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*Original Article*

# Expression of Gli1 and Wnt2B correlates with progression and clinical outcome of pancreatic cancer

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**Abstract:** Objective: The aim of this study was to investigate the expression and clinical significance of Gli1 and Wnt2B in pancreatic cancer. Methods: We have constructed a formalin-fixed paraffin embedded pancreatic tissue microarrays 180 cylindrical tissue cores of human pancreatic cancer and its paracancerous nonmalignant pancreatic specimens (NMPs) from 90 patients. Levels of Gli1 and Wnt2B were measured by immunohistochemistry. We analyzed the correlations between the expression of these factors and clinicopathological parameters including prognosis. Results: The expressions of both Gli1 and Wnt2B in human pancreatic cancer tissues were significantly higher than those of normal pancreatic tissues ($P$=0.000, $P$=0.004 respectively). The analysis showed that the high cytoplasmic expression levels of Gli1 in pancreatic cancer tissues had significant correlation with lymph node metastasis ($P$=0.036) and Wnt2B had significant correlation with perineural invasion ($P$=0.045). Gli1 and Wnt2B have no positive correlation. Survival analysis by Kaplan-Meier demonstrated that elevated Wnt2B expression in cancer tissue predicted worse overall survival (OS) compared with group in lower expression ($P$=0.024). No correlation was found between the expression of Gli1 and overall survival of pancreatic cancer patients ($P$>0.05). Conclusions: In conclusion, these results indicate that the high-expression levels of Gli1 and Wnt2B might play a pivotal role during tumorigenesis of pancreatic cancer, and the high expression of Wnt2B might be associated with poor prognosis.

**Keywords:** Pancreatic cancer, Gli1, Wnt2B, prognosis

## Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in western countries and has the least patient survival rate of any solid cancer. Recently, although the cancer death rates of most malignancies have decreased owing to improvements in early detection and treatment, the overall 5-year survival of patients with pancreatic cancer has increased only slightly from 3% to 5%, because of the early and aggressive local invasion and metastatic potential [1]. Conventional therapeutic approaches have no encouraging impact on curing this disease. Therefore, it is necessary to develop a complete understanding of the complex molecular carcinogenesis of pancreatic cancer. Although many genes have been identified to be involved in pancreatic carcinogenesis, few are reported as uniquely and directly responsible for the tumor. Therefore, identification of more biological markers related to tumor aggressiveness is needed to accurately predict the patient prognosis and to develop novel treatments. In our previous study, RNA was extracted and expression profiles experiment was performed using Agilent human whole genomic oligonucleotide microarrays with 41,000 genes. Through comparison of gene expression profile between 6 pancreatic cancer tissues, differentially expressed genes Gli1 and Wnt2B related to lymph node metastasis (LNM) and perineural invasion (PI) were screened respectively.

Gli1 is a zinc finger transcription factor and a member of the vertebrate Gli family. The Gli transcription factors coordinately regulate Gli-

mediated transcription [2]; Gli1 protein act at the downstream end of the hedgehog pathway where they control transcriptional programs in response to pathway activation. Indeed, the induction of Gli1 mRNA expression by hedgehog signaling is a reliable marker for pathway activity [3]. Hedgehog signaling is an essential pathway during embryonic organ development, the misregulation of which has been implicated in a wide range of different tumors including carcinomas of the stomach [4], pancreas [5], breast [6], prostate [7], small cell lung cancer [8], glioblastoma [9] and melanoma [10].

The Wnt gene family encodes multi-functional signaling glycoproteins that are involved in the regulation of a wide variety of normal and pathological processes, including embryogenesis, epithelial differentiation, and tumorigenesis. Wnt2B is one of the canonical Wnt ligands, Wnt2B stimulates the canonical Wnt pathway [11]. Studies have demonstrated that Wnt2B is overexpressed in various human cancers or in human cancer cell lines, including malignant pleural mesothelioma [11], breast cancer [12], Primary Gastric Cancer [13], and lung cancer [14]. Knockdown of Wnt2B by small interfering RNA can inhibits metastasis and enhances chemotherapy sensitivity in ovarian cancer [16]. An adenoviral vector expressing shRNA targeting Wnt2B had an effective antitumor activity against Wnt2B-overexpressing tumors both in vitro and in vivo [15, 16].

