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Correlation Between RAB27B and p53 Expression and Overall Survival in Pancreatic Cancer

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Objectives: RAB27B is a member of the Rab family GTPases involved in vesicle trafficking, and p53 has recently been implicated in regulating the exosome secretion pathway. Because exosome secretion plays an important role in modulating tumor microenvironment and invasive growth, we hypothesized that RAB27B and p53 expression might be associated with the aggressive behavior in pancreatic ductal adenocarcinoma, one of the most deadly human malignancies.

Methods: We determined protein expression of RAB27B and p53 in 260 pancreatic tissues (186 malignant and 74 normal or benign) by immunohistochemistry analysis on tissue microarray and their correlation with patients' clinical parameters and overall survival.

Results: We found that a high RAB27B protein expression ($RAB27B^+$) was significantly associated with perineural and vascular invasion, as well as distant metastasis. Patients with a high RAB27B expression had significantly poorer overall survival in both univariate and multivariate analyses. A significant correlation between RAB27B and p53 expression was observed.

Conclusions: Our data indicate that RAB27B expression is an independent prognostic marker for pancreatic ductal adenocarcinoma and suggest that RAB27B-regulated exosome secretion pathway represents a novel therapeutic target in pancreatic cancer.

Key Words: RAB27B, p53, pancreatic ductal adenocarcinoma, immunohistochemistry, prognosis

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Pancreatic ductal adenocarcinoma (PDAC), comprising 95% of pancreatic cancer cases, is one of the most aggressive and lethal forms of human malignancy. Worldwide, PDAC is the 12th most common cancer and the seventh leading cause of cancer-related deaths, with an annual incidence of 338,000 and mortality of 330,000 (Cancer Incidence and Mortality Worldwide in 2012, International Agency for Research on Cancer).[1] In China, PDAC is the seventh leading cause of cancer-related death, with an overall 5-year survival rate of 4.1% and a median survival time of 3.9 months.[2] This is because the majority of the patients are diagnosed at an advanced stage who are no longer amenable to surgical resection while there is no effective targeted therapy. Only 10% to 15% of the patients are eligible for curative surgical resection with a slightly longer median survival of 12.6 months.[3] The conventional chemotherapy is for palliative care to reduce symptoms only.[4,5]

In an attempt to identify novel molecular prognostic markers that could also serve as potential mechanistic therapy targets, we investigated RAB27B and p53 protein expression in PDAC, which have been identified as regulators of the exosome secretion pathway. Recently, the exosome secretion signaling pathway has emerged as a novel mechanism for cancer invasion and metastasis.[6] Exosomes are 40- to 100-nm membrane vesicles derived from the multivesicular endosomes and released on fusion with the plasma membrane. Cancer cells use exosomes to communicate with the environment by transporting proteins to the surface and release of growth factors and cytokines to establish invasive tumor growth.[7] It has been demonstrated that exosome messaging contributes to tumor immune escape[8] and metastatic niche preparation.[9]

RAB27B belongs to the Rab family of small GTPases, the master regulator of vesicle fusion and trafficking. It was originally isolated from human platelets. RAB27B RNA is rarely detected in normal tissues, except in testis, but is detected in the melanoma cell line.[10,11] RAB27B protein, however, can be detected in a wide variety of differentiated secretory epithelial cells, including those lining the salivary gland and gastrointestinal, mammary, and prostate tracts.[12] Elevated expression of RAB27B has been observed in breast, bladder, and liver cancers and is associated with aggressive behavior.[13–16] RAB27B protein expression has been shown as a prognostic marker in liver cancer.[16]

p53 is one of the most important tumor suppressor genes, mutated in more than 50% of human malignancies.[17] It regulates DNA repair, cell cycle, and apoptosis and therefore plays an essential role in maintaining cellular genetic stability.[18] Recent studies have also indicated the role of p53 in the regulation of exosome secretion. On one hand, exosomes can stabilize p53 protein to create a tumor-permissive environment[19]; on the other hand, p53 transcribes key regulators of endosomal compartment, thus regulating exosome production and secretion.[20–22] Both p53 protein accumulation and p53 mutation have been detected in PDAC.[23,24] However, it is unclear whether p53 is involved in the regulation of exosome in PDAC.

