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EYA4 inhibits hepatocellular carcinoma by repressing MYCBP by dephosphorylating β-catenin at Ser552

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the fourth leading cause of cancer-related death worldwide. Our previous study showed that EYA4 functioned by suppressing growth of HCC tumor cells, but its molecular mechanism is still not elucidated. Based on the results of gene microassay, EYA4 was inversely correlated with MYCBP and was verified in human HCC tissues by immunohistochemistry and western blot. Overexpressed and KO EYA4 in human HCC cell lines confirmed the negative correlation between EYA4 and MYCBP by qRT-PCR and western blot. Transfected siRNA of MYCBP in EYA4 overexpressed cells and overexpressed MYCBP in EYA4 KO cells could efficiently rescue the proliferation and G2/M arrest effects of EYA4 on HCC cells. Mechanistically, armed with serine/threonine-specific protein phosphatase activity, EYA4 reduced nuclear translocation of β-catenin by dephosphorylating β-catenin at Ser552, thereby suppressing the transcription of MYCBP which was induced by β-catenin/LEF1 binding to the promoter of MYCBP. Clinically, HCC patients with highly expressed EYA4 and poorly expressed MYCBP had significantly longer disease-free survival and overall survival than HCC patients with poorly expressed EYA4 and highly expressed MYCBP. In conclusion, EYA4 suppressed HCC tumor cell growth by repressing MYCBP by dephosphorylating β-catenin S552. EYA4 combined with MYCBP could be potential prognostic biomarkers in HCC.

KEYWORDS
beta-catenin, EYA4, hepatocellular carcinoma, MYCBP, tumor suppressing gene

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer and the fourth leading cause of cancer-related death worldwide.[1] HCC is prevalent in China because of a high rate of hepatitis B virus (HBV) infection. Alcohol abuse and obesity are considered as contributory factors to the increasing incidence of HCC in western countries.[2] Even though surgery, local ablation therapy and transarterial chemoembolization (TACE) have effectively prolonged the life of HCC patients, recurrence of HCC is still an intractable problem that needs to be resolved urgently.[1,3] Molecular-target therapy, such as sorafenib, has been widely used in advanced HCC, but it can

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prolong OS by <3 months only compared with placebo (10.7 months vs 7.9 months).[4] Therefore, better understanding of the molecular mechanisms of HCC carcinogenesis and progression would provide a new therapeutic target, and are of scientific and clinical significance.[5]

Recent studies have shown that aberrant methylation of EYA4 is a potential biomarker in colorectal cancer, urothelial bladder cancer, esophageal squamous cell carcinoma and pancreatic cystic neoplasms.[6-10] In our previous studies, we demonstrated that EYA4 was highly methylated in HCC and related to the prognosis of HCC patients.[11] We also clarified that EYA4 functioned as a tumor suppressor gene in HCC.[12] However, the mechanisms of how EYA4 exerts its tumor suppression effect have not been well defined. In order to explore the mechanisms through which EYA4 suppresses HCC tumorigenesis and progression, a gene expression microarray analysis was done in our previous study.[12] The results showed that MYCBP was downregulated in EYA4 overexpressed cells compared with control. MYCBP is an 11-kDa protein that can associate with MYC and stimulate the activation of transcription.[13,14] Increased expression of MYC can facilitate MYCBP translocation into the nucleus in the S phase of the cell cycle and influence proliferation, differentiation and apoptosis of cells.[13] Recently published papers showed that MYCBP played an important role in lung cancer, gastric cancer, glioma, esophageal squamous cancer and acute myeloid leukaemia.[14-18] However, it still remains unknown as to whether MYCBP plays a role in HCC.

In the present study, we aimed at elucidating the molecular mechanisms of how EYA4 interacts with MYCBP to repress HCC.

2 | MATERIALS AND METHODS

2.1 | Human specimens

Human HCC tissue samples were obtained from 118 HCC patients who underwent surgical resection at the Department of Pancreatobiliary Surgery, The First Affiliated Hospital, Sun Yat-sen University. Written informed consent was obtained from all patients. The study was approved by the Ethical Committee of the First Affiliated Hospital, Sun Yat-sen University. This study conformed to the provisions of the Declaration of Helsinki (as revised in Fortaleza, Brazil, October 2013). Written informed consent was obtained from all patients.

2.2 | Extraction and processing of Gene Expression Omnibus

One Affymetrix HT Human Genome U133A Array dataset (GSE14520) and one Affymetrix Human Genome U133 Plus 2.0

