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ORIGINAL RESEARCH

Grainyhead-Like Genes Family May Act as Novel Biomarkers in Colon Cancer

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Objective: The Grainyhead-like (GRHL) genes family were reported to participate in the development of a number of diseases. This study was designed to investigate the role of *GRHL* genes family in colon cancer (CC).

Methods: In this study, the transcriptional levels of *GRHL* genes family in patients with CC from GEPIA were explored. Meanwhile, the immunohistochemical data of the *GRHL* genes family were also obtained in the HPA database. Additionally, we re-identified the mRNA of these genes via real-time PCR. Furthermore, the association between the levels of *GRHL* genes and stage plot as well as survival condition including overall survival and disease-free survival of patients with CC was analyzed. Finally, by transfecting with specific-siRNA, clone formation assay was performed to observe the role of *GRHL* genes family in the proliferation of SW480 human colon cancer cells.

Results: We found that the mRNA and protein levels of *GRHL1*, *GRHL2* and *GRHL3* were significantly higher in CC tissues than in normal colon tissues. Additionally, *GRHL1*, *GRHL2* and *GRHL3* were significantly associated with the stages of CC. The Kaplan–Meier plotter showed that the low levels of *GRHL1*, *GRHL2* and *GRHL3* conferred a better overall survival of patients with CC while the high levels of *GRHL1* and *GRHL3* were associated with poor disease-free survival. Knockdown of *GRHL1*, *GRHL2* and *GRHL3* siHgnificantly inhibited the ability of colony formation of human colon cancer cells.

Conclusion: Our study demonstrated that *GRHL* genes are involved in the prognosis and survival in patients with CC, the inhibition of which may suppress the proliferation of colon cancer cells.

Keywords: colon cancer, Grainyhead-like genes family, prognosis, biomarkers, bioinformatics

Introduction

Colon cancer (CC) is one of the most prevalent malignant tumors, with a high recurrence and the fourth most common cause of cancer-related deaths globally[1,2]. The prognosis of CC is predominantly determined by the clinicopathological features and tumor stages.[3] However, the heterogeneity of the CC makes it is not easy to predict patient prognosis according to these conventional strategies.[4] Additionally, the 5-year overall survival rate of CC remains still very low.[5] Hence, a set of sensitive prognostic markers and potential drug targets should be identified to improve the accuracy of prognosis and individualized treatments.

The Grainyhead-like (GRHL) family of transcription factors possess 3 members, namely *GRHL1*, *GRHL2* and *GRHL3*, which were firstly discovered in the fruit fly *Drosophila melanogaster*.[6] The 3 transcription factors were found to adopt

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a DNA-binding immunoglobulin fold homologous to the key domain of p53 (a well-known tumor suppressor).[7] The expression of the 3 transcription factors is in a tissue and developmentally specific manner, which is primarily expressed in epithelial tissues of organs including olfactory and oral epithelium, urogenital tract, kidney, digestive tract, lung and myocardium.[8] At present, a great many studies unveiled the roles of GRHL genes in different types of cancer: gastric cancer, squamous cell carcinoma of the skin, breast cancer, prostate cancer, colorectal cancer, hepatocellular carcinoma, clear cell renal cell carcinoma, neuroblastoma, and cervical cancer.[6,9] The roles of GRHL genes in various types of cancer are complicated, and even in some cases appear to be contradictory: sometimes they promote cancer development, sometimes they may serve as tumor suppressors. For example, knockdown of *GRHL2* in colorectal cancer cells could inhibit cell proliferation by targeting ZEB1.[10] Meanwhile, the protein level of *GRHL2* was significantly higher in colorectal cancer tissues, which was positively correlated with tumor size and TNM stage. Furthermore, GRHL2 was also an independent prognostic index for recurrence-free survival and overall survival.[11]

The dysregulated expression levels of GRHL genes and their relationship with clinicopathological features and prognosis have been partly reported in human CC. To the best of our knowledge, bioinformatics analysis has yet been applied to investigate the roles of GRHL genes in human CC. Based on the analyses of thousands of gene expression or variation in copy numbers published online, we analyzed and identified the expression and different GRHL transcription factors in patients with CC in detail to investigate their expression patterns, potential functions, and distinct prognostic values of transcription factors in CC.

Materials and Methods

Ethics Statement

Our study was approved by the Academic Committee of The First People's Hospital of Jiashan and conducted according to the principles expressed in the Declaration of Helsinki. All the datasets mentioned in this study were retrieved from the published literature, indicating that it was confirmed that all written informed consent was obtained. A total of 10 pairs of carcinoma tissues and adjacent tissues from patients diagnosed with CC were collected.

