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Loss of EAF2 Facilitates Pancreatic Cancer Progression *via* Inhibiting the Apoptosis of the Neoplastic Cells

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Abstract

Line of studies indicated that EAF2 was a tumour suppressor gene in various human malignancies. However, little was known about its role in PDAC. In the present study, we examined the expression and the biological significance of EAF2 in PDAC. We found that EAF2 was decreased in both the cell lines and clinical samples of PDAC. Functionally, EAF2 was revealed to be positively associated with apoptosis, but not proliferation and migration of PDAC cells. Meanwhile, EAF2 expression was examined in a larger sample of PDAC, and the data showed that EAF2 expression was decreased associating with nodal stage of the patient. Statistically, the Kaplan-Meier in combined with the COX regression assay showed that both EAF2 and Bax low expression confers the worst prognosis and function as an independent prognostic factor for the patients. Taken together, our data suggested that EAF2 was anti-tumoral and might be a novel therapeutic target for PDAC.

Keywords: EAF2; COX regression; PDAC; Apoptosis

Abbreviations

EAF2: ELL-Associated Factor 2; PDAC: Pancreatic Ductal Adenocarcinoma; hPDE: human Pancreatic Duct Epithelial cells

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Introduction

Pancreatic Ductal Adenocarcinoma (PDAC) is a devastating disease with disappointing prognosis among major human malignancies [1]. As reported, PDAC was the fourth-leading cause of cancer-related death all through the world [1]. A recent report estimated that PDAC would take the place of lung cancer to be the first cause of cancer related death in 2030 if no effective interventions were implemented. Hence, it is urgent to obtain more comprehensive understanding toward the machinery whereby PDAC occurs so as to provide the basis for the intervention strategies.

ELL-Associated Factor 2 (EAF2) is an evolutionarily conserved tumour suppressor expressed in many human tissues and responsible for homeostasis [2]. It had been well established that EAF2 was decreased in various human cancers associating with decreased apoptosis [3,4], enhanced angiogenesis [5] and proliferation [6,7]. For instance, Hahn et al. [4] showed that EAF2 over expression significantly suppress the growth of prostate cancer by inducing apoptosis. Evidence also documented that EAF2 was critical in inhibiting angiogenesis, an essential process for cancer progression, by interacting with pVHL and TSP-1 [8]. Moreover, Ai found that EAF2 involved in DNA repair, as showed that intracellular localized EAF2 in prostate cancer was effective in avoiding prostate cancer cell apoptosis by recruiting Ku70/Ku80 to fix the damaged nucleic acids [8]. Taken together, these data concluded an anti-tumoral role of EAF2 in human malignancies. Despite of these advancements, no study examined the expression and biological significance of EAF2 in PDAC.

In the present study, we found that EAF2 expression was significantly decreased in PDAC in both RNA and protein levels. Functional experiments indicated that EAF2 was positively associated with the apoptosis of PDAC cell lines. Moreover, we also found that EAF2 expression was decreased and responsible for a poor prognosis for PDAC patients.

Table 1: EAF2 expression in the cancerous tissues and the adjacent tissues.

	Number	EAF2		p
		Negative	Positive	
Cancerous tissues	88	53	35	p<0.05
Adjacent tissues	88	18	78	

Materials and Methods

Patients and clinical samples

The study included 88 patients diagnosed as pancreatic cancer at the department of general surgery; Shanghai Sixth People's Hospital from 2009 to 2015. The surgical samples as well as the clinical information of the patients were collected at the hospital. Written informed consents were obtained from the patient and approvals from the Ethics Committees of Shanghai Sixth People's Hospital were obtained for the use of these materials for research purposes.

Immunohistochemistry (IHC)

Briefly, tissue microarray sections were de-waxed and dehydrated in a xylene and alcohol bath solution. Endogenous peroxidase activity was blocked by 10-min incubation in 0.3% hydrogen peroxide. Antigen retrieval was conducted by incubating the slides in 0.01M citrate buffer (pH 6.0) at 98°C for 5 min using a microwave oven. The slides were cooled to Room Temperature (RT) and blocked in normal goat serum at RT for 1 h, followed by incubation with a primary antibody at 4°C overnight. The sections were incubated with a horseradish peroxidase-labeled secondary antibody and visualized using 3,3′-diaminobenzidine.

