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Overexpression of grainyhead-like transcription factor 2 is associated with poor prognosis in human pancreatic carcinoma

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Abstract. Recent studies have demonstrated that the abnormal expression of the grainyhead-like transcription factor 2 (GRHL2) gene contributes to the progression and poor prognosis of cancer through multiple mechanisms, but little is known about its expression status and prognostic value in pancreatic carcinoma (PC). The aim of the present study was to investigate the expression of GRHL2 in PC and to evaluate its clinicopathological and prognostic significance. Immunohistochemistry and western blotting were used to detect the expression of GRHL2 in PC tissues and cell lines, respectively. The expression of GRHL2 was investigated in 92 PC tissue samples by immunohistochemistry. High expression of GRHL2 was significantly associated with histological differentiation (P=0.018) and lymphatic metastasis (P=0.024). A Kaplan-Meier analysis revealed that high expression of GRHL2 was associated with worsened overall survival time (P<0.001). Multivariate analysis indicated that GRHL2 may be an independent prognostic factor for poor overall survival time (P=0.001). Additionally, western blot analysis demonstrated that the GRHL2 protein was highly expressed in PC cell lines. GRHL2 may serve an important role in the tumourigenesis of PC and serve as a potential therapeutic target for the prevention of PC progression.

Introduction

Pancreatic carcinoma (PC) was the fourth most common cause of cancer-associated mortality in the United States in 2014 (1), with only a 5% 5-year survival rate (2). Surgical treatment is the only available therapeutic option with respect to PC (2). Despite certain breakthroughs in treatment in recent years, the majority of patients with PC eventually succumb due to recurrence (3). For this reason, in clinical practice, the determination of an effective biomarker capable of predicting tumour behaviour is of emergent importance.

The grainyhead-like transcription factor 2 (GRHL2) gene is a member of the GRHL family of transcription factors that contain a DNA-binding immunoglobulin fold homologous to the core domain of key tumour suppressor p53 (4,5). Frisch *et al* (6) indicated that GRHL2 suppresses the oncogenic epithelial-mesencyhmal transition, thereby acting as a tumour suppressor. GRHL2 has also been implicated in neural tube closure and in early embryonic development (7,8). A number of studies have revealed that GRHL2 is associated with several types of cancer, including those of the breast, prostate, renal cells, cervix and liver (9-13).

In the present study, the GRHL2 expression in PC tissues was investigated, along with its association with clinicopathological factors and prognosis. GRHL2 may prove to be a novel biomarker for PC.

Materials and methods

Clinical tissue samples. Overall, 92 PC samples and their corresponding adjacent tissues were selected from specimens collected from patients diagnosed at Anhui Provincial Hospital (Hefei, China) between June 2008 and June 2012. Detailed pathological and clinical data (including age, sex, tumour location, nerve invasion, degree of differentiation, histological type, depth of invasion, lymph node metastasis and TNM stage) were obtained from the medical records of each patient. The samples were from 56 male and 36 female patients aged 38-77 years (median, 54 years). Samples were included in the present study based on the 7th edition of the Union for International Cancer Control TNM staging system (14). Patients who had received radiotherapy or chemotherapy prior to surgery were not included. The specimens were fixed in 4% formalin at 37˚C for 2 h and embedded in paraffin for pathological analysis and confirmation of the diagnosis. The clinical follow-up data of the patients were obtained from the PC database of Anhui Provincial Hospital. The present study was approved by the Human Research Ethics Committee of the Anhui Provincial Hospital and each patient provided informed consent.

