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ORIGINAL ARTICLE

The WNT5A/ROR2 signaling pathway in pancreatic ductal adenocarcinoma (PDAC)

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Summary

Purpose: *WNT5A/ROR2signaling pathway has been involved in many human cancers. Its role in pancreatic ductal adenocarcinoma (PDAC) has not been clarified yet. The purpose of this study was to determine the prognostic value of WNT5A expression in conjunction with the ROR2 expression in the same PDAC human tissues.*

Methods: *We retrospectively analyzed by immunohistochemistry the WNT5A and ROR2 expression in117 paraffin-embedded PDAC specimens following surgical pancreatic resection. The prognostic value of WNT5A and ROR2 was assessed using Kaplan-Meier survival curves and multivariate Cox regression models.*

Results: *High ROR2 expression was detected in 65.8% (77/117) of PDAC tumors, in 28.2% (33/117) in tumor-stroma, and in 71.1% (65/90) of normal pancreatic tissue. High WNT5A expression was found in 76.9% (90/117) of tumors, in 59.0% (69/117) of tumor-stroma, and in 83.0% (73/88) of normal pancreatic tissue. Spearman's correlation coefficiency demonstrated weak association between ROR2 and WNT5A expression in tumor (r=0.184; p=0.047), and no association in stroma (r=0.036; p=0.699). Multivariate analysis showed that regional lymph node invasion and differentiation were independent prognostic factors of survival, while ROR2- and WNT5A expression were not.*

Conclusions: *Variable expression patterns for ROR2 and WNT5A were demonstrated in PDAC and normal pancreatic tissues suggesting a role for WNT5A/ROR2 signalling pathway, not only in PDAC but also in the normal pancreatic tissue during inflammation. The lack of prognostic significance for ROR2 and WNT5A expression in our cohort, either alone or in subgroup analysis, underlines the complexity of their role in PDAC, which is highly dependent on the different molecular receptor-ligand tissue contexts.*

Key words: *pancreatic ductal carcinoma, prognosis, ROR2, survival, WNT5A, Wnt signaling pathway*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal malignancies counting as the seventh leading cause of cancer-related death worldwide while still demonstrating the poorest prognosis of all solid tumors[1]. Although radical surgical R0 resection, in combination with adjuvant and/or neoadjuvant treatment, remains the most promising chance of cure, high recurrence rates have been recorded [2], whileunresectable locally advanced and disseminated disease display an unfavorable prognosis, with 5-year survival less than 9%, due to aggressive tumor biology and poor medical treatment efficacy and effectiveness [3]. Alternative therapies, such as molecular therapy, which target specific biological markers that play a significant role in lymph node metastasis and

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tumor progression, may add further therapeutic or prognostic benefits. Identification of such novel targets or prognostic markers requires understanding of the molecular pathways underlying the PDAC tumor biology, which still remains largely unclear. The embryological WNT signaling pathway is important for development and tissue homeostasis, while its dysregulation has been associated with tumorigenesis [4].WNT signaling is either canonical or non-canonical, based on the role played by the effector protein β-catenin [5]. In the canonical WNT signaling, WNT signals activate β-catenin mediated transcription of target genes that promote proliferation and differentiation [6]. In contrast, two distinct, non-canonical WNT signaling pathways, the planar cell polarity (PCP)/convergent extension (CE) pathway, and the Ca^{2+} pathway (summarized in [7]), are activated by non-transforming WNT family members and regulate cell migration and polarity, independently of β-catenin [7].

WNT5A is classified as a non-transforming WNT family member which activates non-canonical WNT signaling by interacting with receptor tyrosine kinase-like orphan receptors (ROR) [8]. ROR2 is a member of the ROR family of receptor tyrosine kinases, acts as a receptor or co-receptor for WNT5A, and has been shown to display essential functions in developmental morphogenesis [9]. WNT5A/ROR2 signaling is known to inhibit the canonical WNT signaling in tumor cells, indicating that WNT5A might act as a tumor suppressor [10]. Indeed, loss or low expression of WNT5A is associated with increased invasion and aggressiveness of many carcinomas [11,12]. However, WNT5A has also demonstrated oncogenic properties based on findings depicting its upregulation in melanoma, lung and breast cancers [13-15]. Meanwhile, ROR2-receptor demonstrates oncogenic properties regardless the presence of a ligand [16]. Therefore, the exact function of WNT5A appears highly dependent on the receptor context and its significance in tumor biology needs to be examined in conjunction with the ROR2 expression.

PDAC is characterized by dysregulation of the canonical WNT/β-catenin signaling pathway, which has been associated with tumor initiation, progression and aggressive tumor biology [17-19]. The canonical WNT signaling is reported activated in up to 65% of PanINs and increased even more frequently in invasive PDAC [20–22]. In contrast, only limited and independent reports exist so far investigating the role of non-canonical WNT5A and its ROR2-receptor in PDAC respectively.WNT5A has been reported to mediate the migration and invasion of pancreatic cancer cells via epithelial-to-mesenchymal transition (EMT) [23,24]. On the other hand, high ROR2 expression in tumor cytoplasm or stromal cells in PDAC, has been significantly associated with malignant attributes and poor patients' survival [25].

The already existing reports onWNT5A mediated aggressive tumor phenotype and ROR2 associated poor prognosis in PDAC stress that downstream activation of WNT5A/ROR2 signaling pathway may significantly contribute to tumor biology. However, the prognostic value of WNT5A expression in conjunction with the ROR2 expression in PDAC has not been investigated yet, and their true clinical significance in combination remains still ambiguous. Thus, the aim of this study was to determine the expression pattern and clinical significance of both WNT5A and ROR2, in the same pancreatic cancer tissues in order to reveal their true prognostic value and to clarify their possible biological implications in PDAC.

