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RESEARCH ARTICLE

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Ascending E2F7a/b ratio facilitates KLF13 transcription in hepatocellular carcinoma and correlates with the abundance of binuclear hepatocytes (ABH) modulation for short-term recurrence

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Abstract

Short-term recurrence after surgery severely threatens patients' lives and leads to dismal outcomes in hepatocellular carcinoma (HCC). Our previous research proposed the abundance of binuclear hepatocytes (ABH) as an independent indicator related to the cytokinesis regulator Anillin and significantly associated with HCC recurrence. The exact mechanism of ABH modulation has not been clearly illustrated. In this study, we intensively investigated the probable regulation mechanism and noticed a contradiction between E2F7 upregulation and ABH attenuation. As we discovered, E2F7 has two isoforms, E2F7a and E2F7b, and we innovatively define a value of the E2F7a/b ratio using a cutoff value of 6.5. E2F7 upregulation in the paracancerous tissues was predominantly presented by the E2F7a isoform, leading to an increase in the E2F7a/b ratio, instead of E2F7b as a main component in non-cancerous tissues, and is associated with short-term recurrence. We further found that KLF13 transcriptionally promotes Anillin expression in HCC and was suppressively impacted by E2F7b, but not by the highly expressed E2F7a. Hence, the ascending E2F7a/b ratio induced significant upregulation of KLF13 and participated in the attenuation of ABH in the paracancerous liver tissues. In conclusion, E2F7 presents a particular expression status in HCC by predominantly upregulating E2F7a rather than E2F7b. The ascending E2F7a/b ratio weakens the suppressive effect on KLF13 transcription and sequentially participates in ABH attenuation, associated with short-term HCC recurrence post-operation.

KEYWORDS

ABH value, E2F7a/b isoforms, hepatocellular carcinoma, KLF13, short-term recurrence

Yian Zhang and Nan Wang contributed equally as the first authors.

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1 | INTRODUCTION

Hepatocellular carcinoma (HCC) involves tumor development, invasion, and metastasis, which are difficult to control.[1] Dismal outcomes and high mortality currently occur in HCC patients, even if radical operations are conducted, due to the potent tumor heterogeneity and aggressive cellular behaviors.[2] Based on the innovative progress in HCC treatment, such as targeted therapy and tumor immunotherapy, many more patients at later stages have obtained the opportunity for operative treatments. However, the overall survival (OS) of HCC patients is far from satisfactory, and one of the major intractable issues is the high rate of short-term recurrence after surgery.[3] The discovery of innovative evaluation methods to warn of the potential tumor recurrence risk is requested for resolution.

Short-term recurrence after surgery occurs in around 20% of HCC patients within 2 years without apparent clinical signs and poses challenges for researchers to develop timely strategies for adjuvant prevention.[4] In recent research, we discovered a gradient attenuation of binuclear hepatocytes from non-cancerous tissues to tumor tissues associated with tumor relapse. Based on this, we developed a concept of the abundance of binuclear hepatocytes (ABH) as an indicator of HCC short-term recurrence.[5]

Human somatic chromosomes are diploid (2n), and only a few organs or tissues, like the liver and myocardium, may contain the cells carrying two or more groups of homologous chromosomes for physical necessities called polyploid cells (4n and 8n).[6] Recent studies demonstrate that the maintenance of the polyploidy portion of hepatocytes can prevent tumor formation by stabilizing the microenvironment induced by chronic inflammation and injury to a certain extent.[7] Hence, quantifying the polyploid hepatocytes could be an innovative approach to assessing HCC prognosis.[8]

From a mechanistic perspective, polyploid hepatocytes are mainly generated via a programming cytokinesis failure after weaning in the infant stage, and this process induces an overwhelming binuclear hepatocyte (4n) constitution of the whole polyploid hepatocyte population.[9] Based on this, we suggest ABH as an index for describing the attenuation of polyploid hepatocytes in the paracancerous liver tissues.

Anillin is a terminal effector anomalously upregulated in the HCC process to abolish cytokinesis failure and lead to the depolyploidization of hepatocytes. E2F7 is a member of the E2F transcription factor family and exerts a suppressive transcriptional effect on the E2F family's target genes, different from its canonical family members (E2F1–E2F6). Our recent study observed the upregulation of E2F7 in HCC and verified its stabilizing effect on Anillin expression by inhibiting the negative regulator upstream of Anillin.[10] Sequentially, we illustrated a couple of participants relevant to the E2F7 pathways regulating liver polyploidy status, including some critical transcriptions (SP1 and SOX4) and non-coding RNA products (microRNA-383 and AKR1B10P1).[11,12]

It seems that all the findings above support the overexpression of E2F7 in the HCC process. However, on the contrary, knocking out E2F7 in hepatocytes was conversely verified to induce significant depolyploidization in the liver and facilitated tumorigenesis under a carcinogenic environment.[13] To resolve this contradiction issue, we investigated the products of E2F7 and noticed two isoforms of E2F7 (E2F7a and E2F7b) transcribed distinctively in normal and tumorous tissues. E2F7b is the main isoform expressed in normal tissues. This predominant expression status was gradiently replaced by E2F7a from normal liver and paracancerous tissues to HCC tumor tissues. The ascending ratio of E2F7a/b in the paracancerous tissues was correlated with dismal clinical-pathological characteristics in HCC patients and associated with short-term recurrence along with the ABH value attenuation. Transcription factor KLF13 is upstream of Anillin and is definitely overexpressed in HCC. By detecting the transcriptional activity of E2F7 isoforms on KLF13, E2F7a was found to be much less suppressive on KLF13 transcription than the E2F7b isoform, and the high ratio of E2F7a/b in the HCC process might be a probable explanation for the contradiction of E2F7 expression. We believe that the E2F7a/b ratio is an innovative molecular pathological indicator that supplements the assessment of HCC recurrence along with the ABH value in clinical practice.

2 | MATERIALS AND METHODS

2.1 | Cell culture and preparation

Three typical HCC cell lines (Huh7, HepG2, and Hep3B) were cultured along with the control normal human hepatic cell LO2 (Shanghai Institutes for Biological Sciences, Chinese Academy of Science, Shanghai, China). Briefly, the cell lines were respectively cultured in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), incubated at 37°C in a humidified environment, with 100 μg/ml streptomycin and 100 U/mL Penicillin in a humidified cell atmosphere, with 5% CO_2. Specifically, for the transfected cells, a medium mixed with G418 (Santa Cruz Biotechnology, Inc.; 400 μg/mL) was used for selection.

2.2 | Clinical specimen preparation

A cohort of paired patients' clinical specimens were collected in the number of 152 cases. All cases were conducted R0 radical resections without receiving any pre- or post-operational treatment, at the Department of General Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (2018 to 2019). For each case, the sample contains both the tumor part and the paracancerous liver tissue at least 1 cm close to the tumor margin and was arranged for hematoxylin–eosin (HE) staining for basic pathological evaluation. Meanwhile, 43 cases of the non-cancerous donor's liver tissues were included for comparison. The study was approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, with included informed consent. The clinicopathological features of the cases were collected, including the preoperative vitamin K Absence-II (PIVKA-II) value, alpha-fetoprotein (AFP), tumor size, the number of lesions, grades, et al. Distinguished by the short-term recurrence within 2 years post-operation, the patients were set into two groups: the Recurrence group and the No-recurrence one.

