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High expression of RAB27A and TP53 in pancreatic cancer predicts poor survival

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Abstract RAB27A is a member of Rab family GTPases involved in cellular vesicle trafficking, and TP53 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 RAB27A and TP53 expression might be associated with aggressive behavior in pancreatic ductal adenocarcinoma (PDAC), one of the most deadly human malignancies. We determined protein expression of RAB27A and TP53 in 265 pancreatic tissues (186 carcinomas and 79 normal or benign tissues) by immunohistochemistry analysis on tissue microarray and found their expression was correlated with patients' clinical parameters and overall survival. We found that RAB27A and TP53 protein expression was significantly higher in cancerous tissues compared to normal and benign tissues. High RAB27A protein expression (RAB27A+) was significantly associated with tumor stage and vascular invasion. No correlation between RAB27A and TP53 expression was observed. Patients with high RAB27A expression and high TP53 expression had a poor overall survival. Our data indicate that RAB27A expression is an independent prognostic marker for PDAC, and RAB27A-regulated exosome secretion pathway may represent a novel therapeutic target in pancreatic cancer.

Keywords RAB27A · TP53 · Pancreatic ductal adenocarcinoma · Prognosis

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Introduction

Pancreatic ductal adenocarcinoma (PDAC), arising from the exocrine pancreas and comprising 95 % of pancreatic cancer cases, is one of the most aggressive and lethal forms of human malignancy. Worldwide, PDAC is the twelfth 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]. The prognosis of PDAC is dismal with an overall survival of less than 5 % in 5 years. This is because that majority of the patients is diagnosed at an advanced stage who are no longer eligible for surgical resection with a median survival of 3.5 months, while only 10–15 % of the patients are eligible for curative surgical resection with a slightly longer median survival of 12.6 months [2]. In addition, there is no effective targeted therapy. Conventional chemotherapy is only for palliative care to reduce symptoms and improve quality of life but is not effective in prolonging overall survival in patients with advanced metastatic disease [3, 4].

To better understand and improve the dismal outcome of PDAC, reliable prognostic markers and novel therapy targets are needed. Recently, the exosome secretion signaling pathway has emerged as a novel mechanism for cancer invasion and metastasis [5]. Exosomes are 40- to 100-nm membrane vesicles derived from the multivesicular endosomes and released upon fusion with the plasma membrane. Cancer cells utilize exosomes to communicate with the environment through delivery of surface proteins and release of growth factors and cytokines to establish invasive tumor growth [6]. It has been demonstrated that exosome messaging contributes to tumor immune escape [7] and metastatic niche preparation [8].

RAB27A belongs to the Rab family of small GTPases, the master regulator of vesicle fusion and trafficking. It was originally isolated from human melanoma cells and melanocytes. Its expression has been detected in most of normal tissues and tumor cell lines [9]. Abnormal expression of RAB27A has been observed in breast, lung, bladder, rectal, prostate, and liver cancers and associated with aggressive tumor behavior [10–15]. In glioma cell lines, ectopic expression of RAB27A increases cell viability, promotes proliferation and invasion, and suppresses apoptosis [16]. In vivo, RAB27A expression has been shown as a prognostic marker in glioma and hepatocellular carcinoma [15, 17].

TP53 is one of the most important tumor suppressor genes, which is mutated in over 50 % of human malignancies [18]. It regulates DNA repair, cell cycle, and apoptosis and therefore plays an essential role in maintaining genetic stability [19]. Recent studies have also indicated the role of TP53 in regulation of exosome secretion: On the one hand, exosomes can stabilize TP53 protein to create a tumor permissive environment [20]; on the other hand, TP53 transcribes key regulators of endosomal compartment, thus regulates exosome production and secretion [21–23]. However, the interaction between RAB27A and TP53 in regulating exosome secretion has not been reported. Both TP53 protein accumulation and TP53 mutation have been detected in PDAC [24, 25].

Thus far, no study has investigated the role of exosome secretory pathway in PDAC and the potential roles by RAB27A and TP53 in the pathway. To determine whether RAB27A could be used as a prognostic marker and a therapeutic target in pancreatic cancer, we analyzed RAB27A and TP53 expression by immunohistochemistry analysis in both benign and malignant pancreatic tissues using tissue microarrays (TMAs). We correlated RAB27A and TP53 expression with clinicopathological characteristics as well as overall survival in patients with pancreatic cancers.