Methods

Patients and tissue samples

Formalin-fixed, paraffin-embedded PDAC samples following pancreatic resection with curative intent and matched, tumor-adjacent normal pancreatic specimens were collected from 117 patients with PDAC, who underwent surgery at the University Hospital of Leipzig, Germany, from 2001 to 2013. No patient received chemotherapy or radiotherapy before the operation. Clinical data were obtained by medical records, and follow-up survival data were gained through telephone investigation. The data included epidemiological data, such as gender and age, as well as histopathological analysis, such as tumor differentiation (grading), and the pTNM classification, lymphatic invasion (L), vascular invasion (V), perineural invasion (Pn), residual tumor (R), and UnionInternationale Contre le Cancer (UICC). Pathological tumor stage (pTNM) and histological differentiation were classified by gastrointestinal experienced pathologist (C.W., K.S.) according to the guidelines of the 7th Edition of TNM staging in pancreatic cancer [26]. Data regarding adjuvant chemotherapy and distant or local relapse were also collected by follow-up. Clinical data were collected prospectively and retrospectively analyzed. This study was approved by the Ethic's Committee of the University Hospital of Leipzig, Germany (reference number: 234/14-ek).

Immunohistochemistry

Paraffin-embedded tissue samples were cut into 5 µm slices and dried for at least 6 h at 37°C, deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide at 4°C and afterwards, non-specific bindings were blocked with 5% normal-goat serum. The slides were incubated with the primary antibody against ROR2 (1:100, PA5-14727, Thermo Fisher Scientific, Darmstadt, Germany) and WNT5A (1:200, MA5 15511, Thermo Fisher Scientific,

Darmstadt, Germany) overnight at 4°C. Horse radish-peroxidase-conjugated Affini-Pure goat anti-rabbit antibody IgG (Dianova, Hamburg, Germany) for ROR2 and anti-mouse antibody IgG (Dianova, Hamburg, Germany) for WNT5A were then applied. Immunoreactivity was visualized with diaminobenzidine (Sigma, Taufkirchen, Germany). Positive control for ROR2 was a renal cell carcinoma sample and for WNT5A a lymph node. Negative control was fatty tissue for both (Suppl. Figure 1A). H&E staining facilitated the recognition of the tumor region, stroma and adjacent normal tissue in all tissue samples (Suppl. Figure 1B).

Evaluation of immunohistochemistry reaction

The slides were evaluated by three independent reviewers (L.R., O.L., G.W.). The evaluation of expression of ROR2 and WNT5A was based on the percentage of positive cells [0% (no positive cells), 35%, 65% and 100% (all cells positive)] and their intensity [0 (no staining), 1 (weak intensity), 2 (moderate intensity) and 3 (strong intensity)]. For each tissue sample, the percentage of cells stained at certain intensity was determined and multiplied by the intensity score to generate an intensity percentage score, which ranged from 0 to 300 [25]. Expression in the tumor, stroma and normal tissue were analyzed. The cut-off value for distinguishing positive and negative expression was set at 65, based on consensus of the three independent experts.

Statistics

The statistical review of the study was performed by a biomedical statistician. X^2 test was conducted to test the correlation of expression of ROR2 and WNT5A with clinicopathological variables. Overall survival (OS) was calculated from the day of first surgery until death or end of follow-up using the Kaplan-Meier method, and the log-rank test was used for survival analysis. Cox regression model was used to perform multivariate analysis. Correlation between ROR2 and WNT5A expression was tested using Spearman's correlation coefficient. For all tests, the significance level for statistical analyses was set at $p<0.05$. All data were analyzed using IBM© SPSS© Statistics (Version 24.0.0, IBM©, Armonk, USA).

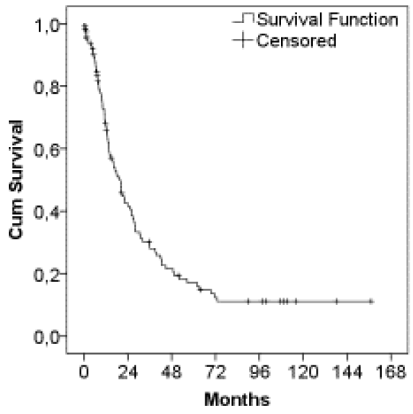

Figure 1. Overall survival of the patient cohort (median survival: 19.5 months).

Table 1. Clinical characteristics of the patient cohort

Variable	*Total n (%)*
Total	117 (100)
Age, years	
Median	66
Min./Max.	40-84
Sex	
Female	45 (38.5)
Male	72 (61.5)
TNM	
pT	
pT1	4 (3.4)
pT2	20 (17.1)
pT3	90 (76.9)
pT4	3 (2.6)
pN	
pN0	35 (29.9)
pN1	82 (70.1)
M	
M0	40 (34.2)
M1	9 (7.7)
Unknown	68 (-)
Lymph vessel invasion (L)	
L0	17 (14.5)
L1	91 (77.8)
Unknown	9 (-)
Vascular invasion (V)	
V0	75 (64.1)
V1	30 (25.6)
Unknown	12 (-)
Perineural invasion (Pn)	
Pn0	15 (12.8)
Pn1	78 (66.7)
Unknown	24 (-)
Resection margin (R)	
R0	91 (77.8)
R1	24 (20.5)
R2	2 (1.7)
Grading (G)	
G1	1 (0.9)
G2	55 (47.0)
G3	61 (52.1)
G4	0 (0)
UICC 7.0	
IA	1 (0.9)
IB	5 (4.3)
IIA	27 (23.1)
IIB	70 (59.8)
III	4 (3.4)
IV	10 (8.5)
Relapse/metastasis	
Yes	41 (35.0)
No	76 (65.0)
Local relapse	
Yes	22 (18.8)
No	95 (81.2)
Chemotherapy	
Yes	50 (42.7)
No	67 (57.3)