Immunohistochemistry and scoring. Immunohistochemistry for GRHL2 was performed on each tissue sample. The tissue

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samples were dissected into 4-μm sections on silanised glass slides. Protein expression was detected with 2-step immunohistochemistry. In brief, deparaffinised and hydrated sections were treated with 0.3% hydrogen peroxide in methanol for 15 min at room temperature in order to block endogenous peroxidase activity, and washed in PBS (3 times for 3 min each), whereby antigen retrieval was conducted in citrate buffer (cat. no. P0081; Beyotime Institute of Biotechnology, Haimen, China; pH 6.0) for 10 min at 100˚C. Following 3 more PBS washes (3 min each), the sections were stained with a GRHL2 monoclonal antibody (cat. no. ab86611, 1:200, Abcam, Cambridge, UK) for 2 h at 37˚C, and washed again with PBS (3 times for 3 min each). Subsequently, the sections were incubated with horseradish peroxidase (HRP) universal IgG antibody polymer (cat. no. sc69786, 1:1,000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 15 min at 37˚C, followed by 3 PBS washes (3 min each). Each section was treated with 50 μl diaminobenzadine working solution (DAB Horseradish Peroxidase Color Development kit; cat. no. P0202; Beyotime Institute of Biotechnology) at room temperature for 3-10 min, followed by a wash in PBS. All sections were counterstained with haematoxylin for 1-2 min at room temperature for the purpose of enabling the morphology of the tissue to be observed using a light microscope.

The expression of GRHL2 in the tumour samples was semi-quantitatively assessed using ImageJ 1.8.0 software (National Institutes of Health, Bethesda, MD, USA). The proportion of positive cells was graded as follows: 0, <1%; 1, 1-30%; 2, 30-70%; and 3, >70%. Intensity of staining was stated as none (score, 0), weak (score, 1), moderate (score, 2) or strong (score, 3). The immunoreactivity was calculated according to the intensity of staining and the percentage of positive cells. The 2 scores were multiplied with each other and the eventual immunostaining score was determined: A final score of 0, 1, 2 or 3 was considered as low expression, and a score of 4, 6 or 9 was classified as high expression. All specimens were evaluated separately by 2 pathologists who were blinded to the clinical data, and discrepancies were resolved by consensus.

Cell lines and culture. PANC-1, BxPC-3 and HPDE6-C7 were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cell lines were grown in Dulbecco's modified Eagle' medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 5% CO_2.

Western blot analysis. The cells were lysed in RIPA cell lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) with 1 mM phenylmethylsulfonyl fluoride at 4˚C for 30 min, with vortexing every 10 min, followed by centrifugation at 13,800 x g for 10 min at 4˚C. BCA kit (cat. no. P0009; Beyotime Institute of Biotechnology) was used to quantify the protein concentration. The supernatant protein solutions were diluted to 4 mg/ml and kept at -80˚C until further use. A total of 50 μg of denatured protein for each sample was separated on a 10% SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% non-fat milk in TBS/Tween-20 for 2 h at room temperature and blotted with primary antibodies of rabbit monoclonal anti-GRHL2 (cat. no. ab86611; 1:200; Abcam) and mouse monoclonal anti-β-actin (cat. no. TA811000; 1:400; OriGene Technologies, Inc., Beijing, China) overnight at 4˚C. Following washing with TBS/Tween-20, the membranes were incubated with anti-rabbit or anti-mouse secondary antibodies, conjugated with HRP (catalog nos. A0208 and A0216; 1:1,000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature. Following 3 washes, the membrane was visualised with an enhanced chemiluminescence system (cat. no. P0018AM; Beyotime Institute of Biotechnology). The housekeeping control in this experiment was β-actin.

Table I. Comparison between GRHL2 protein expression (immunohistochemical staining) in PC and adjacent normal pancreatic tissues.

	GRHL2 expression			
Tissue type	High, n	Low, n	χ^2	P-value
PC	50	42	5.608	0.018
Adjacent	34	58		

GRHL2, grainyhead-like transcription factor 2; PC, pancreatic carcinoma.

Statistical analysis. All statistical analyses were performed using the statistical package SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The χ^2 test was used to analyse the immunohistochemistry results. Survival analysis was performed using Kaplan-Meier survival curves with the log-rank test. A multivariate Cox regression analysis was used to identify significant independent prognostic factors. All P-values were 2-sided and $P<0.05$ was considered to indicate statistically significant differences.