Materials and methods

Human tissue specimens and patient clinical information

A total of 265 formalin-fixed paraffin-embedded (FFPE) tissue samples were collected from 211 patients. These include 186 pancreatic cancers, 54 matched normal surgical margins, and 25 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 types of treatments (radiation therapy, chemotherapy, or immunotherapy) before surgery. Overall survival (OS) 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. All participants signed written informed consent. All data have been anonymized and deidentified.

Tissue microarray (TMA) construction and immunohistochemistry analysis (IHC)

TMA 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 ~70 individual FFPE blocks and arranged in a new recipient paraffin block. A total of four TMAs were made, and four-micron 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 % H2O2. Antigen retrieval was carried out with 0.01 M citrate buffer pH 6.0 and microwave heat induction. RAB27A was detected by mouse monoclonal anti-human RAB27A antibody (dilution 1:200) (Abcam, ab55667), and TP53 was detected by rabbit polyclonal anti-human TP53 antibody (dilution 1:100) (DAKO, M3629). Reactions were detected with Envision+^{TM} peroxidase kit (Dako, Carpinteria, CA, USA). Color development was accomplished by incubating with 3,3′-diaminobenzidine plus (Dako, Carpinteria, CA, USA), 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 RAB27A and TP53 was scored using the semiquantitative H-score method, taking into account both the staining intensity and the percentage of cells at that intensity [26]. The staining intensity was scored as 0 (no staining), 1+ (weak staining), 2+ (moderate staining), or 3+ (intense staining). For each of the four 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 four 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) [27].

Statistical analysis

For statistical analysis, the continuous RAB27A and TP53 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) [27–29].

Student's *t* test and Pearson's Chi-square test were used to determine the statistical significance of differences between comparison groups. The correlation between RAB27A and TP53 protein expression was calculated using Spearman's 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 for the variables. A p value of less than 0.05 was considered statistically significant. All statistical analyses were carried out using the SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL).

Results

RAB27A or TP53 expression in pancreatic tissues

RAB27A protein was localized in the cytoplasm, while TP53 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 significant cutoff point in terms of OS in pancreatic cancers. For RAB27A, the cutoff 90 was selected: Score 0–90 was considered low expression, while 91–300 was considered high expression. For TP53, the cutoff point 60 was selected: Score 0–60 was considered low expression, while 61–300 was considered high expression. For all subsequent analyses, RAB27A and TP53 protein expression levels were considered either as "low" or "high" using these cutoff values.

Although high RAB27A expression was detected in benign pancreatic lesions and normal surgical margins, the

