ANSWERING REVIEWERS

Jan 24, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 15587-Edited-revised.doc).

Title: Low RASSF6 Expression in Pancreatic Ductal Adenocarcinoma is Associated with Poor Survival

Author: Huilin Ye, Doudou Li, Qing Lin, Yu Zhou, Quanbo Zhou, Bing Zeng, Zhiqiang Fu, Wenchao Gao, Yimin Liu, Ruiwan Chen, Zhihua Li, Rufu Chen

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 2429

The manuscript has been improved according to the suggestions of reviewers:

Reviewer #1:

Comments of reviewer #1:

There are several points that need to be addressed: 1) Typos and grammatical errors need to be corrected. 2) Abstract: results: the authors note that the median survival time of strong positive and negative RASSF6 staining groups was 33 months and 11 months. However, survival curve revealed that lower RASSF6 expression was correlated with better overall survival. please check why? Conclusion: low expression of RASSF6 in PDAC is associated with poor survival, so how can it be a biomarker of unfavorable prognosis? 3) Figure 2: the figure purporting to show reduced RASSF6 expression in tumor cells that invaded nerves is unconvincing. Some quantification would be appropriate 4) Results: Page 9: the authors note that the RASSF6 expression were significantly down-regulated in cancer tissues. However, as shown in table 1, higher-stage tumors demonstrated RASSF6 expression much more frequently. Is there any reason? 5) Discussion: Page 10: Please check the result of the relationship between RASSF6 expression and the UICC T stage in PDAC patients (the correlation is positive or negative?) Page 10: "……down-regulation of RASSF6 is a predictor of different aggressive tumor behaviors such as advanced T stage, poorly differentiated carcinoma……". As shown in table 1, no significant association was observed between RASSF6 expression and tumor differentiation (P=0.138). 6) How many patients with a follow up of less than 5 years were included in this study?

Responses----------

1) Typos and grammatical errors need to be corrected.

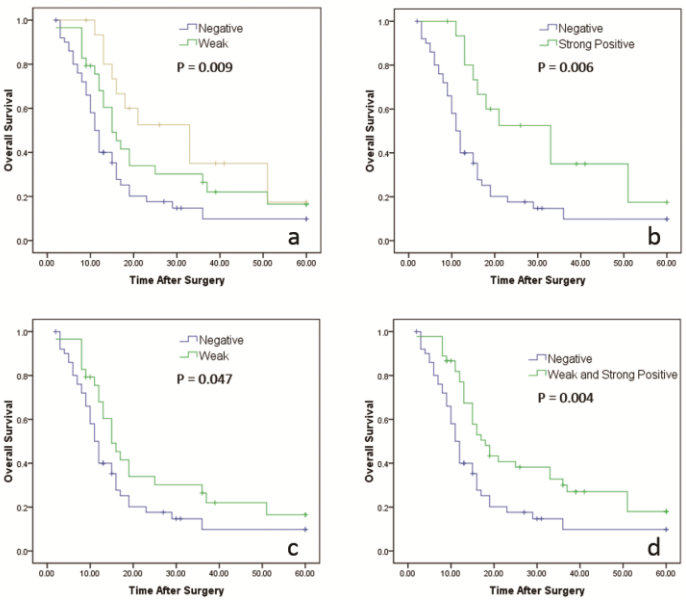
Response: The language was revised by professional English language editing companies.

2) Abstract: results: the authors note that the median survival time of strong positive and negative RASSF6 staining groups was 33 months and 11 months. However, survival curve revealed that lower RASSF6 expression was correlated with better overall survival. please check why? Conclusion: low expression of RASSF6 in PDAC is associated with poor survival, so how can it be a biomarker of unfavorable prognosis?

Response: Many thanks to you for finding this mistake. In reality, lower RASSF6 should be correlated with poor prognosis. In the survival curve (Please find Figure 4, or see below), we can find that negative staining was associated with the poorest survival.