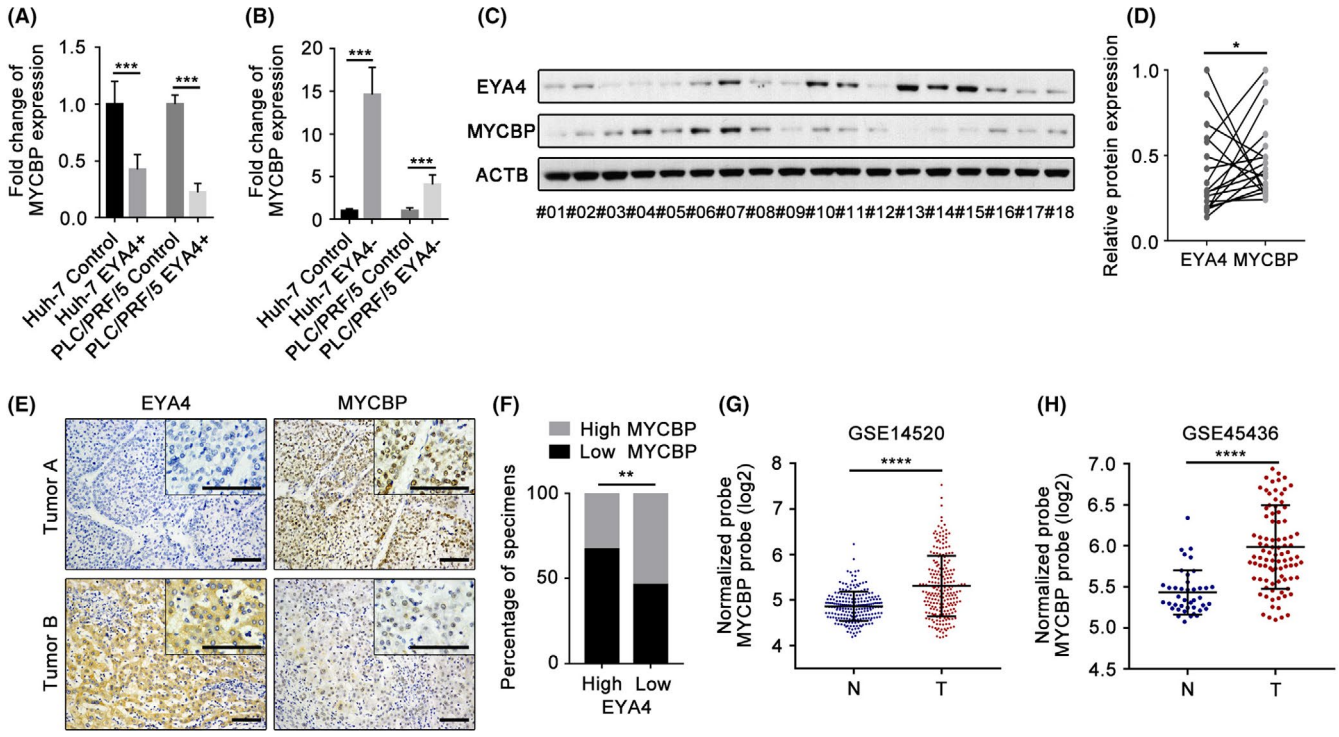

FIGURE 1 EYA4 negatively correlates with MYCBP in human hepatocellular carcinoma (HCC) tissues. A and B, Gene expression microarray results of the related expression level of MYCBP in EYA4 overexpressed (EYA4+) (A) HCC cell lines and EYA4 knockdown (EYA4−) (B) HCC cell line compared with control. C, Western blot results of EYA4 and MYCBP expression in human HCC tissues. D, Paired dot plot shows that based on the results of (E), EYA4 was negatively correlated with MYCBP in human HCC tissues. E, Immunohistochemical staining shows the correlation of EYA4 and MYCBP in HCC patient tissues. F, Bar graphs indicate that in human HCC tissue, MYCBP expression was significantly different in EYA4 highly expressed tissues and EYA4 poorly expressed tissues. G and H, Dot plot shows different expression of MYCBP in HCC patient tissues (T) and non-cancerous human liver tissues (N) from datasets GSE14520 (G) and GSE45436 (H). (Scale bar = 50 μm; **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001)

Array (GSE45436) were selected. The raw data were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/gds/). The array data of GSE14520 included 225 human HCC tissue samples and 220 non-cancerous human liver tissue samples. The dataset of GSE45436 consisted of 93 human HCC tissue samples and 41 non-cancerous human liver tissue samples. The specific analysis method has been described previously.[19]

2.3 | Survival analysis on Gene Expression Profiling Interactive Analysis platform

The online database Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/index.html) was used to generate survival curves, including disease-free survival and overall survival. Survival analysis was carried out on a GEPIA platform based on gene expression. In the present study, liver hepatocellular carcinoma (LIHC) was selected.

2.4 | Histopathology and immunohistochemistry

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Immunohistochemistry (IHC) staining was carried out on 3-μm paraffin sections. The following antibodies were used: rabbit anti-human EYA4 (1:50, Abcam ab102648), rabbit anti-human MYCBP (1:50, Abcam ab66331). IHC score (Figure 1E) is the product of staining intensity × proportion of positive cells. Staining intensity (0, negative; 1, mild; 2, moderate; 3, severe) and proportion of positive cells (0, ≤10%; 1, >10% and ≤25%; 2, >25% and ≤50%; 3, >50% and ≤75%; 4: >75%) were quantified, respectively. Two experienced pathologists scored the stained tissues independently. Percentage of specimens in Figure 1F was based on the IHC score of Figure 1E. From Figure 1E, we obtained the median IHC score of EYA4 and MYCBP. In Figure 1F, HCC patients were first separated into two groups (High EYA4 or Low EYA4) with the median IHC score of EYA4. In each group, HCC patients were separated into High MYCBP or Low MYCBP subgroups based on the median IHC score of MYCBP. Then we obtained the percentage of MYCBP (High/Low) in High EYA4 or Low EYA4 groups separately.

2.5 | Cell culture

Huh-7 and PLC/PRF/5 cell lines were directly obtained from Cell Bank of the Chinese Academy of Science (Huh-7: TCHu182; PLC/PRF/5: TCHu119). Cells were cultured with DMEM (Gibco 41965039) with 10% FBS (Gibco 11550356) in a humidified incubator (37°C, 5% CO_2).

2.6 | Western blot

Western blot was carried out by standard method using the following antibodies: rabbit anti-human EYA4 (1:1000, Abcam ab102648), rabbit anti-human MYCBP (1:500, Abcam ab66331), rabbit anti-human β-catenin (1:1000, CST #8480), rabbit anti-human β-catenin (Ser675) (1:1000, CST #4176), rabbit anti-human β-catenin (Ser552) (1:1000, CST #5651). ImageJ was used to measure the integrated density (Figure 1F) of each blot from Figure 1C. The integrated density reflected the expression level of the blot. Maximum value of the expression level of the blot was set as 100%, and other blots obtained their relative expression levels.

2.7 | Immunofluorescence staining

Cells were seeded on glass coverslips and then fixed with 4% paraformaldehyde for 20 minutes and washed three times with PBS for 5 minutes each. Cells were permeabilized with 0.1% Triton X-100 (Solarbio #T8200) for 10 minutes and washed three times with PBS for 5 minutes each. They were then blocked in 10% goat serum (Thermo Fisher Scientific #16210072) for 1 hour at room temperature. Incubation with β-catenin antibody (1:200, Abcam ab32572) was carried out at 4°C for 16 hours. Nuclei were counterstained with DAPI (Thermo Fisher Scientific #P36931). Images of cultured cells were obtained through using the ZEISS Laser Scanning Confocal Microscope LSM710 with oil ×67 magnification lenses, and ZEN 2.3 software was used.

2.8 | Quantitative real-time PCR

Total RNA was extracted by TRIzol Reagent (Thermo Fisher Scientific 15596026) and reverse-transcribed to cDNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific K1622). Quantitative real-time PCR was done using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific 4367659). Primers for qRT-PCR are listed in Table 1. All primers were obtained from Sangon Biotech.

2.9 | Cell Counting Kit-8 for cell proliferation

Cell Counting Kit-8 (Dojindo CK04) was used for measuring cell proliferation. We dispensed 100 μL cell suspension (5000 cells/well) in a 96-well plate. The plate was preincubated for 24 hours in a humidified incubator (37°C, 5% CO_2) and then incubated for a range of times (12, 24, 48, 72, 96 hours). CCK-8 solution (10 μL) was added to each well of the plate and the plate was incubated for 1 hour. Absorbance at 450 nm was measured using a microplate reader.

2.10 | Colony formation assay

Cells were seeded in 6-well plates at equal densities (200 and 500 cells). They were allowed to form colonies for 2-3 weeks. Medium was changed every week. After 2-3 weeks, we replaced the medium and washed the plates with PBS. Cells were fixed with 2 mL of 4% paraformaldehyde for 20 minutes. After replacing the stained the colonies with 1% crystal violet for 30 minutes, the plates were washed with PBS and allowed to dry. We took pictures of these plates and calculated colony numbers with ImageJ.