BrdU labeling

BrdU labeling reagent and staining kit was purchased from Zymed (Invitrogen). BrdU staining was performed following the protocol provided by the manufacturer.

IHC evaluation

The staining was evaluated in at least five areas at 400x magnification by two independent pathologists blind to the study. The staining was assigned according to the intensity and percentage. In general, staining intensity was classified into1 (negative), 2 (weak), 3 (moderate), and 4 (strong). The percentages were classified into 1 (≤ 25%), 2 (25% to 50%), 3 (50% to 75%), and 4 (75% to 100%). The staining intensity multiple the percentages of positive cells was treated as the final score. A score <8 was regarded as negative expression, and >8 as positive expression.

Real-time-quantitative polymerase chain reaction (RT-qPCR) analysis

RNA was extracted by Trizol reagent as described previously [9]. Reverse transcription was preformed following protocol of applied biosystems. Primers for RT-qPCR were EAF2: F-A G G T G A C C A T A A C T C T G C C A A A T, R-A G C C G A C A T T C T C C A G T A T C A; GAPDH: F-A C A G T C A G C C G C A T C T T C T T, R-G A C A A G C T T C C C G T T C T C A G. GAPDH expression was treated as endogenous control. Relative expression of EAF2 was calculated with the comparative threshold cycle (Ct) (2–ΔΔCt) method.

Western blot analysis

In Brief, cells were washed three times with cold PBS and lysed on ice in RIPA buffer with the protease inhibitor PMSF (Beyotime Biotechnology, China). Protein concentrations were determined by BCA assay (Beyotime Biotechnology, China). A total of 20 μg protein was separated by 10% SDS-PAGE and electro-blotted onto NC membranes using a semi-dry blotting apparatus. After blocking in 3% Bovine Serum Albumin (BSA), the membranes were incubated with primary antibodies overnight at 4°C. The membranes were washed and incubated with secondary antibodies for 1 h at room temperature on a shaker. The protein bands were visualized using a chemiluminescence kit (Thermo Scientific, Hudson, NH, USA). GAPDH was used as controls. The primary antibodies include EAF2, Bax, p53 (CST, Beverly, MA, USA); and GAPDH (Santa Cruz Biotechnology, CA, USA). Image J was used to the quantization of the blots described previously [10].

Cell culture

Bxpc3 and panc1 were purchased from Chinese Academy of Science. Human Umbilical Vein Endothelial Cells (HUVEC) was purchased from promo cell (Promo cell, Heidelberg, Germany). Bxpc3-nc, panc1-ncand cells with EAF2 over expression were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Gibco, Carslbad, CA, USA) and 1.5 μg/mL puromycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All the cells were sub-cultured by trypsin-EDTA.

Vector construction and cell transfection

To induce EAF2 over expression, the full-length cDNA (NC_000003.12) for the human EAF2 gene was obtained by chemical synthesis (Qiangyao Biotech company, shanghai, China) and cloned into a pcDNA3.0 vector (Invitrogen; Thermo Fisher Scientific, Inc.) to generate pcDNA-EAF2 (designated as EAF2-overexpression). The pcDNA3.0 empty plasmid was used as a negative control (designated as EAF2-control). Vector transfection was performed using lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol for 48 h. Stable selection of transfected cells was carried out in the presence of G418 at 1 μg/μl.

Flow cytometry analysis of cell apoptosis and cell cycle

For analysis of cell apoptosis, cells were harvested at 70% to 80% confluence and incubated with reagent containing Annexin V-FITC and propidium iodide (BD Biosciences) for 15 min in dark at Room Temperature (RT). Apoptotic cells were analyzed using FACSCalibur flow cytometer (BD Biosciences). For cell cycle analysis, cells were fixed in 70% ethanol at 4°C overnight and then treated with RNase A (50 μg/ml) and stained with propidium iodide (25 μg/ml) for 30 min at 37°C. Distribution of cell-cycle phases was determined using ModFit software (BD Biosciences).

Statistical analysis

Statistical analyses were performed using SPSS (version 21.0; SPSS Inc., Chicago, USA). The relationships between the clinic-pathological factors and EAF2 were investigated using Pearson χ^2 test. The Spearman's rank test was used to evaluate the correlation between EAF2 and Bax. Kaplan-Meier analysis in combined with COX regression assay was used to demonstrate the prognostic value. Data were considered statistically significant when $p<0.05$.