GEPIA (Gene Expression Profiling Interactive Analysis) Dataset

GEPIA (http://gepia.cancer-pku.cn/index.html) serves as a newly generated interactive web server designed by Zefang Tang, Chenwei Li, and Boxi Kang of Zhang Lab, Peking University, aiming to analyze the RNA sequencing expression data of 9736 tumors and 8587 normal samples from the GTEx projects the TCGA database in a standard processing manner. GEPIA provides customizable functions including tumor/normal differential expression analysis, profiling according to cancer types or pathological stages, patient survival analysis, similar gene detection, correlation analysis, and dimensionality reduction analysis.[12] In this study, we mainly employed the boxplot to detect the mRNA expression of GRHL genes in CC and normal colon tissues.

The Stage Plot and Survival Condition of Patients with Different Levels of GRHL Genes

Similarly, we used the GEPIA database to obtain stage plot, overall survival and disease-free survival information of GRHL genes. The log-rank P value and hazard ratio (HR) with 95% confidence intervals were shown on the plot. $P < 0.05$ was statistically significant.

Human Protein Atlas

The Human Protein Atlas (HPA, https://www.proteinatlas.org/) is a Swedish-based program initiated in 2003 with the aim to map all human proteins in cells, tissues, and organs using the integration of various omics technologies, including antibody-based imaging, mass spectrometry-based proteomics, transcriptomics, and systems biology.[13] By getting immunohistochemical data of patients with or without CC on the basis of HPA, we further verified the protein expression levels of GRHL genes.

Cell Culture and RNA Interference

SW480 human CC cells were acquired from the Cell Bank of Chinese Academy of Sciences. They were cultured in a 95% humidified air/5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone). The siRNA targeting human *GRHL1* (siGRHL1, 5′-CGAATCGTA AACGGTTCA-3′), *GRHL2* (siGRHL2, 5′-CGTAGCTAGT TTCGTAG-3′) and *GRHL3* (siGRHL3, 5′-CGAGCTTATG GGCCAAA-3′) and nonspecific siRNA (siNC) were

purchased from GenePharma Company. siRNAs or siNC were inserted into SW480 human CC cells using Lipofectamine 4000 (Invitrogen) based on the manufacturer's instructions. Th clone formation assays were performed 48 h after transfection.

Colony Formation Assay

The colony-forming ability of SW480 human CC cells transfected with siRNA or siNC was detected using a colony formation assay. In detail, the transfected cells (siGRHL1, siGRHL2, siGRHL3 and siNC) growing in log phase were trypsinized and seeded into six-well plates with a density of 2000 cells per well. The cells were kept in an incubator at 37°C for 7 days. Seven days later, the colonies were washed with phosphate-buffered saline (PBS), fixed with formalin (10%; Beyotime Institute of Biotechnology, Haimen, China) and stained with methyl violet. Finally, the methyl violet dye was washed off with PBS. The number of colonies was counted using a microscope (Olympus IX53; Olympus Corporation, Tokyo, Japan). Colony-inhibition rate=[1−(number of colonies in experimental groups/control group)]×100%; and colony-forming efficiency=(1−colony-inhibition rate) were calculated.

Real-Time PCR

Total RNA was extracted from cell cultures using Trizol (Invitrogen, Grand Island, NY, USA) based on the manufacturer's protocol. Subsequently, the cDNA was amplified by a reverse transcriptional kit (Promega, Madison, WI, USA). The real-time PCR was performed using cDNA as a template and Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) by an Applied Biosystems 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). The relative amount of mRNA was calculated and normalized using GAPDH as internal reference. The primers used in this study are presented in Table 1.

Western Blot

Lysates from CC cells were immunoblotted according to protocol. Briefly, protein extracts were resolved on SDS-PAGE and transferred to PVDF membrane. After blocking with 5% non-fat dry milk, the membrane was incubated overnight at 4°C with primary antibodies: anti-GRHL1, anti-GRHL2, anti-GRHL3 or anti-GAPDH. After incubation with primary antibodies, membranes were incubated with secondary antibodies for 50 min. To confirm equal protein loading, blots with antibody against GAPDH were used. Proteins were visualized with Amersham ECL™ Western blotting system.