TABLE 1 Nucleotide sequences used in the present study

Target gene	Sequence
qRT-PCR	
EYA4	F: 5′-AGTCCCTCCACACCCATCAAAG-3′
	R: 5′-ACACGCTCCAGGTCACTATCAG-3′
MYCBP	F: 5′-AGGAATGTGGCTATGGCTGG-3′
	R: 5′-AGCACTTAGTCCACTGCTGC-3′
GAPDH	F: 5′-GAGTCCACTGGCGTCTTCAC-3′
	R: 5′-ATCTTGAGGCTGTTGTCATACTTCT-3′
siRNAs	
MYCBP	#1: F: 5′-GUAGAAGCUAUUGUAGAA-3′
	#1: R: 5′-UUCUACAAUAGCUUCAUAC-3′
	#2: F: 5′-GGGCAUUAUCAGUGAACUA-3′
	#2: R: 5′-UAGUUCACUGAUAAUGCCCAA-3′
sgRNAs	
EYA4	#1: 5′-CAAGUCCUCAUACUCUUGU-3′
	#2: 5′-CAAACAAUGUCUGCCUAUGC-3′
Mutation	
β-catenin^{S552A}	F: 5′-CCCAGCGCCGTACGgCCATGGGTGGGACAC-3′
	R: 5′-GTGTCCCACCCATGGcCGTACGGCGCTGGG-3′

2.11 | Propidium iodide staining of DNA

Cells were harvested by 0.25% trypsin and washed in PBS. Cells were fixed in 70% ethanol added drop wise to the cell pellet while vortexing and fixed for 30 minutes at 4°C. Cells were washed in PBS twice. Cells were centrifuged at 2000 rpm which removed the ethanol. RNAse (5 μL of 1 mg/mL; Sigma 10109134001) was added. Then, 300 μL propidium iodide (50 μg/mL) was added and cells incubated in the dark for 30 minutes. We ran the flow cytometer, chose single cells and quantitated the percentage of cells in each cell cycle phase by FlowJo.

2.12 | Plasmid sgRNAs and siRNAs

β-Catenin^{S552}A mutation plasmid was constructed with QuickMutation Site-Directed Mutagenesis Kit obtained from Beyotime Biotechnology (D0206). The sequences are shown in Table 1. CRISPR/Cas9 sgRNAs that targeted EYA4 were designed using http://crispor.tefor.net/. The sequences are shown in Table 1. The cloning methods were based on the studies of Sanjana et al and Shalem et al.[20,21] siRNAs targeting MYCBP are shown in Table 1. siRNAs were obtained from Sangon Biotech.

2.13 | Luciferase reporter gene assay

The first 2000 bp/1000 bp/500 bp nucleotides of the 5′ regions of MYCBP were amplified by PCR and cloned into the pGL3-Basic-IRES vector (pGL3-2000, pGL3-1000, pGL3-500). CDS of LEF1 (NM_016269.5) was cloned to pcDNA3.1 (pcDNA3.1-LEF1). The pRL Renilla luciferase control reporter vector and pcDNA3.1-LEF1 were cotransfected with pGL3 (Basic, pGL3-2000, pGL3-1000, pGL3-500 separately) to HEK 293T cells with Lipofectamine 2000 (Thermo Fisher Scientific #11668019) following the manufacturer's instructions. Luciferase and Renilla activities were measured 48 hours after transfection using the Dual Luciferase Reporter Assay System (Promega #E1910) following the manufacturer's instructions.

2.14 | Statistical analysis

Results are given as mean ± standard deviation. Statistical analyses were carried out on PC by using SPSS software package (version 25.0). Student's *t* test was used for comparing the intergroup differences of numerical variables, and chi-squared test was used for comparing the intergroup differences of categorical variables. Correlation analyses were used for assessing the strength of the relationship between EYA4 and MYCBP expression level in human HCC tissues. Survival analyses with log test were used for evaluating the intergroup differences of outcomes in HCC patients grouped by expression of EYA4 and MYCBP. Cox proportional hazards regression analysis was used for screening the prognostic factors of HCC patients. *P*-value <.05 was considered to be statistically significant (*P < .05, **P < .01, ***P < .001).

3 | RESULTS

3.1 | EYA4 negatively correlates with MYCBP in human HCC tissues and cell lines

In our previous study, we carried out gene expression microassay in HCC overexpressed cell lines and HCC knockdown cell lines compared with control. The results showed that MYCBP was poorly expressed in EYA4 overexpressed cell lines and highly expressed in EYA4 knockdown cell lines (Figure 1A,B). These results indicated that EYA4 may be negatively correlated with MYCBP.

To verify the correlations between EYA4 and MYCBP in human HCC tissues, we tested the expression of EYA4 and MYCBP in 18 HCC patient tissues by western blot. The results showed that EYA4 was negatively correlated with MYCBP (r = −.524, P = .025) (Figure 1C,D). In addition, 100 patients who were diagnosed with HCC and had had hepatectomy at The First Hospital of Sun Yet-sen University were randomly selected and protein expression of EYA4 and MYCBP by IHC was carried out. The results showed that 40 tissues expressed a high level of EYA4, whereas 60 tissues expressed a low level of EYA4. In tissues with highly expressed EYA4, 26 (65%) tissues had low MYCBP protein expression. In tissues with poorly expressed EYA4, 32 (53%) tissues had high MYCBP protein expression (P = .0042) (Figure 1E,F). These results indicated that EYA4 and MYCBP are negatively correlated in HCC. By analyzing datasets from GEO, we found that in dataset GSE14520 (containing 220 non-cancerous human liver tissue samples and 225 human HCC tissue samples) and dataset GSE45436 (containing 41 non-cancerous human liver tissue samples and 93 human HCC tissue samples), MYCBP was significantly highly expressed in HCC tissues compared with non-cancerous liver tissues

(Figure 1G,H). This indicated that MYCBP might play an important role in promoting HCC progression. Therefore, we hypothesized that EYA4 functions as a tumor suppressor gene by inhibiting MYCBP expression.

We then verified the gene expression microarray results in human HCC cell lines. We generated EYA4 overexpressed cell lines and EYA4 KO cell lines in Huh7 and PLC/PRF/5, respectively. According to the microarray results, MYCBP was significantly poorly expressed in EYA4 overexpressed cell lines. MYCBP in EYA4 KO cell lines was significantly highly expressed (Figure 2). In Figure 2C, Huh-7 CR-1 and Huh-7 CR-2 both expressed a higher level of MYCBP compared with control. However, Huh-7 CR-1 had a higher level of MYCBP compared with Huh-7 CR-2. We thought this was because of the heterogeneity of tumor cells. Different tumor cells responded a little differently when knocked out EYA4. As MYCBP was upregulated in both CR-1 and CR-2, we considered that it was reasonable to conclude that knocked out EYA4 could increase the expression level of MYCBP.

3.2 | EYA4 suppressed HCC tumor cell growth by inhibiting MYCBP expression

To explore whether MYCBP is the key to mediating the tumor suppressor function of EYA4 in human HCC, we carried out rescue experiments in Hun-7 and PLC/PRF/5 cell lines. Repressed mRNA and protein expression of MYCBP in EYA4 overexpressed cell lines were reversed by transfecting a plasmid carrying the MYCBP gene (Figure 3A). Elevated mRNA and protein expression of MYCBP in EYA4 KO cell lines was reversed by transfecting siRNA of MYCBP (Figure 3J). Cell proliferation, as measured by colony assay and survival curves, were attenuated when EYA4 was overexpressed, but this effect was rescued by transfecting MYCBP (Figure 3B-G). Cell cycle analysis, which also reflects cell proliferation, showed that the G2/M phase was arrested when EYA4 was overexpressed, but this effect was rescued by transfecting MYCBP (Figure 3H,I). Additionally, EYA4 KO cells showed enhanced proliferation ability in colony assay, survival curves and cell cycle analysis. This enhancement was also inhibited by transfecting siRNA of MYCBP (Figure 3K-R). Therefore, these results showed that EYA4 suppressed HCC tumor cell growth by inhibiting MYCBP expression.