2.3 | RT-qPCR assay

RNA isolation was conducted from the specimens, respectively, according to the instructions of the TRIzol reagent (Invitrogen, USA). The first-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (ABI, USA), and the primers of the E2F7a, E2F7b, KLF13, and Anillin were synthesized by Shanghai JIKE Biotech Company (Shanghai, China) (Table S1). Real-time quantitative polymerase chain reaction (RT-qPCR) was conducted according to the TaqMan Gene Expression Assays protocol (ABI, USA).

2.4 | The HE staining assays

HE-stained slides of paracancerous tissues were produced following the conventional pathological examination procedures: specimens were fixed overnight in a 4% paraformaldehyde solution, then dehydrated and cleaned. For paraffin embedding, tissues were infiltrated at 65°C two times (10 and 60 min). Sections were cut into 4 μm thicknesses and deparaffinized in xylene and ethanol before staining with hematoxylin and eosin. For the 43 normal liver tissues, treatments and tests were carried out simultaneously by the same methods. Two experienced pathologists were assigned to review all the slides independently. The histopathologic evaluation of ABH value was conducted following our previous report.[5]

2.5 | The immunohistochemistry assays

The immunohistochemistry (IHC) assay was conducted following our regular methods.[14] Antibodies against E2F7 were purchased (abs132823; 1:1000; Absin). The detection of E2F7 in tissues was practiced by the two assigned pathologists independently and blindly. The specimens were separated into two groups according to the staining intensity grade: no or low staining status (0–1+) and moderate to high staining status (2+–3+).

2.6 | Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was applied to verify the interaction between the transcription factor and the targeted genes' promoter region (E2F7a and E2F7b to KLF13, and KLF13 to Anillin). A total of 5×10^6 cells were cultured in each 10 cm dish and subjected to the protocol of ChIP assay by introducing the ChIP-ITTM Kit (Active Motif). Chromatin was immunoprecipitated with 2 μg of either the transcription factor antibodies (Abcam, USA) or IgG as the negative control. The extracted DNA was analyzed through RT-qPCR using the relative primers (Table S1).

2.7 | Plasmid preparation and cell transfection

The lentiviral vectors pLKO.1 containing shRNA were transfected into cultured Hep3B cells at the exponential phase (JIKE Biochemistry, Shanghai, China) to selectively suppress E2F7a, and the control vectors were assigned. The transfected cells were selected using a medium mixed with G418 (Santa Cruz Biotechnology, Inc.; 400 μg/mL). On the contrary, the lentiviral vector pLV (Addgene, Cambridge, USA) was applied for ectopically expressing E2F7b (pLV- KLF13) for the rescue experiments, and the pLV-Null was set for control.

2.8 | Statistical analysis

Statistical analysis was conducted using SPSS 20.0. *P*-values were calculated following an unpaired Student's *t*-test and Fisher's exact test. The Spearman correlation analysis and Logistic regression analysis were used to

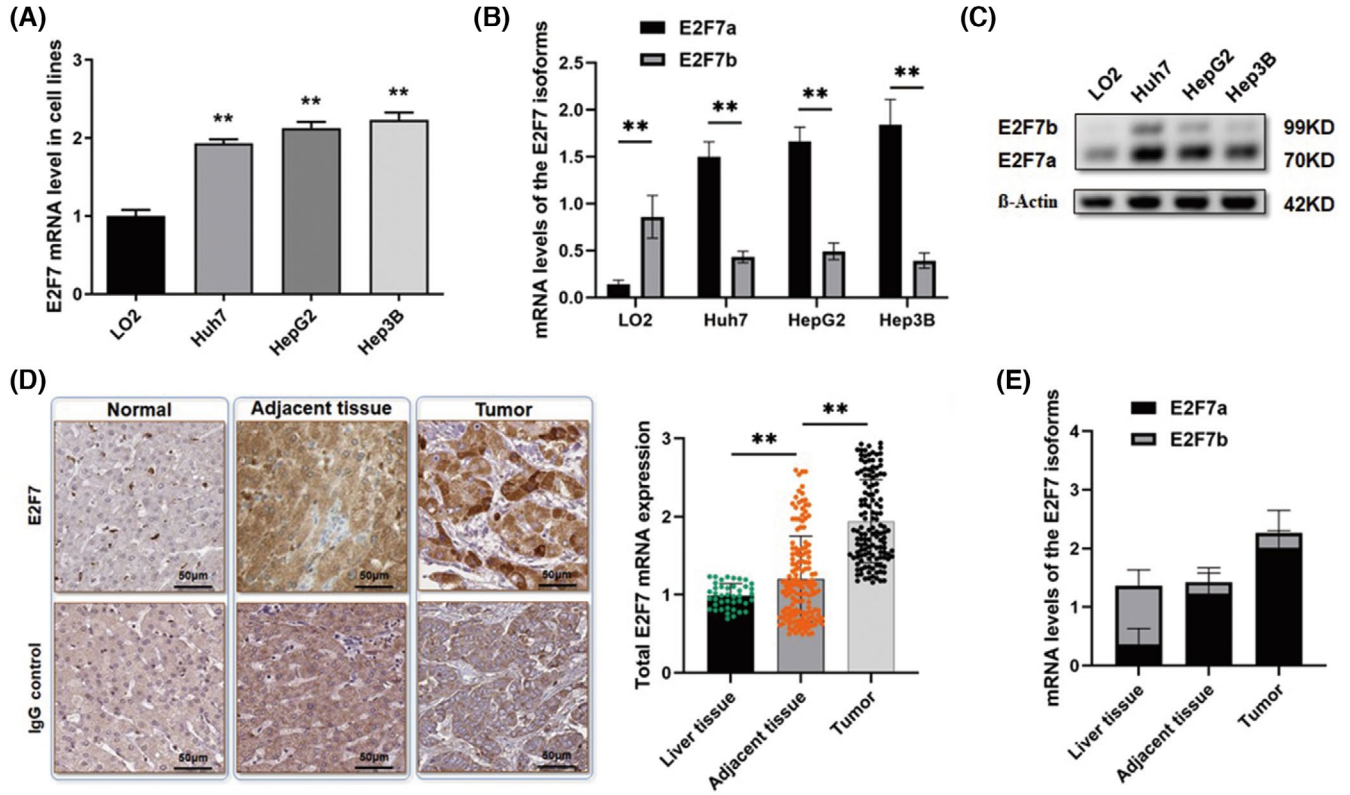


FIGURE 1 The expression profile of E2F7 isoforms in the cell lines and tissues. (A) Total E2F7 expression profile in the cell lines was detected by the RT-qPCR assay. Total E2F7 mRNA was significantly increased in three HCC cell lines, compared with the control LO2 cells (**$p < .01$). (B) Expression status of either E2F7a or E2F7b in cell lines. Both E2F7a and E2F7b were elevated in the HCC cell lines, and E2F7a occupied the main portion of E2F7 composition in tumor cells (**$p < .01$). (C) The western blot analysis. E2F7 presented a significantly high expression in three HCC cell lines, predominantly by the E2F7a isoform. (D) Representative graph of IHC assay (400×). The IgG antibody was used for staining the specimens as a control. An ascending gradient of E2F7 expression from the normal liver, adjacent non-cancerous tissues, and tumor tissues was observed. (E) A total E2F7 mRNA was elevated in gradient from normal liver tissues to the tumor specimens, and E2F7a composed the predominant composition in the HCC specimens.

describe the correlation between the various parameters and the risk of short-term recurrence. Differences were considered statistically significant at *p*-values <.05.