Table 1 Gene-Specific Primers Used in Real-Time PCR

Gene	Forward	Reverse
GAPDH	5-CATGAGAAGTATGACAACAGCCT-3	5-AGTCCTTCCACGATACCAAAGT-3
GRHL1	5-ACGTGTGTAGCTAGTCAAA-3	5-AGTTGCTGATGCTGATCCC-3
GRHL2	5-TTTGTCGTACGATAACCCC-3	5-GTCGATGCTGATCGTAGC-3
GRHL3	5-ACGTAGTTTTACCAGTG-3	5-AACCCTCCTAGGTGTCAG-3

Statistical Analysis

The obtained data were presented as the mean ± SD (standard deviation) and assessed by the two-tailed Student's *t*-test. A difference of $P<0.05$ was considered statistically significant.

Results

Transcriptional Levels of GRHL Genes in Patients with CC

By analyzing 349 normal colon tissues and 275 CC tissues based on GEPIA online website, we found that the mRNA levels of *GRHL1*, *GRHL2* and *GRHL3* were significantly higher in carcinoma tissues than in normal colon tissues ($P<0.05$), indicating that the GRHL family of transcription factors may act as potential markers in the diagnosis of CC (Figure 1A–C).

Immunohistochemical Data of GRHL Genes in Patients with CC

To further obtain the protein expression levels of GRHL genes, we obtained the immunohistochemical data of the 3 genes from HPA. Consistent with the above results based on GEPIA, the immunohistochemical data from HPA confirmed the protein levels of GRHL genes in CC (Figure 2A–C).

Correlation Between GRHL Genes Expression and Tumor Stage in Patients with CC

Subsequently, we analyzed the association between GRHL genes expression levels and tumor stage in patients with

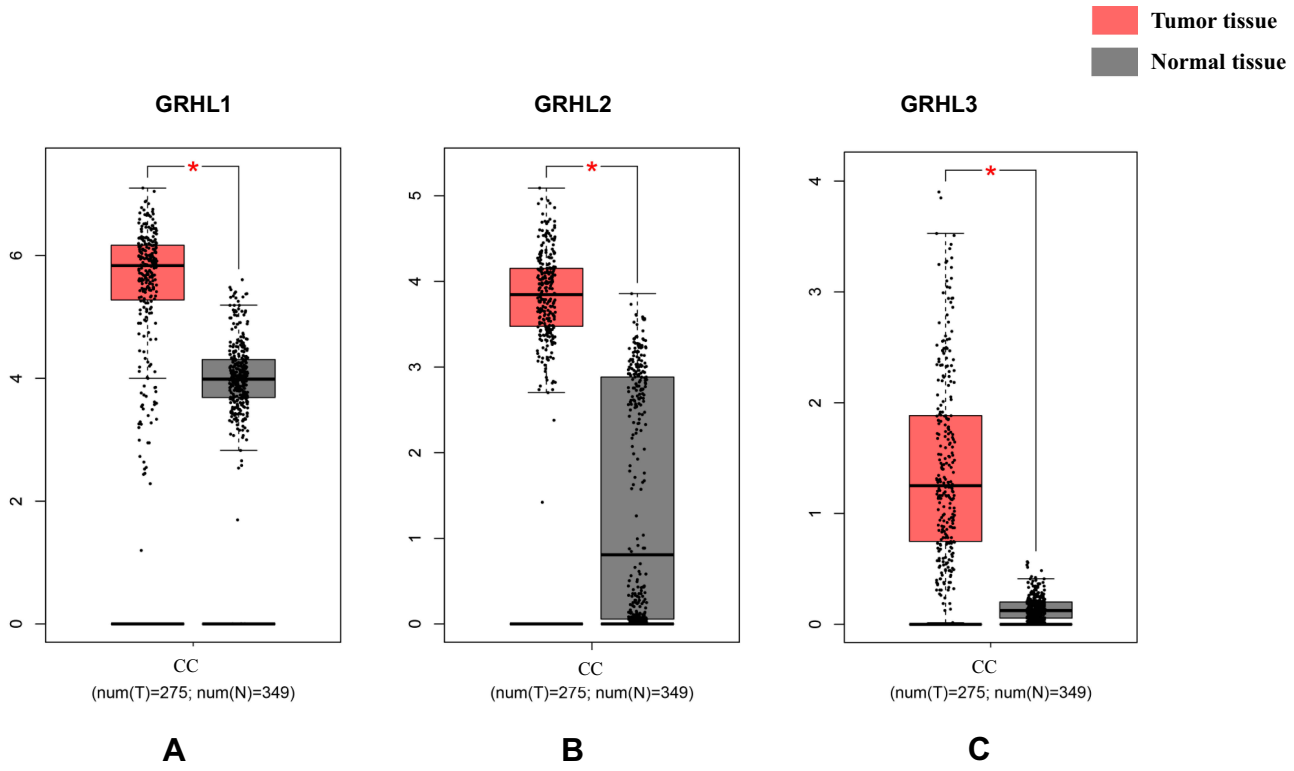

Figure 1 The mRNA expression level of *GRHL1*, *GRHL2* and *GRHL3* (**A**) *GRHL1*; (**B**) *GRHL2*; (**C**) *GRHL3*. *P < 0.05 versus the tumor tissue.

