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Identification of genes involved in the regulation of *TERT* in hepatocellular carcinoma

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Telomerase reverse transcriptase (*TERT*) promotes immortalization by protecting telomeres in cancer cells. Mutation of the *TERT* promoter is one of the most common genetic alterations in hepatocellular carcinoma (HCC), indicating that *TERT* upregulation is a critical event in hepatocarcinogenesis. Regulators of *TERT* transcription are, therefore, predicted to be plausible targets for HCC treatment. We undertook a genome-wide shRNA library screen and identified C15orf55 and C7orf43 as regulators of *TERT* expression in HepG2 cells. Promoter assays showed that C15orf55- and C7orf43-responsive sites exist between base pairs −58 and +36 and −169 and −59 in the *TERT* promoter, respectively. C15orf55 upregulates *TERT* expression by binding to two GC motifs in the SP1 binding site of the *TERT* promoter. C7orf43 upregulates *TERT* expression through Yes-associated protein 1. The expression levels of *C15orf55* and *C7orf43* also correlated with that of *TERT*, and were significantly increased in both HCC tissues and their adjacent non-tumor tissues, compared to normal liver tissues from non-HCC patients. Analysis of 377 HCC patients in The Cancer Genome Atlas dataset showed that overall survival of patients with low levels of *C15orf55* and *C7orf43* expression in tumor tissues was better compared with patients with high levels of *C15orf55* and/or high *C7orf43* expression. These results indicate that C15orf55 and C7orf43 are involved in the incidence and progression of HCC by upregulating *TERT*. In conclusion, we identified C15orf55 and C7orf43 as positive regulators of *TERT* expression in HCC tissues. These genes are promising targets for HCC treatment.

KEYWORDS
C15orf55, C7orf43, hepatocellular carcinoma, telomerase reverse transcriptase, transcription

Abbreviations: AMOTL2, angiomotin-like protein 2; BRD4, bromodomain-containing protein 4; CLEC4C, C-type lectin domain family 4 member C; EGR1, early growth response protein 1; GABPA, GA-binding protein alpha chain; HCC, hepatocellular carcinoma; HIF1, hypoxia-inducible factor 1; LZTR1, leucine zipper like transcription regulator 1; SLC22A16, solute carrier family 22, member 16; TCGA, The Cancer Genome Atlas; TERT, telomerase reverse transcriptase; YAP1, Yes-associated protein 1.

1 | INTRODUCTION

Telomerase, consisting of TERT and telomerase RNA component, adds telomeric repeat DNA sequences (TTAGGG) at the end of eukaryotic linear chromosomes to protect DNA from end-to-end fusion, rearrangement, and translocation.[1,2] Cell mitosis results in progressive shortening of telomeres and leads to cell senescence; therefore, immortal cells constitutively activate telomerase to maintain telomere length and escape from telomere-induced replicative senescence.[3-6] A recent study clarified that increased expression of *TERT* also promotes immortalization by bypassing oncogene-induced senescence.[7] This is consistent with the observation that *TERT* gene expression is activated in more than 90% of cancers, irrespective of tumor type.[8]

Hepatocellular carcinoma is the third leading cause of cancer-related death.[9] Hepatocellular carcinoma and cirrhotic premalignant lesions frequently show increased expression of *TERT*, whereas mature and senescent hepatocytes have low *TERT* expression.[6,10-13] The difference in *TERT* expression between these 2 tissue types suggests that telomerase reactivation plays an important role in hepatocarcinogenesis.[14] Indeed, *TERT* promoter mutation resulting in activation of *TERT* expression is the most frequent genetic alteration in HCC and is seen in 61% of HCC tissues and in 19% of premalignant nodules.[11,15,16] These results indicate that *TERT* is a possible target for HCC therapy.[17] However, a direct telomerase inhibitor was frequently associated with adverse events and failed to improve the prognosis of non-small-cell lung cancer patients in a phase II study.[18] *TERT* expression is the primary determinant regulating the telomerase activity in cancer cells[12] and a number of studies examining *TERT* expression in cancer have been published, most of which have investigated promoter mutations and chromosomal rearrangements.[19] Suppression of *TERT* transcription is a reasonable approach to target telomerase; therefore, it is important to identify the genes that regulate *TERT* transcription.

In the present study, we undertook an unbiased screen of *TERT* promoter activators using a genome-wide shRNA library. This screening system succeeded in identifying 2 previously unrecognized activators of *TERT* transcription. These activators are expected to be novel therapeutic targets for HCC.

2 | MATERIALS AND METHODS

2.1 | Plasmids and lentiviruses

Short hairpin RNA expression vectors for C15orf55, C7orf43, F2, SLC22A16, CLEC4C, and LZTR1, and expression vectors for C15orf55 and C7orf43 and their negative controls (sh-empty and pcDNA6-empty) were used in this study. Firefly luciferase reporter vectors driven by multiple lengths of the *TERT* promoter are described in Figure S1. The lentiviruses were generated based on former shRNA vectors using Second Generation Packaging Mix (Applied Biological Materials, Richmond, Canada) and 293T cells. All constructs were verified by direct DNA sequencing.

2.2 | Cell lines and cell culture

HepG2 cells were maintained in DMEM supplemented with 10% FBS. HepG2 sublines were established by transfection of the cells with pcDNA6-Empty (OE-Empty), pcDNA6-C15orf55 (OE-C15orf55), and pcDNA6-C7orf43 (OE-C7orf43).

2.3 | Short hairpin RNA library screening

A lentivirus-based shRNA library (hGW shRNA Library Module 2; Cellecta inc., Mountain View, CA) was transduced into HepG2 cells, which were doubly transfected with both *TERT* promoter-driven GFP and an exogenous *TERT* expression vector to maintain steady growth of cells. Selection with puromycin and cell-sorting was carried out to detect cells that suppressed *TERT* promoter activity.

2.4 | Patient samples

Liver tumor tissue and adjacent nontumor liver tissue from 65 HCC patients were acquired following surgery for HCC between 2007 and 2011 at the Tottori University Hospital (Yonago, Japan). The study was approved by the ethical committee of Tottori University (1610A118). The data of 377 HCC patients in TCGA dataset (https://cancergenome.nih.gov/) were also examined.

2.5 | Statistical analysis

All statistical analyses were carried out using R software (version 3.1.3; www.r-project.org/). $P < .05$ was considered to indicate significant difference. Additional information is provided in Appendix S1.