Results

Immunohistochemistry of GRHL2 expression in PC tissue samples. For the purpose of evaluating the levels of GRHL2 protein in PC tissue samples, immunohistochemistry was performed on 92 PC specimens and their matched adjacent healthy pancreatic tissues. Representative images of high and low expression of GRHL2 in PC and adjacent normal tissue samples are presented in Fig. 1. The GRHL2 staining was primarily observed in the cytoplasm of the tumour cells. High expression of GRHL2 was observed in the majority of the PC tissues (50/92 samples) and in a number of the non-cancerous pancreatic tissues (34/92 samples). The number of PC tissue samples with high GRHL2 expression was significantly higher than the number of non-cancerous tissue samples of the same phenotype (P=0.018; Table I).

Western blot analysis of the GRHL2 protein level in the PC cell lines. The results of the western blotting were in agreement with those of the immunohistochemistry. As demonstrated in Fig. 2, the amount of GRHL2 protein in the PC cell lines (PANC-1

Table II. Associations between GRHL2 protein expression (immunohistochemical staining) in pancreatic carcinoma and various clinicopathological variables.

		GRHL2 expression			
Variables	Total, n	Low (n=42)	High (n=50)	χ^2	P-value
Sex				0.035	0.852
Male	56	26	30		
Female	36	16	20		
Age, years				0.207	0.649
≤60	44	19	25		
>60	48	23	25		
Tumour diameter, cm				0.372	0.542
≤4	47	20	27		
>4	45	22	23		
Tumour location				0.589	0.443
Head and neck	50	21	29		
Body and tail	42	21	21		
Nerve invasion				2.170	0.141
Negative	56	29	27		
Positive	36	13	23		
Differentiation				5.571	0.018[a]
Well/moderate	63	34	29		
None/poor	29	8	21		
T classification				0.058	0.809
T1+T2	69	31	38		
T3+T4	23	11	12		
M classification				0.032	0.858
M0	88	40	48		
M1	4	2	2		
N classification				5.124	0.024[a]
N0	66	35	31		
N1	26	7	19		
TNM stage				0.049	0.825
I-II	86	39	47		
III-IV	6	3	3		

[a]P<0.05. GRHL2, grainyhead-like transcription factor 2; TNM, Tumour-Node-Metastasis.

and BxPC-3) was higher compared with that from the normal pancreatic cell line (HPDE6-C7).

Association of GRHL2 expression with patient clinicopathological parameters. In order to evaluate the biological significance of GRHL2 in PC, the associations between PC tissue GRHL2 levels and clinicopathological parameters, including age, sex, tumour size, tumour location, nerve invasion, lymphatic metastasis, pathological grade, depth of invasion and TNM stage, were analyzed. As demonstrated in Table II, the increased GRHL2 expression was associated with higher tumour differentiation (P=0.018) and N classification (P=0.024). By contrast, no significant association was identified between the expression of GRHL2 and any of the other parameters tested. These findings imply that GRHL2 may serve an important role in the progression of PC.

Association of GRHL2 expression with PC prognosis. A Kaplan-Meier survival curve was plotted in order to compare the OS time and the GRHL2 expression data (Fig. 3). Patients whose samples revealed high expression of GRHL2 presented with a worse prognosis than those with low GRHL2 expression (P<0.001; Fig. 3). Multivariate survival analysis further revealed the high GRHL2 levels in the tumour tissue to be an independent prognostic marker for poor OS time (P=0.001; Table III).