CC based on GEPIA. The results demonstrated that the expression levels of the 3 transcription factors displayed a strong correlation with the tumor stage in patients with CC (Figure 3A–C).

Correlation Between GRHL Genes Expression and Survival Condition in Patients with CC

Meanwhile, we further analyzed the potential association between the expression levels of GRHL genes expression levels and the survival condition of patients with CC. The Kaplan–Meier showed that the levels of 3 transcription factors displayed significant correlation with the overall survival and disease-free survival of patients with CC. In detail, the low level of 3 genes may contribute to better overall survival of CC (Figure 4A–C) while the high level of *GRHL1* and *GRHL3* may contribute to better disease-free survival (Figure 4D–F) (P<0.05).

Re-Identification of the Expression of GRHL Genes

To enhance the reliability of database, we detected the mRNA expression levels of GRHL genes in carcinoma tissues and adjacent tissues from patients with CC using real-time PCR, the results (Figure 5) of which showed further validated the previous hypothesis from bioinformatics analysis.

Knockdown of GRHL Genes Reduces Proliferation of Human CC Cells

Finally, a colony formation assay and a CCK8 assay were performed to explore the effects of *GRHL1*, *GRHL2* and *GRHL3* on proliferation ability. Western blot showed that the 3 genes were successfully knocked down by siRNA Figure 6A. As shown in Figure 6B and C, the proliferation ability and the relative colony number were significantly declined after theses 3 genes were inhibited in CC cells (P<0.05). Also, we performed the same experiments to detect the proliferation ability in another cell line LoVo, the results of which further proved the anti-proliferation ability of GRHL genes (Supplementary materials).

Discussion

The dysregulation of *GRHL* transcription factors has been reported in various types of cancers. Although the role of *GRHL* transcription factors in the carcinogenesis and prognosis of some certain cancers has been partially unveiled,