3 | RESULTS

3.1 | Identification of C15orf55 and C7orf43 as activators of the *TERT* promoter

To identify genes that activate *TERT* transcription, we carried out an shRNA screen of HepG2 human HCC cells that were genetically modified to express exogenous *TERT* to compensate for the decreased expression of endogenous *TERT* by shRNA (Figure 1A). One hundred clones with low *TERT* promoter activity were obtained after shRNA-transduction of HepG2 cells. From these clones, 6 genes were identified as potential activators of *TERT* transcription (Tables S1 and S2). The expression of *CLEC4C* was not high enough to be detected in HepG2 cells by RT-PCR; therefore, this gene was omitted from further analysis (data not shown). After confirmation of successful shRNA-induced downregulation of the genes *F2*, *C15orf55*, *C7orf43*, and *LZTR1* (Figure 1B), shRNA-induced downregulation experiments selected C15orf55 and C7orf43 as activators

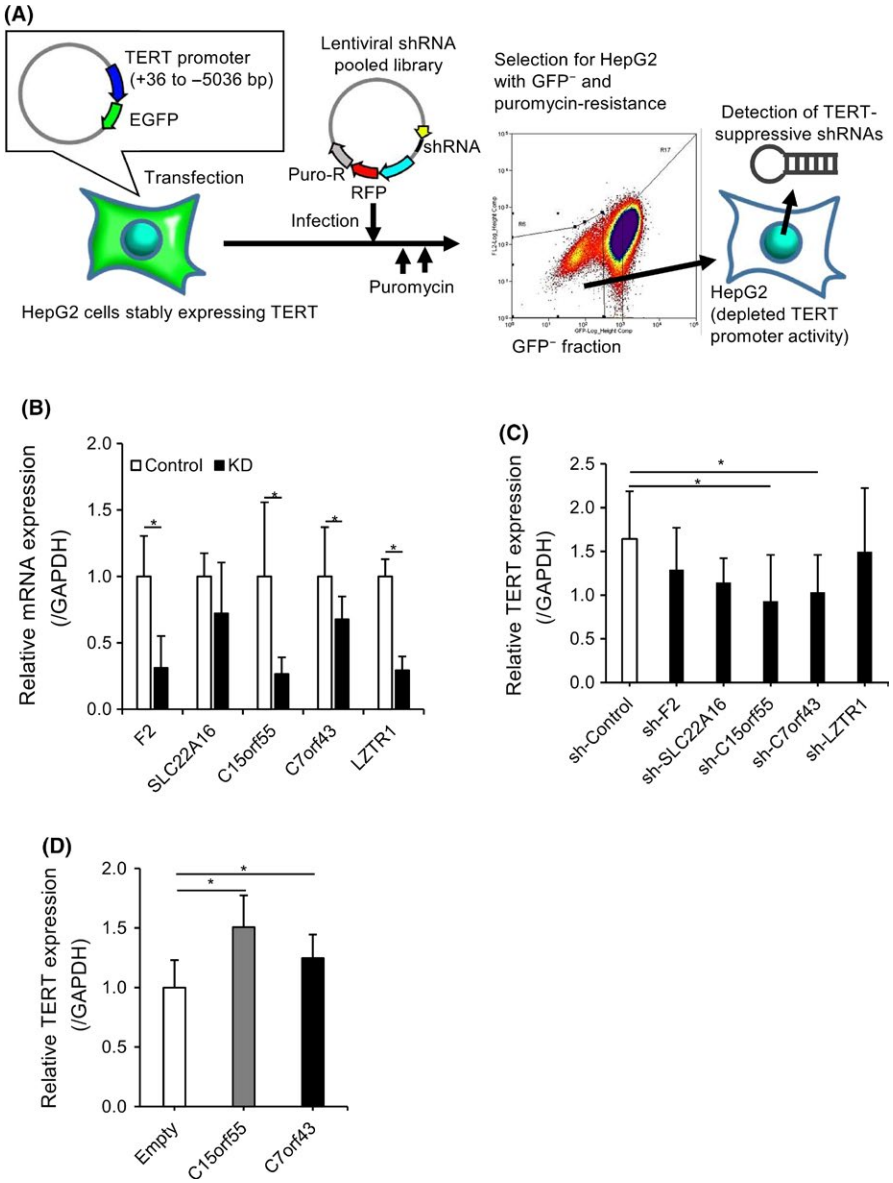

FIGURE 1 Lentiviral shRNA screening identified C15orf55 and C7orf43 as activators of *TERT* expression. A, Outline of the shRNA screening strategy. B, Efficiency of shRNA gene knockdown (KD). All genes except *SLC22A16* were significantly knocked down by shRNAs. C, Downregulation of *TERT* mRNA levels by shRNA KD. Two shRNAs, to *C15orf55* and *C7orf43*, produced significant suppression of *TERT* expression. D, Upregulation of *TERT* mRNA levels by transient overexpression of C15orf55 and C7orf43. Results expressed as the mean ± SD, n = 3. **P* < .05

of *TERT* transcription (Figure 1C, Table S3). The transient expression of C15orf55 induced significantly higher levels of *TERT* mRNA expression than control vector in all HCC cell lines including HepG2, HLE, HuH7, HuH6, and PLC/PRF/5 (Figures 1D and S2). C7orf43 also showed significantly higher *TERT* mRNA expression in these cell lines, except for that in HuH7 cells, in which *TERT* expression was not significantly, but slightly increased (Figures 1D and S2). Stably transfected OE-C15orf55 and OE-C7orf43 clones showed significantly higher *TERT* expression compared with OE-Empty (Figures 2A and S3). Telomerase activity was also increased in OE-C15orf55 and OE-C7orf43 cells compared with OE-Empty control cells (Figure 2B).

3.2 | Effect of C15orf55 and C7orf43 on HepG2 cell proliferation

As TERT promotes cell proliferation through several pathways,[20-22] we attempted to investigate the effect of C15orf55 and C7orf43 on cell proliferation. Both OE-C15orf55 and OE-C7orf43 clones grew faster in a plane culture, and formed larger colonies in a methylcellulose medium than OE-Empty clone did (Figures 3A and S4). The population-doubling time of OE-C15orf55 and OE-C7orf43 clones was shorter than that of OE-Empty (OE-C15orf55, 29.8 hours; OE-C7orf43, 30.0 hours, and OE-Empty, 37.1 hours; Figure 3A). The shRNA-mediated knockdown of *C15orf55* or *C7orf43* in HepG2 cells resulted in a significant reduction in the number of colonies, compared with that of control shRNA (Figure 3B,C).

3.3 | C15orf55 and C7orf43 recognize different promoter sites to activate *TERT* expression

To identify the sites responsible for C15orf55- and C7orf43-induced activation of the *TERT* promoter, we generated luciferase reporter constructs containing 5, 1.2, 0.7, 0.4, 0.2, and 0.1 kb of the *TERT* promoter (#1 to #7). HepG2 cells were cotransfected with these

