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EYA4 functions as tumor suppressor gene and prognostic marker in pancreatic ductal adenocarcinoma through β-catenin/ID2 pathway

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ABSTRACT

Eye absent homolog 4 (EYA4) was initially found as key gene in controlling eye development in *Drosophila*. We recently found that EYA4 was an independent prognostic factor in hepatocellular carcinoma. Its biological functions in malignancies remained unknown. The present study aimed at investigating its biological functions, molecular mechanisms and prognostic values in pancreatic ductal adenocarcinoma (PDAC). Overexpression of EYA4 in PDAC cells inhibited proliferation and invasion in vitro and tumor growth in vivo. Depletion of EYA4 in PDAC cells enhanced proliferation and invasion in vitro and tumor growth in vivo. Mechanistically, armed with the serine/threonine-specific protein phosphatase activity, EYA4 dephosphorylated β-catenin at Ser675, blocked β-catenin nuclear translocation and inhibited ID2 transactivation. Consistently, EYA4 expression inversely correlated with the levels of p-Ser675-β-catenin and ID2 in tissues. EYA4 expression in PDAC tissues was significantly reduced as compared with adjacent non-tumoral tissues. EYA4 expression was an independent prognostic factor in PDAC, with a lower EYA4 level in association with shorter long-term survival and disease-free time. We showed that EYA4 functioned as tumor suppressor gene in PDAC via repressing β-catenin/ID2 activation, and was an independent prognostic factor in PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most devastating diseases among human solid malignancies, with a median survival of 5–6 months and an overall 5-year survival rate of less than 5% [1]. Some clinicopathological features, including tumor size, lymph node ratio, vascular status and tumor differentiation grades, have been shown as prognostic factors for PDAC [2,3]. However, the molecular prognostic markers of PDAC have not been well-documented. Identification of its molecular prognostic markers is of clinical and scientific values. They helped stratify the high-risk patients for tumor recurrence and metastasis, reveal the pivotal genes in carcinogenesis and tumor progression as well as offer the potential molecular therapeutic targets.

Eyes absent homolog 4 (EYA4) gene was one member of EYA gene family, which contained four members, i.e. EYA1, EYA2, EYA3 and EYA4. EYA genes were initially found as key genes in regulating eye development in *Drosophila* [4]. They encoded proteins containing a highly conserved 271-amino acid C-terminal motif named as Eya domain (ED), which was required and indispensable for the interaction of EYA proteins with other proteins [5]. EYA proteins possessed specific dual protein phosphatase activity (N-terminal serine/threonine-specific phosphatase activity and C-terminal tyrosine-specific phosphatase activity) [6,7].

Recently, EYA genes were found to play important roles in immune response induction, apoptosis regulation and tumor metastasis [6,8–10]. Chung et al. [11] found that EYA4 promoter was frequently methylated in colorectal cancer. Silencing of EYA4 by its promoter hypermethylation was detected in approximate 83% (33/40) of esophageal adenocarcinomas tissues but not in normal esophageal tissues [12]. By using DNA methylation microarray analysis, our previous study showed that EYA4 gene was markedly hypermethylated in hepatocellular carcinoma (HCC) as compared with adjacent non-tumoral tissues. Consistently, EYA4 expression was reduced in HCC tissues in comparison with the adjacent non-tumoral tissues. Further multi-variate analysis revealed that EYA4 expression level in HCC was an independent prognostic factor, being a lower EYA4 expression in association with a worse disease-free and overall survival [13]. However, the underlying molecular mechanisms of EYA4 gene as a prognostic factor in malignancies have not been reported.

Abbreviations: EYA4, eyes absent homolog 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LEF, lymphoid enhancer factor; PCR, polymerase chain reaction; PDAC, pancreatic ductal adenocarcinoma; Scr, scramble shRNA; Ser675, serine 675; TCF, T cell factor.