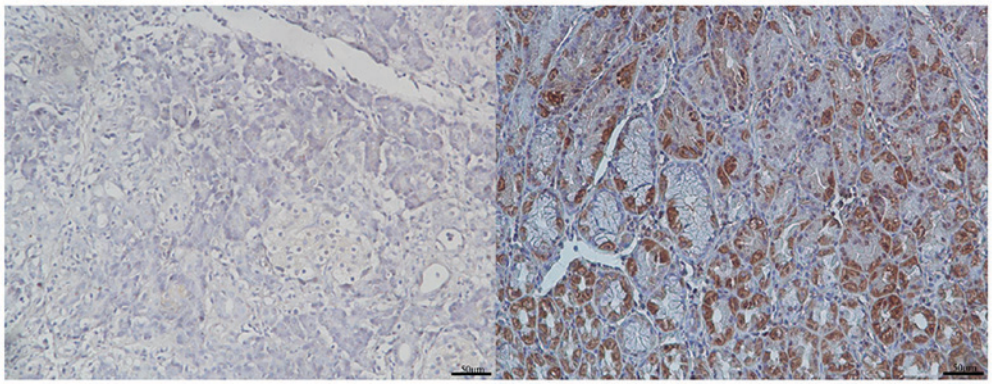

Figure 1. Immunohistochemical staining for grainyhead-like transcription factor 2 in pancreatic cancer (right panel) and adjacent normal tissue (left panel). Scale bar, 50 μm.

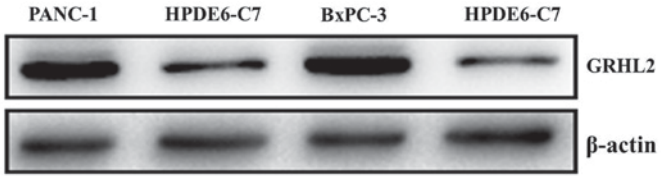

Figure 2. Western blot analysis of GRHL2 protein in 2 pancreatic cancer cell lines (PANC-1 and BxPC-3) and 1 normal pancreatic cell line (HPDE6-C7). GRHL2, grainyhead-like transcription factor 2.

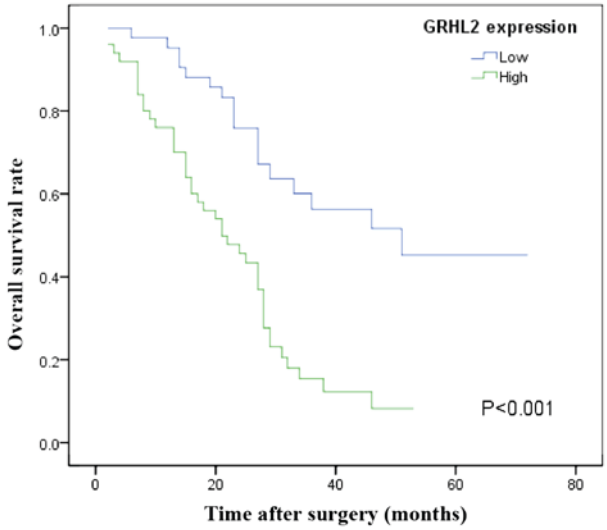

Figure 3. Kaplan-Meier analysis of overall survival times of PC patients according to tumour GRHL2 expression. GRHL2, grainyhead-like transcription factor 2.

Discussion

GRHL was first identified in the fruit fly *Drosophila melanogaster* (15). To date, a total of 3 members of the GRHL family, termed GRHL1-3, have been detected in mammals (16,17). GRHL2, an important member of the GRHL family, is quintessential in embryonic neural tube development (7). A number of studies have revealed that altered expression of GRHL2 is associated with development, progression, tumourigenesis and poor prognosis in a variety of different cancer types, including colorectal cancer, oral squamous cell carcinoma and liver cancer (4,11,18,19). Quan *et al* (18) investigated the GRHL2 expression in 171 colorectal cancer and paired normal colon mucosa samples, and revealed that GRHL2 is an independent prognostic factor for OS, as well as recurrence-free survival times. Butz *et al* (11) studied the GRHL2 expression in 593 clear cell renal cell carcinoma and 389 normal kidney specimens, and demonstrated that GRHL2 expression was associated with higher chances of disease recurrence and that the disease-free survival times of GRHL2-positive patients were lower.