3 | RESULTS

3.1 | Components and expression profile of the gradient upregulated E2F7 concerning HCC

For HCC cell lines, E2F7 expression was detected compared with the control LO2 cells. The total E2F7 mRNA level in three HCC cell lines (Huh7, HepG2, and Hep3B) is much higher than that in the LO2 cells (Figure 1A). The mRNA level of either E2F7a or E2F7b was investigated, as shown in the histogram (Figure 1B). Even though E2F7b mRNA was elevated in the three HCC cell lines, E2F7a occupied the main portion of E2F7 composition in tumor cells. At the protein status, since the protein products of E2F7a contain 728 amino acids, and E2F7b contains 911 ones, two bands were detectable by the Western blot assay, respectively indicating E2F7a (at 77KD) and E2F7b (at 99KD) (Figure 1C).

For specimens, the IHC assay indicated an ascending gradient of E2F7 expression from normal liver, adjacent non-cancerous tissues, and tumor tissues (Figure 1D). However, this assay provided no valuable information on the E2F7a and E2F7b distribution. In this study, we used the RT-qPCR assay to describe the isoform expression. As Figure 1E demonstrated, a total E2F7 mRNA was elevated in gradient from normal liver tissues to the tumor specimens, and E2F7a composed the predominant composition.

3.2 | Stratification by E2F7a/b ratio is associated with HCC clinicopathological features

According to the average expression of E2F7a and E2F7b, we established the calculation of the E2F7a/b

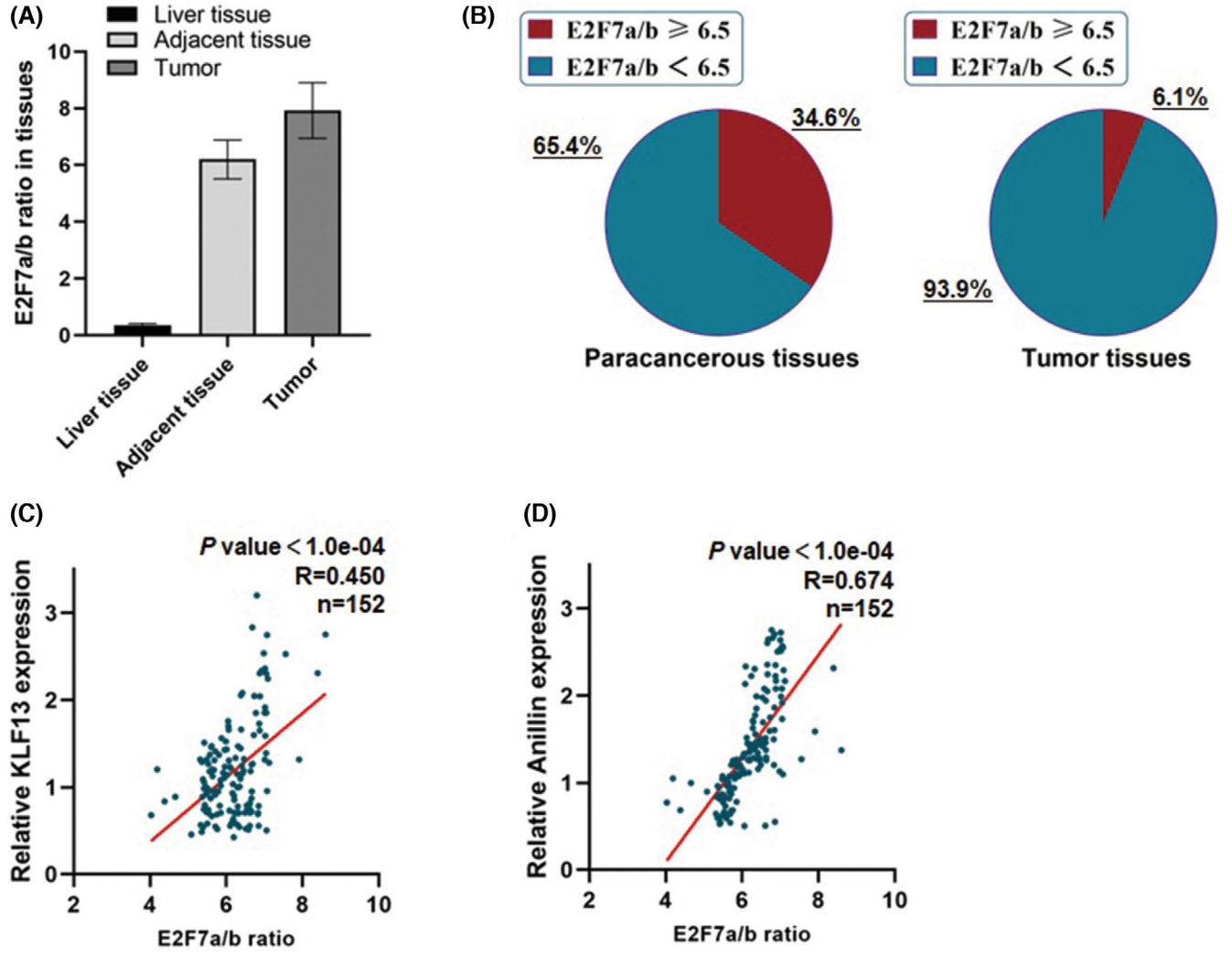


FIGURE 2 The distribution of E2F7 isoforms in tissues assessed by the E2F7a/b ratio. (A) The E2F7a/b ratio was calculated. According to the data, a significant difference in the distribution of the E2F7a/b ratio was observed: 0.354 (ranging from 0.280 to 0.576; Median: 0.347) in normal liver tissues; 6.2 (ranging from 4.808 to 8.602; Median: 6.209) in the paracancerous tissue; 7.9 (ranging from 0.280 to 0.576; Median: 9.696) in the tumor. (B) 34.6% of cases presented a higher E2F7a/b ratio over 6.5 in the paracancerous liver tissues, whereas the values were 93.9% of the patients in the tumor tissues. (C) The E2F7a/b ratio and KLF13 expression level were positively correlated in the paracancerous liver tissues ($p < 1.0e\text{-}4$, $R = 0.450$). (D) The E2F7a/b ratio and Anillin expression level were positively correlated in the paracancerous liver tissues ($p < 1.0e\text{-}4$, ($R = 0.674$).

ratio for each case, from the normal liver tissues, paracancerous tissues, to the tumor tissues. On data, the E2F7a/b ratio was 0.354 (ranging from 0.280 to 0.576; Median: 0.347) in normal liver tissues; was 6.2 (ranging from 4.808 to 8.602; Median: 6.209) in the paracancerous tissue; and 7.9 (ranging from 0.280 to 0.576; Median: 9.696) in the tumor (Figure 2A,B). The increased amplitude of the E2F7a/b ratio occurred in the paracancerous tissues, and we set a cutoff value of 6.5 to separate the E2F7a/b ratio into high and low groups.