Thus far, no study has investigated the role of RAB27B and the presumed role of the exosome secretory pathway in PDAC. To determine whether RAB27B could be used as a prognostic marker and a therapeutic target in pancreatic cancer and whether RAB27B and p53 coordinated in influencing PDAC behavior, we analyzed RAB27B and p53 expression by immunohistochemistry (IHC) analysis in both benign and malignant pancreatic tissues using tissue microarrays (TMAs). We correlated RAB27B and p53 expression with clinicopathological characteristics as well as overall survival (OS) in patients with pancreatic cancer.

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The authors declare no conflict of interest.

MATERIALS AND METHODS

Human Tissue Specimens and Patient Clinical Information

A total of 260 formalin-fixed paraffin-embedded tissue samples were collected from 209 patients. These include 186 pancreatic cancers, 51 matched normal surgical margins, and 23 benign pancreatic lesions. All tissue blocks were obtained from the Department of Pathology, Affiliated Hospital of Nantong University, from 2003 to 2010. Clinical characteristics of cancer patients were extracted from their medical record, including age, sex, tumor location, differentiation grade, perineural and vascular invasion, and tumor stage. None of the cancer patients received any type of treatment (radiation therapy, chemotherapy, or immunotherapy) before surgery. *Overall survival* was defined as the period from initial biopsy–confirmed diagnosis to death. Patients who were alive at the last follow-up date were censored from the analysis. The study protocol was approved by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University, Jiangsu, China.

TMA Construction and IHC Analysis

Tissue microarray was generated using the manual Tissue Microarrayer System Quick Ray (UT06; UNITMA, Korea) in the Department of Clinical Pathology, Nantong University Hospital, Jiangsu, China. Specifically, core tissue biopsies (2 mm in diameter) were taken from approximately 70 individual formalin-fixed paraffin-embedded blocks and arranged in a new recipient paraffin block. A total of 4 TMAs were made, and 4-μm sections were cut and placed on super frost-charged glass microscope slides to generate TMA slides.

Tissue sections were deparaffinized and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2. Antigen retrieval was carried out with 0.01 M citrate buffer pH 6.0 and microwave heat induction. RAB27B was detected by rabbit polyclonal antihuman RAB27B antibody (dilution, 1:100) (ab104083; Abcam), and p53 was detected by rabbit polyclonal antihuman p53 antibody (dilution, 1:100) (M3629; Dako, Carpinteria, Calif). Reactions were detected with EnVision+ peroxidase kit (Dako). Color development was accomplished by incubating with 3,3′-diaminobenzidine plus (Dako), counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene, and coverslipped with permanent mounting media.

All cases were reviewed and scored without knowledge of clinical characteristics. The expression of RAB27B and p53 was scored using the semiquantitative H-score method, taking into account both the staining intensity and the percentage of cells at that intensity.[25] The staining intensity was scored as 0 (no staining), 1+ (weak staining), 2+ (moderate staining), or 3+ (intense staining). For each of the 4 staining intensity scores, the percentage of cells stained at the respective intensity was determined and multiplied by the intensity score to yield an intensity percentage score. The final staining scores were then calculated from the sum of the 4 intensity percentage scores. Thus, the staining score had a minimum value of 0 (no staining) and a maximum of 300 (100% of cells with 3+ staining intensity).