Results

EAF2 expression was decreased in pancreatic tissues

To begin with, we detected the expression of EAF2 in five PDAC patients in mRNA levels using RT-PCR. As exhibited, EAF2 was more

Table 2: Univariate and multivariate survival analysis of PDAC patients.

Factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Gender-Male/Female	1.12	1.28-1.53	0.78			
Age->60/ ≤ 60	1.25	0.79-2.77	0.75			
T-stage-T3/ ≤ T2	1.22	0.80-2.59	0.99			
N-stage-N0/N1	0.55	0.43-1.90	0.23			
Tumorlocation-Head-Neck/Body-Tail	0.82	0.59-2.43	0.78			
Lymphvascular invasion-Yes/No	1.33	0.88-3.33	0.25			
Nuclear grade- ≤ II/>II	0.56	0.39-1.83	0.55			
Jaundice-No/Yes	0.99	0.45-1.94	0.99			
Pain-No/Yes	0.87	0.33-1.87	0.28			
EAF2-Negative/Positive	3.58	0.85-5.33	0.045	3.25	1.22.-3.16	0.012
Bax-Negative/Positive	0.78	0.97-4.63	0.59			
EAF2/-Bax-/All others	3.11	0.96-6.76	<0.001	2.78	1.45-5.80	0.002

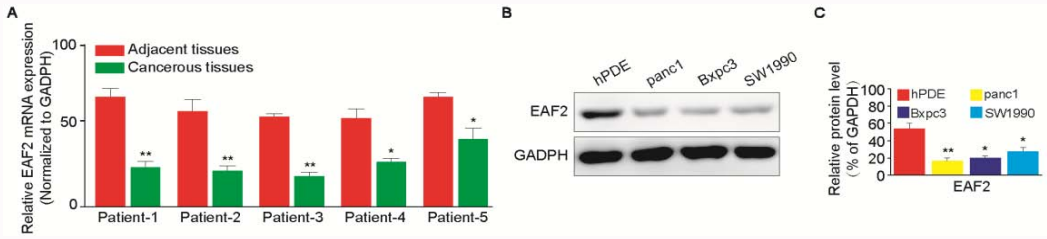

Figure 1: EAF2 was decreased in PDAC. A) EAF2 expression between the cancerous tissues and the adjacent tissues in RNA levels using RT-PCR. B) EAF2 expression in the PDAC cell lines and human Pancreatic Ductal Epithelial Cells (hPDE). C) Relative protein level was quantitated using Image J (*P<0.05 and **P<0.01).

abundance in the adjacent tissues compared to the paired cancerous tissues in all patients (Figure 1A). Meanwhile, its protein expression level in cell line of PDAC was detected using western blot. We found that all the studied cell lines, including pan1, Bxpc3 and SW1990, exhibited lower EAF2 expression compared to hPDE (Figure 1B and 1C). Based on these data, we concluded that EAF2 expression was decreased in PDAC.

EAF2 was ineffective on PDAC cell proliferation and invasion

Then, the biological role of EAF2 in PDAC cells was investigated. To attain this, we initially constructed EAF2 over expression cells (Figure 2A). As we seen, the proliferation and invasion of EAF2 over expression cells showed no obvious change compared to the control cells (Figure 2C and 2D). After that, we examined cell cycle and invasion-related markers of the treated cells. The data showed that cell cycles between EAF2 over expression cells and the normal control were nearly identical (Figure 2E and 2F). Consistently, specific invasion and migration markers, such as MMP-9 and MMP-3, also exhibited no significant change compared to the normal control (Figure 2G). These data indicated that EAF2 had no effect on the proliferation and invasion of PDAC cells.

EAF2 over expression increased the apoptosis of PDAC cells

Previously, shattered reports suggested that EAF2 was positively associated with the apoptosis of prostate cancer [4]. To reveal whether EAF2 functions in the same manner in PDAC, we examined the apoptosis of PDAC cells upon EAF2 over expression. We found that cells with EAF2 over expression exhibited increased apoptosis compared to the control (Figure 3A). Subsequently, we examined the expression of apoptosis-related markers, and found that cells with EAF2 over expression showed increased Bax and p53 expression (Figure 3B). Taken together, these data indicated that EAF2 had a positive association with the apoptosis of PDAC, and that the anti-tumoral property of EAF2 might depend on its anti-apoptosis capacity.