Evidence shows that aberrant disorders of Hedgehog and Wnt pathway are often associated with wide variety of human cancers. However, as key roles in these two pathway, Gli1 and Wnt2B's expression in human pancreatic cancer tissues and the correlation of these two proteins with clinicopathological parameters is not very clearly reported so far. Therefore, to clarify the tumor biology of Gli1 and Wnt2B in human pancreatic cancer, we performed a comprehensive clinical study on the expression of Gli1 and Wnt2B, in relation to the clinical feathers.

## Materials and methods

### *Case selection*

In total, 90 cases of formalin-fixed, paraffin-embedded (FFPE) pancreatic cancer and 90 paracancerous blocks were obtained from the Biobank in National Engineering Centre for Biochip at Shanghai. All tissues specimens were obtained for the present study with patient informed consent for retention and analysis of their tissue for research purpose, and Ethical approval for the study was obtained from the Ethical Committee of Biobank Centre Related Hospitals. Tissue samples, mainly adenocarcinoma, were retrieved from 90 patients who underwent pancreatic surgical resection between 2004 and 2008. The pancreatic diagnosis was confirmed and histological subtype determined by pathologist and a database was established noting clinical parameters such as age, gender, location, tumor size, histology, stage, lymph node metastasis and Overall survival (OS). The stage was updated according to current American Joint Committee on cancer (AJCC) guidelines. Overall survival was measured from time of definitive operation to death from pancreatic cancer. This report includes the follow-up data up end by December, 2011.

### *Tissue microarray construction*

Each tissue microarray (TMA) was assembled with 0.6 mm cores punched from distinct regions of each FFPE primary tumor and arrayed in duplicate. Tumor regions to be sampled were reviewed by study pathologist (MR), who was blinded to specimen protein expression status. Representative tumor regions and its paracancerous nonmalignant pancreatic specimens (NMPs) were selected from each tissue block and 2 tissue cores were taken from each region using an automated tissue arrayer (Beecher Instruments, Sun Prarie, WI). Cores were transferred to individual recipient blocks. In all cases, cores taken normal adjacent pancreas were also used as internal controls. Sections were stained with H&E to confirm the presence of tumor within each core. Representative tumor regions and its paracancerous nonmalignant pancreatic specimens (NMPs) were selected for each tissue block.

### *Immunohistochemistry staining and scoring*

The following antibodies were used: a rabbit polyclonal antibody for Gli1 (Novus Biologicals, expression in cytoplasm) diluted at 1:500, a rabbit polyclonal antibody for Wnt2B (Novus Biologicals, expression in cytoplasm and nuclei) diluted at 1:50. Pancreatic cancer tissue microarrays were immunohistochemically stained as previous described [18]. TMA slides were deparaffinized, rehydrated through graded alcohol, washed with Tris-buffered saline, and processed using a streptavidin-biotin-peroxidase

**Table 1.** Clinicopathological characteristics of the pancreatic cancer

| Characteristics | No. of patients | % |
|---|---|---|
| Age (years) | | |
| Median | 62 | |
| Range | 38-85 | |
| Gender | | |
| Male | 57 | 63% |
| Female | 33 | 37% |
| Tumor size | | |
| Median (cm) | 4.0 | |
| Range (cm) | 0.5-14 | |
| Tumor differentiation | | |
| Moderate/Well | 57 | 71% |
| Poor | 23 | 29% |
| Miss | 10 | |
| Lymph node metastasis | | |
| 0 | 52 | 58% |
| 1 | 38 | 42% |
| Perineural invasion | | |
| 0 | 20 | 37% |
| 1 | 34 | 63% |
| Miss | 36 | |
| AJCC Stage | | |
| Stage I | 40 | 45% |
| Stage II | 48 | 53% |
| Stage III | 0 | 0 |
| stage IV | 2 | 2% |