The correlation between the E2F7a/b ratio and the patients' clinicopathological features was detected. Simultaneously, we also analyzed the expression profiles of KLF13 and Anillin (Figure S1A; Table 1). As Table 1 shows, no significant correlation was observed between the E2F7a/b ratio and age, gender, HBsAg status, or tumor encapsulation. Notably, a potent trend toward larger tumor dimensions, advanced TNM stages, severe tumor microsatellite formation, venous invasion, and higher levels of AFP and PIVKA-II was discovered in patients of the high E2F7a/b ratio group, which was consistent with the relatively higher expression of KLF13 and Anillin. Notably, the E2F7a/b ratio was correlated with the expression level of either KLF13 or Anillin. This means there exists a potential regulatory mechanism between them (Figure 2C,D).

TABLE 1 Correlation between E2F7a/b ratio, KLF13, Anillin, and clinicopathological features in 152 paired HCC specimens.

	E2F7a/b ratio ≥6.5			KLF13 level			Anillin level		
Clinicopathologic parameters	**No ($n=99$)**	**Yes ($n=53$)**	**p***	**Low ($n=39$)**	**High ($n=113$)**	**p***	**Low ($n=44$)**	**High ($n=108$)**	**p***
Age (years)									
≤50	61	38	.284	21	78	.118	27	72	.576
>50	38	15		18	35		17	36	
Gender									
Male	59	40	.074	20	79	.051	25	74	.192
Female	40	13		19	34		19	34	
Diameter (cm)									
≤5	66	17	.001	29	54	.005	31	52	.019
>5	33	36		10	59		13	56	
TNM stage									
I–II	59	18	.004	26	51	.026	31	46	.011
III–IV	40	35		13	62		13	52	
Tumor encapsulation									
Absent	46	28	.498	20	54	.715	18	56	.283
Present	53	25		19	59		26	52	
Tumor microsatellite									
Absent	58	12	.001	27	43	.001	35	35	.001
Present	41	41		12	70		9	73	
Venous invasion									
No	60	22	.028	27	55	.040	35	47	.001
Yes	39	31		12	58		9	61	
HBsAg									
Negative	17	10	.826	11	16	.054	13	14	.002
Positive	82	43		28	98		31	94	
AFP (ng/mL)									
≤400	47	15	.025	31	31	.001	32	30	.001
>400	52	38		9	82		12	78	
PIVKA-II									
Positive	64	46	.004	19	91	.001	20	90	.001
Negative	35	7		20	22		24	18	

Note: The E2F7a/b ratio (in paracancerous tissues), along with the expression level of KLF13 and Anillin (in tumor), which associated with clinicopathologic features in 152 HCC patients, including age, gender, tumor size, tumor stage (AJCC), tumor encapsulation, tumor microsatellite formation, vein invasion, HBsAg status, AFP level, and especially, preoperation PIVKA-II. Statistically, significance was assessed by Fisher's exact test.

*$p < .05$.

3.3 | The high E2F7a/b ratio in the paracancerous tissues is related to short-term recurrence after surgery

We assessed the clinicopathological features from the 152 HCC cases and screened out the information associated with the short-term recurrence. Forty-three patients out of the 152 cases experienced short-term recurrences within 2 years after surgery. Refer to the analysis of the distribution of the E2F7a/b ratio separately in the groups that recurred or not; 93.02% (40/43) of cases in the recurred group provided an E2F7a/b ratio exceeding 6.5 (6.88 on average; Median: 6.84). As for the patients without recurrence, the average value of the E2F7a/b ratio was 5.8 (Median:), and only 14.14% (14/99) of cases presented a value greater than 6.5 (Figure 3A,B).

As we reported before, ABH value is a specific predicting indicator for short-term recurrence using a cutoff