Decreased EAF2 expression was a risk factor for PDAC patients

Finally, we examined EAF2 expression and its prognostic value in the clinical samples of PDAC. We found that EAF2 expression in PDAC ranged from negative to strong staining with negative in predominance (Figure 4A, Table 1). Statistically, the data indicated that decreased EAF2 expression confers a poor prognosis (Figure 4B), and functions as an independent prognostic factor for PDAC (Table 2). Additionally, we revealed that decreased EAF2 expression was significantly associated the clinical stage, but not other parameters of the patients (Table 3).

Correlated EAF2 and Bax promotes PDAC progression synergistically

Since we had showed that EAF2 expression was positively associated with the apoptosis of PDAC cells, we examined whether this exists in the clinical samples of PDAC. To this end, we examined Bax expression in PDAC samples using immunohistochemistry. We found that Bax was elevated in PDAC (Figure 5A). Then, we examined whether EAF2 and Bax was correlated in these samples. We observed

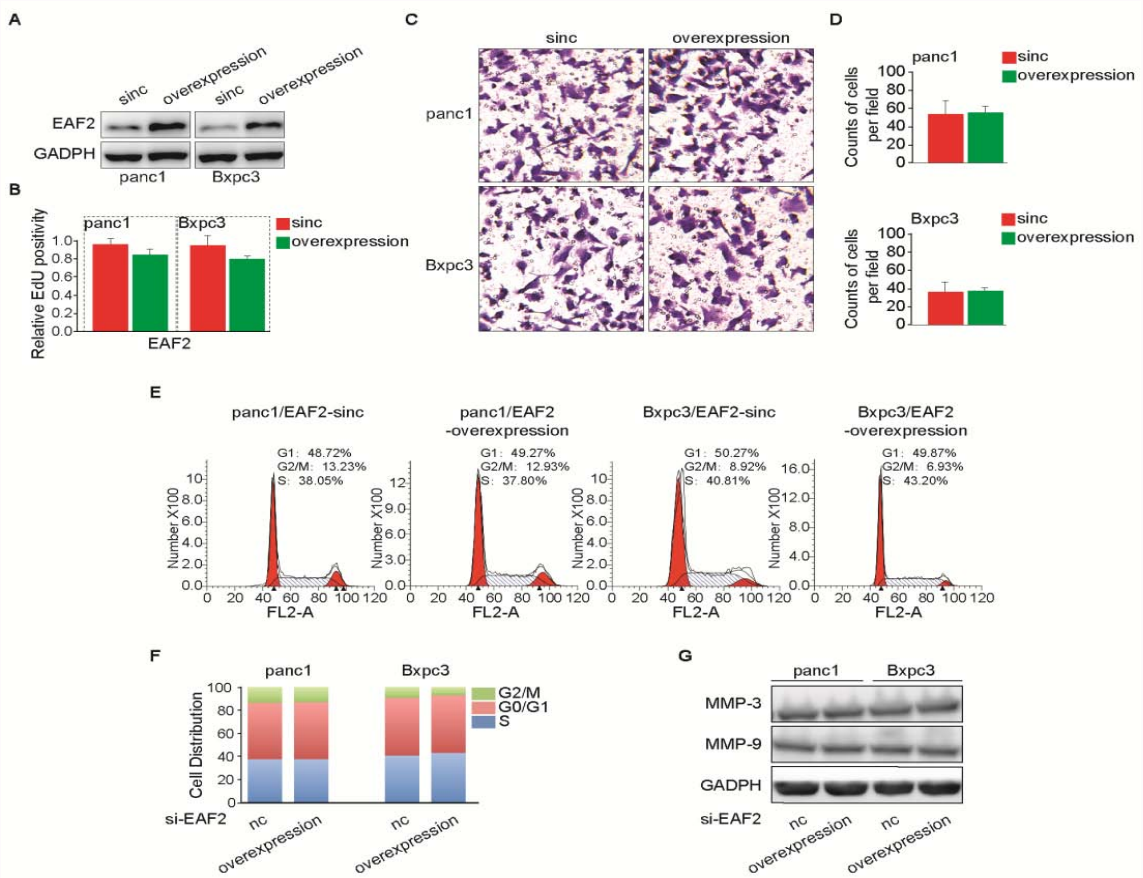

Figure 2: EAF2 showed no effect on the proliferation and invasion of PDAC cells. A: the detection of EAF2 expression upon EAF2 over expression. B: Relative protein level was quantitated using Image J (*P<0.05 and **P<0.01). C: The migration of PDAC cells upon EAF2 over expression. D: Comparison of invasion capacity of PDAC cells upon EFA2 over expression. E-F: Cell cycle analysis of PDAC cells upon EAF2 over expression. G: MMP-3 and MMP-9 expression of PDAC cells upon EAF2 over expression.