In the present study, the GRHL2 protein expression levels were examined in samples of patients with PC who had undergone surgery, along with the potential association between GRHL2 expression levels and clinicopathological characteristics of the tumour. Furthermore, GRHL2 overexpression was determined in 2 human PC cell lines. An association between increased GRHL2 expression and lymphatic metastasis and tumour differentiation was identified, while a χ^2 test revealed that there were significantly more PC tissue samples with high GRHL2 protein levels compared with the corresponding adjacent non-cancerous tissue samples. Additionally, the OS time in patients with low levels of GRHL2 was increased compared with that of patients with elevated GRHL2 levels. The Kaplan-Meier and multivariate analyses suggested that the expression level of GRHL2 was a potential independent prognostic predictor of poor OS time. It was revealed that the increased expression level of GRHL2 was associated with the prognosis of PC.

In summary, the present study suggests that elevated GRHL2 protein levels may be a malignant phenotypic characteristic of PC. Furthermore, this is the first report of GRHL2 protein expression being a novel independent prognostic marker for PC. Further research, including cell function and pathway experiments, is therefore required for the purpose of exploring the molecular mechanism of GRHL2, along with investigating its potential as a therapeutic target in PC. A limitation of the present study is the small sample size from a single centre. Future studies with larger sample sizes are required to further validate the association between GRHL2 expression and prognosis.

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Not applicable.

Table III. Univariate and multivariate analyses of the clinicopathological parameters and overall survival time of patients with pancreatic carcinoma.

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Sex						
Male	1.270	0.759-2.123	0.363			
Female						
Age, years						
≤60	1.319	0.794-2.191	0.285			
>60						
Tumour size (diameter), cm						
≤4	1.115	0.672-1.851	0.674			
>4						
Differentiation						
Well/moderate	1.721	1.022-2.901	0.041[a]	1.216	0.646-2.290	0.545
None/poor						
Tumour location						
Head and neck	0.977	0.587-1.627	0.930			
Body and tail						
Nerve invasion						
Positive	1.531	0.921-2.544	0.100			
Negative						
T classification						
T1+T2	1.019	0.558-1.861	0.950			
T3+T4						
M classification						
M0	1.173	0.365-3.768	0.789			
M1						
N classification						
N0	2.301	1.371-3.861	0.002[a]	1.780	0.952-3.326	0.071
N1						
TNM stage						
I-II	1.425	0.569-3.566	0.450			
III-IV						
GRHL2 level						
Low	3.247	1.847-5.708	<0.01[a]	2.901	1.547-5.439	0.001[a]
High						