complex method. Antigen retrieval was performed by microwaveheating sections in 10 mm sodium citrate buffer (pH 6.0) for 10 minutes. After quenching of endogenous peroxidase activity and blocking of nonspecific binding, 2 antibodies were added, then slides were incubated at 4°C overnight. The corresponding secondary biotinylated rabbit antibody was used at a special dilution for 30 minutes at 37°C. After further washing with Tris-buffered saline, sections were incubated with StrepABC complex/horseradish peroxidase (1:1000, DAKO) for 30 minutes at 37°C. Chromogenic immunolocalization was performed by exposure to 0.05% 3, 3-diaminobenzidine tetrahydrocloride. Slides were counterstained with hematoxylin before dehydration and mounting.

Scoring: The staining intensity of Gli1 and Wnt2B was graded on a scale of 0 to 3 (0, none; 1, weak; 2, intermediate and 3 strong). The expression was assessed according to the percentage of staining as follows: 0 points for no staining; 1 point for <25%; 2 points for 26~50%; 3 points for 51~75%; 4 points for 76~100%. The total score was the product of the scores for the intensity and positive rate of staining (staining index = intensity + positive rate). For data analysis, staining index scored as 0~3 were considered low expression, and the staining index >3 were considered high expression. The immunostained slides were examined by two authors without knowledge of the patient characteristics. Cases with discrepancies were jointly reevaluated until a consensus was reached.

*Statistical analysis*

Analysis was performed with SPSS 17.0 software for windows. The differences in the expression levels of Gli1 and Wnt2B between cancer tissues and paracancerous tissues were analyzed by Wilcoxon. The relationships between expression levels and clinicopathological parameters were studied using the $\chi^2$-test. Correlation between protein expression (immunohistochemical scores) were evaluated with the Spearman rank order correlation test. The Kaplan-Meier method was used to estimate the survival function. $P<0.05$ was considered statistically significant.

## Results

*Patient and tumor characteristics*

As shown in **Table 1**. Of the 90 patients, 57 were male and 33 were female, and the median age of the patients was 62 years (range: 38-85 years). The median tumor size was 4 cm (range 0.5 cm-14 cm). 57 tumors were well differentiated or moderate differentiation, 23 were poorly differentiated and 10 cases were missed. 38 of 90 cases were with lymph node metastasis (LNM), and 52 cases have no LNM. According to updated American Joint Committee on cancer (AJCC) standers, 40 patients were in stage I, 48 patients in stage II, none in stage III and 2 patients in stage IV.

*Immunohistochemical analysis of Gli1 and Wnt2B protein expression in pancreatic cancer and nonmalignant speciments of the pancreas*

Immunohistochemical staining showed that Gli1 was mainly located in the cytoplasm of pancreatic cancer cell. The cytoplasm expression level was higher in cancer tissues than in

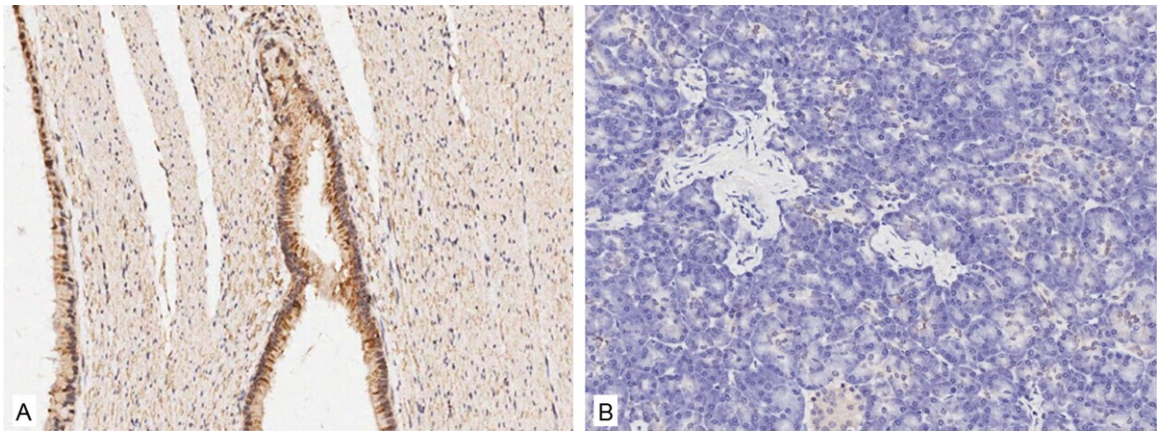


**Figure 1.** Panel A showed the high expression of Gli1 in the cancer tissues. Panel B showed the low expression in the paracancerous tissues. All images were taken at 200x magnification.