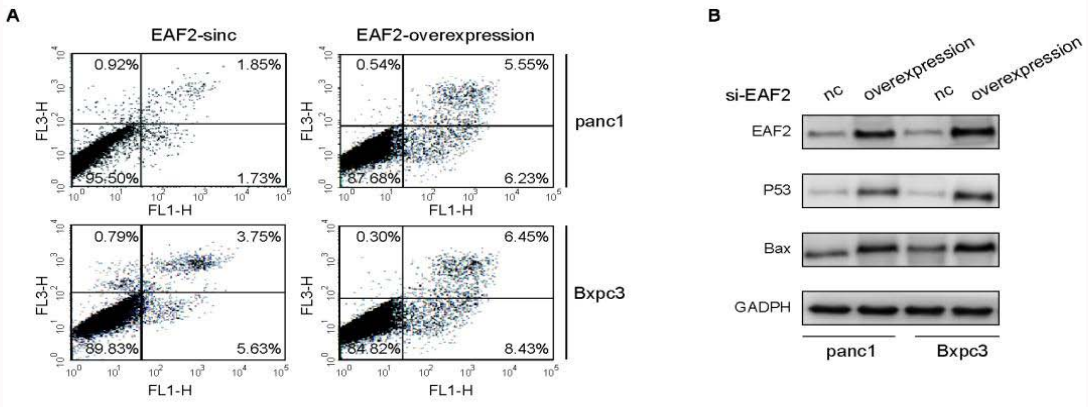

Figure 3: EAF2 facilitates the apoptosis of PDAC cells. A: The comparison of PDAC cell apoptosis upon EAF2 over expression. B: The expression of apoptosis markers of PDAC cells upon EAF2 over expression.

under the microscope that patients with decreased EAF2 expression exhibited low Bax expression, and vice versa (Figure 5B). Then, the correlation assay showed that EAF2 expression was positively associated with Bax with statistical significance in PDAC (Figure 5C). Finally, Kaplan-Meier assay and COX regression assay were conducted to evaluate their prognostic value in PDAC. We showed that patients with both decreased EAF2 and Bax expression exhibited the worst prognosis among all the patients (Figure 5D). Overall, these data indicated that EFA2 and Bax were intrinsic interacted, and that they had synergistic effect in promoting PDAC progression.

Discussion

In the present study, we had examined the expression and biological significance of EAF2 in PDAC. The data demonstrated that EAF2 was decreased and positively associated with apoptosis in PDAC. Our study expands the anti-tumoral role of EAF2 in human

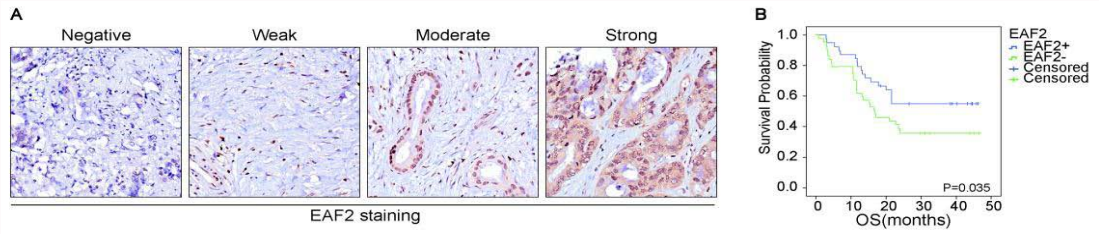

Figure 4: Decreased EAF2 confers poor prognosis for PDAC. A: The representative images of EAF2 expression in PDAC. B: Survival analysis of PDAC patients based on EAF2 expression.