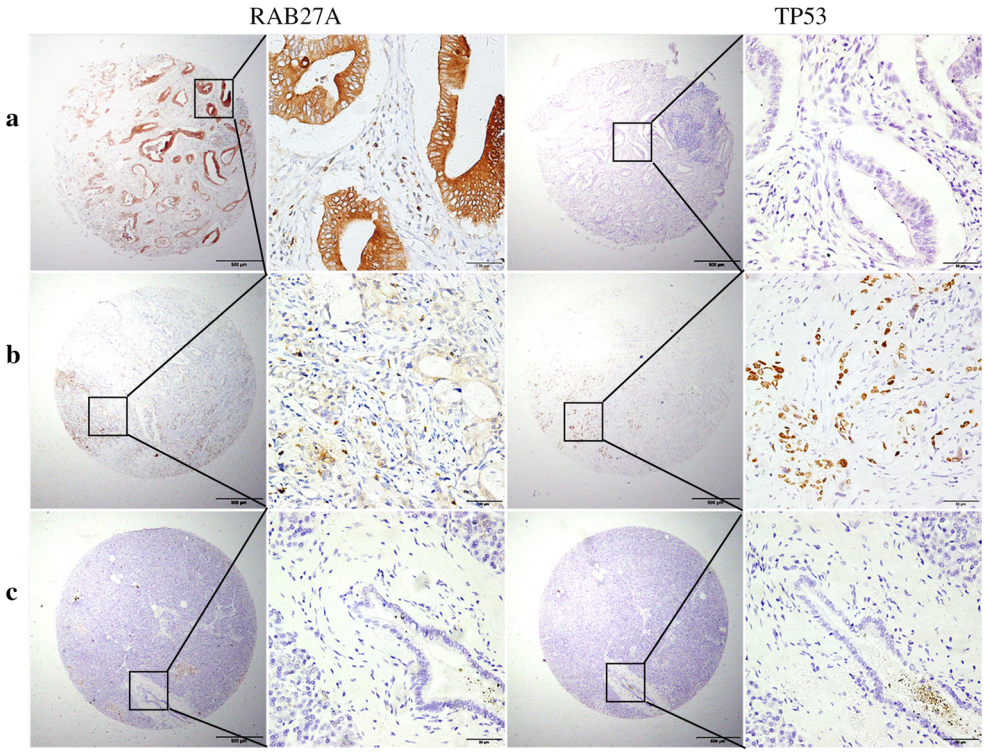

Fig. 1 Representation of RAB27A and TP53 protein expression in pancreatic benign and malignant tissues on TMA sections. **a** Pancreatic cancer with high RAB27A expression and no TP53 expression; **b** pancreatic cancer with low RAB27A expression and high TP53 expression; **c** benign pancreatic ductal epithelium with no RAB27A expression and no TP53 expression; *column 1* and *2* are RAB27A staining with ×40 (*bar* 500 μm) and ×400 (*bar* 50 μm) magnification, respectively, and *column 3* and *4* are TP53 staining with ×40 (*bar* 500 μm) and ×400 (*bar* 50 μm) magnification, respectively

Table 1 RAB27A and TP53 expression in pancreatic benign and malignant tissues

Groups	No	RAB27A			TP53			RAB27A/TP53		
		High expression (%)	Pearson χ^2	p value	High expression (%)	Pearson χ^2	p value	RAB27A+/TP53+ (%)	Pearson χ^2	p value
			6.744	0.034*		13.136	0.001*		9.687	0.008*
Benign pancreatic lesion	25	4 (16.00)			2 (8.00)			0 (0.00)		
Normal surgical margin	54	14 (25.93)			2 (3.70)			0 (0.00)		
Pancreatic cancer	186	72 (38.30)			44 (23.66)			21 (11.29)		

RAB27A+ represents high RAB27A expression, TP53+ represents high TP53 expression, and RAB27A+/TP53+ represents high RAB27A and high TP53 expression

frequency of high RAB27A expression (RAB27A+) was significantly higher in pancreatic cancers ($p = 0.034$) (Table 1). Similarly, the frequency of high TP53 expression (TP53+) was also significantly higher in cancers ($p = 0.001$) than in benign lesions and normal surgical margins. Interestingly, high RAB27A and TP53 coexpression (RAB27A+/TP53+) was only detected in pancreatic cancers, and none of benign pancreatic lesions and normal surgical margins had RAB27A+/TP53+ staining, although not all cancers show coexpression of RAB27A and TP53.

Association of RAB27A and TP53 expression with clinicopathological characteristics in pancreatic cancers

Next, we examined the correlation between RAB27A or TP53 protein expression and clinical parameters among pancreatic cancer patients. High RAB27A expression was significantly associated with vascular invasion ($p = 0.016$) and tumor stage ($p = 0.021$), especially with tumor size ($p = 0.008$) and distant metastasis ($p = 0.008$), while high TP53 expression was significantly associated with tumor stage ($p = 0.041$), especially lymph node ($p = 0.036$) and distant metastasis ($p = 0.003$), and marginally associated with perineural invasion ($p = 0.067$) (Table 2). High RAB27A and TP53 coexpression (RAB27A+/TP53+) was significantly associated with vascular invasion ($p = 0.015$), tumor stage ($p = 0.001$), especially tumor size ($p = 0.003$) and distant metastasis ($p < 0.001$), marginally associated with perineural invasion ($p = 0.074$). No correlation between RAB27A and TP53 expression was detected.

Prognostic value of RAB27A and TP53 protein expression in pancreatic cancer

We also determined prognostic factors in pancreatic cancers using both univariate and multivariate analysis. High RAB27A expression (HR 2.427, 95 % CI 1.268–4.643; $p = 0.007$) was significantly associated with poor OS in univariate analysis, as well as high TP53 expression (HR 2.795, 95 % CI 1.450–5.384; $p = 0.002$), and high RAB27A/high TP53 (RAB27A+/TP53+) coexpression (HR 3.808, 95 % CI 1.625–8.923; $p = 0.002$). Differentiation (tumor grade) was also significantly associated with poor OS in univariate analysis (HR 2.106, 95 % CI 1.015–4.369; $p = 0.045$). But regional lymph node metastasis was marginally associated with poor OS (HR 1.759, 95 % CI 0.931–3.324; $p = 0.082$). In multivariate analysis, only high RAB27A expression and high TP53 expression remained significantly associated with poor OS (HR 2.938, 95 % CI 1.236–6.986; $p = 0.015$ and HR 3.340, 95 % CI 1.347–8.282; $p = 0.009$, respectively) (Table 3) (Fig. 2).