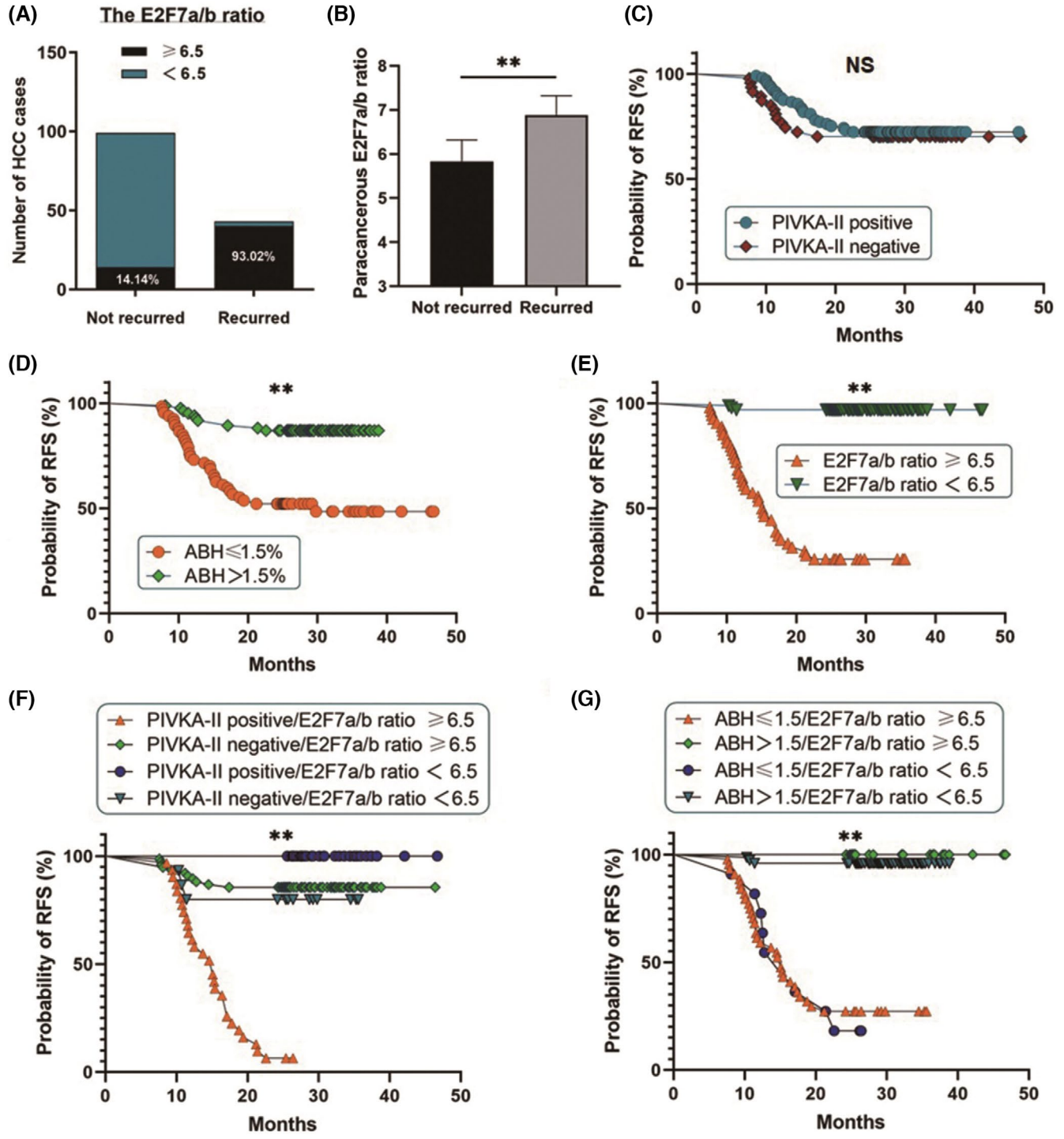


FIGURE 3 The high E2F7a/b ratio in the paracancerous tissues is related to short-term recurrence after surgery. (A and B) Statistic of the number of cases concerning the levels of E2F7a/b ratio in the paracancerous tissues. The E2F7a/b ratio was over 6.5 in most of the paracancerous liver tissues (93.02%, 40/43) from the patients who recurred. For the patients without short-term recurrence, the percentage is 14.14% (14/99) (**$p < .01$). (C) The Kaplan–Meier plot indicates that there was no significant correlation between the short-term recurrence incidence and the status of PIVKA-II (NS ≥0.05). (D) The Kaplan–Meier plot was generated. The patients with an ABH value below 1.5% in the paracancerous tissues showed a significantly lower RFS in the 152 cases of HCC patients (**$p < .01$). (E) By setting a cutoff value of the E2F7a/b ratio at 6.5, the Kaplan–Meier plot shows a significant difference in the RFS among the HCC patients. A high E2F7a/b ratio is correlated with more frequent short-term recurrence after surgery (**$p < .01$). (F) RFS rate was increased potently according to the E2F7a/b ratio, no matter the PIVKA-II status (**$p < .01$). (G) By assessing through combining ABH value and the E2F7a/b ratio, the RFS rate of the 152 patients was significantly differentiated specifically and sensitively (**$p < .01$).

value of 1.5%. Similarly, PIVKA-II is regarded as a serum HCC-specific biomarker indicating poor prognosis and recurrence.[15] Thus, we compared the significance of these two indexes and the E2F7a/b ratio, respectively, in the 152 cases with HCC short-term recurrence within 2 years. As Figure 3C shows, even though an expected inclination exists, there is no significant relationship between PIVKA-II and short-term recurrence observed. Noteworthily, for ABH value below 1.5% and the E2F7a/b ratio below 6.5, a much more significant correlation with HCC short-term recurrence was observed (Figure 3D–F).

3.4 | The E2F7a/b ratio is a promising independent indicator for predicting HCC short-term recurrence and is consistent with the ABH value

According to our observation, we set 8 variables (including E2F7a/b ratio, ABH, PIVKA-II, AFP, tumor diameter, tumor microsatellite, venous invasion, and tumor encapsulation) for the univariate regression analysis. As shown in Figure 4A, the E2F7a ratio and ABH are the two variables that present the most significant relationship to the short-term recurrence in HCC patients, with ORR of

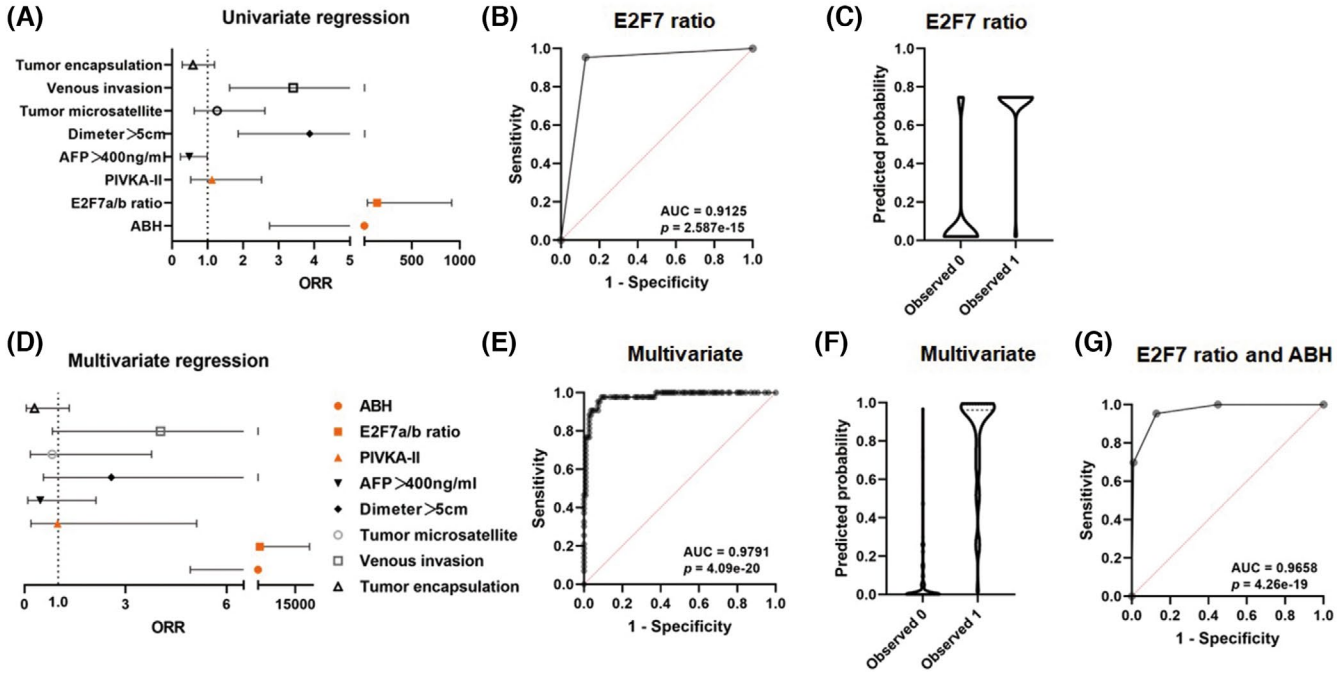
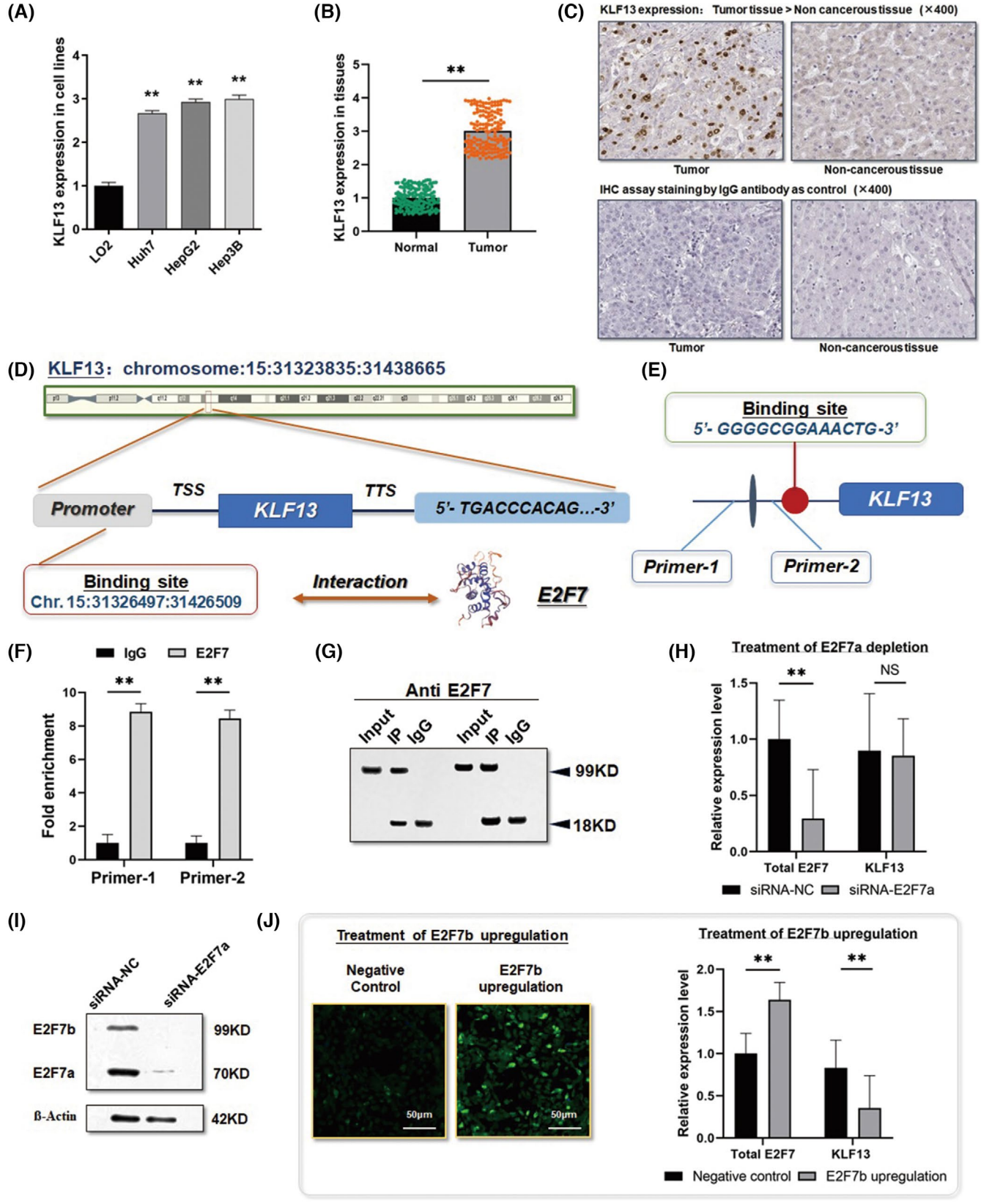