Statistical Analysis

For statistical analysis, the continuous RAB27B and p53 expression data from IHC were first converted into dichotic data (low vs high) using specific cutoff values, which were selected to be significant in terms of OS using the X-tile software program (The Rimm Lab at Yale University; http://www.tissuearray.org/rimmlab).[26,27]

Student *t* test and Pearson χ^2 test were used to determine the statistical significance of differences between comparison groups. The correlation between RAB27B and p53 protein expression was calculated using the Spearman test. The cumulative patient survival was estimated using the Kaplan-Meier method, and a log-rank test was used to compare the survival curves. A Cox proportional hazards model was used to calculate univariate and multivariate hazard ratios (HRs) for the variables. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using the SPSS 19.0 statistical software package (SPSS Inc, Chicago, Ill).

RESULTS

RAB27B or p53 Expression in Pancreatic Tissues

RAB27B protein was primarily localized in the cytoplasm and, in rare occasions, also present in the nuclei, whereas p53 protein was localized in the nuclei (Fig. 1). Using the X-tile software program for TMA data analysis (http://www.tissuearray.org/rimmlab), we first identified a significant cutoff point in relationship to OS in pancreatic cancers. For RAB27B, the cutoff 90 was selected: scores 0 to 90 were considered low expression, whereas scores 91 to 300 were considered high expression. For p53, the cutoff point 50 was selected: scores 0 to 50 were considered low expression, whereas scores 51 to 300 were considered high expression. For all subsequent analyses, RAB27B and p53 protein expression levels were considered either "low" or "high" using these cutoff values.

Although the frequency of high RAB27B expression was higher in pancreatic cancers than in benign pancreatic lesions and normal surgical margins, the difference was not statistically significant (Table 1). The frequency of high p53 expression ($p53^+$) was significantly higher in cancers ($P = 0.001$) than in normal surgical margins and benign pancreatic lesions. No case of high RAR27B and high p53 coexpression ($RAB27B^+/p53^+$) was detected.

Association of RAB27B and p53 Expression With Clinicopathologic Characteristics in Pancreatic Cancers

Next, we examined the correlation between RAB27B or p53 protein expression and clinical parameters among pancreatic cancer patients. A high RAB27B expression was significantly associated with perineural invasion ($P = 0.043$), vascular invasion ($P = 0.021$), and distant metastasis ($P = 0.037$), whereas a high p53 expression was significantly associated with vascular invasion ($P = 0.047$), tumor stage ($P = 0.014$), especially regional lymph node metastasis ($P = 0.005$), and distant metastasis ($P = 0.003$) (Table 2) and marginally associated with perineural invasion ($P = 0.086$) and tumor size ($P = 0.061$). High RAB27B and high p53 coexpression ($RAB27B^+/p53^+$) was significantly associated with distant metastasis ($P = 0.002$) and marginally associated with perineural invasion ($P = 0.083$), vascular invasion ($P = 0.068$), and tumor size ($P = 0.093$). Interestingly, a significant correlation between RAB27B and p53 expression was observed ($P = 0.026$).

Prognostic Value of RAB27B and p53 Protein Expression in Pancreatic Cancer

We also determined prognostic factors in pancreatic cancers using both univariate and multivariate analyses. A high RAB27B expression (HR, 2.435; 95% confidence interval [95% CI],