3.3 | EYA4 mediates MYCBP by dephosphorylating β-catenin at S552

We subsequently aimed to illuminate the mechanism of how EYA4 mediates MYCBP in HCC cells. MYCBP was reported as a direct target of the β-catenin/LEF-1 pathway through its LEF-1 binding sites in the MYCBP promoter. EYA4 is known as a phosphatase. In our previous studies, EYA4 could dephosphorylate β-catenin at S675 in pancreatic cancers. β-Catenin, the stabilization of which can be modified by phosphorylation/dephosphorylation at S33/S37, T41/S45, S552 and S675, is a key point in Wnt signal pathways. Therefore, we hypothesized that EYA4 may mediate MYCBP by dephosphorylating β-catenin. To verify this hypothesis, we carried out western blot in EYA4 overexpressed cells and EYA4 KO cells. The results showed that phospho β-catenin S552 was decreased in EYA4 overexpressed cells and increased in EYA4 KO cells, but the total β-catenin did not change, nor did phospho β-catenin S675 (Figure 4A). This result indicated that EYA4 can dephosphorylate β-catenin at S552. To investigate whether the phosphorylation/dephosphorylation switch of β-catenin S552 can influence the expression level of MYCBP, we cloned plasmids of β-catenin without (WT) or with S552A mutation and transfected the plasmids to EYA4 overexpressed cells, respectively. When transfected with the same amount of total β-catenin, the MYCBP protein level was significantly higher in cells transfected with WT β-catenin than in cells transfected with S552A mutation β-catenin (Figure 4B).

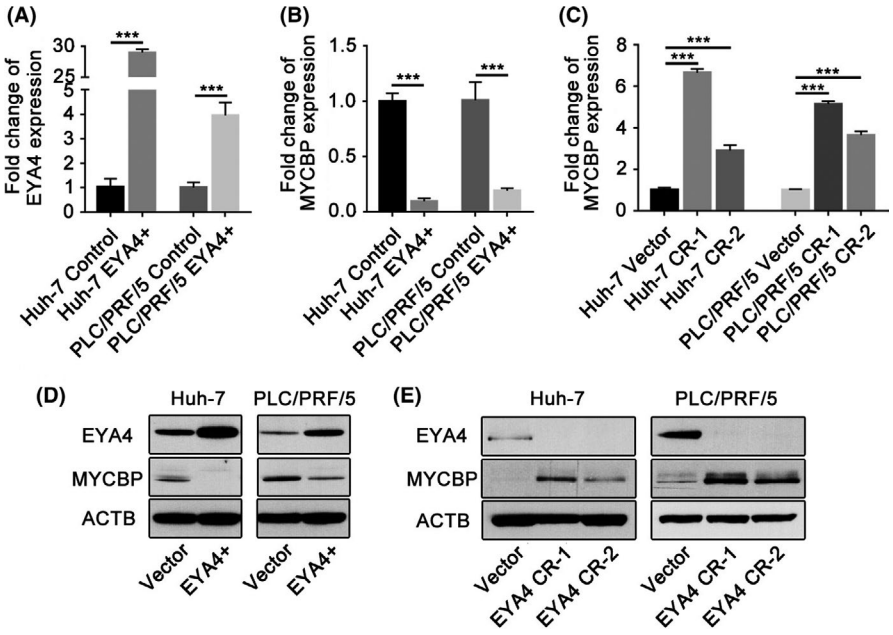

FIGURE 2 EYA4 negatively correlates with MYCBP in human hepatocellular carcinoma (HCC) cell lines. A-C, qPCR results show the relative expression levels of EYA4 (A) and MYCBP (B,C) in EYA4 overexpressed (EYA4+) HCC cell lines (A,B) and EYA4 KO (EYA4 CR-1/CR-2) HCC cell lines (C). D and E, Western blot results show the expression of EYA4 and MYCBP in EYA4 overexpressed HCC cell lines (D) and EYA4 KO HCC cell lines (E). (***$P < .001$)