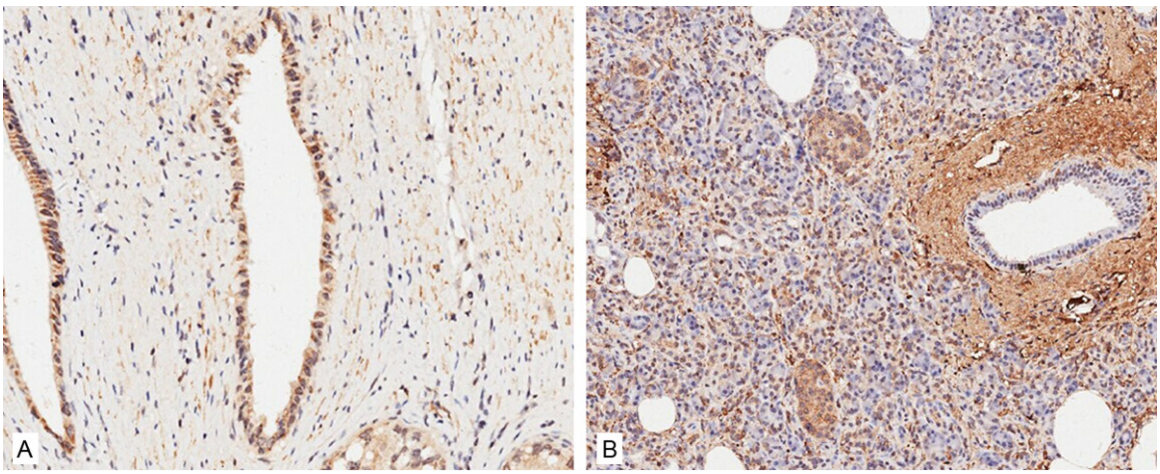


**Figure 2.** Panel A showed the high expression of Wnt2B in pancreatic cancer tissue. Panel B showed the low expression of Wnt2B in the paracancerous tissues. All images were taken at 200x magnification.

paracancerous tissues ($P$<0.001) (**Figure 1**). Wnt2B was mainly located in the cytoplasm, a little was seen in the nucleus. The cytoplasm expression level was higher in cancer tissues than in paracancerous tissues ($P$<0.01) (**Figure 2**).

*Correlations of Gli1 and Wnt2B expression with clinicopathological features*

**Table 2** shows the results of statistical analysis of the correlations between Gli1 and Wnt2B protein expression and the clinicopathological factors of the patients with pancreatic cancer. The analysis showed that the cytoplasm high expression of GLi1 was significantly correlated with lymph node metastasis ($P$=0.036) and high expression of Wnt2B was correlated with perineural invasion ($P$=0.045), but not with other factors, including age, gender, location, size, and Tumor differentiation.

*Correlation between Gli1 and Wnt2B*

In pancreatic cancer tissues, there was not significant positive correlation between the expression of Gli1 and Wnt2B ($P$>0.05), by spearman's correlation.

*Survival analysis*

We examined the postoperative survival of the 90 patients who underwent curative surgery, in relation of Gli1 and Wnt2B expression.