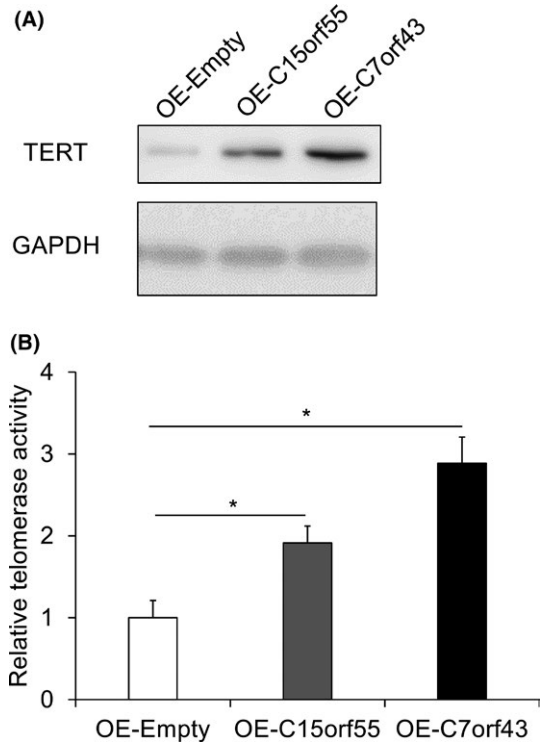

FIGURE 2 HepG2 cells with stable C15orf55 (OE-C15orf55) or C7orf43 (OE-C7orf43) expression have activated *TERT* and telomerase expression. A, Western blot analysis for OE-C15orf55 and OE-C7orf43. B, Quantitative telomerase repeat amplification protocol assay. Results are expressed as the mean ± SD, n = 3. *$P < .05$

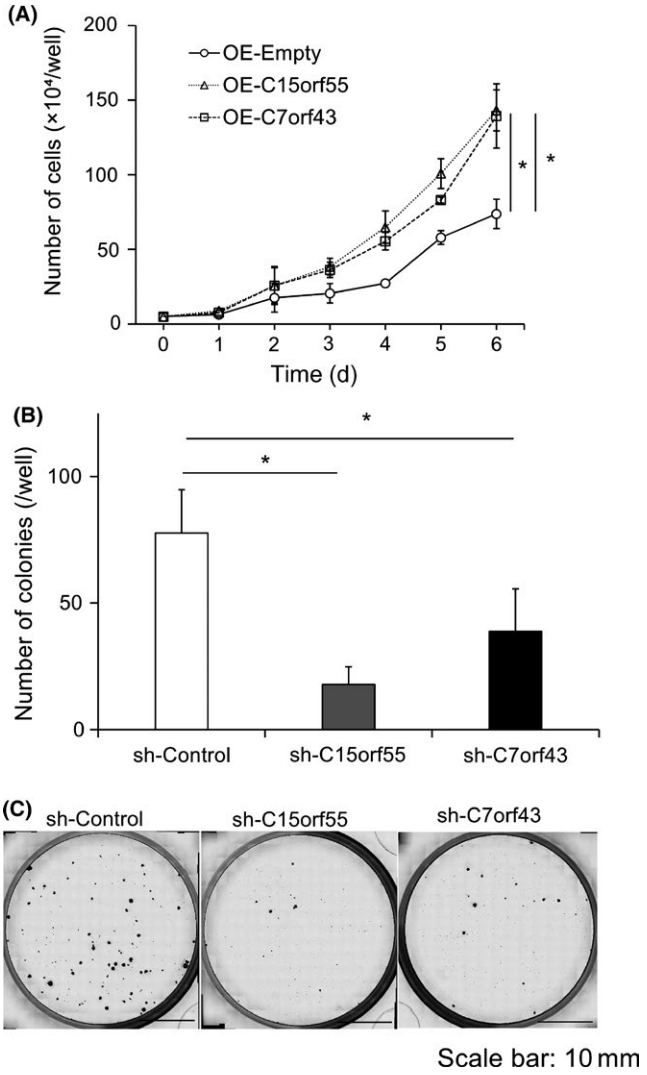

FIGURE 3 Overexpression of C15orf55 or C7orf43 promotes cell proliferation, whereas knockdown (KD) of C15orf55 or C7orf43 inhibits colony formation ability. A, Cell proliferation plots for cells stably expressing each gene. n = 4. B, Colony formation assay using shRNA lentivirus. Number of colonies 20 days after induction of shRNAs using lentivirus; n = 3. C, Typical colonies in the colony formation assay. Scale bar = 10 mm. Results are expressed as the mean ± SD. *$P < .05$

reporter vectors and C15orf55 or C7orf43 expression vectors, and luciferase activity was measured. Transfection of C15orf55 significantly activated promoters #1 to #7 of the *TERT* gene. C7orf43 transfection also activated promoters #1 to #6, but failed to activate promoter #7 (Figure 4A,B). A double transfection of C15orf55 and C7orf43 activated the *TERT* promoter to a greater extent than a single transfection of each one (Figure 4C), suggesting that C15orf55 and C7orf43 independently activate the *TERT* promoter at different sites. These results indicate that C15orf55- and C7orf43-responsive sites exist between base pairs −58 and +36, and −169 and −59 of the *TERT* promoter, respectively.

3.4 | Involvement of SP1 in *TERT* promoter activation by C15orf55

The region of the *TERT* promoter responsible for C15orf55-induced activation (−58 to +36) contains 2 GC motifs, a region to which SP1 binds to induce *TERT* expression.[23] To investigate whether C15orf55 regulates *TERT* transcription through SP1 on the GC motifs, 2 reporter vectors carrying 1 or the other of the GC motifs were constructed (Figure S1B). C15orf55 was shown to activate *TERT* expression through both GC motifs (Figure 5A). Furthermore, mithramycin, a competitive inhibitor of SP1 for binding to the GC motif,[24] prevented C15orf55-mediated activation of the *TERT* promoter (Figure S5A). The siRNA-mediated SP1 knockdown canceled C15orf55-induced activation of the TERT promoter (Figure S5B,C). Consistent with this observation, significantly more SP1 was recruited to the GC motifs in the *TERT* promoter in OE-C15orf55 cells than in OE-Empty cells (Figure 5B). These data indicate that C15orf55 activates *TERT* expression through SP1 on the promoter (Figure 5F).

3.5 | Involvement of YAP1 in *TERT* promoter activation by C7orf43

Although our luciferase assay indicated that the region from −169 to −59 bp of the *TERT* promoter is responsible for C7orf43-induced activation of the promoter, only a few transcription factors that