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Recently, Kim et al. reported that EYA4 transfection could suppress the growth of the transfected colorectal cancer cells in vitro and in vivo, and its tumor suppressive roles required upregulation of Dickkopf-1 (DKK-1), a well-known negative upstream regulator of Wnt signaling [14]. However, whether EYA4 exerted tumor suppressive functions via Wnt signaling pathway remained unknown. β-catenin, one major effector of Wnt/Wingless signaling pathway, had a critical role in cancer development and progression. In the absence of Wnt stimulation, cytoplasmic β-catenin formed a complex with axin and glycogen synthase kinase-3β (GSK-3β). GSK-3β could directly interact with and phosphorylate β-catenin, and trigger β-catenin degradation through E3 ubiquitin ligase β-transducin repeat-containing protein (β-TrCP)-dependent ubiquitin/proteasome pathway [15]. By contrast, Wnt stimulation abolished GSK-3β-dependent β-catenin phosphorylation and induced translocation of β-catenin into the nucleus, where β-catenin bound to TCF4 family transcription factors, which ultimately increased the expression of downstream gene such as ID2 [16]. The correlations between EYA4 and the downstream target genes of Wnt signaling pathway, including β-catenin/TCF4 and ID2, have not been documented.

The present study aimed at investigating biological effects and molecular mechanisms of EYA4 gene on the PDAC cells, and assessing its prognostic values of in the PDAC.

Materials and methods

For detailed experimental materials, methods and relevant references, please see the supplementary experimental procedures. These experiments were performed as described previously with some modifications.

Results

EYA4 functioned as a putative tumor suppressor gene in PDAC cells

To evaluate the biological effects of EYA4 gene on PDAC cells, we constructed stably EYA4-overexpressing PDAC cell lines by using EYA4-expressing plasmids or vector plasmids (negative control) in SW-1990 and Capan-2 cells, designated as SW-1990/EYA4+, SW-1990/Vector, Capan-2/EYA4+ and Capan-2/Vector, respectively, as well as stably EYA4-knocked down PDAC cell lines using EYA4-specific short hairpin RNA (shRNA) or scrambled RNA (negative control) in SW-1990 and Capan-2 cells, designated as SW-1990/EYA4–, SW-1990/Scr, Capan-2/EYA4– and Capan-2/Scr, respectively. EYA4 expression was markedly elevated in SW-1990/EYA4+ and Capan-2/EYA4+, and significantly depressed in SW-1990/EYA4– and Capan-2/EYA4– as compared with their corresponding parental and control cells (Fig. 1A). SW-1990/EYA4+ and Capan-2/EYA4+ cells had remarkably lower capacities in cell proliferation as compared with their corresponding parental and vector-control cells (Fig. 1B). Clonogenic formation assays revealed that SW-1990/EYA4 + and Capan-2/EYA4+ cells had much lower capacities in clonogenic formation as compared with their corresponding parental and vector-control cells (Fig. 1C). Moreover, transwell invasion assays illustrated that SW-1990/EYA4+ and Capan-2/EYA4+ cells had strikingly decreased abilities in cellular invasiveness as compared with their corresponding parental and vector-control cells (Fig. 1D). To further explore the biological effects of EYA4 overexpression on PDAC cells in vivo, subcutaneously implanted tumor models in nude mice were established using SW-1990/EYA4+ , Capan-2/EYA4+ cells and their corresponding parental and control cells. The results showed that growth of SW-1990/EYA4+ and Capan-2/EYA4+ cells in vivo was markedly suppressed as compared with their corresponding parental and control cells (Fig. 1E, F and Fig. S1A, B). The above-stated data demonstrated that overexpression of EYA4 could markedly suppress the growth of PDAC cells in vitro and in vivo.

On the contrary, SW-1990/EYA4– and Capan-2/EYA4– cells had markedly elevated capacities in cell proliferation (Fig. S2A, B), clonogenic formation (Fig. S2C), cellular invasiveness in vitro (Fig. S2D), and greater tumor growth in vivo (Fig. S2E, F and Fig. S1C, D) as compared with their corresponding parental and control cells. It implied that depletion of EYA4 could markedly promote the growth of PDAC cells in vitro and in vivo. These data collectively demonstrated that EYA4 represented as a putative tumor suppressor gene in PDAC.