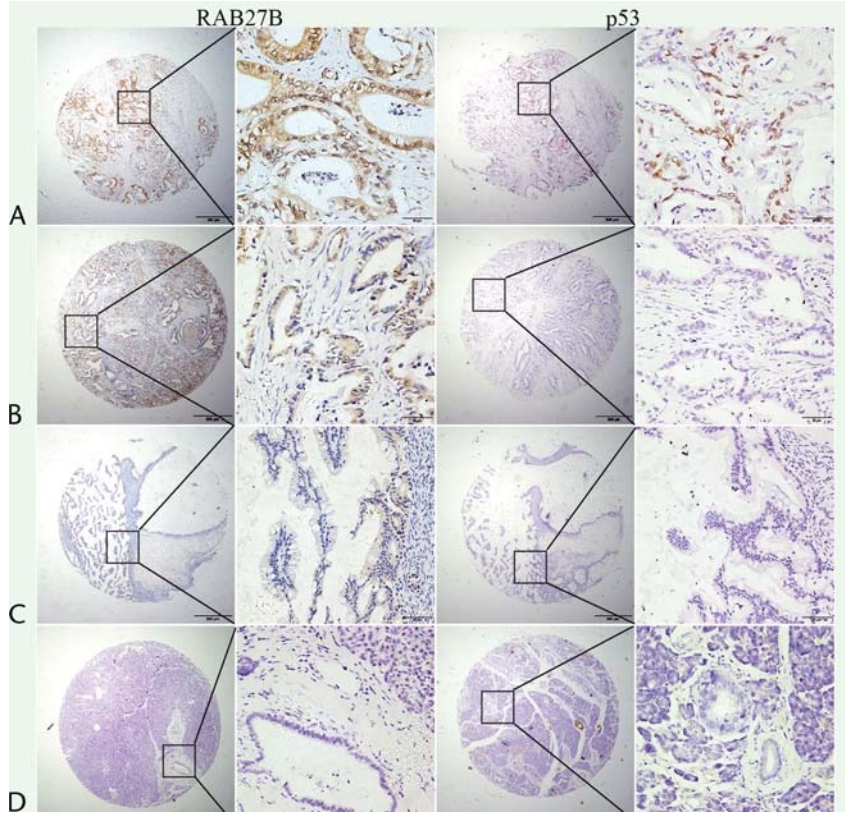

FIGURE 1. Representation of RAB27B and p53 protein expression in pancreatic benign and malignant tissues on TMA sections. Row A, pancreatic cancer with high RAB27B expression and high p53 expression. Row B, pancreatic cancer with high RAB27B expression and no p53 expression. Row C, benign pancreatic lesion with low RAB27B expression and no p53 expression. Row D, normal pancreatic ductal epithelium with no RAB27B expression and no p53 expression. Columns 1 and 2 are RAB27B staining with ×40 (scale bar, 500 μm) and ×400 (scale bar, 50 μm) magnification, respectively, and columns 3 and 4 are p53 staining with ×40 (scale bar, 500 μm) and ×400 (scale bar, 50 μm) magnification, respectively.

1.235–4.804; $P = 0.01$) was significantly associated with poor OS in univariate analysis and so do a high p53 expression (HR, 2.657; 95% CI, 1.296–5.446; $P = 0.012$) and a high RAB27B and high p53 ($RAB27B^+/p53^+$) coexpression (HR, 4.383; 95% CI, 1.730–11.103; $P = 0.002$). Differentiation (grading) was marginally associated with poor OS in univariate analysis (HR, 2.007; 95% CI, 0.898–4.485; $P = 0.09$), as well as regional lymph node metastasis (HR, 1.903; 95% CI, 0.954–3.794; $P = 0.068$). In multivariate analysis, only a high RAB27B expression remained significantly associated with poor OS (HR, 2.610; 95% CI, 1.097–6.210; $P = 0.03$), whereas a high p53 expression was only marginally associated with poor OS (HR, 2.506; 95% CI, 0.921–6.821; $P = 0.072$) (Table 3; Fig. 2).

DISCUSSION

In this study, we have determined RAB27B and p53 protein expression in pancreatic tissues by IHC analysis on TMA. We found that a high RAB27B protein expression and a high p53 protein expression were associated with vascular invasion as well as distant metastasis. We also detected a significant correlation between RAB27B and p53 expression. In both univariate and

TABLE 1. RAB27B and p53 Expression in Pancreatic Benign and Malignant Tissues

		RAB27B			p53			RAB27B/p53		
		High Expression, %	Pearson χ^2	*P*	High Expression, %	Pearson χ^2	*P*	High Expression, %	Pearson χ^2	*P*
Groups	No		3.960	0.138		13.007	0.001*		4.350	0.114
Benign pancreatic lesion	23	5 (21.74)			3 (13.04)			3 (13.04)		
Normal surgical margin	51	14 (27.45)			1 (1.96)			1 (1.96)		
Pancreatic cancer	186	72 (38.30)			44 (23.66)			21 (11.29)		

$RAB27B^+$ represents high RAB27B expression, $p53^+$ represents high p53 expression, and $RAB27B^+/p53^+$ represents high RAB27B and high p53 expression.