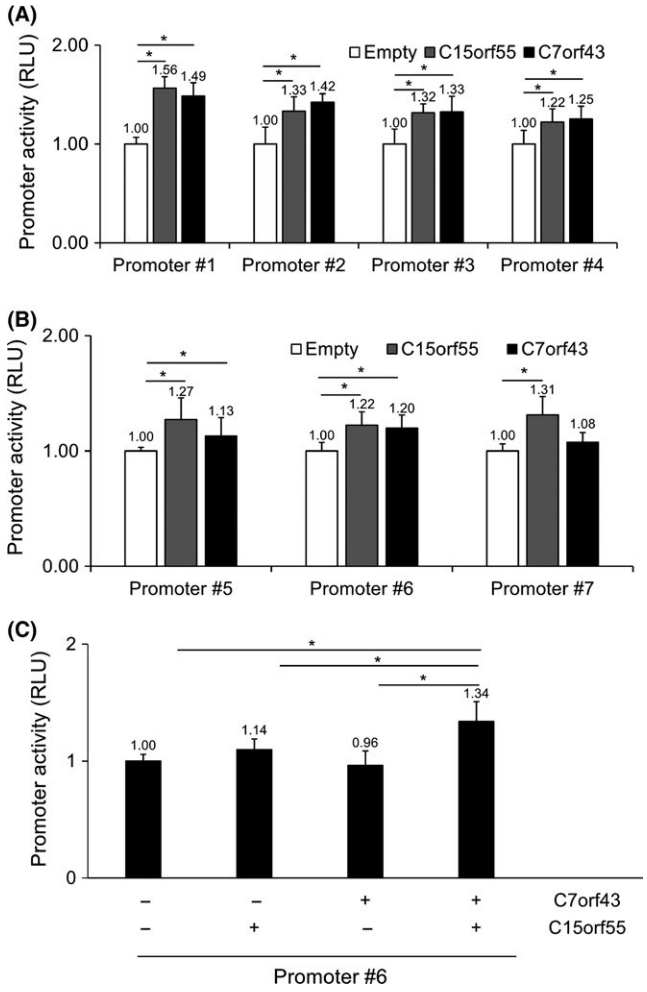

FIGURE 4 C15orf55 and C7orf43 interact with different sites in the *TERT* promoter. A,B, Luciferase reporter assays to determine the response sites of each gene. Expression vectors were cotransfected into HepG2 cells with a luciferase reporter plasmid containing the designated length of *TERT* promoter. Data are normalized with the control in each assay. C, Luciferase reporter assay to show a synergic effect of C15orf55 and C7orf43. Half the amount of each expression vector used in the reporter assays shown in A and B were cotransfected. Average values are shown above each bar charts. Results are expressed as the mean ± SD; n = 3. *P < .05. RLU, relative light unit

bind to this region have been reported, including GABPA, E2F1, HIF1, and paired box family 8.[19] To determine the site responsible for C7orf43-induced activation, this region was divided into 6 sequences (fragments #1-#6, Figure S1C). Luciferase reporter assays using these reporter vectors revealed that C7orf43 activated fragment #3 containing unknown transcription factor binding sites, and fragment #6 containing the C228T mutation-dependent GABPA-binding site (Figure 5C).[25] The JASPAR program indicated that transcription factor EGR1 might bind to fragment #3 (JASPAR 2018 database, http://jaspar.genereg.net/). Yes-associated protein 1 is commonly associated with EGR1 and GABPA.[26,27] Moreover, C7orf43 interacts with *AMOTL2*, which is a suppressor of YAP1 and a target gene of the Hippo pathway.[28,29] To evaluate the effect of C7orf43 overexpression on YAP1 activity, the expression and nuclear localization of YAP1 were examined in OE-C7orf43 cells. As expected, the protein levels of YAP1 and AMTOL2 were upregulated, whereas C7orf43 did not affect YAP1 phosphorylation, which represses YAP1 activity through the cytosolic retention of YAP1 (Figure 5D).[30] Nuclear localization of YAP1 was also significantly increased in OE-C7orf43, compared to OE-Empty cells, as indicated by immunofluorescent staining and immunoblotting of nuclear extracts (Figures 5E and S6). In addition, it was shown that C7orf43-induced activation of *TERT* promoter was canceled by siRNA-mediated knockdown of YAP1 (Figure S7). Our data indicate that C7orf43 activation of YAP1 is dominant over AMOTL2-mediated suppression of YAP1. Taken together, these data indicate that C7orf43 activates the *TERT* promoter via YAP1 (Figure 5F).

3.6 | C15orf55 and C7orf43 have clinical significance for tumorigenesis and patient survival

To determine the clinical importance of *C15orf55* and *C7orf43*, gene expression was examined in HCC specimens collected by our institution. The clinical and pathological characteristics of 65 patients are shown in Table 1. The gene expression of *C15orf55*, *C7orf43*, and *TERT* is also shown (Figures 6, 7A-D, S8, and S9). Increased expression levels of *C15orf55* and *C7orf43* were seen in nontumor liver tissues as well as tumor tissues, compared with normal liver tissues from non-HCC patients (Figure 6A,B). In the nontumor liver tissues, expression of *C15orf55* was significantly associated with a lower Child-Pugh score (P = .046; Figure 6C). Although it did not reach statistical significance, *C7orf43* expression also showed a tendency to be associated with liver cirrhosis (P = .197; Figure 6D). The expression levels of *C15orf55* and *C7orf43* were significantly correlated with *TERT* expression levels both in nontumor and tumor tissues of HCC patients (Figure 7A-D). Although the detection rate of *TERT* mRNA in tumor and nontumor tissue appears to be low, *TERT* mRNA levels were also reported to be very low or undetectable in previous studies.[31,32] Given the existence of the correlation in nontumor tissues in which the frequency of *TERT* promoter mutation is quite low,[11] C15orf55 and C7orf43 could play a role in *TERT* transcription independently from the mutation. These data indicate that C15orf55 and C7orf43 are involved in tumor progression, and also in the reactivation of telomerase toward tumorigenesis in damaged livers without the *TERT* promoter mutation. In agreement with this notion, C15orf55 and C7orf43 activated the WT *TERT* promoter to the same extent as mutant promoters, including the C228T mutation (Figure S10).

To further investigate the impact of C15orf55 and C7orf43 on the prognosis of HCC patients, survival data from TCGA database, in which 377 patients are enrolled, were analyzed because the small population enrolled at our institution has limited statistical power to estimate patients' prognosis (Figure S11). The HCC patients with high levels of *C15orf55* and/or *C7orf43* expression tended to have a poorer prognosis compared with those with low expression levels of both *C15orf55* and *C7orf43* (Figures 7E,F and S11).