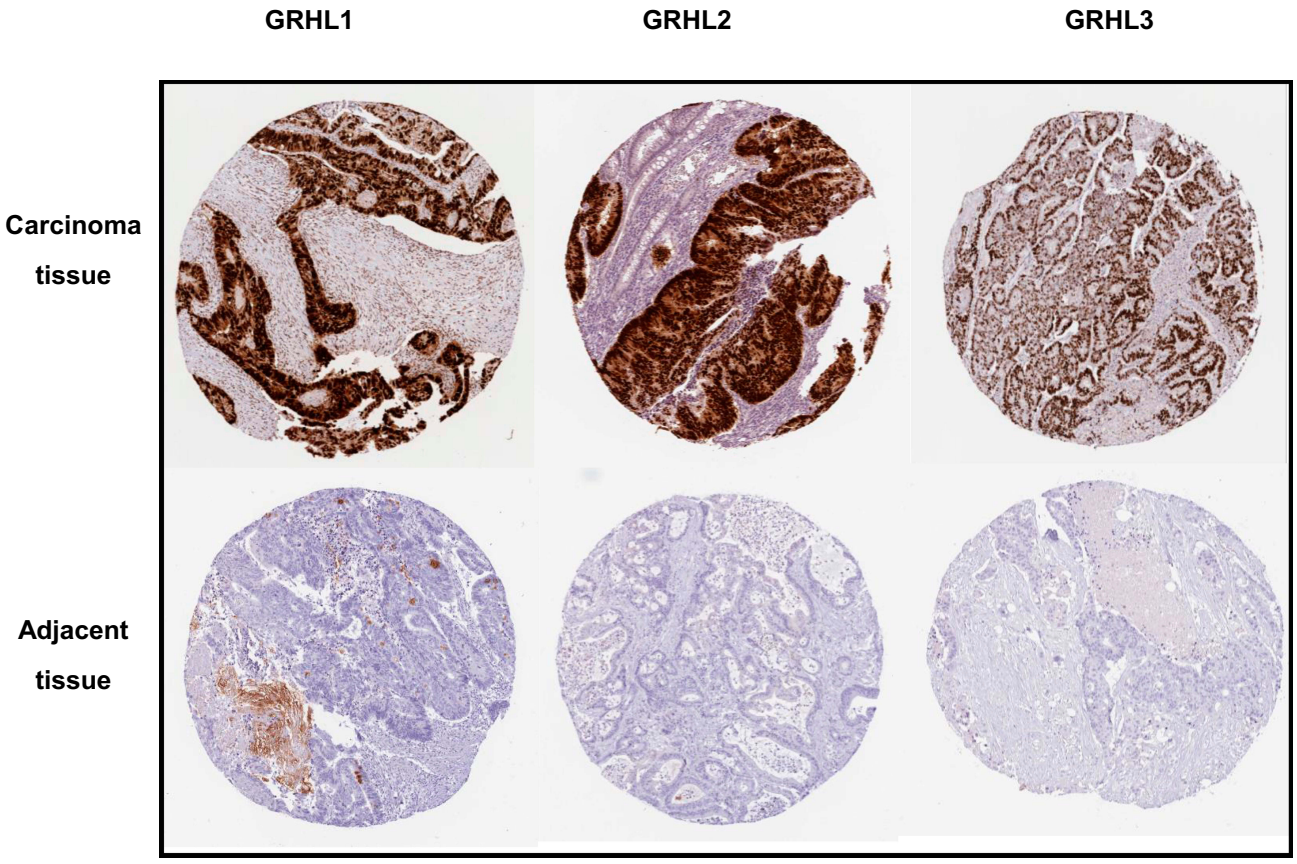

Figure 2 The immunohistochemical staining of GRHL genes in carcinoma tissues and adjacent tissues in patients with CC obtained from HPA database.

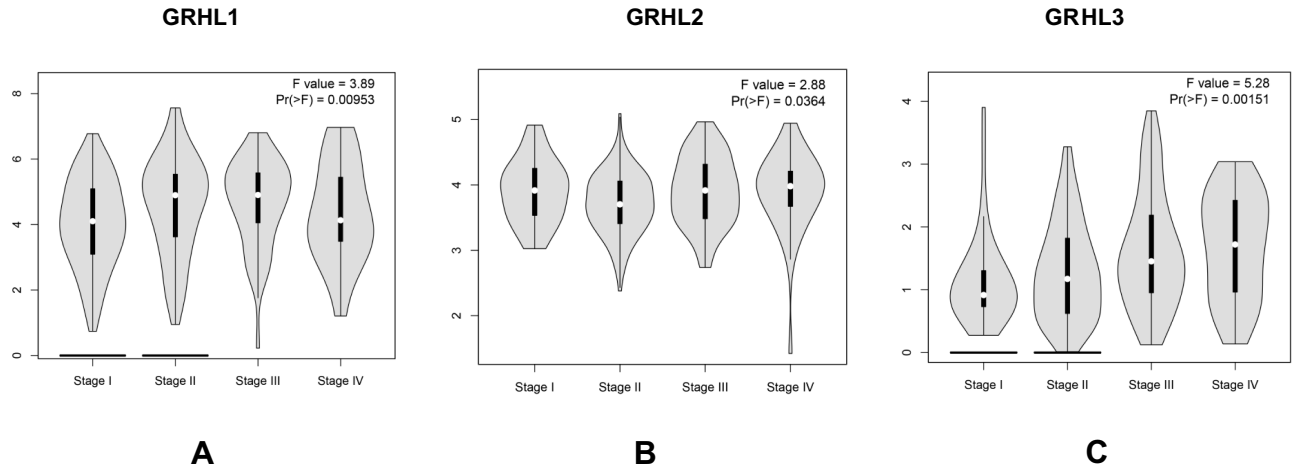

Figure 3 The relationship between the level of GRHL genes and tumor stages in patients with CC. (**A**) *GRHL1*; (**B**) *GRHL2*; (**C**) *GRHL3*.

further bioinformatics analysis of CC on GRHL has yet to be performed till now. To our knowledge, our study is the first time to investigate the mRNA and protein expression as well as prognostic values of different members in GRHL genes family in CC. We sincerely hope that our study will contribute to available knowledge, improve treatment designs, and enhance the accuracy of prognosis for patients with CC.