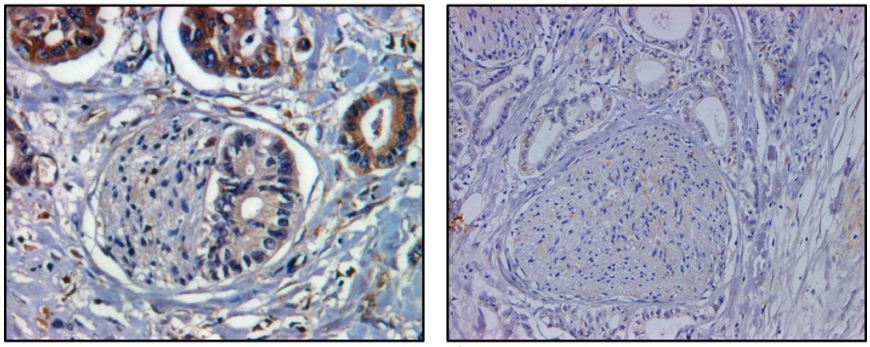
In the manuscript for review, we made a mistake in the "Results" part of the abstract. The last sentence of the "Result" in the previous manuscript was "**Survival curve revealed that lower RASSF6 expression was correlated with better overall survival (P=0.009).**" But, in fact, it should be **"Survival curve revealed that increased RASSF6 expression was correlated with better overall survival (P=0.009)."**

Fig 4.



3) Figure 2: the figure purporting to show reduced RASSF6 expression in tumor cells that invaded nerves is unconvincing. Some quantification would be appropriate

Response: We thank you for bringing this to our attention. In the previous manuscript, we concluded from Figure 2 (please see below, it is deleted in the revised paper) that tumor cells that invaded nerves showed reduced RASSF6 level. **We removed this observation from our revised manuscript because:** 1) As suggested by reviewer, this observation is unconvincing. Meanwhile, we fell it is arbitrary. 2) This finding does not affect the main conclusion that RASSF6 expression is associated with survival. 3) Correlation between RASSF6 expression and neural invasion was analyzed by $\chi 2$ test as shown in Table 1. In the aspect of prognostic significance, this result is much more important than the finding that reduced RASSF6 expression in tumor cells that invaded nerves. 4) There lack a appropriate quantification method to analyze such a micro pathological result.



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4) Results: Page 9: the authors note that the RASSF6 expression were significantly down-regulated in cancer tissues. However, as shown in table 1, higher-stage tumors demonstrated RASSF6 expression much more frequently. Is there any reason?

Response: Thanks for helping to revise this.

First, we correct a mistake that the number of RASSF6 positive patients in pT3 should be 11, but not 13 in the previous manuscript. The p value of 0.047 is right because when we analyzed the data by SPSS, we entered the right patient number “11” but not “13” of pT3.

The p value of 0.047 just suggested a statistically significant between groups, it does not point out the trend of data. We made a wrong conclusion from the date. In fact, the data suggests that higher-stage tumors demonstrated RASSF6 expression less frequently, because (please find the Table below): as it is shown in the Table below, the RASSF6 positive proportion in pT3 is only 14%, although it is 0% in pT1, the percentage is up to 50% in pT2. Similarly, the RASSF6 negative proportion in pT1 is 60%, which is close to the proportion of 56% in pT3 patients, but the proportion in pT2 is only 30%. We should note that pT1 group just includes 5 cases, therefore, this group contribute much less to the statistical result. If we put pT1 and pT2 in a same group, the negative percentage of pT1+pT2 is less than pT3 (40% vs. 56%), and the positive percentage of pT1+pT2 is higher than pT3 (33% vs. 14%), indicating a increased negative proportion and decreased positive proportion in pT3 compared with pT1+pT2.

When using Pearsonχ^2 test, the p value is 0.047. Thanks for the reviewer bring an important issue to our attention that the sample size of both pT1 and pT2 is small. Therefore, we added a statistical result of Fisher's exact Test which showed a p value of 0.076. Because the Fisher's exact Test is more suit for data including groups of low sample size, and it showed no statistical significance, we made a more conservative conclusion that "In addition, RASSF6 expression seemed to be associated with the UICC T stage. We found that higher-stage tumors demonstrated RASSF6 expression less frequently with a significant difference at the boundary".

T-stage	Total	Negative	Weak	Positive
pT1	5	3,60%	2, 40%	0, 0%
pT2	10	3, 30%	2, 20%	5, 50%
pT1+pT2	15	6, 40%	4, 27%	5, 33%
pT3	81	45, 56%	25,	11, 14%

			31%	

At last, although we note that the RASSF6 expression were significantly down-regulated in cancer tissues, it does not mean that RASSF6 expression must be lower in larger tumors. The expression of oncogene/tumor suppressor gene is some kind of the biological property of tumor itself, which may not change a lot with the tumor proliferation. May genes/proteins which were found decreased/increased in tumor tissues did not show correlation with tumor size. For example, the HMGA1/HMGA2 in hepatic cancer (Piscuoglio et al, Histopathology 2012, 60, 397–404. DOI: 10.1111/j.1365-2559.2011.04121.x), lncRNA RP11-462C24.1 in colorectal cancer (Shi et al, Med Oncol (2014) 31:31 DOI. 10.1007/s12032-014-0031-7), hENT1 in pancreatic cancer (Greenhalf et al, J Natl Cancer Inst. 2014 Jan;106(1):djt347), they all showed increased or decreased in tumor tissues, but their expression level did not show correlation with tumor size, T stage, and so on (Please find screenshot below).