FIGURE 4 The E2F7a/b ratio is a promising independent indicator for predicting HCC short-term recurrence. (A) For the Univariate logistic regression analysis, the E2F7a/b ratio and ABH value respectively demonstrated a consistently significant relationship with HCC short-term recurrence, independently. (B) The E2F7a/b ratio over 6.5 indicates a higher risk of HCC short-term recurrence with significance (AUC: 0.9125, $p = 2.587e\text{-}15$). (C) The predicted probability of the E2F7a/b ratio. (D) For the Multivariate logistic regression analysis, the E2F7a/b ratio presented a consistently significant relationship with HCC short-term recurrence along with the ABH value. (E) Multivariate logistic regression yielded a model with high prediction strength (AUC: 0.9791, $p = 4.09e\text{-}20$). (F) The predicted probability of the Multivariate logistic regression model. (G) The combination of the E2F7a/b ratio and ABH value provides a model with high prediction strength (AUC: 0.9658, $p = 4.26e\text{-}19$).

FIGURE 5 E2F7b isoform exerts the suppressive function on KLF13 transcription. (A) Transcription factor KLF13 mRNA was significantly elevated in HCC cell lines compared with the control LO2 cells (**$p < .01$). (B) KLF13 presented a high mRNA level in HCC tumor tissues compared with the non-cancerous tissues (**$p < .01$). (C) Representative graph of IHC assay (400×). KLF13 was significantly upregulated in tumors. (D) The promoter region of KLF13 was detected as a potential binding site for E2F7 (5′-GGGGCGGAAACTG-3′, chromosome: 15:31326497:31426509) was predicted. (E) Two primers (Primer 1 and Primer 2) were synthesized for the ChIP assay. (F) As the histograms of the ChIP assay showed, the equivalent region of the promoter upstream of the KLF13 gene was directly bound by KLF13. IgG was used as the negative control (**$p < .01$). (G) The Western blot assay demonstrated that the corresponding band of the participated E2F7 was indicated E2F7b (99KD), but not E2F7a. (H and I) Suppression of E2F7a significantly decreased the total expression of E2F7 in HCC cells, but no significant change in KLF13 expression was observed (**$p < .01$). (J) The effect of E2F7b introduction into the HCC cells was validated through immunofluorescence detection. Upregulation of E2F7b potently decreased the expression of KLF13 mRNA (**$p < .01$).



139.1 (95%CI: 37.32 to 915.40) and 5.899 (95%CI: 2.74 to 13.52) respectively. The E2F7a/b ratio showed a promising predictive value for HCC short-term recurrence (AUC: 0.9125, $p = 2.587e\text{-}15$, Figure 4B,C). Simultaneously, the multivariate logistic regression was performed on the 8 variables. The model in this study significantly distinguishes short-term recurrent HCC patients (AUC: 0.9791, $p = 4.09e\text{-}20$, Figure 4D,E). The negative predictive power

and positive predictive power are 87.16% and 95.35%, respectively (Figure 5F). Notably, we combined the E2F7a/b ratio and ABH for predicting the HCC short-term recurrence and obtained a quite close result that the AUC is 0.9658 ($p = 4.26e-19$) (Figure 4G). Thus, according to our data, we suggest that the E2F7a/b ratio is an independent risk factor for HCC short-term recurrence and could be combined with ABH for precise prediction clinically.

3.5 | E2F7b, but not E2F7a, exerts the suppressive function on KLF13 transcription

The expression profile of KLF13 was validated as upregulated in HCC cell lines and tumor tissues in our series of studies (Figure 5A–C). The results above (Table 1) indicated that high expression of KLF13 was also correlated with dismal clinicopathological features of the patients. We wonder about the relationship between KLF13 and E2F7. Thus, we engaged a fragment of 3000 bp from the promoter sequence of the KLF13 gene to predict potential binding sites for transcription factors. Data analyzed using the Database of Human Transcription Factor Targets (https://guolab.wchscu.cn/AnimalTFDB4/#/) demonstrated E2F7 as a candidate binding to the gene promoter region at a specific sequence (5′-GGGGCGGAAACTG −3′, chromosome: 15:31326497:31426509) with significance ($p = 4.39e-06$) (Figure 5D,E). Following this, the ChIP assay was carried out to determine the direct interaction between E2F7 and the KLF13 gene. The definite interaction between the E2F7 and KLF13 upstream sequence was validated through the ChIP assay with two pairs of primers (Primer-1 and Primer-2) (Figure 5F). Interestingly, the Western blot assay demonstrated that the corresponding band of the participated E2F7 was indicating E2F7b (99KD), but not E2F7a (Figure 5G).