The *GRHL1* transcription factor is tissue-specific and is predominantly expressed in the kidney. The *GRHL1* gene is located at the chromosomal position 2p25 in humans.[14] In a mouse model, the loss of *GRHL1* significantly

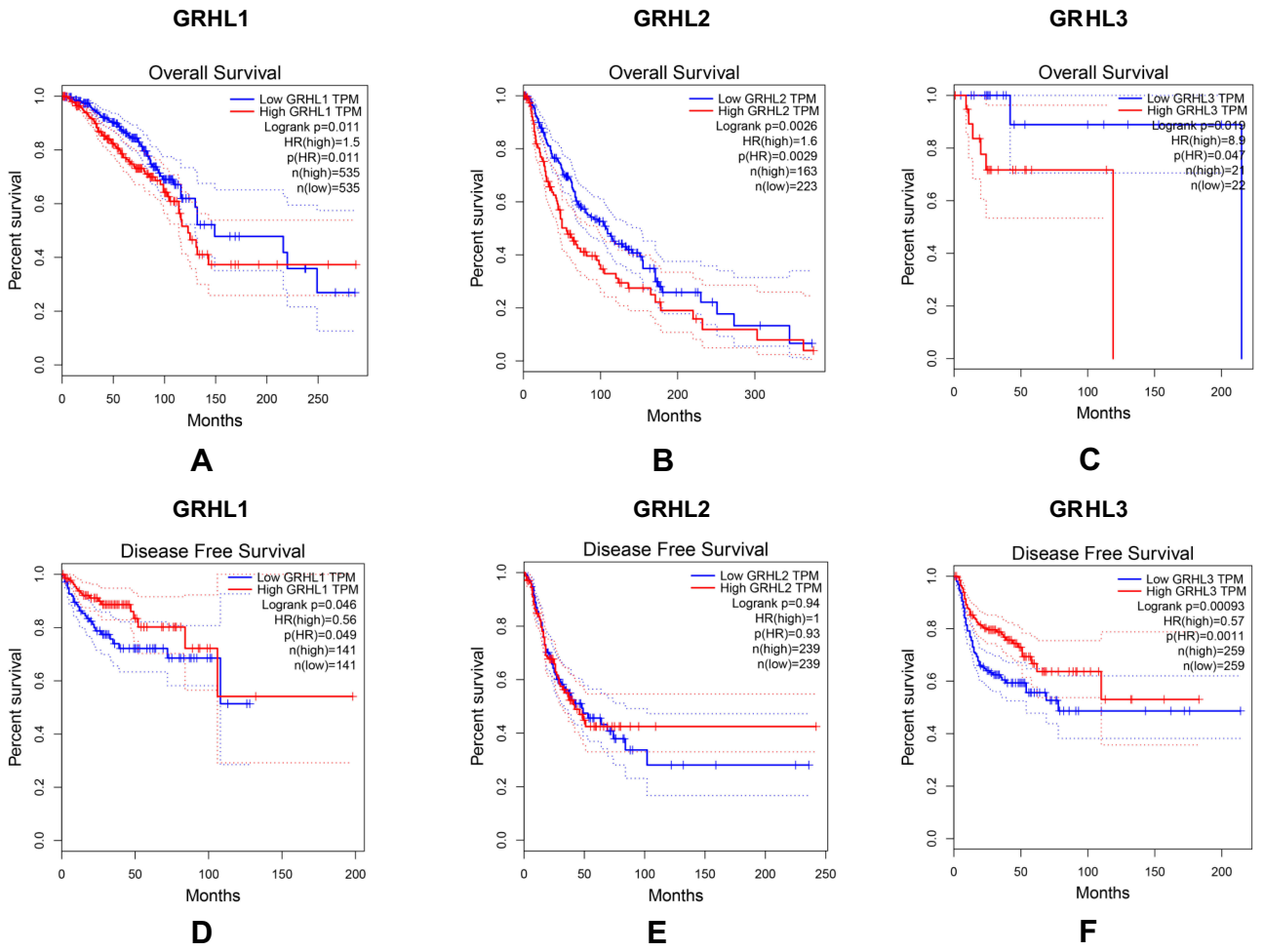

Figure 4 The relationship between the level of GRHL genes and overall survival (**A**–**C**) as well as disease free survival (**D**–**F**) in patients with CC; P (HR) < 0.05 versus the low GRHL TPM.

affected the heart rate but had no effects on blood pressure.[15] *GRHL1* was also reported to serve as a tumor suppressor by affecting mild chronic skin inflammation and aberrant terminal differentiation of keratinocytes in the squamous cell carcinoma of the skin.[16] Additionally, high levels of *GRHL1* expression were also associated with a favorable prognosis for patients with neuroblastoma. In vitro experiments further revealed that GRHL1 could inhibit the development of neuroblastoma by regulating MYCN and HDAC3.[17] In our study, we found that the mRNA and protein expression of *GRHL1* was significantly higher in patients with CC, the higher level of which may be associated with tumor stage and survival condition.