All 90 enrolled patients had a complete follow-up record. The median survival time was 31 months (range: 0~87 months). Patients with

**Table 2.** Association of Gli1 and Wnt2B protein expression with the clinicopathological parameters

| Clinicopathological features | Gli1 | | | Wnt2B | | |
|---|---|---|---|---|---|---|
| | high | low | *P* value | high | low | *P* value |
| | N=55 | N=35 | | N=17 | N=73 | |
| Age (years) | | | | | | |
| <60 | 27 | 14 | 0.399 | 10 | 31 | 0.223 |
| ≥60 | 28 | 21 | | 7 | 42 | |
| Gender | | | | | | |
| Male | 32 | 25 | 0.204 | 12 | 45 | 0.491 |
| Female | 23 | 10 | | 5 | 28 | |
| Tumor location | | | | | | |
| Head | 35 | 26 | 0.292 | 11 | 50 | 0.763 |
| Body/rear | 20 | 9 | | 6 | 23 | |
| Tumor size (cm) | | | | | | |
| <4 | 20 | 14 | 0.729 | 6 | 28 | 0.815 |
| ≥4 | 35 | 21 | | 11 | 45 | |
| Tumor differentiation | | | | | | |
| Moderate/Well | 36 | 21 | 0.581 | 10 | 47 | 0.537 |
| poor | 13 | 10 | | 6 | 17 | |
| Lymph node metastasis | | | | | | |
| No | 27 | 25 | 0.036 | 10 | 42 | 0.923 |
| Yes | 28 | 10 | | 7 | 31 | |
| Perineural invasion | | | | | | |
| No | 6 | 14 | 0.065 | 3 | 17 | 0.045 |
| Yes | 19 | 15 | | 14 | 20 | |
| Miss | 30 | 6 | | | 0 | 36 |
| AJCC stage | | | | | | |
| I | 21 | 19 | 0.176 | 6 | 34 | 0.480 |
| II | 32 | 16 | | 10 | 38 | |

low cytoplasmic expression levels of Wnt2B achieved better survival than patients with high expression levels (*P*=0.025). However, there was no statistically significant relationship in overall survival between patients with high or low expression of Gli1 (*P*>0.05) (**Figure 3**).

## Discussion

Pancreatic cancer is a highly aggressive disease with poor long term survival. Despite improvements in surgical and chemotherapeutic approaches during the past decades, pancreatic cancer continues to have a dismal prognosis, with an average overall 5-year survival rate of less than 5%. To date, surgical resection is still the only potentially curative therapeutic option, however, because of the lack of early symptoms, the vast majority of patients with metastatic disease, rendering their malignancy inoperable. Understanding the molecular mechanisms underlying pancreatic cancer development is an essential first step in early diagnosis of pancreatic cancer.

According to our study, Gli1 maybe contribute to lymph node metastasis. The presence or absence of lymph node metastases is known to be an important prognostic factor for patients with pancreatic cancer. Numerous studies have demonstrated that patients with lymph node metastases have a significantly wo-|rse survival than do patients with node-negative disease [17-19]. In Pawlik's study, there were 187 (20.7%) of the 905 patients who had negative peripancreatic lymph nodes (N0), whereas 718 (79.3%) of the 905 patients had lymph node metastases (N1). The median number of lymph nodes evaluated in the N0 group was 15 versus 18 in the N1 group (*P*=0.12). Patients with lymph node metastases had a shorter median overall survival (16.5 months) compared with patients with negative lymph nodes (25.3 months; P=0.001) [20]. Fortner et al [21] published 58% had regional lymph node metastasis in their study, higher than our result 42% (38 cases in 90).

However, for some cancers, perineural invasion (PNI) may be one of the major route of metastatic spread. Pancreatic cancer is one of the very few cancers that spread along nerves. PNI is considered as an important factor of aggressive tumor behavior and it is associated with local recurrence and poor outcome of pancreatic cancer [22]. In our study, there are 63% (34 cases in 54) cases with perineural invasion. Wnt2B maybe contribute to PNI.