*$P < 0.05$, statistically significant.

TABLE 2. Association of High Expression of RAB27B and p53 With Clinicopathological Characteristics in Pancreatic Cancer Patients

		RAB27B High Expression, %	Pearson χ^2	*P*	p53 High Expression, %	Pearson χ^2	*P*	High Expression, %	Pearson χ^2	*P*
Total	169	82 (48.52)			39 (23.08)			25 (14.79)		
Age			1.611	0.204		1.462	0.227		1.506	0.220
≤60 y	66	28 (42.42)			12 (18.18)			7 (10.61)		
>60 y	103	54 (52.43)			27 (26.21)			18 (17.48)		
Sex			0.102	0.750		0.098	0.754		0.081	0.777
Male	99	53 (43.80)			22 (22.22)			14 (14.14)		
Female	70	29 (41.43)			17 (24.29)			11 (15.71)		
Tumor location			2.288	0.130		0.058	0.810		0.061	0.805
Head	90	48 (53.33)			20 (22.22)			13 (14.44)		
Body and/or tail	50	20 (40.00)			12 (24.00)			8 (16.00)		
Unknown	29	14			7			4		
Differentiation			0.808	0.369		0.113	0.737		0.039	0.844
Well and middle	131	66 (50.38)			31 (23.66)			19 (14.50)		
Poor	38	16 (42.11)			8 (21.05)			6 (15.79)		
Perineural invasion			4.084	0.043*		2.941	0.086		3.014	0.083
No	16	2 (12.50)			1 (6.25)			0 (0.00)		
Yes	61	24 (39.34)			16 (26.23)			10 (16.39)		
Unknown	92	56			22			15		
Vascular invasion			5.333	0.021*		3.938	0.047*		3.333	0.068
No	50	11 (22.00)			7 (14.00)			3 (5.77)		
Yes	20	10 (50.00)			7 (35.00)			4 (20.00)		
Unknown	99	61			25			18		
T-Primary tumor			0.120	0.942		5.603	0.061		1.639	0.441
T1-T2	93	45 (48.39)			20 (21.51)			13 (13.98)		
T3	50	23 (46.00)			10 (20.00)			7 (14.00)		
T4	20	9 (45.00)			9 (45.00)			5 (25.00)		
Unknown	6	5			0			0		
N-Regional lymph nodes			1.723	0.189		7.818	0.005*		2.820	0.093
N0	120	53 (44.17)			22 (18.33)			15 (12.50)		
N1	43	24 (55.81)			17 (39.53)			10 (23.26)		
Unknown	6	5			0			0		
M-Distantmetastasis			4.344	0.037*		9.067	0.003*		14.725	0.002*
M0	156	71 (45.51)			34 (21.79)			21 (13.46)		
M1	7	6 (85.71)			5 (71.43)			4 (57.14)		
Unknown	6	5			0			0		
TNM stage			1.067	0.785		10.599	0.014*		4.102	0.251
Stage 1a and stage 1b	74	37 (50.00)			13 (17.57)			10 (13.51)		
Stage 2a	33	13 (39.39)			5 (15.15)			3 (9.09)		
Stage 2b	35	17 (48.57)			11 (31.43)			6 (17.14)		
Stage 3 and stage 4	21	10 (47.62)			10 (47.62)			6 (28.57)		
Unknown	6	5			0			0		
p53			4.928	0.026*						
Low	130	57 (43.85)								
High	39	25 (64.10)								
RAB27B						4.928	0.026*			
Low	87				14 (16.09)					
High	82				25 (30.49)					

*$P < 0.05$, statistically significant.

multivariate analyses, we found that a high RAB27B expression was significantly associated with patients' poor OS.