Discussion

In this study, we have determined RAB27A and TP53 protein expression in pancreatic tissues by immunohistochemistry analysis on tissue microarray. We found that both RAB27A and TP53 protein expressions were significantly higher in cancerous tissues than in normal and benign tissues. Both high RAB27A protein expression and high TP53 protein expression were associated with tumor stage and distant metastasis. High RAB27A protein expression was also associated with vascular invasion, though we did not detect correlation between RAB27A and TP53 expression. In both univariate analysis and multivariate analysis, we found that high RAB27A expression and high TP53 expression were significantly associated with patients' poor overall survival.

To the best of our knowledge, this is the first study investigating RAB27A protein expression as well as its prognostic value in PDAC. Our data are consistent with studies of RAB27A in other types of cancer. Dong et al. [15] demonstrated higher RAB27A expression in primary

Table 2 Association of high expression of RAB27A and TP53 with clinicopathological characteristics in pancreatic cancer patients

Groups	No.	RAB27A			TP53			RAB27A+/TP53+		
		High expression (%)	Pearson χ^2	p value	High expression (%)	Pearson χ^2	p value	High expression (%)	Pearson χ^2	p value
Total	186	72 (38.30)			44 (23.66)			21 (11.29)		
Age			0.211	0.646		0.986	0.321		0.220	0.639
≤60 years	71	26 (36.62)			14 (19.72)			9 (12.86)		
>60 years	115	46 (40.00)			30 (26.09)			12 (10.43)		
Gender			0.032	0.859		0.503	0.478		0.448	0.504
Male	110	42 (38.18)			24 (21.82)			11 (10.00)		
Female	76	30 (39.47)			20 (26.32)			10 (13.16)		
Tumor location			0.581	0.446		0.013	0.909		0.211	0.646
Head	99	38 (38.38)			22 (22.22)			10 (10.10)		
Body and/or tail	56	25 (44.64)			12 (21.43)			7 (12.50)		
Unknown	31	9			10			4		
Differentiation			1.087	0.297		0.085	0.771		0.124	0.725
Well and middle	145	59 (40.69)			35 (24.14)			17 (11.72)		
Poor	41	13 (31.71)			9 (21.95)			4 (9.76)		
Perineural invasion			0.002	0.962		3.352	0.067		3.182	0.074
No	18	8 (44.44)			1 (5.56)			0 (0.00)		
Yes	71	32 (45.07)			18 (25.35)			11 (15.19)		
Unknown	97	32			23			10		
Vascular invasion			5.797	0.016*		0.727	0.394		5.884	0.015*
No	58	21 (36.21)			9 (15.52)			3 (5.17)		
Yes	21	14 (66.67)			5 (23.81)			5 (23.81)		
Unknown	107	37			28			13		
T—primary tumor			9.578	0.008*		4.504	0.105		11.653	0.003*
T1–T2	99	28 (28.28)			21 (21.21)			7 (7.07)		
T3	61	29 (47.54)			14 (22.95)			7 (11.48)		
T4	21	12 (57.14)			9 (42.86)			7 (11.60)		
Unknown	5	3			0			0		
N—regional lymph nodes			2.657	0.103		4.380	0.036*		0.566	0.452
N0	133	46 (34.59)			27 (20.30)			14 (10.53)		
N1	48	23 (47.92)			17 (35.42)			7 (14.58)		
Unknown	5	3			0			0		
M—distant metastasis			6.992	0.008*		8.786	0.003*		14.725	<0.001*
M0	174	63 (36.21)			39 (22.41)			17 (9.77)		
M1	7	6 (85.71)			5 (71.43)			4 (57.14)		