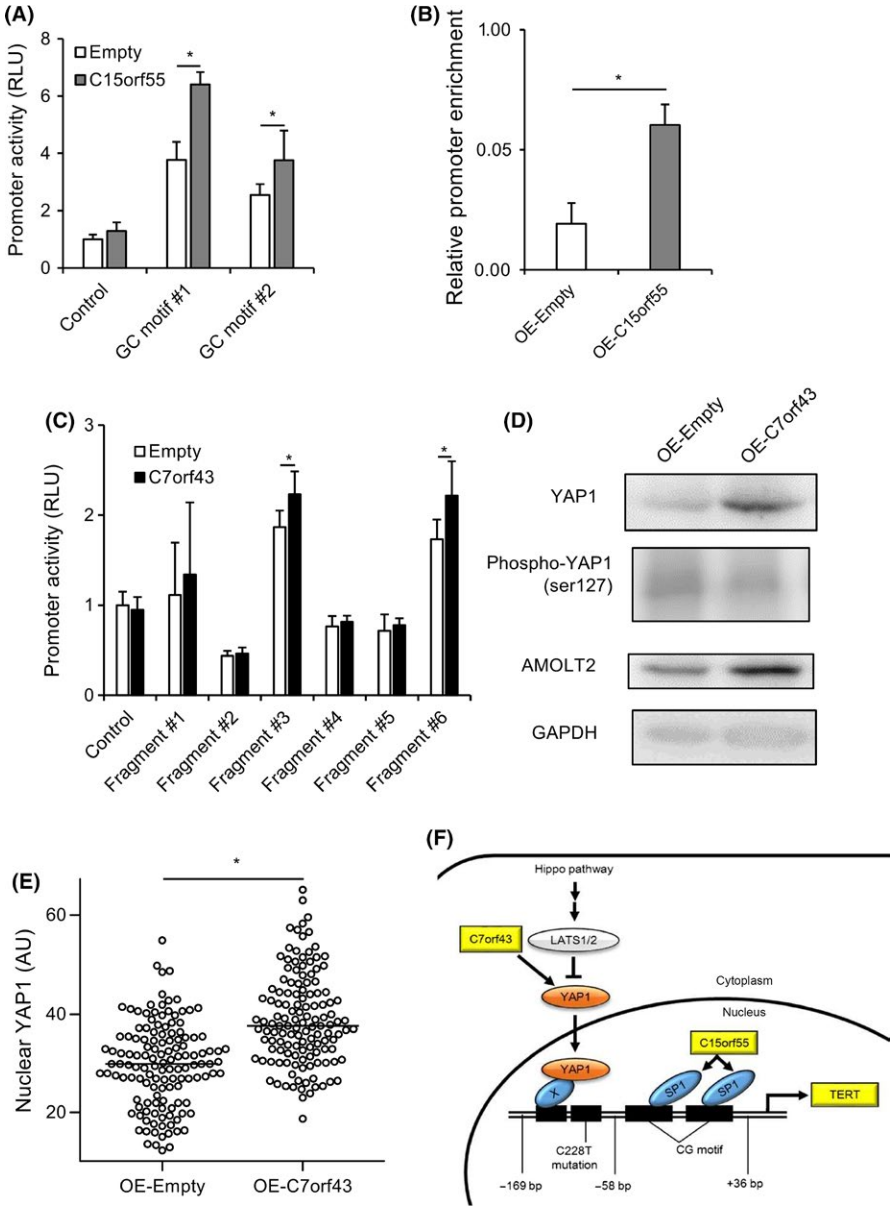

FIGURE 5 C15orf55 and C7orf43 activate the *TERT* promoter through SP1 and Yes-associated protein 1 (YAP1), respectively. A, Reporter assay using luciferase driven by GC motifs of the *TERT* promoter region. B, Recruitment of SP1 to the GC motifs in the *TERT* promoter. Chromatin immunoprecipitation was carried out using an anti-SP1 antibody. Quantitative PCR was carried out with primers for two GC motifs in the *TERT* promoter. Promoter enrichment is shown relative to input. C, Reporter assay to detect C7orf43 response site. Fragments #3 and #6 are shown to have C7orf43 responsiveness. D, Western blot analysis for YAP1 and YAP1-related protein. E, Intensity of nuclear YAP1 by immunofluorescence analysis of OE-Empty and OE-7orf43 cells. F, Schematic diagram depicting *TERT* activation by C15orf55 and C7orf43. Results are expressed as the mean ± SD; n = 3. *$P < .05$

4 | DISCUSSION

Expression of *TERT* is critical for the reactivation of telomerase, which plays an important role in the development and proliferation of cancer cells through telomere protection and avoidance of senescence. However, the mechanism by which hepatocytes acquire telomerase reactivation at the initiation of cancer remains largely unclear. Interestingly, a small subpopulation of hepatocytes with elevated *Tert* expression and telomerase activity exists in the liver of healthy mice to drive the regeneration and renewal of the liver,[33] indicating that telomerase reactivation could be an intrinsic function in progenitor hepatocytes rather than resulting from genomic change, such as mutation or methylation.

In the present study, we used a lentivirus-based shRNA library. The pooled shRNA library is a valuable tool for gene screening. It has identified NQQ1 as a potential drug target for host-directed tuberculosis therapy, *ATP1A1* as a gene that regulates aurilide B cytotoxicity, and the deubiquitinating enzyme, USP5, which modulates cell cycle regulators.[34-36] The shRNA screening system has also identified HIF1α as a *TERT* regulator using mouse ES cells.[37]

In this study in a HCC cell line, we identified two genes, *C15orf55* and *C7orf43*, as activators of *TERT* transcription through SP1 and YAP1, respectively. Although the involvement of these genes in *TERT* transcription and in the development and progression of HCC has not been reported, we found that expression of *C15orf55* and *C7orf43* was upregulated and correlated with *TERT* expression in tumor tissues and surrounding liver tissues from HCC patients. The Cancer Genome Atlas database also showed that expression levels of *C15orf55* and *C7orf43* in HCC are significantly correlated with poor prognosis of HCC patients. Consistent with this observation, overexpression of either *C15orf55* or *C7orf43* enhanced the proliferation of HepG2 cells in accordance with

TABLE 1 Characteristics of 65 patients who underwent surgery for hepatocellular carcinoma between 2007 and 2011

Characteristic	n	Characteristic	n
Number of patients	65	Serosal invasion (n = 63)	
Age, y	70 (62-76)	Negative	41
Gender		Positive	22
Male	57	Portal vein invasion (n = 63)	
Female	8	Negative	49
Etiology		Positive	24
HBV	28	Hepatic vein invasion (n = 63)	
HCV	13	Negative	59
Non-B/non-C	24	Positive	4
Total bilirubin, mg/dL	0.6 (0.5-0.8)	Bile duct invasion (n = 63)	
Albumin, g/dL	3.9 (3.7-4.2)	Negative	61
AST, IU/L	34 (24-46)	Positive	2
ALT, IU/L	28 (19-38)	TNM stage	
AFP, ng/mL	8.2 (3.4-71.4)	IA	7
Child-Pugh score		IB	22
5	51	II	23
6	11	IIIA	7
7	3	IIIB	6
Number of tumors		Fibrosis stage (f; n = 61)	
1	52	0	12
2	12	1	18
≥3	1	2	10
Tumor size, cm	4.0 (2.5-7.0)	3	5
Survival period, y	4.3 (2.9-7.3)	4	16
Differentiation		Gene expression	
Well	2	*C15orf55* in tumor	8.6 (1.0-98.0)
Moderate	58	*C15orf55* in nontumor	3.30 (0.04-37.38)
Poor/Undifferentiated	5	*C7orf43* in tumor	10.7 (1.7-92.2)
Capsular invasion (n = 63)		*C7orf43* in nontumor	2.2 (0.4-23.4)
Negative	52	*TERT* in tumor (n = 27)	0.6 (0.1-1.1)
Positive	11	*TERT* in nontumor (n = 16)	0.07 (0.01-0.31)

Values for age, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), α-fetoprotein (AFP), tumor size, survival period, and gene expression are provided as the median and interquartile range. Relative gene expression levels of *C15orf55* and *C7orf43* to *ACTB* were normalized against that in normal liver. Relative gene expression levels of *TERT* to *ACTB* were normalized against that in tumor.
HBV, hepatitis B virus; HCV, hepatitis C virus.

increased TERT and telomerase activity. Telomerase promotes cell proliferation by stabilizing telomeres.[20] Moreover, TERT itself controls the activity and expression of cell proliferation-related factors such as pRB, E2F, β-catenin, and p65.[21,22] Taking these reports into consideration, the upregulation of TERT is a critical mechanism underlying the C15orf55- and C7orf43-induced cell proliferation. Our findings suggest that *C15orf55* and *C7orf43* are previously unrecognized factors for the reactivation of telomerase in HCC. These genes are potential therapeutic targets for a novel telomerase-directed therapy. An intervention targeting C15orf55 and/or C7orf43 might be beneficial in preventing carcinogenesis and tumor progression.