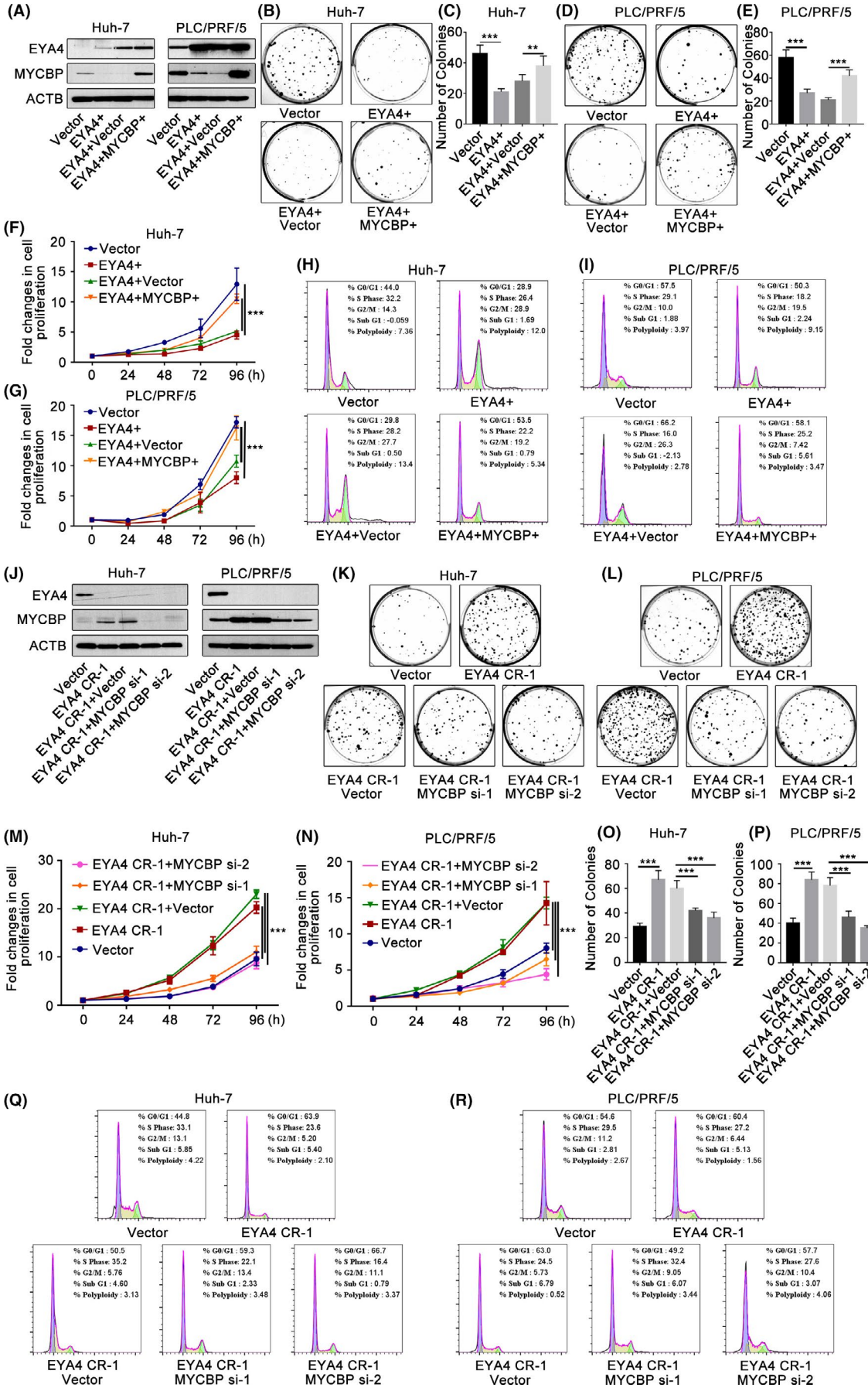
(A)
Huh-7
PLC/PRF/5
EYA4
MYCBP
ACTB
Vector
EYA4+
EYA4+Vector
EYA4+MYCBP+
(B)
Huh-7
Vector
EYA4+
EYA4+ Vector
EYA4+ MYCBP+
(C)
Huh-7
Number of Colonies
(D)
PLC/PRF/5
(E)
PLC/PRF/5
(F)
Huh-7
Fold changes in cell proliferation
96 (h)
(G)
PLC/PRF/5
(H)
Huh-7
% G0/G1 : 44.0
% S Phase: 32.2
% G2/M : 14.3
% Sub G1 : -0.059
% Polyploidy : 7.36
% G0/G1 : 28.9
% S Phase: 26.4
% G2/M : 28.9
% Sub G1 : 1.69
% Polyploidy : 12.0
% G0/G1 : 29.8
% S Phase: 28.2
% G2/M : 27.7
% Sub G1 : 0.50
% Polyploidy : 13.4
% G0/G1 : 53.5
% S Phase: 22.2
% G2/M : 19.2
% Sub G1 : 0.79
% Polyploidy : 5.34
EYA4+Vector
EYA4+MYCBP+
(I)
PLC/PRF/5
% G0/G1 : 57.5
% S Phase: 29.1
% G2/M : 10.0
% Sub G1 : 1.88
% Polyploidy : 3.97
% G0/G1 : 50.3
% S Phase: 18.2
% G2/M : 19.5
% Sub G1 : 2.24
% Polyploidy : 9.15
% G0/G1 : 66.2
% S Phase: 16.0
% G2/M : 26.3
% Sub G1 : -2.13
% Polyploidy : 2.78
% G0/G1 : 58.1
% S Phase: 25.2
% G2/M : 7.42
% Sub G1 : 5.61
% Polyploidy : 3.47
(J)
Huh-7
PLC/PRF/5
EYA4
MYCBP
ACTB
Vector
EYA4 CR-1
EYA4 CR-1+Vector
EYA4 CR-1+MYCBP si-1
EYA4 CR-1+MYCBP si-2
(K)
Huh-7
Vector
EYA4 CR-1
EYA4 CR-1 Vector
EYA4 CR-1 MYCBP si-1
EYA4 CR-1 MYCBP si-2
(L)
PLC/PRF/5
(M)
Huh-7
EYA4 CR-1+MYCBP si-2
EYA4 CR-1+MYCBP si-1
EYA4 CR-1+Vector
EYA4 CR-1
Vector
Fold changes in cell proliferation
96 (h)
(N)
PLC/PRF/5
(O)
Huh-7
Number of Colonies
(P)
PLC/PRF/5
(Q)
Huh-7
% G0/G1 : 44.8
% S Phase: 33.1
% G2/M : 13.1
% Sub G1 : 5.85
% Polyploidy : 4.22
% G0/G1 : 63.9
% S Phase: 23.6
% G2/M : 5.20
% Sub G1 : 5.40
% Polyploidy : 2.10
% G0/G1 : 50.5
% S Phase: 35.2
% G2/M : 5.76
% Sub G1 : 4.60
% Polyploidy : 3.13
% G0/G1 : 59.3
% S Phase: 22.1
% G2/M : 13.4
% Sub G1 : 2.33
% Polyploidy : 3.48
% G0/G1 : 66.7
% S Phase: 16.4
% G2/M : 11.1
% Sub G1 : 0.79
% Polyploidy : 3.37
EYA4 CR-1 Vector
EYA4 CR-1 MYCBP si-1
EYA4 CR-1 MYCBP si-2
(R)
PLC/PRF/5
% G0/G1 : 54.6
% S Phase: 29.5
% G2/M : 11.2
% Sub G1 : 2.81
% Polyploidy : 2.67
% G0/G1 : 60.4
% S Phase: 27.2
% G2/M : 6.44
% Sub G1 : 5.13
% Polyploidy : 1.56
% G0/G1 : 63.0
% S Phase: 24.5
% G2/M : 5.73
% Sub G1 : 6.79
% Polyploidy : 0.52
% G0/G1 : 49.2
% S Phase: 32.4
% G2/M : 9.05
% Sub G1 : 6.07
% Polyploidy : 3.44
% G0/G1 : 57.7
% S Phase: 27.6
% G2/M : 10.4
% Sub G1 : 3.07
% Polyploidy : 4.06

FIGURE 3 EYA4 suppressed hepatocellular carcinoma (HCC) tumor cell growth by inhibiting MYCBP expression. A, Western blot results show the expression level of EYA4 and MYCBP in EYA4 overexpressed (EYA4+) human HCC cell lines after transfected empty plasmids (Vector) or MYCBP overexpressed (MYCBP+) plasmids (rescue experiments). B-E, Colony assay results show the different colony numbers in the rescue experiments of EYA4 overexpressed cell lines. F and G, CCK-8 assay shows the proliferation differences in the rescue experiments of EYA4 overexpressed cell lines. H and I, Cell cycle analysis shows the different G2/M percentages in the rescue experiments of EYA4 overexpressed cell lines. J, Western blot results show the expression level of EYA4 and MYCBP in EYA4 KO (EYA4 CR-1/CR-2) human HCC cell lines after transfected control siRNA (Vector) or MYCBP siRNAs (MYCBP si-1/si-2) (rescue experiments). K,L,O,P, Colony assay results show the different colony numbers in the rescue experiments of EYA4 KO cell lines. M and N, CCK-8 assays show the proliferation differences in the rescue experiments of EYA4 KO cell lines. Q and R, Cell cycle analysis shows the different G2/M percentages in the rescue experiments of EYA4 KO cell lines

Consist with our hypothesis, these results confirmed that EYA4 mediates MYCBP by dephosphorylating β-catenin at S552.