EYA4 repressed ID2 expression

The molecular mechanisms underlying tumor suppressive effects of EYA4 in PDAC were unknown. We first used gene expression microarray to analyze the influence of EYA4 overexpression or depletion on global gene expression by comparing the differential gene expression between SW-1990/EYA4+ and SW-1990/Vector, Capan-2/EYA4+ and Capan-2/Vector, SW-1990/EYA4– and SW-1990/Scr, Capan-2/EYA4– and Capan-2/Scr (Fig. 2A). The full names of top 23 upregulated and downregulated genes were listed in Table S1. Among them, *ID2* (encoding inhibitor of DNA binding 2) had the greatest fold change in gene expression (Table S2). Using qRT-PCR, we confirmed that forced expression of EYA4 resulted in consistent downregulation of ID2 in PDAC cells (Fig. 2B). In addition, forced expression of EYA4 led to a significant reduction of ID2 protein expression, whereas silencing of EYA4 markedly increased the levels of ID2 (Fig. 2C).

To confirm the roles of EYA4 in regulating ID2 expression, we upregulated EYA4 in SW-1990/EYA4– and Capan-2/EYA4– cells by concurrent transfection of EYA4-expressing plasmids. Concurrent transfection of EYA4-expressing plasmids repressed ID2 protein level which was elevated by EYA4 shRNA (Fig. S3). To evaluate the regulative roles of EYA4 on ID2 expression in vivo, we examined ID2 expression in subcutaneous tumor xenografts in nude mice. It was confirmed that ID2 expression was greatly decreased in EYA4-overexpressing tumors (Fig. 2D, left panel), whereas ID2 expression was markedly elevated in EYA4-knockdown xenograft tumors (Fig. 2D, right panel).

CDKN2B (encoding cyclin-dependent kinase inhibitor 2B, P15) [17] and SREBF1 (encoding sterol regulatory element binding transcription factor 1) [18] were two well-known downstream targets of ID2. To further confirm whether EYA4 regulated ID2 expression and its downstream genes, expressions of CDKN2B and SREBF1 were examined in EYA4-knockdown PDAC cells. The results showed that expressions of CDKN2B and SREBF1 were dramatically elevated in PDAC cells in which EYA4 was knockdown (Fig. S4). Additionally, we depleted ID2 expression with siRNA in EYA4-knockdown PDAC

Fig. 1. EYA4 inhibited proliferation, clonogenic formation, invasiveness and tumorigenicity of PDAC cells. A: Western blotting analysis of EYA4 protein levels in EYA4-overexpressing SW-1990/EYA4+ (left panel) and Capan-2/EYA4+ (right panel) cells. B: Cell proliferation curves of SW-1990/EYA4+ (left panel) and Capan-2/EYA4+ (right panel) cells. Experiments were performed three times and data are expressed as mean ± s.d. (*$P < 0.05$). C: Colony formation assays of SW-1990/EYA4+ (top panel) and Capan-2/EYA4+ (bottom panel) cells. A total of 1000 indicated SW-1990 and Capan-2 cells were cultured in six-well plates. After ten days, the growing colonies were stained (left panel) and scored (right panel). Experiments were performed three times and data are expressed as mean ± s.d. (*$P < 0.05$ versus untreated and vector, respectively). D: Representative pictures and quantification of Matrigel invasion assays in SW-1990/EYA4+ (top panel) and Capan-2/EYA4+ (bottom panel) cells. Scale bar = 100 μm. Data are expressed as mean ± s.d. (*$P < 0.05$ versus untreated and vector, respectively). E and F: Representative images and tumor volume of xenografts excised from the tumor-bearing mice. SW-1990/EYA4+ (E) and Capan-2/EYA4+ (F) cells (1×10^7) were subcutaneously inoculated into the right flank of nude mice (6 mice per group). The mice were sacrificed and the tumors were excised and photographed on day 29. Data are presented as mean ± s.d. (*$P < 0.05$).