C15orf55, also known as *NUTM1*, is a testis-specific protein with totally unknown function.[38] However, it is not surprising that C15orf55 promotes carcinogenesis and proliferation of cancer cells because the fusion of *C15orf55* with *BRD4*, which encodes a bromodomain-containing protein, is commonly found in a rare carcinoma arising in midline organs (OMIM: 608963).[39,40] Although the domain corresponding to C15orf55 of the C15orf55-BRD4 fusion protein activated p300 histone acetyltransferase and chromatin remodeling,[40] no attenuation of C15orf55-induced promoter activation was found in a reporter assay using C646, a specific inhibitor of CBP/P300 (data not shown). These data suggest that C15orf55 activates the *TERT* promoter in a CBP/P300-independent manner.

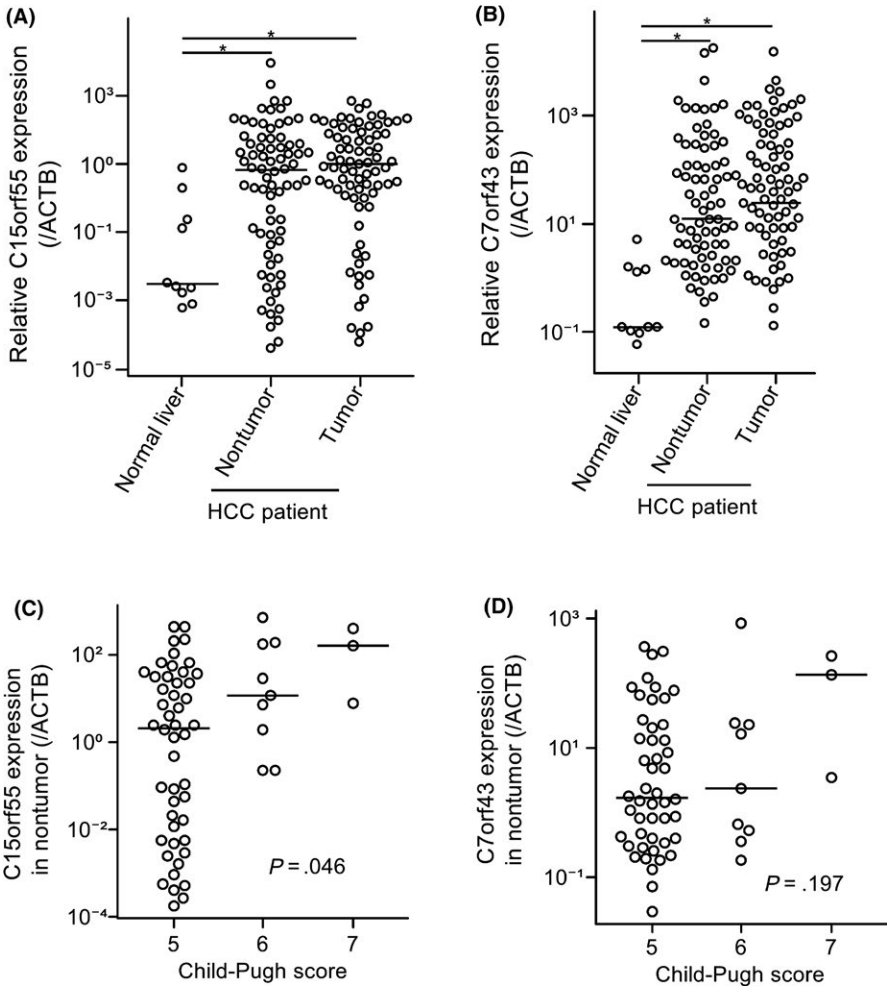

FIGURE 6 C15orf55 and C7orf43 are upregulated in both tumor and nontumor tissues from hepatocellular carcinoma (HCC) patients. A, B, Comparison between normal liver from benign liver disease patients and tumor-adjacent tissue (nontumor) or tumor from HCC patients. C,D, Relationship between gene expression and Child-Pugh score, a clinical surrogate marker for the degree of cirrhosis and hepatitis. Relative gene expression levels of *C15orf55* and *C7orf43* to *ACTB* were normalized against that of normal liver

Instead, we found that SP1 is a critical transcription factor for C15orf55-induced activation of the *TERT* promoter. SP1 plays an important role in the transcriptional regulation of *TERT*.[22] Although a number proteins interact with SP1, little is known about a mechanism that regulates SP1 to activate the transcription of *TERT*. In this study, C15orf55 was identified as a novel upstream factor of SP1.

C7orf43, the other regulator of *TERT* transcription identified in our study, is also an uncharacterized protein. We found that C7orf43 activates YAP1, the main downstream effector of the Hippo signaling pathway, whose activation negatively regulates YAP1. *YAP1* is suggested to be an oncogene in humans because its upregulation and nuclear localization have been observed in multiple types of human cancer.[41-47] Moreover, knockdown or deletion of Hippo pathway components, such as Mst1/2, in liver lead to the activation of YAP1 and the development of HCC.[48] Before this study, interaction between YAP1 and C7orf43 was unknown; however, an interactome analysis showed that C7orf43 interacts with AMOTL2.[49] The latter suppresses YAP1 by tethering it in the cytoplasm.[28] Note that *AMOTL2* is also the target gene of YAP1 as a negative feedback regulator of the Hippo pathway.[27,28] As shown in Figure 5C, C7orf43 induced AMOTL2 expression, but YAP1 was still activated, suggesting that the negative feedback regulation might be abrogated by C7orf43. Therefore, we reason that C7orf43 functions as a suppressor of AMOTL2, thereby indirectly activating YAP1.

Expression of *C15orf55* and *C7orf43* was upregulated in nontumor tissues and in tumor tissues of HCC, and was correlated with *TERT* expression. Because hepatocytes in the nontumor tissue have a much lower mutation rate in the *TERT* promoter than HCC cells,[11] this finding indicates that both C15orf55 and C7orf43 induce *TERT* transcription in hepatocytes in cirrhotic livers in a mutation-independent manner. Consistently, a reporter assay showed that C15orf55 and C7orf43 significantly activated the WT *TERT* promoter (without C228T mutation) (Figure S10). However, activation of the WT *TERT* promoter by C7orf43 was significantly lower than that of the C228T mutant promoter, suggesting that C7orf43 also activates the *TERT* promoter in a mutation-dependent manner. This is consistent with C7orf43 activating both fragments #3 and #6, the latter containing the C228T mutation-dependent GABPA-binding site. Although mutation of the *TERT* promoter plays critical roles in carcinogenesis, a recent study by Chiba et al showed that *TERT* promoter mutation does not necessarily confer sufficient activation to telomerase to immortalize precancerous cells in the early step of carcinogenesis.[50] Their finding indicates that additional factors that increase *TERT* transcription are required to fully activate telomerase