3.4 | EYA4 reduced the nuclear translocation of β-catenin followed by suppression of transcription of MYCBP

In order to clarify how β-catenin promotes the transcription of MYCBP, we carried out the following experiments. β-Catenin belongs to the Wnt/β-catenin pathway, and β-catenin functions by translocating to the nucleus, binding with TCF1/LEF1 transcription factors and then inducing the transcription of downstream genes. So, we first tested the location of β-catenin by immunofluorescence (IF) and the protein level of β-catenin by western blot in EYA4 KO and control HCC cell lines. The results showed that more β-catenin accumulated in the nucleus in KO EYA4 HCC cells than in the control (Figure 4C,D). This indicated that EYA4 reduced the nucleus translocation ability of β-catenin. MYCBP is reported as a direct target of β-catenin/LEF1 through its LEF1-binding site in the MYCBP promoter. We found that there are three LEF1 putative binding sites in the −2000 bp 5′ region of MYCBP (from −136 to −142, from −260 to −266, from −937 to −943). We therefore carried out a Luciferase reporter gene assay to determine whether LEF1 can bind to the promoter region of MYCBP. We cloned four different pGL3 plasmids (pGL3 Basic, pGL3 −2000/+82, pGL3 −1000/82, pGL3 −500/82) and then cotransfected them separately with pcDNA3.1-LEF1 plasmid following the protocol of the Luciferase reporter gene assay. Results showed that the relative signal was significantly increased when transfected with pGL3 −2000/+82, pGL3 −1000/82 and pGL3 −500/82 compared to pGL3 Basic (Figure 4E) which signified that LEF1 could bind to the promoter region of MYCBP and start the transcription. In summary, EYA4 reduced the nuclear translocation of β-catenin followed by suppressing the transcription of MYCBP which was induced by β-catenin/LEF1 binding to the promoter of MYCBP.

3.5 | EYA4 combined with MYCBP as potential prognostic biomarkers in HCC

In our previous studies, we showed that EYA4 was an independent prognostic biomarker in HCC. EYA4 expression was positively related to disease-free survival (DFS) and overall survival (OS) of HCC patients. In the present study, we reconfirmed the prognostic value of EYA4. EYA4 was an independent prognostic biomarker for DFS and OS of HCC patients (Table 2). HCC patients with highly expressed EYA4 had longer survival time compared with poorly expressed EYA4 patients, with a median DFS of 31 months vs 19 months (P = .019) (Figure 5A) and a median OS of 51 months vs 42 months (P = .033) (Figure 5D). Meanwhile, in the present study, it was proven that MYCBP is an independent prognostic biomarker for DFS and OS of HCC patients (Table 2) and that MYCBP was inversely related to DFS and OS of HCC patients. HCC patients with highly expressed MYCBP had a shorter survival time compared with poorly expressed MYCBP patients, with a median DFS of 17 months vs 32 months (P = .002) (Figure 5B) and a median OS of 38 months vs 54 months (P = .002) (Figure 5E). In addition, from the GEPIA platform, we also found that HCC patients with highly expressed MYCBP had shorter DSF and OS compared with HCC patients with poorly expressed MYCBP (P < .05) (Figure 5G,H). We then divided HCC patients into three groups: EYA4+MYCBP−, EYA4+MYCBP+/EYA4−MYCBP−, and EYA4−MYCBP+ and compared DFS, OS, 3-year survival rate and 5-year survival rate, respectively. Patients in the EYA4+MYCBP− group had the longest median DFS, OS and the highest 3-year and 5-year survival rates (38 months, 60 months, 75.8%, 17.6%) whereas EYA4−MYCBP+ group patients had the shortest median DFS, OS and the lowest 3-year and 5-year survival rates (15 months, 33 months, 37.7%, 11.7%) and EYA4+MYCBP+/EYA4−MYCBP− group patients were in the middle (26 months, 54 months, 60.7%, 12.1%) (median DFS: P = .005; medium OS: P < .001) (Figure 5C,F). These results indicated that EYA4 combined with MYCBP could be potential prognostic biomarkers in HCC.

4 | DISCUSSION

In our previously published studies, we explained that EYA4 was poorly expressed in HCC tumor tissue compared with pericarcinomatous tissue. We also showed that EYA4 is a tumor suppressor gene in HCC. To explore the mechanism of how EYA4 functions, we carried out a comparative study of the transcriptome profiles of human HCC cell lines (Huh7 and PLC/PRF/5) overexpressing EYA4 with that of control by gene expression microarray analysis. Results showed that MYCBP was significantly poorly expressed in EYA4 overexpressed cell lines compared with control.

In order to verify the correlation between EYA4 and MYCBP, EYA4 overexpressed and KO cell lines were established in Huh-7 and PLC/PRF/5 cell lines, respectively. IHC assay was also carried