Table 2 continued

Groups	No.	RAB27A			TP53			RAB27A+/TP53+		
		High expression (%)	Pearson χ^2	p value	High expression (%)	Pearson χ^2	p value	High expression (%)	Pearson χ^2	p value
Unknown	5	3			0			0		
TNM stage			9.733	0.021*		8.242	0.041*		15.623	0.001*
Stage 1a and stage 1b	81	22 (27.16)			15 (18.52)			5 (6.17)		
Stage 2a	38	17 (44.74)			7 (18.42)			4 (10.53)		
Stage 2b	40	17 (45.24)			12 (30.00)			4 (10.00)		
Stage 3 and stage 4	22	13 (59.09)			10 (45.55)			8 (36.36)		
Unknown	5	3			0			0		
TP53			1.975	0.160						
Low	142	51 (35.92)								
High	44	21 (47.73)								
RAB27A						1.975	0.160			
Low	114				23 (20.18)					
High	72				21 (29.17)					

* $p < 0.05$

hepatocellular carcinoma than in matched normal adjacent tissues; significant association of RAB27A expression with tumor stage, differentiation grade, and OS. In bladder cancer, both RAB27A and its effectors are abnormally expressed [12]; in glioma, RAB27A was highly expressed in tumor tissues when compared to normal brain tissues and patients with high RAB27A expression had significantly worse prognosis [17]; in breast cancer, RAB27A confers the invasive and metastatic phenotypes by promoting the secretion of insulin-like growth factor II (IGF-II) [10]. Similarly, our observation on TP53′s prognostic value in PDAC is consistent with previous reports from pancreatic cancer and other cancers [30–33].

RAB27A was originally isolated as a novel Rab family small GTP-binding protein from human melanoma cells and melanocytes, implicated in melanosome production [9]. Mutations in RAB27A gene cause a rare human hereditary disease called Griscelli syndrome, which is characterized by silver hair (dysfunction in melanosome transport in melanocytes) and immunodeficiency (dysfunction in granule exocytosis by cytotoxic T lymphocytes) [34, 35]. Because RAB27A is frequently dysregulated in tumor cells, several studies investigated its role in exosome secretion in tumor cells and its influence on tumor microenvironment and invasive tumor growth and metastasis. Silencing RAB27A reduces exosome secretion in breast cancer cells [36] and lung cancer cells [11]. RAB27A is a key component of the secretory pathway in prostate cancer cells (18,413,239). Webber et al. [14] showed that inactivating RAB27A could eliminate TGFβ1 containing exosomes and abolish tumor-promoting stromal myofibroblast differentiation in prostate cancer. Interestingly, a recent study by Bobrie et al. [8] indicates that RAB27A can modify tumor microenvironment and promote tumor progression through both exosome-dependent and exosome-independent pathways, raising the possibility that RAB27A participates in additional tumor-promoting signaling pathways.

Recent studies suggest that besides regulating genomic instability, TP53 protein also regulates exosome secretion. Lespagnol et al. [37] demonstrated that DNA damage-induced exosome secretion is TP53 dependent, as well as senescence-associated exosome release [38]. Mechanistically, it has been shown that TP53 protein responds to stress signals by regulating the transcription of a variety of genes, including TSAP6, playing a role in exosome production and sorting [23, 39]. In addition, TP53 also regulates Chmp4C, Caveolin-1, DRAM, which are involved in the regulation of endosomal compartment [21, 22]. Our data do not support the hypothesis that TP53 regulates exosome secretion in PDAC through RAB27A, but could not rule out the possibility of weak or indirect interaction between RAB27A and TP53 due to the nature of our study design.

Table 3 Univariate and multivariate analysis of prognostic markers for overall survival in pancreatic cancer patients