Results

Patient cohort

In total, tissue samples were collected from 117 patients with PDAC, who underwent pancreatic resection at the University Hospital of Leipzig, Germany, from 2001 to 2013. At the time of surgery, patients' ages ranged from 40 to 84 years, with a median age of 66 years. There were 45 females (38.5%) and 72 males (61.5%). None of the patients had received neoadjuvant treatment prior to surgery. The surgical procedures included Kausch-Whipple operations in 53 patients, pylorus-preserving pancreaticoduodenectomy (PPPD) in 50 patients, left pancreatectomy in 12 patients, and total pancreatectomy in 2 patients. In 9 patients with intraoperatively detected oligometastasis (M1), the operation was still performed along with removal of the oligometastatic site due to younger age (5 patients with single liver metastasis and 4 patients with interaortocaval lymph node metastasis). According to the decision of the interdisciplinary tumor board, based on the final pathology report, 50 (43%) patients received adjuvant chemotherapy. The median follow-up reached 100 months (8 years). The median OS was 19.5 months with a 95% confidence interval (CI) of 14.4-24.6 months (Figure 1). Patients who received adjuvant chemotherapy demonstrated improved OS. The 5-year survival rate following curative resection reached 16%. These findings correlate with the statistics reported in patients able to undergo a successful curative resection, in which median survival time ranges from 12-19 months, and the 5-year survival rate is 15-20% [27]. Patients' characteristics are summarized in Table 1.

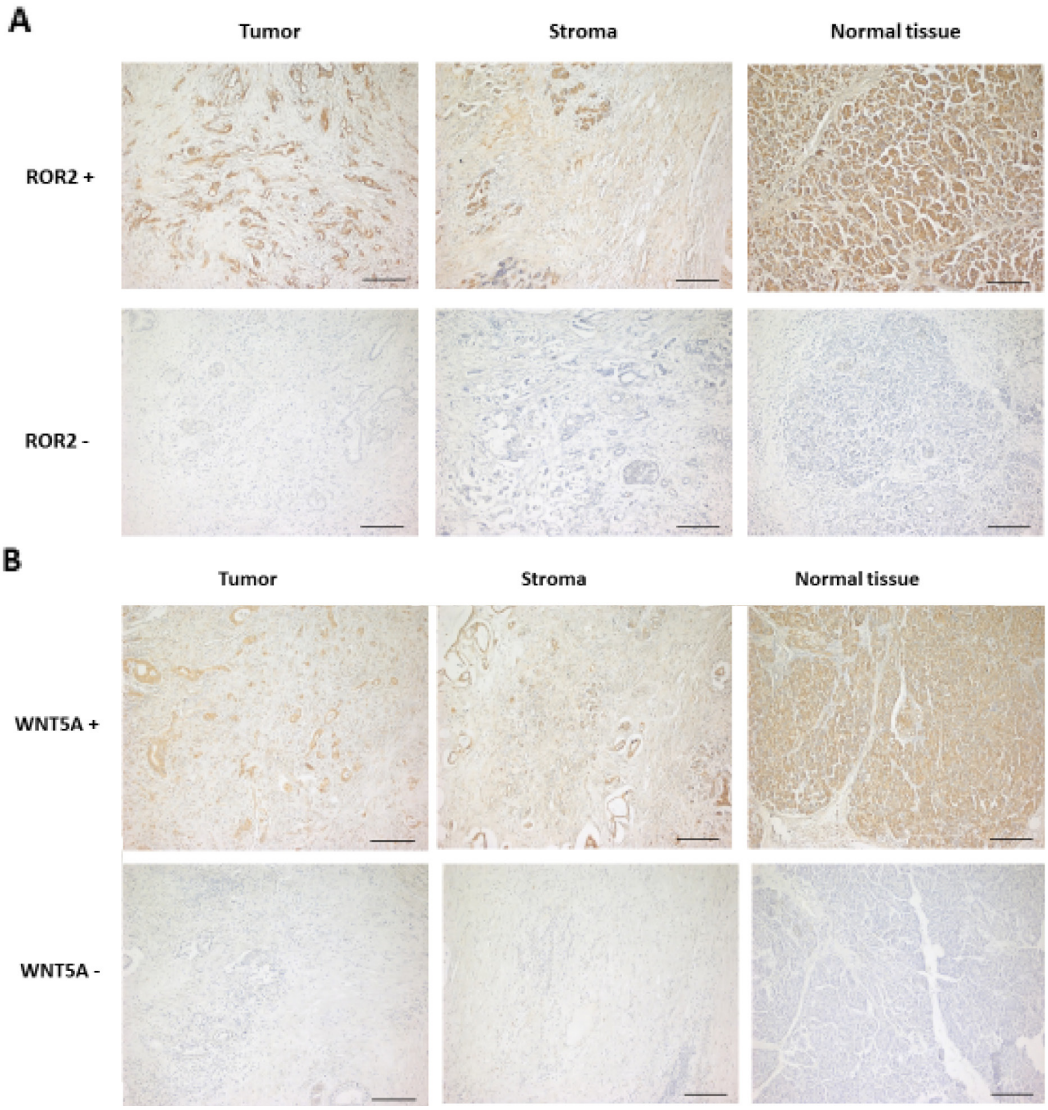

Figure 2. Representative pattern of ROR2 expression **(A)** and WNT5A expression **(B)** in PDAC, stroma and adjacent normal pancreatic tissue, as determined by IHC. Bar 200 μm.