Table 3. Protein marker expression related to stage (pT, pN), and tumour grade

	HMGA1	HMGA2
pT stage		
pT1–2	25.7 ± 28.7; 15.0	16.0 ± 28.4; 0.0
pT3–4	27.1 ± 31.3; 10.0	16.9 ± 28.9; 0.0
P-value	0.83	0.952
pN stage		
pN0	18.3 ± 22.8; 5.0	8.4 ± 19.0; 0.0
pN1	32.7 ± 33.6; 20.0	21.5 ± 32.4; 0.0
P-value	0.012	0.039
Tumour grade		
G1–2	22.2 ± 27.3; 10.0	12.0 ± 23.3; 0.0
G3	37.3 ± 35.1; 30.0	26.8 ± 36.0; 5.0
P-value	0.009	0.008

↑ **Piscuoglio et al, Histopathology 2012, 60, 397–404. DOI: 10.1111/j.1365-2559.2011.04121.x Shi et al, Med Oncol (2014) 31:31 DOI. 10.1007/s12032-014-0031-7**

Table 2 Correlations between RP11-462C24.1 expression and clinical characteristics

Characteristics	Number of case	RP11-462C24.1 expression				*P* value
		High ($n = 32$)	%	Low ($n = 54$)	%	
Age (years)	86					0.132
<60	43	13	40.6	30	55.6	
≥60	43	19	59.4	24	44.4	
Gender						0.300
Male	63	25	78.1	38	70.4	
Female	23	7	21.9	16	29.6	
Tumor size (cm)						0.180
<4	31	14	43.8	17	31.5	
≥4	55	18	56.2	37	68.5	
Depth of invasion						0.372
T1,T2	16	7	21.9	9	16.7	
T3,T4	70	25	78.1	45	83.3	
Tumor stage						0.070
I and II	23	12	37.5	11	20.4	
III and IV	63	20	62.5	43	79.6	
Metastasis						0.011*
Absent	36	19	59.4	17	31.5	
Present	50	13	40.6	37	68.5	

* $P < 0.05$

↑ **Shi et al, Med Oncol (2014) 31:31 DOI. 10.1007/s12032-014-0031-7**

Table 2. Relationship between hENT1 level and patient or tumor characteristics*

Characteristic	Number	hENT1 H score, median (IQR)	Low hENT1 (<48), No.	High hENT1 (≥48), No.	*P*
Resection margin					
Negative	219	47 (3–83)	114	105	.53†
Positive	161	50 (10–80)	78	83	
Lymph node status					
Negative	78	47 (17–88)	42	36	.53†
Positive	302	49 (5–80)	150	152	
Stage					
1	22	39 (23–60)	14	8	.27‡
2	93	50 (10–83)	44	49	
3	233	48 (10–75)	120	113	
4	11	17 (0–50)	8	3	
Tumor grade					
Well	32	58 (19–95)	14	18	.42‡
Moderate	249	50 (10–83)	121	128	
Poor	94	45 (0–73)	52	42	
Local invasion					
No	198	50 (23–80)	96	102	.47†
Yes	168	47 (0–79)	88	80	
Maximum tumor diameter					
<30 mm	178	50 (23–85)	83	95	.18†
≥30 mm	202	45 (0–80)	109	93	
Diabetes					
No	287	49 (3–79)	143	144	.85‡
IDDM	45	47 (0–85)	22	23	
NIDDM	29	47 (17–85)	16	13	
Sex					
Male	226	48 (5–88)	115	111	.92†
Female	154	48 (12–78)	77	77	
Age, y					
<64	189	50 (13–85)	84	105	.02†
≥64	191	45 (5–76)	108	83	

* IDDM = insulin-dependent diabetes mellitus; IQR = interquartile range; NIDDM = noninsulin-dependent diabetes mellitus.

† Fisher exact test, two-sided value.

‡ $\chi 2$ test, two-sided value.