GRHL2 plays vital roles in embryonic neural tube closure, epidermal integrity, and wound healing processes. Mountainous evidence has disclosed that *GRHL2* may be a novel proto-oncogene which could regulate epithelial plasticity by suppressing endothelial–mesenchymal transition in several types of tumor.[18] For example, GRHL2 expression level was positively associated with CD133 and E-cadherin in primary pancreatic ductal adenocarcinoma and was highly expressed in liver metastatic pancreatic ductal adenocarcinoma tissues.[19] Meanwhile, Yang et al[20] have identified 6 genes involving *FN1*, *CDH2*, *CTNNB1*, *CITED2*, as well as *CTNNA3* as GRHL2-related genes together with *GRHL2* in breast cancer metastasis, the expression levels of which were associated with clinical features. On the contrary, other studies also revealed that *GRHL2* may serve as a tumor suppressor in cervical cancer, sarcoma, gastric cancer, and clear cell renal cell carcinoma.[21] Exogenous *GRHL2* transduced into gastric cancer cells obviously suppressed the proliferation and enhanced apoptosis. At the same time, over-expression of *GRHL2* significantly reduced the protein expression levels of c-Myc and Bcl-2.[22] Our study demonstrated that *GRHL2* may serve as

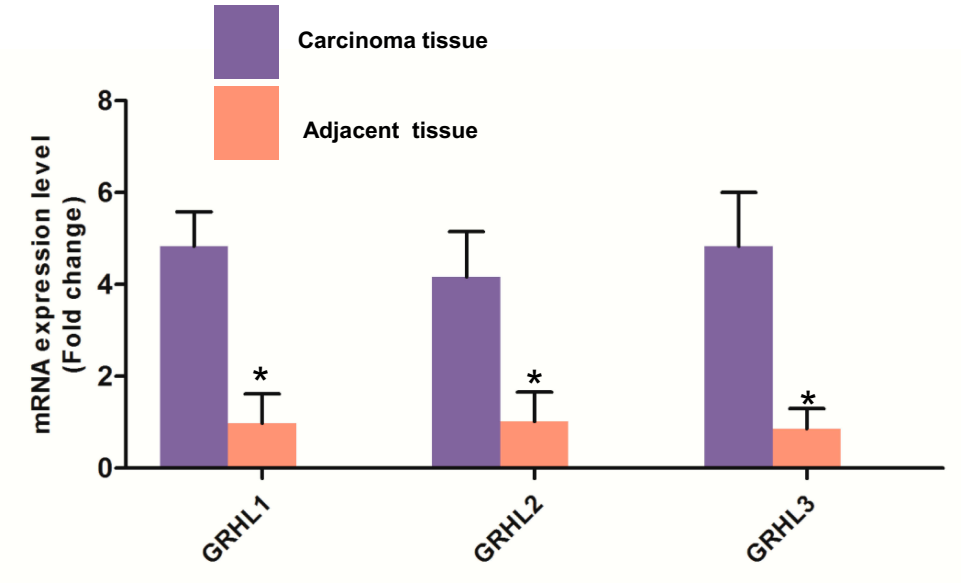

Figure 5 Re-identification of GRHL genes expression of carcinoma tissues and adjacent tissues from patients diagnosed with CC using real-time PCR. *P < 0.05 versus the carcinoma group.