Expression of ROR2 and WNT5A in PDAC

To analyze the expression of ROR2 and WNT5A in PDAC, we performed immunohistochemistry in all 117 tumor samples along with the adjacent normal tissue, if available (n=90 in ROR2 and n=88 in WNT5A). Positive staining of ROR2 and WNT5A were primarily localized in the cytoplasm of tumor cells, in the stroma and in normal pancreatic tissue. For statistical analysis, we dichotomized the expression of both ROR2 and WNT5A at a cut-off value of 65, based on tabular analysis. Tissues with protein staining of 65 or less were considered negative, otherwise they were labelled as positive. High ROR2 expression was found in 65.8% (77/117) of tumors, in 28.2% (33/117) in tumor-stroma, and in 71.1% (65/90) of normal pancreatic tissue. The expression in tumor cells was significantly higher than in stromal tissue (p<0.001). High WNT5A expression was found in 76.9% (90/117) of tumors, 59.0% (69/117) in stroma, and 83.0% (73/88) in normal tissue. Similarly, expression in tumor cells was significantly higher than in stromal tissue (p<0.001). Representative immunohistochemical staining for ROR2 and WNT5A in PDAC tissues is shown in Figure 2A and Figure 2B, respectively. Calculation of Spearman's correlation coefficient demonstrated weak association between ROR2 and WNT5A expression in the tumor (r=0.184; p=0.047) and no association in the stroma (r=0.036; p=0.699) (data not shown).

Association between ROR2 and WNT5A expression with clinicopathological parameters

To further elucidate the role of ROR2 and WNT5A in PDAC, we analyzed the associations between their expression patterns and major clinicopathological parameters. As shown in Tables 2 and 3, no significant associations were detected between the positive ROR2 and WNT5A expression in all three investigated tissue types (tumor, stroma and normal tissue) with any clinical parameter, respectively.

Survival analysis

Univariate analysis showed differences for OS between the following groups: differentiation grade (G), tumor depth of invasion (pT), regional lymph node metastasis (pN), lymphatic (L) and vessel invasion (V) along with recurrence and metastasis (Table 4). No significant association with OS was

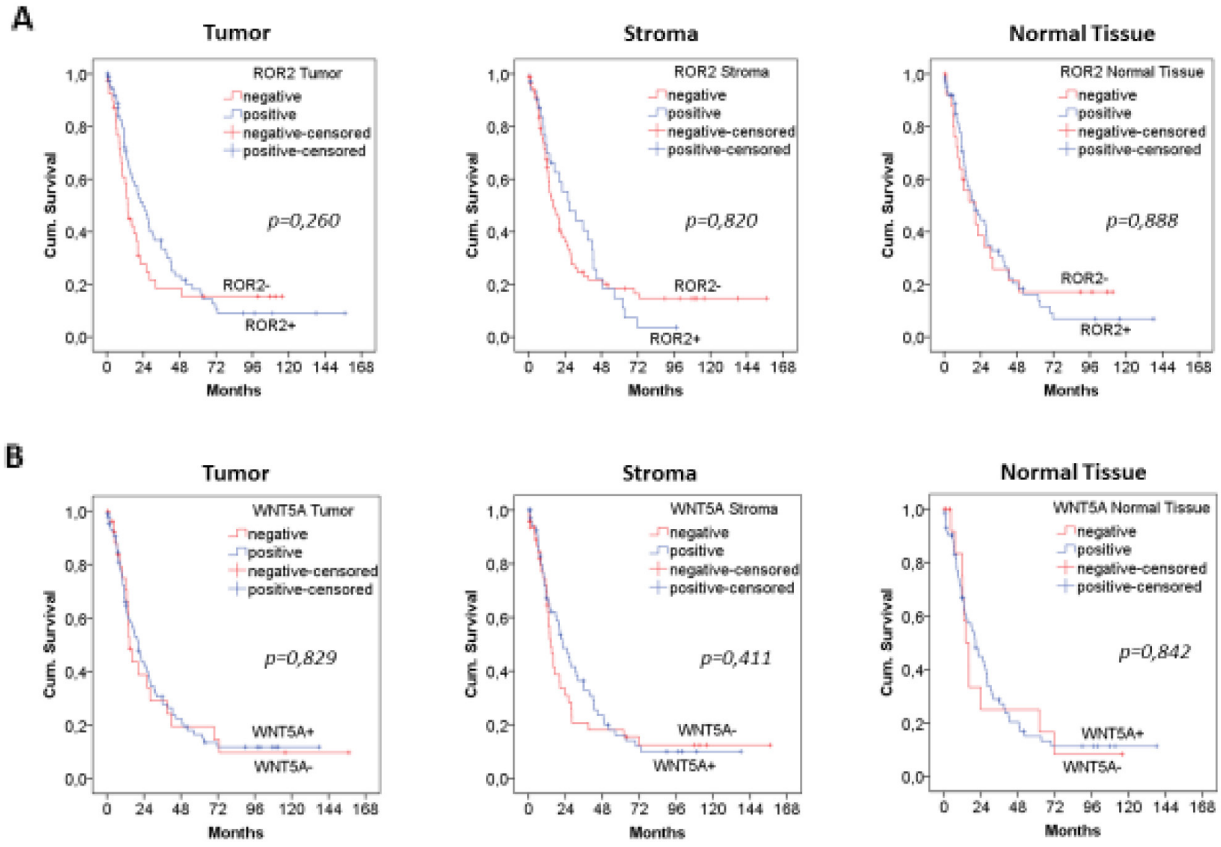

Figure 3. Survival analysis of PDAC patients with Kaplan-Meier method and log-rank test. Overall survival rates in patients with ROR2 negative and positive expression **(A)** and WNT5A negative and positive expression **(B)** in tumor cells, stroma and adjacent normal tissue.