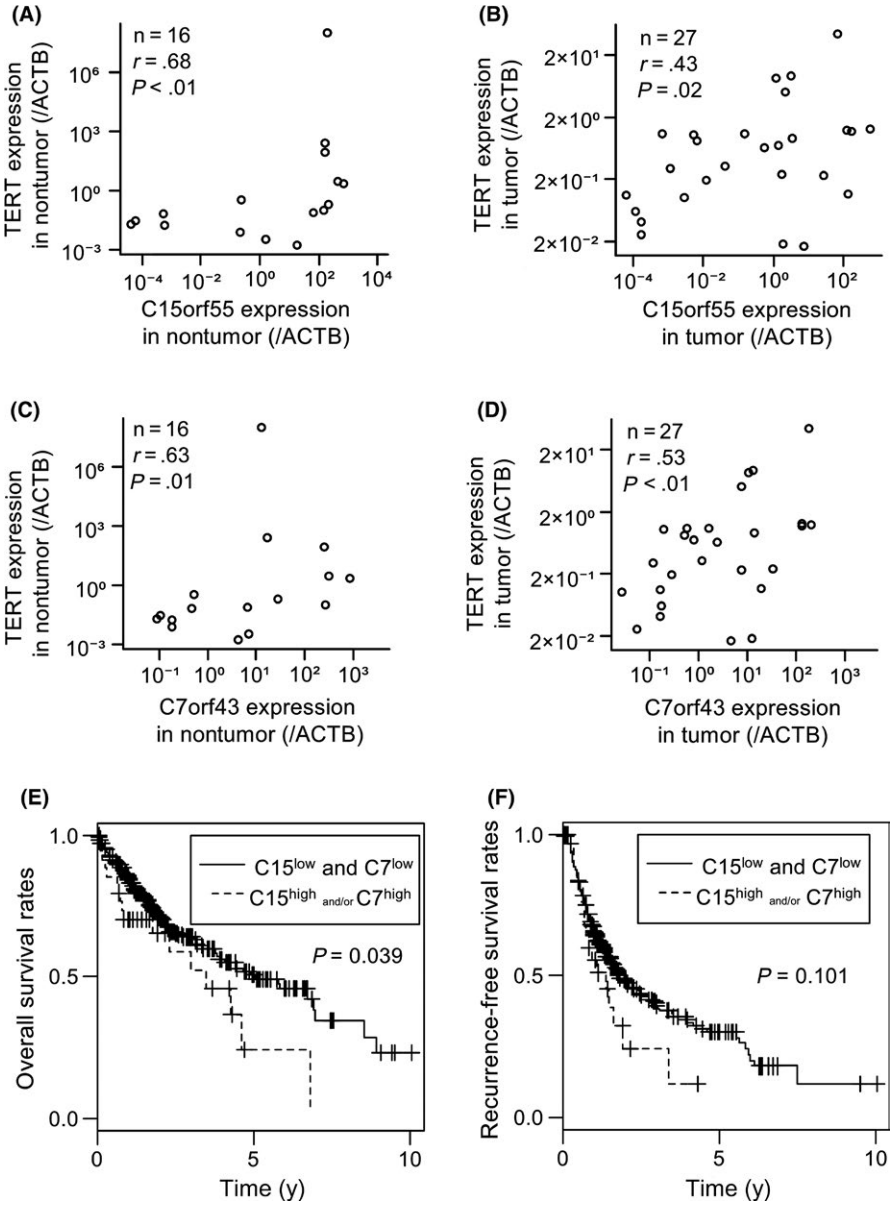

FIGURE 7 Expression of *C15orf55* and *C7orf43* correlates with *TERT* expression and patient survival. A-D, Gene expression and *TERT* expression in nontumor (A,C) and tumor (B,D) in patients with hepatocellular carcinoma. E,F, Survival curves of hepatocellular carcinoma patients from The Cancer Genome Atlas database. Overall survival rates (A), and recurrence-free survival rates (B) are shown for patients with low *C15orf55* and *C7orf43* expression ($C15^{low}$ and $C7^{low}$), and high *C15orf55* and/or high *C7orf43* expression ($C15^{high}$ and/or $C7^{high}$). Relative gene expression levels of *TERT* to *ACTB* were normalized against that in the tumor

and to immortalize the cells.[50] Our data suggest that C15orf55 and C7orf43 might be critical factors for the reactivation of telomerase in HCC, in addition to the promoter mutation.

Consistent with our in vitro observations, *C15orf55* and *C7orf43* had a negative impact on patients' prognosis. Our findings indicate that upregulation of these genes induces tumor progression through telomerase reactivation, which strongly associates with poor prognosis of HCC patients.[51,52] Based on these results, further studies to reveal the precise mechanisms in which these genes act could lead to the development of a novel telomerase inhibitor. Interestingly, it is also possible that C15orf55 and C7orf43 themselves promote cell proliferation in a TERT-independent manner, because C15orf55 and C7orf43 activate SP1 and YAP1, respectively, both of which have various functions for cell proliferation other than TERT.[46,48,53] Therefore, it is worth investigating whether C15orf55 or C7orf43 has tumorigenic potential independent of *TERT*. The mechanism of how these genes activate SP1 and YAP1 could provide useful information for a novel HCC therapy.

A recent study by Lin et al showed that a small population of hepatocytes expressing high levels of TERT ($TERT^{high}$) in healthy liver is involved in hepatocyte renewal.[33] Their transcriptome analysis revealed that several genes related to SP1 and YAP1 are significantly

upregulated in TERT^{high} hepatocytes, including *SP1* (1.67 ± 0.18 fold change compared with TERT^{low} hepatocytes), *AMOTL2* (1.65 ± 0.26), and the target gene of YAP1, *CYR61* (1.45 ± 0.31).[33] In combination with their report, the present study indicates that C15orf55 and C7orf43 function as intrinsic TERT activators in hepatocytes. Investigation of C15orf55 and C7orf43 expression and activation of SP1 and YAP1 in TERT^{high} cells could provide information for the prevention of hepatocarcinogenesis.

In conclusion, the present study identified 2 novel activators of *TERT* expression through the *TERT* promoter. Our results provide new insights into the molecular background of telomerase reactivation in HCC as well as in hepatocytes. Functional analysis of the factors related to C15orf55 and C7orf43 should be undertaken to further our understanding of the molecular mechanisms of hepatocarcinogenesis. C15orf55 and C7orf43 are also promising targets for the treatment and prevention of HCC.

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CONFLICT OF INTEREST

We declare the following conflict of interest: GS has more than 5% of the total shares of KanonCure Inc. The other authors have no competing interests.

