways that affect the development of tumor.

Of these genes, the role of NPR3 in cancer is controversial. Studies have shown that NPR3 is increased in the non-tumor patients of hypertension, diabetes, obesity and without exercise [32]. Thus, NPR3 may play a potential role in cancers related to metabolic syndrome, especially digestive tract tumors such as gastrointestinal cancer, liver cancer, pancreatic cancer and so on. It has been explained before that the effects of NPR3 on the occurrence and development of solid tumors are contradictory, which is still not clear in gastric cancer. In our model, NPR3 was a factor that represents a worse prognosis which prompted us to further explore the role of NPR3 in gastric cancer. NPR3 over-expression could promote migration in AGS, which was consistent with the bioinformatics analysis of GSCA that NPR3 was a risk factor of GC and could activate EMT pathway. IRS demonstrated that NPR3 were higher in the tumor tissues than that in the paracancerous tissues. High IRS was a risk factor for vascular invasion (OR: 5.056, 95%CI: 1.159–22.060, p = 0.031) and severe stage (OR: 5.100, 95%CI: 1.336–19.470, p = 0.017). Furthermore, we found that the expression of NPR3 in the tumor was positively correlated with the collagen expression in the TME. These results indicate that NPR3 plays a role in promoting cancer in gastric cancer.

In conclusion, we constructed a LMR model predicting the prognosis of GC patients based the PFI. In addition, we further demonstrated that NPR3 could promote migration and vascular invasion in GC. However, follow-up studies still need to further explore the clinical usability of this model and the specific mechanism of NPR3 involved in the malignant biological regulation of gastric cancer.

**Abbreviations**

GC, gastric cancer; ESD, endoscopic submucosal dissection; CD36, fatty acid transporter; SLC27, fatty acid transport protein family; FASN, fatty acid synthase; SREBP, sterol regulatory element binding proteins; TME, tumor microenvironment; NPR3, natriuretic peptide receptor 3; NP, natriuretic peptide hormone; LMR, lipid metabolism-related; LASSO, least absolute shrinkage and selection operator
regression; time-ROC, time-dependent receiver operating characteristic curve; PFI, progression-free interval; GSEA, Gene Set Enrichment Analysis; DEGs, differentially expressed genes; FC, fold change; GO, Gene ontology; KEGG, Kyoto Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis; PAM, Partitioning Around Medoids; OS, overall survival; DSS, disease specific survival; WGCNA, Weighted correlation network analysis; TOM, topological overlap matrix; DCA, decision curve analysis; ssGSEA, single sample gene set enrichment analysis; FDR, false discovery rate; NES, normalized enrichment score; HPA, The Human Protein Atlas; GSCA, Gene Set Cancer analysis; IHC, Immunohistochemistry; IRS, immunoreactivity score; CAMs, cell adhesion molecules; MAPK, mitogen-activated protein kinases; TGF-β, transforming growth factor beta; peroxisome PPAR, proliferators-activated receptors; EMT, epithelial–mesenchymal transition; ACLY, ATP citrate lyase; ACS, acetyl-CoA Synthases; NHEJ, non-homologous end joining pathway; DSBs, DNA double strand breaks.

Declarations

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Author contributions: Xuan Wang & Quan Zhou: study design/conception, collection and interpretation of data, manuscript writing; Hongzhen Li: manuscript modification; Pin Wang & Wei Zhang & Huiming Guo: supervision; Xiaoping Zou: study design/conception, supervision, manuscript writing.

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Conflict of interest: all authors declared that there are no potential conflicts of interest in this study.

Ethical Approval: this study was approved by the ethic committees of Nanjing Drum Tower Hospital and conformed to the principles of the World Medical Association Declaration of Helsinki, as revised in 2013.

Consent for publication: not applicable in the declarations section.

Availability of data and materials: the datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Informed Consent: The study involving human tissues from Nanjing Drum Tower Hospital were reviewed and approved by the Ethical Committee of Medical Research. As it is a retrospective study, we applied for exemption from informed consent.

References


Figures
Figure 1: A: volcano map of 153 DEGs (375 GC tissue vs 32 paracancerous tissue); B: heat map of top 50 DEGs; C-D: KEGG analysis of 776 LMR genes and 153 DEGs; E-F: GO analysis of 776 LMR genes and 153DEGs.

See image above for figure legend.
Figure 2: Consensus clustering. A: Area under distribution curve; B: Cumulative distribution curve; C: Consistency between K clustering-groups. D: heat map of Consistency between K clustering group.

Figure 2

See image above for figure legend.
Figure 3: A: determination of soft-thresholding power; B: clustering dendrogram; C: correlation between modules and clinical features; D: univariate cox regression of top 10 mRNA, based on P value sequence.

Figure 3

See image above for figure legend.
Figure 4: A: λ and 12 genes in LASSO regression; B-C (12 genes): time-AUC in training set and validation set, respectively; D-E (6 genes): time-AUC in training set and validation set, respectively; F-G (6 genes): cut-off value = -1.205, KM curve in training set and validation set; H-I (6 genes): DCA curve in training set and validation set.

Figure 4

See image above for figure legend.
Figure 5: cut-off value of risk = -1.205. A-H: KM curve of subgroup analysis. A: age $\geq$ 60;  B: age < 60;  C: male;  D: female;  E: stage 1 or 2;  F: stage 3 or 4;  G: grade 1 or 2;  H: grade 3. 1-J: identified up-regulated pathway in low (1) and high (2) risk score.

Figure 5

See image above for figure legend.
Figure 6: A: NPR3 expression level of mRNA and protein in 6 cell lines; B: NPR3 over-expression efficiency in AGS; C: wound healing test of AGS migration (1% FBS); D: transwell test of AGS migration. Note: NC: negative control, OE: over expression. *: p < 0.05; **: p < 0.01; ***: p < 0.001; ****: p < 0.0001.

Figure 6

See image above for figure legend.
Figure 7: A: immunohistochemical intensity scoring standard of NPR3; B: common expression situation of NPR3 and collagen fiber in GC tissue and paracancerous tissue; C: relationship between vascular invasion and semi-quantitative immunoreactivity score (IRS) of NPR3; D: NPR3 expression-related pathway of KEGG (TCGA, GC patients, N = 375).

Figure 7

See image above for figure legend.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
• SubTableS16FigureS13.docx