Table 2. Association of ROR2 expression with clinicopathological parameters in PDAC (*p<0.05)

ROR2		*Tumor*			*Stroma*				*Normal tissue*		
		Expression		*p value*	*Expression*		*p value*		*Expression*		*p value*
		negative (%)	*positive (%)*		*negative (%)*	*positive (%)*			*negative (%)*	*positive (%)*	
Total No.	117	40	77		84	33		90	26	64	
Age				0.491			0.756				0.609
≤60 years	40	12(30.0)	28(70.0)		28(70.0)	12(30.0)		31	10(32.3)	21(67.7)	
>60 years	77	28(36.4)	49(63.6)		56(72.7)	21(27.3)		59	16(27.1)	43(72.9)	
Sex				0.878			0.897				0.849
Female	45	15(33.3)	30(66.7)		32(71.1)	13(28.9)		36	10(27.8)	26(72.2)	
Male	72	25(34.7)	47(65.3)		52(72.2)	20(27.8)		54	16(29.6)	38(70.4)	
pT				0.561			0.907				0.059
pT1 & pT2	24	7(29.2)	17(70.8)		17(70.8)	7(29.2)		14	1(7.1)	13(92.9)	
pT3 & pT4	93	33(35.5)	60(64.5)		67(72.0)	26(28.0)		76	25(32.9)	51(67.1)	
pN				0.660			0.954				0.451
N0	35	13(37.1)	22(62.9)		25(71.4)	10(28.6)		27	6(22.2)	21(77.8)	
N1	82	27(32.9)	55(67.1)		59(72.0)	23(28.0)		63	20(31.7)	43(68.3)	
M				0.273			1.000				0.663
M0	40	23(57.5)	17(42.5)		34(85.0)	6(15.0)		32	12(37.5)	20(62.5)	
M1	9	3(33.3)	6(66.7)		8(88.9)	1(11.1)		6	3(50.0)	3(50.0)	
Unknown	68							52			
L				0.939			0.772				1.000
L0	17	6(35.3)	11(64.7)		12(70.6)	5(29.4)		13	4(30.8)	9(69.2)	
L1	91	33(36.3)	58(63.7)		67(79.0)	24(29.0)		71	22(31.0)	49(69.0)	
Unknown	9							6			
V				0.609			0.724				0.225
V0	75	29(38.7)	46(61.3)		55(73.3)	20(26.7)		62	17(27.4)	45(72.6)	
V1	30	10(33.3)	20(66.7)		23(76.7)	7(23.3)		19	8(42.1)	11(57.9)	
Unknown	12							9			
Pn				0.836			0.752				0.262
Pn0	15	6(40.0)	9(60.0)		12(80.0)	3(20.0)		10	1(10.0)	9(90.0)	
Pn1	78	29(37.2)	49(62.8)		57(73.1)	21(26.9)		62	20(32.3)	42(67.7)	
Unknown	24							18			
Grading				0.403			0.744				0.508
G1 & G2	56	17(30.4)	39(69.6)		41(73.2)	15(26.8)		43	11(25.6)	33(74.4)	
G3	61	23(37.7)	38(62.3)		43(70.5)	18(29.5)		47	15(31.9)	32(68.1)	
UICC 7.0				0.553			0.918				0.940
IIA	27	11(40.7)	16(59.3)		19(70.4)	8(29.6)		20	6(30.0)	14(70.0)	
IIB	70	24(34.3)	46(63.9)		50(71.4)	20(28.9)		55	17(30.9)	38(69.1)	
Other	20							15			
Relapse/metastasis				0.688			0.808				0.394
Yes	41	15(36.6)	26(63.4)		30(73.2)	11(26.8)		32	11(34.4)	21(65.6)	
No	76	25(32.9)	51(67.1)		54(71.1)	22(28.9)		58	15(25.9)	43(74.1)	
Local relapse				0.216			0.914				0.559
Yes	22	10(45.5)	12(54.5)		16(72.7)	6(27.3)		17	6(35.3)	11(64.7)	
No	95	30(31.6)	65(68.4)		68(71.6)	27(28.4)		73	20(27.4)	53(72.6)	

Table 3. Association of WNT5A expression with clinicopathological parameters in PDAC (*p<0.05)