To further confirm the exact isoform of E2F7 controlling the transcription of KLF13, we respectively suppressed E2F7a or upregulated E2F7b in Hep3B cells. As expected, even though losing E2F7a reduced the main portion of E2F7 expression in Hep3B cells, the treatment induced no significant KLF13 mRNA decrease; on the contrary, by upregulating E2F7b, the mRNA level of KLF13 was sequentially decreased (Figure 5H–J).

3.6 | E2F7a/b ratio modifies Anillin expression in the way of regulating KLF13 transcription

Since the E2F7a/b ratio is associated with ABH value, we further discussed the impact of the E2F7a/b ratio on the expression of Anillin, which is the terminal effector working on ABH changes. Using the same methods applied in the upper section, a potential binding site for KLF13 on the sequence of the promoter region of Anillin was noted (5′-GGCCCCGCCTCCT-3′, chromosome: 7:36389716:36389728) with significance ($p = 7.55e-06$) (Figure 6A,B). The direct interaction between them was further verified following ChIP assay through the paired Primer-3 and Primer-4 (Figure 6C).

Meanwhile, the treatment of suppressing E2F7a did not impair the mRNA expression of Anillin, while upregulating E2F7b led to a tendency for Anillin decrease (Figure 6D,E). We believe that it is the loss of E2F7b, but not E2F7a, that gives out the key point to partly explain the phenotype induced by knocking out E2F7 in HCC as presenting a rise in Anillin and the consequential attenuation of ABH. The definition of the E2F7a/b ratio seems to support and supplement our study and understanding of E2F7, Anillin, and the polyploidy mechanism of hepatocytes.

4 | DISCUSSION

HCC is one of the major malignancies challenging researchers and clinical doctors with aggressive biological characteristics, strong growth ability, severe invasiveness, and high motility.[16] Radical surgery is the fundamental treatment option for HCC patients to achieve reliable survival. However, over 60% of HCC cases suffer recurrence in a short time after surgery, even if some rapid progress in treatment has been achieved to a certain extent based on targeted therapy and tumor immunotherapy.[17] Unpredictable tumor progression and the high rate of tumor recurrence strongly limit the improvement of OS and recurrence-free survival (RFS). The innovative and practical methods for the evaluation of tumor recurrence as early as possible are valuable for prolonging the disease-free period and better prognosis.

In our previous research, we focused on the distinctive organ characteristics of the polyploid hepatocytes in the liver. Polyploid cells compose almost 90% of the portion of hepatocytes in rodents and about 50% of human beings, which can present as binuclear or mononuclear (tetraploids, octoploids) based on different mechanisms.[18] Notably, the dominant cytokinesis failure process results in the main portion of polyploid hepatocytes being binuclear.[19] Thus, the binuclear hepatocytes indicate the macroscopical status of polyploidy in the liver, and we developed the concept of the ABH value mentioned above.

According to our reported literature, the attenuation of binuclear hepatocytes occurs not only in the tumor tissue but also in the paracancerous liver tissues at an earlier stage

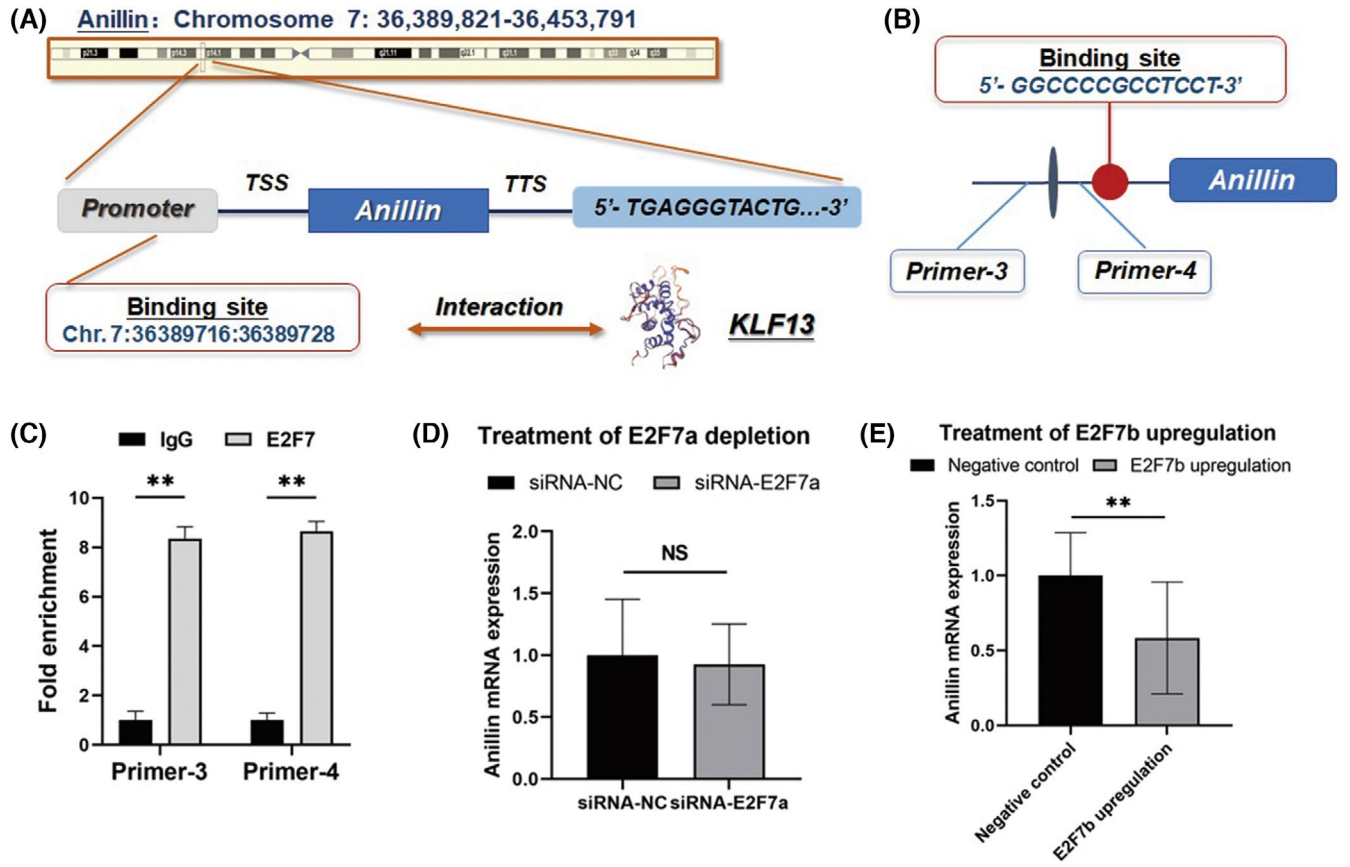


FIGURE 6 E2F7a/b ratio modifies Anillin expression in the way of regulating KLF13 transcription. (A) The promoter region of KLF13 was detected as a potential binding site for E2F7 (5′-GGCCCCGCCTCCT-3′, chromosome: 7:36389716:36389728) was predicted. (B) Two primers (Primer 3 and Primer 4) were synthesized for the ChIP assay. (C) The ChIP assay demonstrated that the equivalent region of the promoter upstream of the Anillin gene was bound by KLF13. IgG was used as the negative control (**$p < .01$). (D and E) The treatment of suppressing E2F7a did not impair the mRNA expression of Anillin; Upregulating E2F7b led to a tendency for Anillin decrease (**$p < .01$).