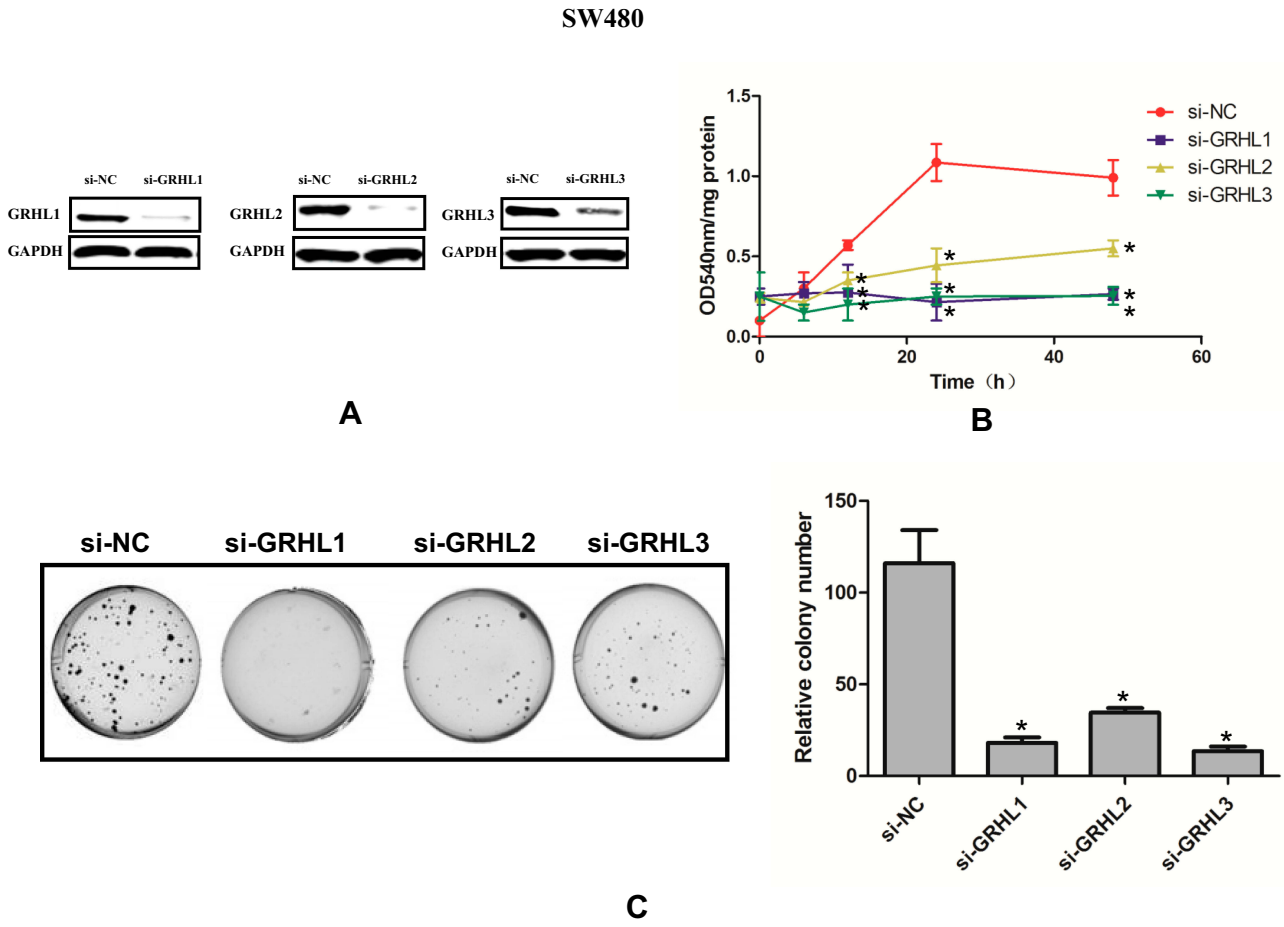

Figure 6 Knockdown of GRHL genes reduces proliferation of human SW 480 CC cells. (**A**) The representative Western blot images in indicated groups. (**B**) The OD540 of different groups at different time points using CCK8 assay. (**C**) Colony formation images in indicated groups. P < 0.05 versus the si-NC group.

a potential proto-oncogene in CC and is involved in the proliferation of CC cells, at least proliferation ability.

GRHL3 is very important for epidermal development and homeostasis in a wide range of species.

GRHL3 deficiency could induce epidermal keratinocyte hyperproliferation during embryogenesis by targeting PTEN directly in squamous cell carcinoma.[23] In colorectal cancer cells and tissues, the *GRHL3* expression at both mRNA and protein levels was significantly increased. Knockdown of *GRHL3* with siRNA obviously repressed colorectal cancer cell viability, proliferation, and migration, apart from promoting cell cycle arrest at G0/G1 phase and inducing cell apoptosis.[24] In accordance with this study, our study also proved the expression level of *GRHL3* was correlated with prognosis and tumor stage of patients with CC. More importantly, *GRHL3* also affected the proliferation of CC cells, which was evidenced by the altered colony formation ability of CC cells.

Conclusion

Taken together, in this study, we systemically analyzed the expression and prognostic value of *GRHL* genes family in CC and provided a thorough understanding of the heterogeneity and complexity of the molecular biological properties of CC. Our studies indicated that the increased expression of *GRHL1*, *GRHL2* and *GRHL3* in CC tissues might play a vital role in CC oncogenesis; therefore, they may be promising diagnostic biomarkers for CC. Additionally, the levels of *GRHL1*, *GRHL2* and *GRHL3* were significantly associated with survival and tumor stages of the patients with CC, suggesting that Pitx1 could be potential therapeutic targets for CC.

Disclosure

The authors report no conflicts of interest in this work.

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