Variable	Univariate analysis			Multivariate analysis		
	HR	*p* value	95 % CI	HR	*p* value	95 % CI
RAB27A expression						
High versus low	2.427	0.007*	1.268–4.643	2.938	0.015*	1.236–6.986
TP53 expression						
High versus low	2.795	0.002*	1.450–5.384	3.340	0.009*	1.347–8.282
RAB27A/TP53 expression						
RAB27A+/TP53+ versus non-RAB27A+/TP53+	3.808	0.002*	1.625–8.923	0.589	0.450	0.149–2.327
Gender						
Female versus male	1.560	1.198	0.793–3.069			
Age (years)						
≤60 versus > 60	0.857	0.630	0.458–1.604			
Tumor location						
Head versus body and/or tail	0.578	0.144	0.277–1.206			
Differentiation						
Well and middle versus poor	2.106	0.045*	1.015–4.369	1.908	0.119	0.846–4.300
Perineural invasion						
Yes versus no	1.480	0.594	0.350–6.253			
Vascular invasion						
Yes versus no	1.505	0.333	0.658–3.443			
T—primary tumor						
T1–T2 versus T3 versus T4	1.205	0.347	0.817–1.777			
N—regional lymph nodes						
N0 versus N1	1.759	0.082	0.931–3.324			
M—distant metastasis						
M0 versus M1	1.439	0.621	0.340–6.101			
TNM stage						
I versus IIa versus IIb versus III–IV	1.091	0.527	0.833–1.428	0.881	0.431	0.644–1.206

* $p < 0.05$

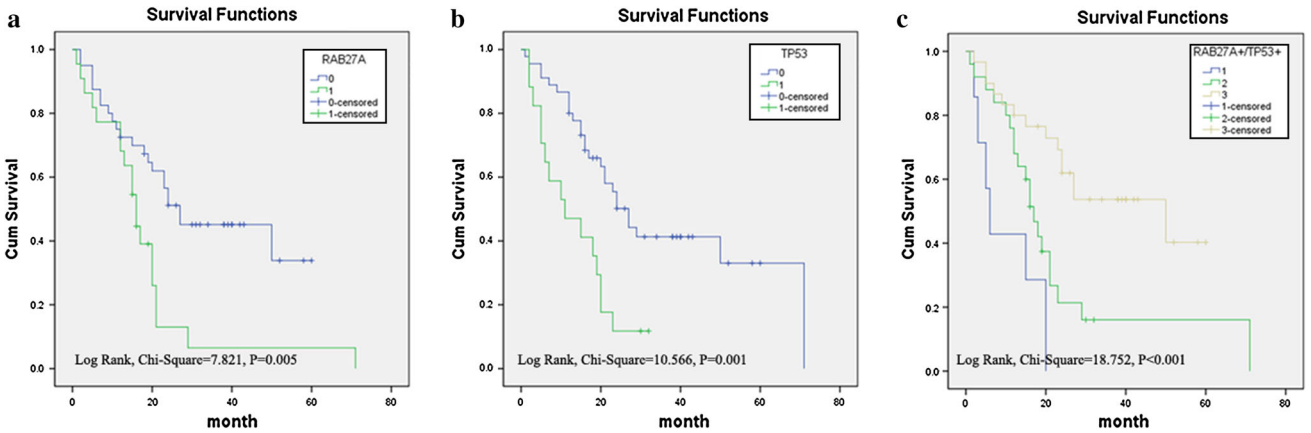

Fig. 2 Survival curves of pancreatic cancer by the Kaplan–Meier method and the log-rank test. **a** Overall survival curves of RAB27A+ (*green line*, 1) and RAB27A- (*blue line*, 0); **b** overall survival curves of TP53+ (*green line*, 1) and TP53- (*blue line*, 0); **c** overall survival curves of RAB27A+/TP53+ (*blue line*, 1), RAB27A+/TP53- or RAB27A-/TP53+ (*green line*, 2), RAB27A-/TP53+ (*yellow line*, 3)

Our study has several limitations: First, it is a retrospective observational study, the use of archived convenient samples could introduce bias, and thus, the conclusions might not be applicable to the general population. Larger prospective studies are needed to confirm our findings. Secondly, TMA technology utilizes small sections

of tissue blocks to analyze target protein expression, and the expression pattern might not represent the expression pattern of the whole tissue block, thus introducing potential biases in the data. Thirdly, IHC data are subjective and semiquantitative, and additional objective methods are needed to evaluate and confirm RAB27A and TP53 expression in tumor cells. Finally, we do not know whether and how RAB27A protein influences the tumor microenvironment in PDAC. Future in vitro studies are needed to investigate the mechanism of RAB27A and TP53-mediated exosome secretion in pancreatic cancer development.

In conclusion, we have shown that high RAB27A protein expression is an independent prognostic marker in PDAC. Because of the essential role of RAB27A in exosome secretion, future research is warranted to investigate whether RAB27A plays a key role shaping tumor microenvironment and whether RAB27A is a valid novel therapy target in metastatic pancreatic cancer.

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Conflict of interest None.

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