To the best of our knowledge, this is the first study investigating RAB27B protein expression as well as its prognostic value in PDAC. Our data are consistent with studies of RAB27B in other types of cancer. In our study, although RAB27B expression was higher in malignant tissues (38.30%) than in normal (21.74%) and benign lesions (27.45%), the difference was not statistically significant ($P = 0.114$). This is similar to the study reported by Dong et al[16] where they did not detect a higher RAB27B

TABLE 3. Univariate and Multivariate Analyses of Prognostic Markers for OS in Pancreatic Cancer Patients

	Univariate Analysis			Multivariate Analysis		
Variable	**HR**	***P***	**95% CI**	**HR**	***P***	**95% CI**
RAB27B expression high vs low	2.435	0.010*	1.235–4.804	2.610	0.030*	1.097–6.210
p53 expression high vs low	2.657	0.012*	1.296–5.446	2.506	0.072	0.921–6.821
RAB27B/p53 expression $RAB27B^+/p53^+$ vs non- $RAB27B^+/p53^+$	4.383	0.002*	1.730–11.103	0.845	0.861	0.193–3.846
Sex						
Female vs male	1.780	0.126	0.850–3.724			
Age, y						
≤60 vs >60	0.684	0.272	0.347–1.347			
Tumor location						
Head vs body and/or tail	0.578	0.170	0.264–1.265			
Differentiation						
Well and middle vs poor	2.007	0.090	0.898–4.485	1.885	0.165	0.771–4.609
Perineural invasion						
Yes vs no	1.281	0.739	0.299–5.486			
Vascular invasion						
Yes vs no	1.529	0.346	0.633–3.692			
T-Primary tumor						
T1-2 vs T3 vs T4	1.184	0.422	0.784–1.786			
N-Regional lymph nodes						
N0 vs N1	1.903	0.068	0.954–3.794			
M-Distant metastasis						
M0 vs M1	1.551	0.554	0.363–6.632			
TNM stage						
I vs IIa vs IIb vs III-IV	1.095	0.531	0.824–1.454	1.014	0.931	0.771–4.609

*$P < 0.05$, statistically significant.

expression in primary hepatocellular carcinoma than in nonneoplastic liver, although a high RAB27B expression was associated with tumor stage and OS. The presence of RAB27B expression in the matched normal surgical margin samples suggests that abnormal RAB27B expression is an early event for PDAC. Future studies including normal pancreatic tissues are needed to determine whether RAB27B expression can be used as an early detection marker. In bladder cancer, both RAB27B and its effectors are abnormally expressed[15]; in breast cancer, both RAB27B RNA and protein are significantly upregulated in cancer cells when compared with normal tissues,[13,28] and a high RAB27B expression is associated with poor prognosis in breast cancer patients.[14,28,29]

RAB27B was originally isolated as a novel Rab family small GTP-binding protein from human platelets[30] and later from melanoma cells and melanocytes, implicated in melanosome production.[10] The function of RAB27B in tumorigenesis is best studied in breast cancer. It has been shown that RAB27B expression was significantly higher among estrogen receptor (ER)–positive breast cancer,[13] which is consistent with in vitro data that estrogen regulates the expression of RAB27B in breast cancer cell lines.[31] Expression of RAB27B mRNA and protein was associated with lymph node metastasis and differentiation grade in ER-positive human breast tumors.[13] Mechanistically, it has been shown that increased expression of RAB27B promotes cell cycle transition,