WNT5A		*Tumor*			*Stroma*			*Normal tissue*		
		Expression		*p value*	*Expression*		*p value*	*Expression*		*p value*
		negative (%)	*positive (%)*		*negative (%)*	*positive (%)*		*negative (%)*	*positive (%)*	
Total No.	117	27	90		48	69		88	15	73	
Age				0.200			0.815				0.596
≤60 years	40	12(30.0)	28(70.0)		17(42.5)	23(57.5)		30	6(20.0)	24(80.0)	
>60 years	77	15(19.5)	62(80.5)		31(40.3)	46(59.7)		58	9(15.5)	49(84.5)	
Sex				0.862			0.327				0.421
Female	45	10(22.2)	35(77.8)		21(46.7)	24(53.3)		33	7(21.2)	26(78.8)	
Male	72	17(23.6)	55(76.4)		27(37.5)	45(62.5)		55	8(14.5)	47(85.5)	
pT				0.060			0.142				0.700
pT1 & pT2	24	9(37.5)	15(62.5)		13(54.2)	11(45.8)		14	3(21.4)	11(78.6)	
pT3 & pT4	93	18(19.4)	75(80.6)		35(37.6)	58(62.4)		74	12(16.2)	62(83.8)	
pN				0.658			0.333				0.538
pN0	35	9(25.7)	26(74.3)		12(34.3)	23(65.7)		26	3(11.5)	23(88.5)	
pN1	82	18(22.0)	64(78.0)		36(43.9)	46(56.1)		62	12(19.4)	50(80.6)	
pM				0.440			1.000				1.000
pM0	40	10(25.0)	30(75.0)		15(37.5)	25(62.5)		31	4(12.9)	27(87.1)	
pM1	9	1(11.1)	8(88.9)		3(33.3)	6(66.7)		6	1(16.7)	5(83.3)	
Unknown	68							51			
L				0.235			0.304				0.698
L0	17	6(35.3)	11(64.7)		9(52.9)	8(47.1)		13	3(23.1)	10(76.9)	
L1	91	20(22.0)	71(78.0)		36(39.6)	55(60.4)		69	12(17.4)	57(82.6)	
Unknown	9							6			
V				0.942			0.618				1.000
V0	75	18(24.0)	57(76.0)		31(41.3)	44(58.7)		61	11(18.0)	50(82.0)	
V1	30	7(23.3)	23(76.7)		14(46.7)	16(53.3)		119	3(15.8)	16(84.2)	
Unknown	12							8			
Pn				0.499			0.122				1.000
Pn0	15	4(26.7)	11(73.3)		9(60.0)	6(40.0)		11	1(9.1)	10(90.9)	
Pn1	78	15(19.2)	63(80.8)		30(38.5)	48(61.5)		60	10(16.7)	50(83.3)	
Unknown	24							17			
Grading				0.177			0.130				0.995
G1 & G2	56	16(28.6)	40(71.4)		27(48.2)	29(51.8)		41	7(17.1)	34(82.9)	
G3	61	11(18.0)	50(82.0)		21(34.4)	40(65.6)		47	8(17.0)	39(83.0)	
UICC 7.0				0.751			0.075				0.727
IIA	27	5(18.5)	22(81.5)		7(25.9)	20(74.1)		19	2(10.5)	17(89.5)	
IIB	70	15(21.4)	55(78.4)		32(45.7)	38(54.3)		54	10(18.5)	44(81.5)	
Other	20							15			
Relapse/metastasis				0.111			0.132				0.042
Yes	41	6(14.6)	35(85.4)		13(31.7)	28(68.3)		32	2(6.2)	30(93.8)	
No	76	21(27.6)	55(72.4)		35(46.1)	41(53.9)		56	13(23.2)	43(76.8)	
Local relapse				0.966			0.990				0.726
Yes	22	5(22.7)	17(77.3)		9(40.9)	13(59.1)		18	2(11.8)	15(88.2)	
No	95	22(23.2)	73(76.8)		39(41.1)	56(58.9)		71	13(18.3)	58(81.7)	

found for high expression in tumor, stroma and normal pancreatic tissue of either ROR2 (Figure 3A) or WNT5A (Figure 3B). Multivariate analysis identified the regional lymph node invasion and the grading to be independent prognostic factors of survival. Patients with lymph node invasion showed a hazard ratio (HR) of 3.00, thus a 3 times increased risk to cancer-related death than patients without lymph node invasion. Poorly differentiated tumors (G3) showed a 1.6 higher risk to cancer-related death than tumors of G1 or G2 grading. ROR2 and WNT5A levels of expression could not be identified as independent prognostic parameters. Further subgroup analysis of possible combinations of ROR2 and WNT5A expression patterns also failed to demonstrate significant differences in OS (Figure 4A). Finally, in order to evaluate whether ROR2 and WNT5A expression patterns may influence response to adjuvant chemotherapy, we performed subgroup analyses only for patients who had received adjuvant chemotherapy (n=50). In this subgroup analysis, both ROR2 and WNT5A expression failed to demonstrate any prognostic value in the setting of adjuvant chemotherapy (Figures 4B and 4C).

Discussion

To our knowledge, this is the first study to analyze simultaneously the prognostic value of ROR2 and WNT5A expression patterns in PDAC patients, in an effort to reveal the true role of the non-canonical ROR2/WNT5A signaling pathway in this malignancy. We demonstrated variable expression patterns for ROR2 and WNT5A in tumor, stroma and normal pancreatic tissues, which, however,

Table 4. Univariate and multivariate analysis of prognostic factors for 5-year survival in PDAC (*p<0.05)

Variable	Univariate analysis			*Multivariate analysis*		
	HR	*95% KI*	*p value*	HR	*95% KI*	*p value*
ROR2 Tumor						
Negative (ref) vs. Positive	0.78	0.50-1.20	0.261	0.75	0.45-1.25	0.272
WNT5A Tumor						
Negative (ref) vs. Positive	0.95	0.57-1.56	0.829	0.89	0.53-1.49	0.648
ROR2 Stroma						
Negative (ref) vs. Positive	0.95	0.60-1.50	0.820	0.83	0.49-1.42	0.500
WNT5A Stroma						
Negative (ref) vs. Positive	0.83	0.54-1.28	0.412	0.86	0.55-1.35	0.517
Sex						
Female (ref) vs. Male	1.13	0.72-1.61	0.597			
Alter						
≤ 60 (ref) vs > 60	1.28	0.81-2.00	0.288			
Grading						
G1 & G2 (ref) vs. G3	1.84	1.18-2.85	0.007	1.58	1.00-2.50	0.050
pT						
pT1&pT2 (ref) vs. pT3&pT4	1.79	1.05-3.05	0.033	1.23	0.70-2.18	0.474
pN						
N0 (ref) vs. N1	3.16	1.87-5.33	<0.001	3.00	1.70-5.28	<0.001
pM						
M0 (ref) vs. M1	1.70	0.79-3.67	0.172			
L						
L0 (ref) vs. L1	2.47	1.22-4.97	0.012			
V						
V0 (ref) vs. V1	1.90	1.15-3.02	0.011			
Pn						
Pn0 (ref) vs. Pn1	0.93	0.50-1.73	0.815			
Recurrence/Metastasis						
Yes (ref) vs. No	0.47	0.30-0.74	0.001			
Local Recurrence	0.75	0.45-1.23	0.248			