of tumorigenesis. This phenotype can also be explained and is consistent with the findings from Zhu et al.,[20] that under chronic liver injury like hepatitis, the generation of more polyploid hepatocytes helps to maintain the ability to regenerate liver tissues and prevent tumor formation, and the loss of polyploidy could be regarded as a disadvantage microenvironment weakening tumor prevention. Anillin is the terminal effector controlling cytokinesis in hepatocytes and presents as a pivotal regulator of hepatocyte polyploidization.[21] Overexpression of Anillin significantly promotes cell division and leads to the sharp descent of binuclear hepatocytes, and knocking down Anillin could potently inhibit tumor formation without impairing normal hepatocyte generation.[22,23] As we discovered, high Anillin effectively facilitates HCC tumor growth and is correlated with worse RFS and OS for HCC patients (Figure S1B), which aligns with our findings that ABH reduction facilitates HCC recurrence (Figure S1C). Further corresponding study revealed that anomalous expression of Anillin in HCC could be effectively induced by knocking down the upstream regulator E2F7.[11,24] Definitely, by intensively discussing the follow-up data from the real-world patient about recurrence and ABH value information, we confirmed that the raising of Anillin in the paracancerous liver tissues is a sensitive signal significantly associated with the decline of ABH and short-term recurrence after surgery. Accordingly, E2F7 and Anillin are hopeful candidates for indicating related liver microenvironment profiles and tumor processes.

E2F7 and E2F8 are two suppressive transcription factors among the E2F family, and this profile makes them independently exert the limiting effect against most of their cognates and maintain the E2F family's transcription function in balance.[25]

Based on the structure of the E2F7 protein, it should have to form dimers, especially the homodimers as the main mode intracellular, to conduct the transcription regulation.[26] The excessively high expression of E2F7 has been reported in numerous malignancies, such as gliomas, non-small-cell lung cancer, pancreatic cancer, and colorectal cancer, and E2F7 commonly exerts a positive effect on tumor cell proliferation and motility.[27] According

to the literature, there are two E2F7 transcripts regarded as conservative isoforms generating effector proteins with different amino compositions (E2F7a and E2F7b).[28] However, most of the previous studies conventionally discussed E2F7 without distinguishing these two isoforms, and the definite difference between E2F7a and E2F7b has not been illustrated. As observed from the literature, there inevitably exists controversy on the function of E2F7 in human malignancies, including HCC. For example, overexpression of E2F7 in HCC promotes tumor growth and even induces mTOR inhibitor resistance in the patients after liver transplantation via different pathways[29]; whereas, knocking out E2F7 induced tumor formation in the livers of the mice models. On this point, E2F7 exactly plays a role in inhibiting HCC.[30] We believe that the contradiction of E2F7's function and expression profiles may concern the loss of balance of the isoform ratio.

The predominant expression mode of E2F7 in hepatocytes is E2F7b, whose mRNA contains 2600 bp of nucleotides and is translated into a longer protein product than E2Fa. As we observe, the E2F7a/b ratio in the hepatocytes is below 0.3 on average. In the tumor microenvironment, the ratio ascends in a gradient from the non-cancerous liver tissue, paracancerous tissues (1 cm close to the tumor margin), to the tumor tissue. Although few kinds of literature have ever discussed the regulation of different E2F7 isoforms, it is reported that E2F7b mRNA is targeted for degradation in the early G1 phase and increases after entering the S phase, while E2F7a expression is consistent across cell cycles.[28] The increased E2F7a/b ratio may reflect the dysregulated cell proliferation in a precancerous tissue environment, which requires further studies to confirm.

This change in the E2F7a/b ratio is consistent with the gradient increase of Anillin we proved previously. By setting a cutoff value of 6.5 for the E2F7a/b ratio, the separated group of the cases in this study presented a significant differentiation in the short-term recurrence after surgery. In the regression analysis, both the E2F7a/b ratio and ABH value were screened out as the independent risk factors for HCC short-term recurrence, and the integration of these two indexes provided us with innovative factors for the assessment postoperation.

More importantly, the increase of E2F7a in HCC is overwhelming and takes the place of the prominent E2F7b expression status. This phenomenon strongly prompts a persuasive hypothesis that even though the E2F7 level is elevated significantly in HCC, the transcription modulation induced by it should change in some way based on the composition of E2F7a and E2F7b. Logically, this theory can reliably illustrate the former reports about the preventive effect on HCC formation induced by E2F7 based on the total E2F7 depletion without being impacted by the E2F7a/b ratio.

To investigate this probable mechanism, we focused on KLF13, which is also an effective transcription factor remarkably overexpressed in HCC and was predicted as a potential upstream regulator of Anillin. As acknowledged, KLF13 belongs to the family of Krüppel-like factors (KLF) containing conserved zinc finger domains for regulating transcriptional activity. KLF13 has been reported as highly expressed in oral cancer cells and significantly promotes cell proliferation.[31] Recently, it has been described as a promoter in HCC overexpressed and mediating and enhancing HMGCS1-related cholesterol biosynthesis.[32] Briefly, we first validated the direct interaction between KLF13 and the promoter region of the Anillin gene. The upregulation of KLF13 efficiently promotes the transcription of Anillin. Secondly, E2F7b, but not E2F7a, was verified to exert the suppressive function on KLF13 transcription. The ectopic increase of E2F7b in the Hep3B cells significantly induced a decrease in KLF13 mRNA expression. On the contrary, by depleting E2F7a in the Hep3B cells, even though the total level of E2F7 expression was turned down with significance, no KLF13 mRNA level change and consequential Anillin expression modulation were observed. All these findings indicated that the E2F7a isoform has no practical effect on the KLF13 gene, and the ratio of E2F7a/b could be considered a representative marker index for describing the microenvironment intrahepatically during the HCC process and specially applied for assessing the short-term recurrence after surgery.

In summary, to further observe the early stages of the microenvironment for evaluating the HCC recurrence, we developed the detection of the E2F7a/b ratio. The gradient ascending of the E2F7a/b ratio not only explains the expression contradiction of E2F7 as a regulator in HCC-related depolyploidization, but also presents a much more precise strategy to indicate the attenuation of binuclear hepatocytes in the paracancerous microenvironment by integrating with the ABH value for the risk evaluation for short-term HCC recurrence after surgery. The findings of this study may be valuable for designing individual adjuvant therapy clinically for the HCC patients for better overall survival.

AUTHOR CONTRIBUTIONS

YFZ and JQW wrote the article; YQL and FJH contributed to the data analysis and biomolecular experiments; NW and XCF were in charge of the pathological experiments and data mining; XCF and YFZ also worked on the collection of clinicopathological features YJC and JQW designed and directed the study.

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DISCLOSURES

No potential competing interests were disclosed.

DATA AVAILABILITY STATEMENT

Data from this study is available on request from the authors.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Informed consent was obtained, and the study was approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine, following the Declaration of Helsinki.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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