[a]P<0.05. HR, hazard ratio; CI, confidence interval; TNM, Tumour-Node-Metastasis; GRHL2, grainyhead-like transcription factor 2.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CW conceived and designed the study. JP and LZ analyzed and interpreted the patient data. GW, JP and LZ contributed to the acquisition of data, data analysis and data interpretation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Human Research Ethics Committee of the Anhui Provincial Hospital and each patient provided informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2017. CA Cancer J Clin 67: 7-30, 2017.
2. Wolfgang CL, Herman JM, Laheru DA, Klein AP, Erdek MA, Fishman EK and Hruban RH: Recent progress in pancreatic cancer. CA Cancer J Clin 63: 318-348, 2013.
3. Groot VP, van Santvoort HC, Rombouts SJ, Hagendoorn J, Borel Rinkes IH, van Vulpen M, Herman JM, Wolfgang CL, Besselink MG and Molenaar IQ: Systematic review on the treatment of isolated local recurrence of pancreatic cancer after surgery; re-resection, chemoradiotherapy and SBRT. HPB (Oxford) 19: 83-92, 2017.
4. Mlacki M, Kikulska A, Krzywinska E, Pawlak M and Wilanowski T: Recent discoveries concerning the involvement of transcription factors from the Grainyhead-like family in cancer. Exp Biol Med (Maywood) 240: 1396-1401, 2015.
5. Kokoszynska K, Ostrowski J, Rychlewski L and Wyrwicz LS: The fold recognition of CP2 transcription factors gives new insights into the function and evolution of tumor suppressor protein p53. Cell Cycle 7: 2907-2915, 2008.
6. Frisch SM, Farris JC and Pifer PM: Roles of Grainyhead-like transcription factors in cancer. Oncogene 36: 6067-6073, 2017.
7. Ray HJ and Niswander LA: Grainyhead-like 2 downstream targets act to suppress epithelial-to-mesenchymal transition during neural tube closure. Development 143: 1192-1204, 2016.
8. Rifat Y, Parekh V, Wilanowski T, Hislop NR, Auden A, Ting SB, Cunningham JM and Jane SM: Regional neural tube closure defined by the Grainy head-like transcription factors. Dev Biol 345: 237-245, 2010.
9. Yang X, Vasudevan P, Parekh V, Penev A and Cunningham JM: Bridging cancer biology with the clinic: Relative expression of a GRHL2-mediated gene-set pair predicts breast cancer metastasis. PLoS One 8: e56195, 2013.
10. Danila DC, Anand A, Schultz N, Heller G, Wan M, Sung CC, Dai C, Khanin R, Fleisher M, Lilja H and Scher HI: Analytic and clinical validation of a prostate cancer-enhanced messenger RNA detection assay in wholeblood as a prognostic biomarker for survival. Eur Urol 65: 1191-1197, 2014.
11. Butz H, Szabo PM, Nofech-Mozes R, Rotondo F, Kovacs K, Mirham L, Girgis H, Boles D, Patocs A and Yousef GM: Integrative bioinformatics analysis reveals new prognostic biomarkers of clear cell renal cell carcinoma. Clin Chem 60: 1314-1326, 2014.
12. Torres-Reyes LA, Alvarado-Ruiz L, Pina-Sanchez P, Martínez-Silva MG, Ramos-Solano M, Olimón-Andalón V, Ortiz-Lazareno PC, Hernández-Flores G, Bravo-Cuellar A, Aguilar-Lemarroy A and Jave-Suarez LF: Expression of transcription factor grainyhead-like 2 is diminished incervical cancer. Int J Clin Exp Pathol 7: 7409-7418, 2014.
13. Tanaka Y, Kanai F, Tada M, Tateishi R, Sanada M, Nannya Y, Ohta M, Asaoka Y, Seto M, Shiina S, *et al*: Gain of GRHL2 is associated with early recurrence of hepatocellular carcinoma. J Hepatol 49: 746-757, 2008.
14. Sobin LH, Gospodarowicz MK and Wittekind C (eds): Pancreas. International Union against Cancer (UICC) TNM Classification of Malignant Tumours, 7th edition. Wiley-Blackwell, New York, NY, pp132-135, 2010.
15. Dynlacht BD, Attardi LD, Admon A, Freeman M and Tjian R: Functional analysis of NTF-1, a developmentally regulated Drosophila transcription factor that binds neuronal cis elements. Genes Dev 3: 1677-1688, 1989.
16. Ting SB, Wilanowski T, Cerruti L, Zhao LL, Cunningham JM and Jane SM: The identification and characterization of human sister-of-mammalian grainyhead (SOM) expands the grainyhead-like family of developmental transcription factors. Biochem J 370: 953-962, 2003.
17. Wilanowski T, Tuckfield A, Cerruti L, O'Connell S, Saint R, Parekh V, Tao J, Cunningham JM and Jane SM: A highly conserved novel family of mammalian developmental transcription factors related to drosophila grainyhead. Mech Dev 114: 37-50, 2002.
18. Quan Y, Xu M, Cui P, Ye M, Zhuang B and Min Z: Grainyhead-like 2 promotes tumor growth and is associated with poor prognosis in colorectal cancer. J Cancer 6: 342-350, 2015.
19. Zhao Q, Caballero OL, Davis ID, Jonasch E, Tamboli P, Yung WK, Weinstein JN, Kenna Shaw for TCGA research network, Strausberg RL and Yao J: Tumor-specific isoform switch of the fibroblast growth factor receptor 2 underlies the mesenchymal and malignant phenotypes of clear cell renal cell carcinomas. Clin Cancer Res 19: 2460-2472, 2013.