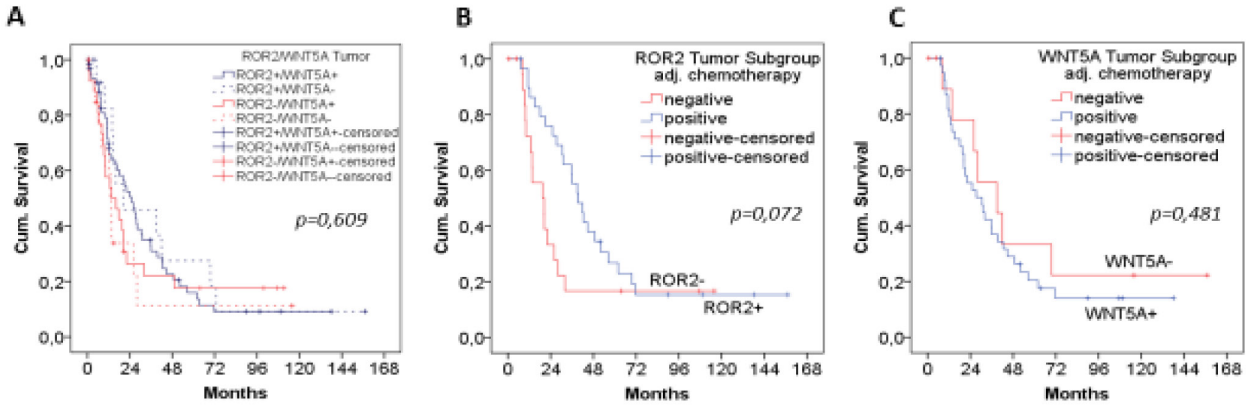

Figure 4. Subgroup analysis of overall survival in PDAC patients with different combinations of ROR2- and WNT5A-expression patterns **(A)** and in PDAC patients, who had received adjuvant chemotherapy with ROR2 negative and positive expression **(B)** or WNT5A negative and positive expression **(C)**.

failed to demonstrate any type of correlation to each other. High WNT5A expression was found in 76.9% of PDAC tissues. This is in accordance with a previous study displaying a positive WNT5A expression in 81.3% of pancreatic cancer tissues [24]. On the other hand, the positive expression rate of ROR2 in our study was higher, up to 65% of the examined PDCA tissues, compared to a previous reported rate of 45.7% [25]. Noteworthy, we demonstrated elevated expression rates for ROR2 and WNT5A also in the adjacent normal pancreatic tissue, up to 71% and 83%, respectively. Regarding their clinical significance, expression patterns of ROR2 and WNT5A in our analysis failed to correlate with clinicopathological parameters or to demonstrate a prognostic significance in survival, even in the subgroup analysis. In contrast, regional lymph node invasion and differentiation levels were defined as independent prognostic factors of survival.

ROR2 is a co-receptor for WNT5A and exerts its function predominantly via the non-canonical WNT signalling [9]. On the other hand, WNT5A functions are highly dependent on the receptor context, and its role in tumor biology is highly influenced by the presence of the ROR2 receptor. Therefore, the prognostic significance of ROR2 should be evaluated only in conjunction with WNT5A and *vice versa*. The already reported high expression rates of WNT5A and ROR2 in PDAC along with their association with aggressive tumor phenotype and poor prognosis has been shown in independent studies, respectively [24,25]. These findings underlay the activation of the WNT5A/ROR2 signaling pathway, which may contribute to the PDAC tumor promotion. However, WNT5A-mediated activation of the ROR2 receptor is claimed to mediate inhibition of the canonical Wnt/β-catenin signalling [28], whose activation has also been strongly associated with PDAC carcinogenesis and tumor progression [18,22]. Therefore, expression patterns indicative of WNT5A/ROR2 signaling activation, such as double positive cancers (ROR2+/WNT5A+), should be expected to be associated with better prognosis rather than poorer as has been reported previously. In an effort to clarify this uncertain background, our study aimed to evaluate the ROR2 and WNT5A expressions in combination in the same PDAC tissues and to facilitate a more confident conclusion of their prognostic value. Although high expression rates of ROR2 and WNT5A were demonstrated in 65% and 76.9% of the PDAC tissues respectively, our study failed to confirm any association with clinicopathological parameters or to demonstrate a prognostic impact for the expression of both.

Previous reports support an oncogenic role for ROR2 and WNT5A positive expression in PDAC [24,25], which, however, could not be confirmed further in our study. The lack of prognostic significance can be attributed to the complexity of the ROR2 and WNT5A functions, which are highly dependent on the receptor-ligand context and vary under different molecular conditions. Even the study of Bo et al [24], which supported that WNT5A up regulation promotes EMT and metastasis in pancreatic cancer via activation of thecanonical WNT/β-catenin signalling and independently of ROR2receptor, could not finally demonstrate a prognostic significance for WNT5A expression. On the other hand, Huang and colleagues recently supported that high ROR2 expression is associated with poor prognosis without, however, to evaluate WNT5A expression patterns or other downstream molecules of the ROR2 receptor in their cohort [25]. Regarding other malignancies, many studies have attempted to determine the prognostic values

of ROR2 and WNT5A in combination, supporting the viewpoint that double positive cancers (ROR2+/WNT5A+) might have significantly worse survival than those with double negative cancers (ROR2-/WNT5A-) [29-34], while other studies suggest independent roles for WNT5A as tumor suppressor and for ROR2 receptors mediator of WNT5A independent tumor promotion [10,35,36]. This variety in WNT5A and ROR2 molecular functions in regard to tumor progression surely reflects the differences in the receptor-ligand context or cell context of cancer cells even in PDAC and can in part explain the lack of significance, even in the subgroup analysis.