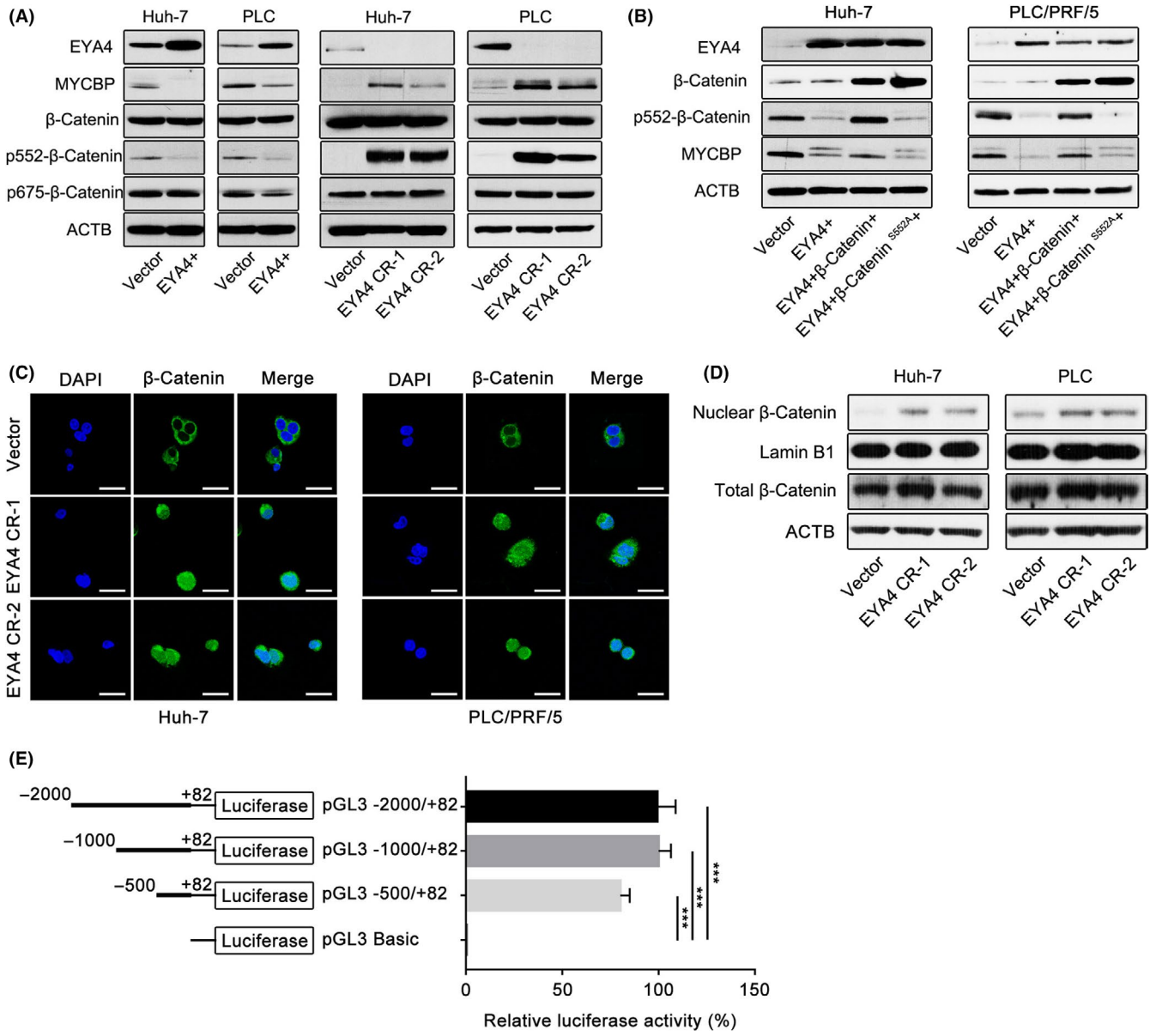

FIGURE 4 EYA4 mediates MYCBP by dephosphorylating β-catenin at S552. A, Western blot assay shows the expression level of EYA4, MYCBP, β-catenin, p552-β-catenin and p675-β-catenin in EYA4 overexpressed (EYA4+) human hepatocellular carcinoma (HCC) cell lines (A) and EYA4 KO (EYA4 CR-1/CR-2) human HCC cell lines (B). B, Western blot assay shows the expression level of EYA4, MYCBP, β-catenin and p552-β-catenin in EYA4 overexpressed human HCC cell lines after transfection of WT (EYA4 + β-catenin+) or S552A mutated β-catenin (EYA4 + β-catenin^{S552A}+) plasmids. C, Immunofluorescence assay results show the location of β-catenin in EYA4 KO (EYA4 CR-1/CR-2) human HCC cell lines and control. Nucleus was dyed by DAPI. D, Western blot assay shows the protein level of β-catenin in the nucleus and in the whole cell. E, Luciferase reporter gene assay shows the relative signal level after cotransfection with pRL Renilla luciferase control reporter vector, pcDNA3.1-LEF1 and pGL3 (Basic, pGL3-2000, pGL3-1000, pGL3-500 separately) to HEK 293T cells

out on 100 randomly selected human HCC tissues for verification of EYA4 and MYCBP. Corresponding to the microarray data and verified by both human HCC cell lines and tissues, EYA4 negatively correlated with MYCBP. To determine the role of MYCBP in HCC, we analyzed two HCC datasets from GEO. We found that MYCBP was more highly expressed in HCC tissues compared with non-cancerous liver tissues, which indicated that MYCBP might play a cancer-promoting role in HCC. Therefore, we hypothesized that EYA4 might suppress HCC tumor cell growth by inhibiting MYCBP. To verify this hypothesis, we carried out a rescue experiment to confirm whether EYA4 can mediate MYCBP. Transfections of MYCBP-overexpressed plasmids in EYA4 overexpressed cell lines and MYCBP siRNAs in EYA4 KO cell lines were done to counteract inhibition of MYCBP by EYA4. Overexpressing MYCBP in EYA4 overexpressed cells could effectively diminish the suppression of cell proliferation and G2/M arrest induced by EYA4 overexpression. Meanwhile, knockdown of MYCBP in EYA4 KO cells could efficiently reverse the tumor-promotion effect on

TABLE 2 Prognostic factors in hepatocellular carcinoma patients

Variable	Overall survival			Disease-free survival		
	HR	95% CI	*P*-value*	HR	95% CI	*P*-value*
Univariate						
Gender						
Female	1.000	Reference	.829	1.000	Reference	.389
Male	1.067	0.592-1.921		1.300	0.716-2.360	
Age (y)						
Male <40/Female <50	1.000	Reference	.348	1.000	Reference	.549
Male ≥40/Female ≥50	0.785	0.473-1.302		1.175	0.694-1.991	
HBV						
Negative	1.000	Reference	.236	1.000	Reference	.486
Positive	0.665	0.339-1.305		0.789	0.405-1.536	
Child-Pugh class						
A	1.000	Reference	.256	1.000	Reference	.825
B	1.535	0.733-3.216		1.092	0.502-2.377	
Liver cirrhosis						
Not present	1.000	Reference	.861	1.000	Reference	.218
Present	0.951	0.542-1.670		0.736	0.451-1.199	
AFP						
≤100 ng/mL	1.000	Reference	.224	1.000	Reference	.224
>100 ng/mL	1.338	0.837-2.140		1.265	0.811-1.973	
Tumor size						
Single tumor ≤5 cm/2-3 tumors ≤3 cm each	1.000	Reference	.050	1.000	Reference	.084
Single tumor >5 cm/2-3 tumors one >3 cm	1.618	0.993-2.637		1.500	0.948-2.374	
No. of tumors						
One only	1.000	Reference	.084	1.000	Reference	.364
More than one	1.787	1.094-2.921		1.250	0.772-2.026	
TNM						
I/II	1.000	Reference	.002	1.000	Reference	.007
III/IV	2.120	1.303-3.450		1.885	1.191-2.983	
PVTT						
Not present	1.000	Reference	.363	1.000	Reference	.435
Present	1.339	0.714-2.513		1.270	0.697-2.314	
LNM						
Not detected	1.000	Reference	.190	1.000	Reference	<.001
Detected	2.710	0.611-12.023		8.721	3.350-22.701	
EYA4						
IHC score <6	1.000	Reference	.015	1.000	Reference	.043
IHC score ≥6	0.538	0.327-0.886		0.622	0.392-0.989	
MYCBP						
IHC score <6	1.000	Reference	.010	1.000	Reference	.004
IHC score ≥6	1.893	1.168-3.068		1.936	1.230-3.046	
Multivariate						
TNM						
I/II	1.000	Reference	<.001	1.000	Reference	<.001
III/IV	2.845	1.704-4.750		2.120	1.266-3.548	

(Continues)

TABLE 2 (Continued)

Variable	Overall survival			Disease-free survival		
	HR	95% CI	*P*-value*	HR	95% CI	*P*-value*
LNM						
Not detected				1.000	Reference	<.001
Detected				6.249	2.246-17.389	
EYA4						
IHC score <6	1.000	Reference	.010	1.000	Reference	.019
IHC score ≥6	0.516	0.313-0.852		0.569	0.356-0.910	
MYCBP						
IHC score <6	1.000	Reference	.002	1.000	Reference	.002
IHC score ≥6	2.294	1.371-3.838		2.127	1.316-3.438	

AFP, alpha fetoprotein; 95% CI, 95% confidence interval; HBV, hepatitis B virus; HR, hazard ratio; IHC, immunohistochemistry; LNM, lymph node metastasis; PVTT, portal vein tumor thrombosis.
*$P < .05$ as significant.