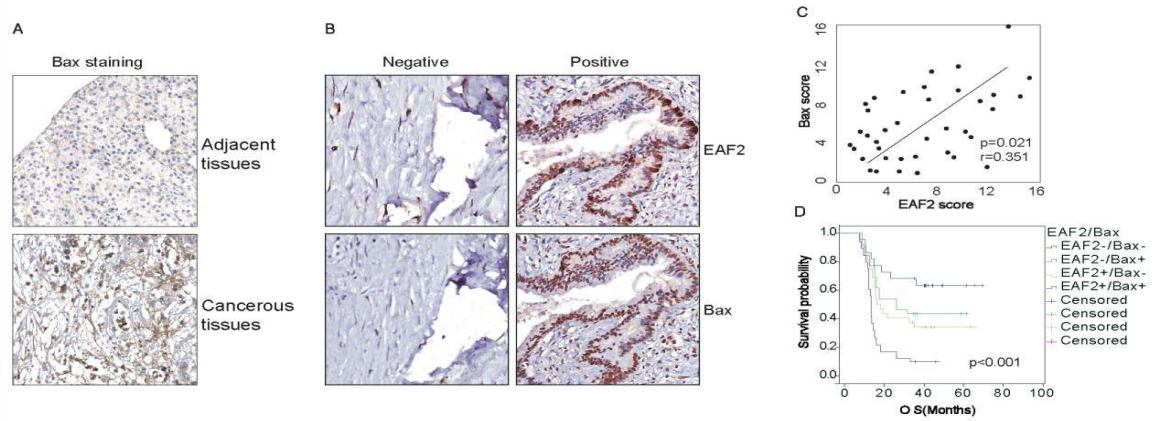

Figure 5: Decreased EAF2 and Bax expression promote PDAC progression synergistically. A: The representative images of Bax expression in PDAC. B: The representative images of EAF2 and Bax expression in serial sections of a single patient. C: The correlation assay between EAF2 and Bax in all the PDAC patients. D: The survival analysis of PDAC patients based on EAF2 and Bax expression.

Table 3: The correlation between EAF2 expression and the clinical parameters of the patients.

Clinical parameters	Negative	Positive	p
Gender-Male	35	21	0.56
Gender-Female	18	14	
Aged ≤ 60	24	17	0.76
Aged >60	29	18	
T stage ≤ T2	43	31	0.35
T stage T3	10	4	
Nodal stage N0	26	26	0.02
Nodal stage N1	27	9	
Tumor location-Body/Tail	15	15	0.16
Tumor location-Head/Neck	38	20	
Without Lymphvascular invasion	28	22	0.35
With Lymphvascular invasion	25	13	
Nuclear graded ≤ II	42	31	0.26
Nuclear graded >II	11	4	
Without Jaundice	34	28	0.11
With Jaundice	19	7	
Without pain	25	14	0.51
With pain	28	21	

malignancies, suggesting that EAF2 might be a novel therapeutic target for the management of PDAC.

Previously, EAF2 was initially identified as androgen Unregulated gene 19 (U19) with tumour suppressor capacity in prostate cancer [11]. With time passed by, mounting studies revealed that homozygous or heterozygous deletion of U19/EAF2 resulted in high rates of lung adenocarcinoma, hepatocellular carcinoma, and B-cell lymphoma [12], which further re-confirmed the anti-tumoral role of EAF2 in human cancers. Fortunately, we also showed that EAF2 was a tumour suppressor gene in PDAC, a conclusion consistent with the prior studies.

Mechanistically, various studies indicated that decreased EAF2 in prostate cancer was responsible for uncontrolled cell growth *via* trans-activating RAS/BRAF/ERK signaling, and that targeted inhibition of the signaling might resolve the proliferation of the neoplastic cells [13]. Furthermore, they showed in mice model that U19/EAF2 deletion did not affect the cell cycle of prostate cancer through a mechanism independent of the cell cycle machinery [14]. What interested is that we showed EAF2 was inhibitive in PDAC cell cycle, suggesting that the role of EAF2 in cell proliferation might be tissue related.

Meanwhile, the positive role of EAF2 in cell apoptosis had been well established by Zhou Wang in early 2003 [15]. They showed that the over expression of EAF2 in 12 surveyed cell lines induced apoptosis, and the expression of EAF2 in xenograft prostate tumors markedly induced apoptosis and inhibited tumour growth *in vivo* [15]. Our conclusion that EAF2 inhibits PDAC progression by promoting cell apoptosis was obviously consistent with these prominent discoveries. However, there was still much to know about how EAF2 interacts with apoptosis after a comprehensive literature review.