↑ **Greenhalf et al, J Natl Cancer Inst. 2014 Jan;106(1):djt347**

5) Discussion: Page 10: Please check the result of the relationship between RASSF6 expression and the UICC T stage in PDAC patients (the correlation is positive or negative?) Page 10: "……down-regulation of RASSF6 is a predictor of different aggressive tumor behaviors such as advanced T stage, poorly differentiated carcinoma……". As shown in table 1, no significant association was observed between RASSF6 expression and tumor differentiation (P=0.138).

Response: Thanks for pointing out our mistakes. As discussed in the above response, the relationship between RASSF6 expression and the UICC T stage in PDAC patients seemed be negative. Chi-square test showed a p value of 0.047, but Fisher's exact test showed a p value of 0.076; in view of the small sample size of pT1, using Fisher's exact test might be more appropriate, but chi-square test showed a p of < .05， therefore, we changed a conservative conclusion that "In addition, RASSF6 expression seemed to be associated with the UICC T stage, we found that higher-stage tumors demonstrated RASSF6 expression less frequently with a boundary statistical difference (Table 1)".

6) How many patients with a follow up of less than 5 years were included in this study?

Response: Thanks for the detailed suggests. PDAC cases between Jan. 2000 and Jun. 2012 were involved in this study. From Sep. 2009 to around Sep. 2014 (The time of this study as well as analysis was conducted), most patients died and six patients survived. It means that 6 of the 96 (6%) cases was followed less than 5 years.

Reviewer #2:

Comments of reviewer #2:

Abstract (Results): You say "Survival curve revealed that lower RASSF6 expression was correlated with better overall survival" but above you expose that the median survival time of strong positive cases (33 months) is higher than the median survival time of negative cases (11 months) Results (Expression of RASSF6 in tumor cells and correlation with clinicopathological parameters): You write "higher stage tumors demonstrated RASSF6 expression much more frequently" however, in the discussion you determine that using IHC "RASSF6 protein expression was significantly lower in higher T stage tumors" Discussion:

You expose "down-regulation of RASSF6 is a predictor of different agressive tumor behaviours such as advanced T stage, poorly differentiated carcinoma, perineural invasion, and poor survival outcome" but below you write that in your data the RASSF6 expression don′ t tend to be negative in poorly differentiated tissues.

Responses----------

(1) You say "Survival curve revealed that lower RASSF6 expression was correlated with better overall survival" but above you expose that the median survival time of strong positive cases (33 months) is higher than the median survival time of negative cases (11 months)

Response: Thanks to point out this for us. In the manuscript for review, we made a mistake in the “Results” part of the abstract. The last sentence of the “Result” in the previous manuscript was “**Survival curve revealed that lower RASSF6 expression was correlated with better overall survival (P=0.009).**” But, in fact, it should be **“Survival curve revealed that increased RASSF6 expression was correlated with better overall survival (P=0.009).”**

(2) Results (Expression of RASSF6 in tumor cells and correlation with clinicopathological parameters): You write "higher stage tumors demonstrated RASSF6 expression much more frequently" however, in the discussion you determine that using IHC "RASSF6 protein expression was significantly lower in higher T stage tumors"

Response: Thanks for pointing out this mistake. In the reviewers’ comments, both reviewers point out this problem. The correlation between RASSF6 level and T stage should be positive, but the p value was boundary statistical significance. We revised the result and conclusion. Please find the More detailed reason and response **in the response to the fourth question of the first reviewer.**

(3) Discussion: You expose "down-regulation of RASSF6 is a predictor of different agressive tumor behaviours such as advanced T stage, poorly differentiated carcinoma, perineural invasion, and poor survival outcome" but below you write that in your data the

RASSF6 expression don′ t tend to be negative in poorly differentiated tissues.

Response: We are sorry that we made a mistake. The correlation between RASSF6 and tumor differentiation was not statistical significance. We have revised this mistake.

The manuscript has also been improved according to the suggestions of editors:

1. A running title has been added.
2. Author contributions has been added.
3. A ethic approval document of PDF format has been provided, and statement has been mentioned in the manuscript text.
4. Institutional animal care and use committee and Animal care and use statement

   Response: *We did not conduct any animal experiment in this study, therefore, we think we might not need provide the "Institutional animal care and use committee" and "Animal care and use statement".*
5. Data sharing statement of PDF format has been provided.
6. Key words has been provided.
7. Core tip less than 100 words has been provided.
8. The format of reference numbers has been revised.
9. A revised statement of "Statistical analysis" was provided.
10. A "COMMENTS" including section of background, research frontiers, innovations and breakthroughs, applications, and peer review was provided.
11. All authors abbreviation names and manuscript title have been listed in the last of this manuscript.
12. Decomposable figures were provided.

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

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