An interesting finding in our study is the high rates of positive ROR2 and WNT5A expression in the normal pancreatic tissues. In adult tissues, expression levels of WNT5A and ROR2 are in general decreased [37]. Thus, the majority of oncological reports tend to speculate the potential of ROR2 and WNT5A as therapeutic targets based on their differential expressions within human malignancies and on the low to absent expression in normal tissues [38]. However, the expression in normal tissues is often neglected in reporting. Therefore, the positive expression of WNT5A and ROR2 in the adjacent normal pancreatic tissue as found here, signifies their role in pancreatic tissue physiology and inflammation. The roles of WNT5A ROR2 axis in normal cells has been demonstrated, involving diverse cellular functions, including cell polarization, migration and stemness[37]. Such prominent cellular processes in the adjacent to PDAC normal pancreatic tissue may dictate the tumor microenvironment in favour of tumor progression and merit further investigation.

Our study has some limitations: first, it is a mainly retrospective study and its findings might not be applied to the general population. Larger prospective studies are needed to confirm or to exclude the prognostic value of WNT5A and ROR2 in PDAC. Second, we utilized immunohistochemistry (IHC), which only assesses protein expression of WNT5A and ROR2 in pancreatic tissues and, as such, provide rather limited information on the functional level without allowing conclusions on their molecular interactions. Third, the IHC data are semiquantitative and additional methods are needed to evaluate and confirm the expression level of ROR2 and WNT5A in tumor cells.

In conclusion, this is the first study - to our knowledge - to examine the concomitant effect of ROR2 and WNT5A on the prognosis of patients with PDAC. We demonstrated variable expression patterns for ROR2 and WNT5A in PDAC, stroma and adjacent normal pancreatic tissues, suggesting a role for WNT5A/ROR2 signaling pathway in the pancreatic tissue physiology and PDAC tumor biology. However, we could not demonstrate a prognostic significance for ROR2 and WNT5A expression in our cohort, either alone or in further subgroup analyses, indicating the complexity of their role in PDAC according to the molecular context. However, because the sample size of this study was relatively small, the correlation between ROR2 and WNT5A in PDAC needs to be further evaluated in studies with larger sample size, and *in vitro* and *in vivo* studies should be performed to clarify the underlying mechanism of correlation.

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Conflict of interests

The authors declare no conflict of interests.

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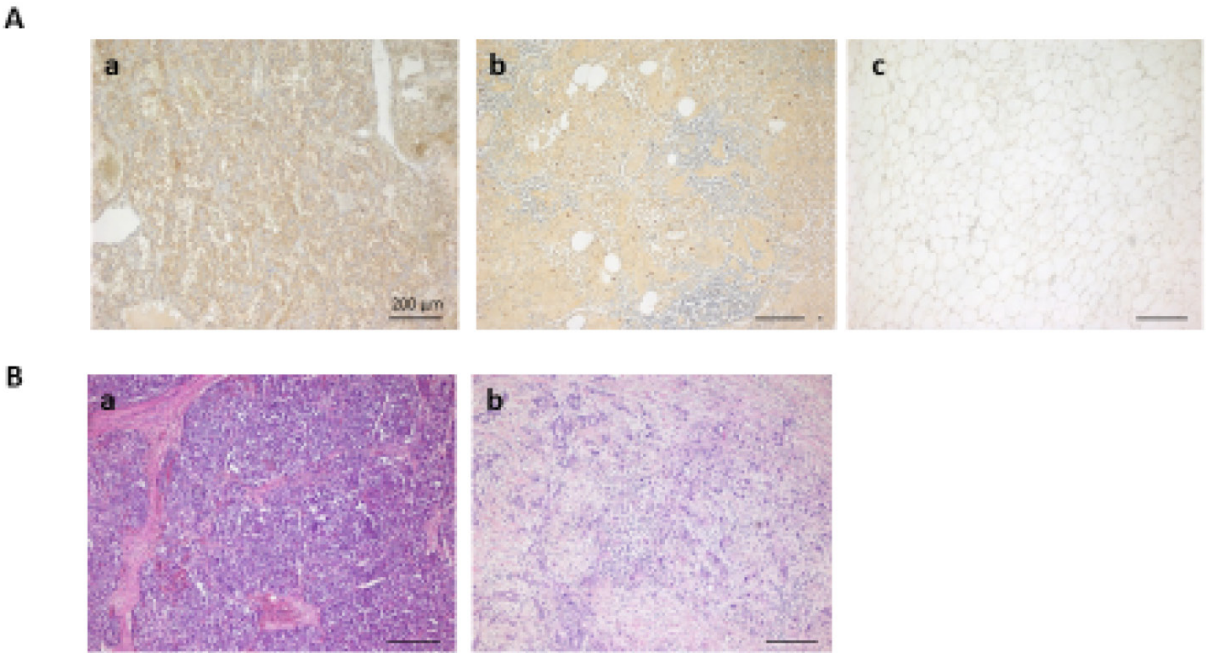

Supplementary Figure 1. A: Positive & negative controls, (a) renal cell carcinoma: positive staining for ROR2. (b) Histiocytes in lymph nodes: Positive staining for WNT5A. (c) Fatty tissue: negative staining for ROR2 and WNT5A. **B:** H&E staining of normal pancreatic tissue and tumor tissue.