To be honest, there were also limitations of our study. First, the conclusion that EAF2 was positive associated with apoptosis in PDAC was merely descriptive without mechanistic illustration. In addition,

our clinical samples in the study were too small, which rendered that our conclusion was not persuasive as expected. Finally, since apoptosis is characterized as Bax, p53 positive, it is inappropriate to simply treat Bax positive cells as apoptosis cell in the 'Result' section.

Conclusion

Our data indicated that EAF2 expression was decreased in PDAC. Also, we showed that EAF expression was positively associated with apoptosis, and they had synergistic effect on promoting PDAC progression. Taken together, our data suggested that EAF2 might be a novel therapeutic target for PDAC.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018;68(1):7-30.
2. Simone F, Luo RT, Polak PE, Kaberlein JJ, Thirman MJ. ELL-Associated Factor 2 (EAF2), a functional homolog of EAF1 with alternative ELL binding properties. Blood. 2003;101(6):2355-62.
3. Li Y, Takahashi Y, Fujii S, Zhou Y, Hong R, Suzuki A, et al. EAF2 mediates germinal centre B-cell apoptosis to suppress excessive immune responses and prevent autoimmunity. Nat Commun. 2016;7:10836.
4. Hahn J, Xiao W, Jiang F, Simone F, Thirman MJ, Wang Z. Apoptosis induction and growth suppression by U19/EAF2 is mediated through its ELL-binding domain. Prostate. 2007;67(2):146-53.
5. Pascal LE, Ai J, Rigatti LH, Lipton AK, Xiao W, Gnarra JR, et al. EAF2 loss enhances angiogenic effects of Von Hippel-Lindau heterozygosity on the murine liver and prostate. Angiogenesis. 2011;14(3):331-43.
6. Qiao Z, Wang D, Hahn J, Ai J, Wang Z. Pirin down-regulates the EAF2/U19 protein and alleviates its growth inhibition in prostate cancer cells. Prostate. 2014;74(2):113-20.
7. Qiu X, Pascal LE, Song Q, Zang Y, Ai J, O'Malley KJ, et al. Physical and functional interactions between ELL2 and RB in the suppression of prostate cancer cell proliferation, migration, and invasion. Neoplasia. 2017;19(3):207-15.
8. Xiao W, Ai J, Habermacher G, Volpert O, Yang X, Zhang AY, et al. U19/EAF2 binds to and stabilizes von hippel-lindau protein. Cancer Res. 2009;69(6):2599-606.
9. Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe against Cancer program. Leukemia. 2003;17(12):2318-57.
10. Zhang J, Li XY, Wang YZ, Zhang QD, Gu SY, Wu X, et al. ROCK is involved in vasculogenic mimicry formation in hepatocellular carcinoma cell line. PLoS One. 2014;9(9): e107661.
11. Su F, Pascal LE, Xiao W, Wang Z. Tumor suppressor U19/EAF2 regulates thrombospondin-1 expression via p53. 2010;29(3):421-31.
12. Xiao W, Zhang Q, Habermacher G, Yang X, Zhang AY, Cai X, et al. U19/EAF2 knockout causes lung adenocarcinoma, B-cell lymphoma, hepatocellular carcinoma and prostatic intraepithelial neoplasia. Oncogene. 2008;27(11):1536-44.
13. Su F, Correa BR, Luo J, Vencio RZ, Pascal LE, Wang Z. Gene expression profiling reveals regulation of ERK phosphorylation by androgen-induced tumor suppressor U19/EAF2 in the mouse prostate. Cancer Microenviron. 2013;6(3):247-61.
14. Xiao W, Zhang Q, Habermacher G, Yang X, Zhang AY, Cai X, et al. U19/EAF2 knockout causes lung adenocarcinoma, B-cell lymphoma, hepatocellular carcinoma and prostatic intraepithelial neoplasia. Oncogene. 2008;27(11):1536-44.
15. Xiao W, Zhang Q, Jiang F, Pins M, Kozlowski JM, Wang Z. Suppression of prostate tumor growth by U19, a novel testosterone-regulated apoptosis inducer. Cancer Res. 2003;63(15):4698-704.