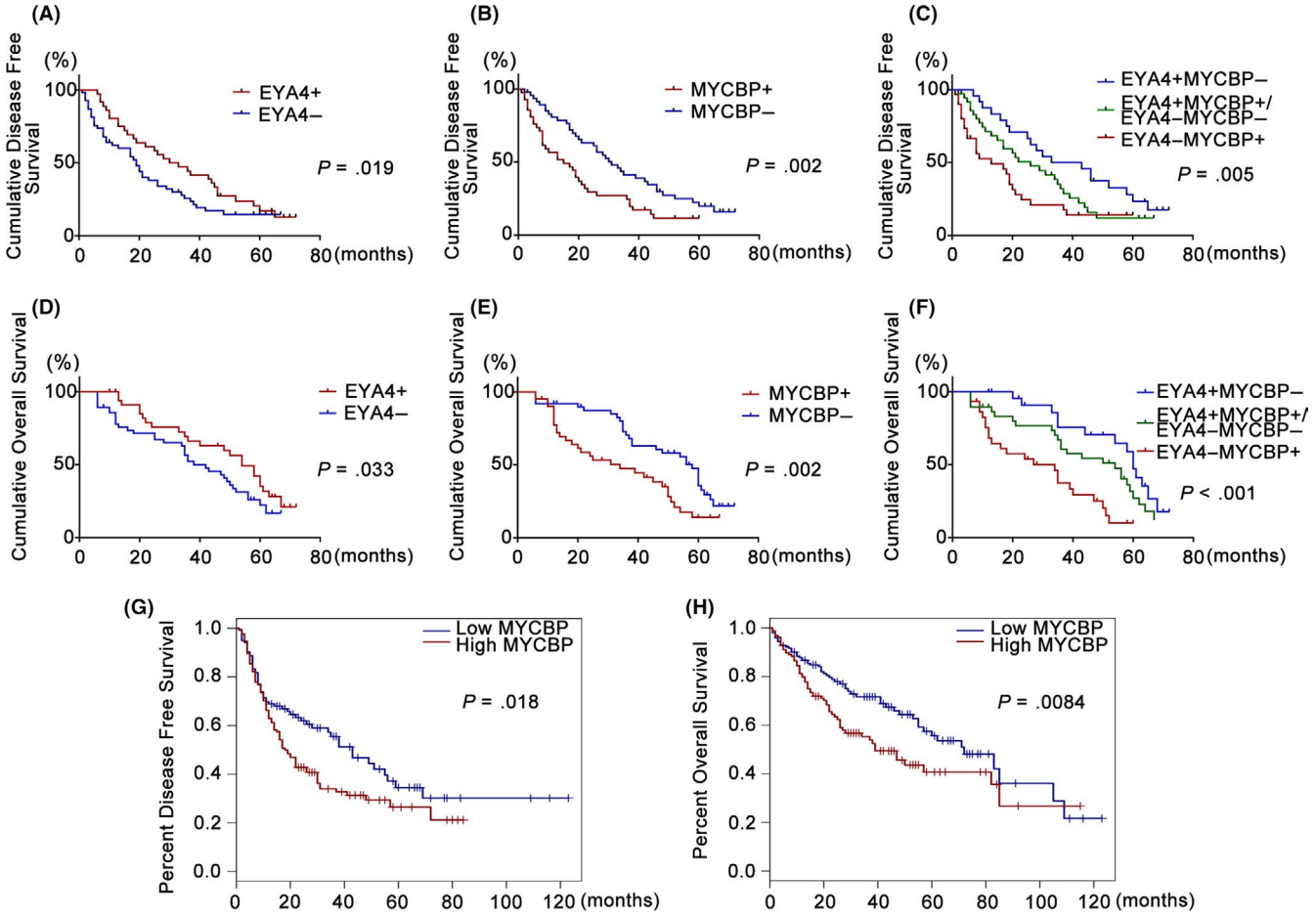

FIGURE 5 Survival curves of hepatocellular carcinoma (HCC) patients divided by EYA4 and MYCBP expression. A-C, Disease-free survival curves of HCC patients divided by EYA4 expression (A, EYA4+, highly expressed EYA4; EYA4–, poorly expressed EYA4), MYCBP expression (B, MYCBP+, highly expressed MYCBP; EYA4–, poorly expressed MYCBP) and combination of EYA4 and MYCBP expression (C). D-F, Overall survival curves of HCC patients divided by EYA4 expression (D), MYCBP expression (E) and combination of EYA4 and MYCBP expression (F). G and H, Disease-free survival (G) and overall survival (H) of HCC patients from online database GEPIA

cell proliferation and cell cycle induced by EYA4 KO. These results indicated that EYA4 suppresses HCC progression by mediating MYCBP.

MYCBP was reported as a direct target of the β-catenin/LEF-1 pathway.[15,22] EYA4 was shown to mediate the Wnt pathway by dephosphorylating β-catenin Ser675 in pancreatic cancer.[15,23] Therefore, we hypothesized that EYA4 may mediate MYCBP by dephosphorylating β-catenin. β-Catenin is one of the core proteins in the Wnt signaling pathway, and it was shown that phosphorylation at Ser552 and Ser675 of β-catenin could activate the β-catenin mediated signaling pathway.[24-26] In this study, in EYA4 overexpressed cells and EYA4 KO cells, the total level of β-catenin and p675-β-catenin did not change, but the level of p552-β-catenin was decreased in EYA4 overexpressed cells and increased in EYA4 KO cells. To investigate whether p552-β-catenin can facilitate transcription of MYCBP, we established β-catenin overexpressed plasmids with (WT) or without S552A mutation, a negative phosphorylated mutation of β-catenin at Ser552.[27] Then we transfected WT and S552A plasmids in EYA4 overexpressed cells. Results showed that the transcription level of MYCBP was higher in cells transfected with control plasmids than in S552A mutated plasmids. These results indicated that EYA4 downregulates MYCBP by dephosphorylating β-catenin at Ser552. β-Catenin functioned by translocating to the nucleus, binding with TCF1/LEF1 transcription factors and then inducing transcription of downstream genes. By IF and western blot assay, we found that β-catenin accumulated in the nucleus in EYA4 KO human HCC cell lines compared to the control. This proved that EYA4 reduced the nucleus translocation ability of β-catenin. Luciferase reporter gene assay proved that LEF1 could bind to the promotor region of MYCBP and start transcription. In summary, we demonstrated that EYA4 reduced the nuclear translocation of β-catenin by dephosphorylating β-catenin at Ser552, therefore suppressing the transcription of MYCBP which was induced by β-catenin/LEF1 binding to the promoter of MYCBP.

Our previous study showed that EYA4 was an independent prognostic factor in HCC. Higher expression of EYA4 was associated with a better DFS and OS.[12] In the present study, we reconfirmed the prognostic effect of EYA4 and also showed that MYCBP was an independent prognostic factor in HCC. Higher expression of MYCBP was associated with a worse DFS and OS. MYCBP combined with EYA4 could be prognostic biomarkers in HCC patients. HCC patients in the EYA4+MYCBP− group had the longest DFS and OS and the highest 3-year and 5-year survival rates, whereas EYA4−MYCBP+ group patients had the shortest DFS and OS and the lowest 3-year and 5-year survival rates and EYA4+MYCBP+/EYA4−MYCBP− group patients were in the middle.

In summary, in the present study, we showed that EYA4 could suppress HCC tumor cell growth. EYA4 suppressed cell proliferation and induced G2/M arrest in HCC cell lines by repressing MYCBP. EYA4 reduced the nuclear translocation of β-catenin by dephosphorylating β-catenin at Ser552, thereby suppressing the transcription of MYCBP which was induced by β-catenin/LEF1 binding to the promoter of MYCBP. EYA4 combined with MYCBP could be prognostic biomarkers in HCC patients.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

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