In these study, we found that the expressions of Gli1 in human pancreatic cancer tissues was significantly higher than those of normal pancreatic tissues (*P*=0.000). Gli1 is a transcrip-

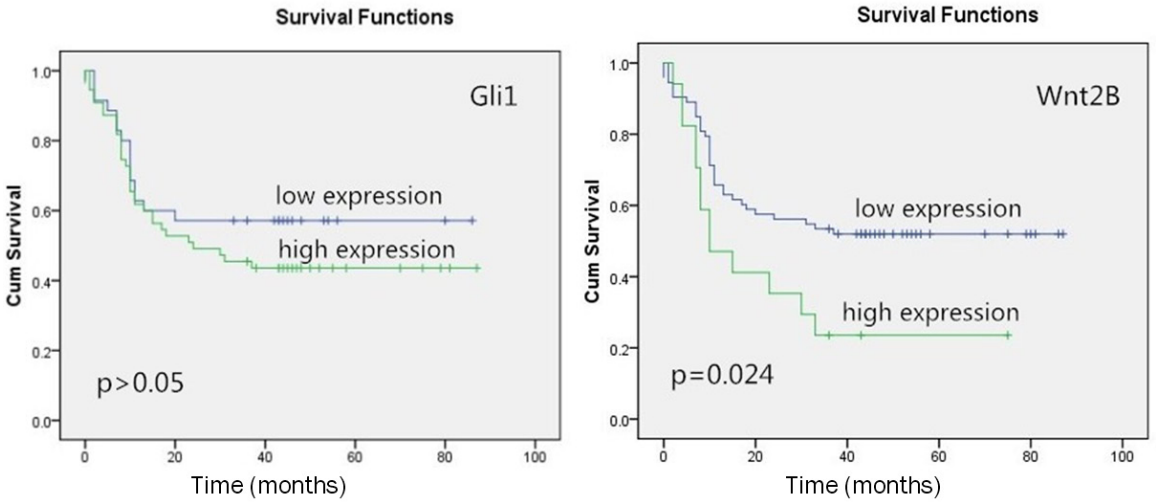


**Figure 3.** Kaplan-Meier curves showing no significant association between the expression level of Gli1 and overall survival (p>0.05), high level expression of Wnt2B correlated with poor survival (p=0.024).

tional factor and considered as one of the indicators for Hedgehog pathway activation [23]. By immunohistochemistry, overexpression of Gli1 was observed in pancreatic cancer when compared with that in nonmalignant specimens of the pancreas. Aberrant expression of Gli1 has been implicated in a large number of cancers, including pancreatic cancer, and is often associated with poor prognosis. There were significant differences of the cytoplasmic expression of Gli1 between cancer and paracancerous tissues. These results implied that Gli1 may be contributed to the tumorigenesis of pancreatic cancer. Over-expression of Gli1 was associated with lymph node metastasis, and Wnt2B with perineural invasion in patients with pancreatic cancer, respectively.

Since the abnormal activation of the Wnt signaling pathway is involved in the pathogenesis of various tumors, the Wnt gene encoding the multifunctional glycoproteins is concerned with the regulation of a wide variety of normal and pathologic processes including embryogenesis, differentiation and carcinogenesis, etc. In our study, Wnt2B in human pancreatic cancer tissues was significantly higher than those of normal pancreatic tissues (*P*=0.004), and high expression of Wnt2B may indicate a poor overall survival. Wnt2B may be contributed to the tumorigenesis and progression of pancreatic cancer.

Gli1 and Wnt2B both contribute to the tumorigenesis of pancreatic cancer. Previous study had indicated that Wnt and Hedgehog signaling pathways play central roles in embryogenesis and tumorigenesis, Hedgehog signaling pathway cause an elevation of Gli1 expression and transcriptional activity [21], and stimulate Wnt pathway, but our study did not found a correlation between the expression of Gli1 and wnt2B (*P*>0.05). This would indicate there may be another way to stimulate Wnt pathway, but not the elevate of wnt2B, or maybe there is an mediator between Gli1 and Wnt2B. The WNT and Hedgehog signal pathways share Gli1 as a transcription factor, and a novel mechanism has also been discovered by which WNT signaling induces expression of an RNA-binding protein that stabilizes Gli1 mRNA, increasing Gli1 expression/transcriptional activity [24].

In conclusion, these results indicate that the high-expression levels of Gli1 and Wnt2B might play a pivotal role during tumorigenesis and progression of pancreatic cancer, and the high expression of Wnt2B might be associated with poor prognosis. Cross-talk between WNT and Hedgehog signaling in pancreatic cancer need further research.

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## Disclosure of conflict of interest

None.

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