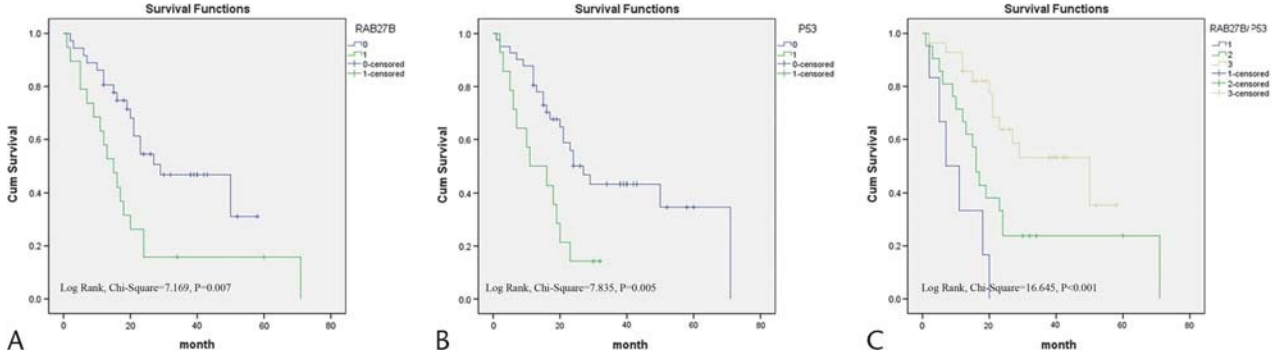

FIGURE 2. Survival curves of pancreatic cancer by the Kaplan-Meier method and the log-rank test. A, Overall survival curves of $RAB27B^+$ (green line, 1) and $RAB27B^-$ (blue line, 0). B, Overall survival curves of $p53^+$ (green line, 1) and $p53^-$ (blue line, 0). C, Overall survival curves of $RAB27B^+/p53^+$ (blue line, 1), $RAB27B^+/p53^-$ or $RAB27B^-/p53^+$ (green line, 2), and $RAB27B^-/p53^+$ (yellow line, 3).

proliferation, and cell invasion and confers invasive growth and metastasis in ER-α–positive breast cancer cells by regulating the secretion of heat shock protein 90α, which is required for matrix metalloproteinase-2 activation.[13]

Recent studies suggest that, in addition to regulating genomic instability, p53 protein regulates exosome secretion. Lespagnol et al[32] demonstrated that DNA damage–induced exosome secretion is p53 dependent and so is senescence-associated exosome release.[33] Mechanistically, it has been shown that p53 protein responds to stress signals by regulating the transcription of a variety of genes, including TSAP6, thus playing an important role in exosome production and sorting.[22,34] In addition, p53 regulates Chmp4C, caveolin-1, and DRAM, which are involved in the regulation of endosomal compartment.[20,21] Our finding of a significant correlation between RAB27B and p53 expression in PDAC suggests that RAB27B's function in regulating the exosome secretion in PDAC might be dependent on p53 expression. In other words, there may be a functional coordination between RAB27B and p53. Further mechanistic studies are needed to determine whether p53 directly or indirectly supports the expression or activity of RAB27B.

Our study has several limitations. First, it is a retrospective observational study, which might suffer from sampling biases; the conclusions need to be confirmed in larger prospective studies. Second, we determined p53 overexpression as a surrogate marker for p53 mutation; however, not all p53 mutations lead to p53 overexpression. Future studies are needed to confirm our observation by determining p53 mutation in PDAC. Third, IHC data are semiquantitative; additional methods are needed to evaluate and quantify RAB27B and p53 expression levels in tumor cells. Finally, we do not know whether and how RAB27B protein influences the tumor microenvironment in PDAC. Future in vitro studies are needed to investigate the mechanisms of RAB27B- and p53-mediated exosome secretion in pancreatic cancer development.

In conclusion, we have shown that a high RAB27B protein expression is an independent prognostic marker in PDAC. Because of the essential role of RAB27B in exosome secretion, future research is warranted to investigate whether RAB27B plays a key role in shaping the tumor microenvironment and whether RAB27B is a valid novel therapeutic target in metastatic pancreatic cancer.

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