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REFERENCES

1. Donati B, Valenti L. Telomeres, NAFLD and chronic liver disease. *Int J Mol Sci*. 2016;17:383.
2. Griffith JDCL, Rosenfield S, Stansel RM, Bianchi A, Moss H, de Lange T. Mammalian telomeres end in a large duplex loop. *Cell*. 1999;97:503-514.
3. Deng Y, Chan SS, Chang S. Telomere dysfunction and tumour suppression: the senescence connection. *Nat Rev Cancer*. 2008;8:450-458.
4. Calado RT, Young NS. Telomere diseases. *N Engl J Med*. 2009;361:2353-2365.
5. Shay JW, Wright WE. The reactivation of telomerase activity in cancer progression. *Trends Genet*. 1996;12:129-131.
6. Shay JW, Wright WE. Role of telomeres and telomerase in cancer. *Semin Cancer Biol*. 2011;21:349-353.
7. Patel PL, Suram A, Mirani N, Bischof O, Herbig U. Derepression of hTERT gene expression promotes escape from oncogene-induced cellular senescence. *Proc Natl Acad Sci U S A*. 2016;113:E5024-E5033.
8. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science*. 1994;266:2011-2015.
9. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin*. 2010;60:277-300.
10. Zucman-Rossi J, Villanueva A, Nault JC, Llovet JM. Genetic landscape and biomarkers of hepatocellular carcinoma. *Gastroenterology*. 2015;149:1226-1239.
11. Nault JC, Calderaro J, Di Tommaso L, et al. Telomerase reverse transcriptase promoter mutation is an early somatic genetic alteration in the transformation of premalignant nodules in hepatocellular carcinoma on cirrhosis. *Hepatology*. 2014;60:1983-1992.
12. Nakayama JTH, Tahara E, Saito M, et al. Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. *Nat Genet*. 1998;18:65-68.
13. Tahara HNT, Kitamoto M, Nakashio R, et al. Telomerase activity in human liver tissues: comparison between chronic liver disease and hepatocellular carcinomas. *Cancer Res*. 1995;55:2734-2736.
14. Nault JC, Zucman-Rossi J. TERT promoter mutations in primary liver tumors. *Clin Res Hepatol Gastroenterol*. 2016;40:9-14.
15. Totoki Y, Tatsuno K, Covington KR, et al. Trans-ancestry mutational landscape of hepatocellular carcinoma genomes. *Nat Genet*. 2014;46:1267-1273.
16. Fujimoto A, Furuta M, Totoki Y, et al. Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. *Nat Genet*. 2016;48:500-509.
17. Llovet JM, Hernandez-Gea V. Hepatocellular carcinoma: reasons for phase III failure and novel perspectives on trial design. *Clin Cancer Res*. 2014;20:2072-2079.
18. Chiappori AA, Kolevska T, Spigel DR, et al. A randomized phase II study of the telomerase inhibitor imetelstat as maintenance therapy for advanced non-small-cell lung cancer. *Ann Oncol*. 2015;26:354-362.
19. Ramlee MK, Wang J, Toh WX, Li S. Transcription regulation of the human telomerase reverse transcriptase (hTERT) gene. *Genes (Basel)*. 2016;7:50. https://doi.org/10.3390/genes7080050
20. Greider CW. Telomerase activity, cell proliferation, and cancer. *Proc Natl Acad Sci U S A*. 1998;95:90-92.
21. Smith LL, Coller HA, Roberts JM. Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. *Nat Cell Biol*. 2003;5:474-479.
22. Wu XQ, Huang C, He X, et al. Feedback regulation of telomerase reverse transcriptase: new insight into the evolving field of telomerase in cancer. *Cell Signal*. 2013;25:2462-2468.
23. Kyo S, Takakura M, Taira T, et al. Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). *Nucleic Acids Res*. 2000;28:669-677.
24. Sleiman SF, Langley BC, Basso M, et al. Mithramycin is a gene-selective Sp1 inhibitor that identifies a biological intersection between cancer and neurodegeneration. *J Neurosci*. 2011;31:6858-6870.
25. Bell RJ, Rube HT, Xavier-Magalhaes A, et al. Understanding TERT promoter mutations: a common path to immortality. *Mol Cancer Res*. 2016;14:315-323.
26. Zagurovskaya M, Shareef MM, Das A, et al. EGR-1 forms a complex with YAP-1 and upregulates Bax expression in irradiated prostate carcinoma cells. *Oncogene*. 2009;28:1121-1131.
27. Wu H, Xiao Y, Zhang S, et al. The Ets transcription factor GABP is a component of the hippo pathway essential for growth and antioxidant defense. *Cell Rep*. 2013;3:1663-1677.
28. Wang W, Huang J, Chen J. Angiomotin-like proteins associate with and negatively regulate YAP1. *J Biol Chem*. 2011;286:4364-4370.
29. Dupont S, Morsut L, Aragona M, et al. Role of YAP/TAZ in mechanotransduction. *Nature*. 2011;474:179-183.
30. Zhao B, Li L, Lu Q, et al. Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein. *Genes Dev*. 2011;25:51-63.

31. Nault JC, Mallet M, Pilati C, et al. High frequency of telomerase reverse-transcriptase promoter somatic mutations in hepatocellular carcinoma and preneoplastic lesions. *Nat Commun*. 2013;4:2218.
32. Ko E, Seo HW, Jung ES, Kim BH, Jung G. The TERT promoter SNP rs2853669 decreases E2F1 transcription factor binding and increases mortality and recurrence risks in liver cancer. *Oncotarget*. 2016;7:684-699.
33. Lin S, Nascimento EM, Gajera CR, et al. Distributed hepatocytes expressing telomerase repopulate the liver in homeostasis and injury. *Nature*. 2018;556:244-248.
34. Li Q, Karim AF, Ding X, et al. Novel high throughput pooled shRNA screening identifies NQO1 as a potential drug target for host directed therapy for tuberculosis. *Sci Rep*. 2016;6:27566.
35. Takase S, Kurokawa R, Arai D, et al. A quantitative shRNA screen identifies ATP1A1 as a gene that regulates cytotoxicity by aurilide B. *Sci Rep*. 2017;7:2002.
36. Kaistha BP, Krattenmacher A, Fredebohm J, et al. The deubiquitinating enzyme USP5 promotes pancreatic cancer via modulating cell cycle regulators. *Oncotarget*. 2017;8:66215-66225.
37. Coussens M, Davy P, Brown L, et al. RNAi screen for telomerase reverse transcriptase transcriptional regulators identifies HIF1alpha as critical for telomerase function in murine embryonic stem cells. *Proc Natl Acad Sci U S A*. 2010;107:13842-13847.
38. French CA. Demystified molecular pathology of NUT midline carcinomas. *J Clin Pathol*. 2010;63:492-496.
39. French CA. Pathogenesis of NUT midline carcinoma. *Annu Rev Pathol*. 2012;7:247-265.
40. Reynoird N, Schwartz BE, Delvecchio M, et al. Oncogenesis by sequestration of CBP/p300 in transcriptionally inactive hyperacetylated chromatin domains. *EMBO J*. 2010;29:2943-2952.
41. Chan SW, Lim CJ, Guo K, et al. A role for TAZ in migration, invasion, and tumorigenesis of breast cancer cells. *Cancer Res*. 2008;68:2592-2598.
42. Cordenonsi M, Zanconato F, Azzolin L, et al. The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. *Cell*. 2011;147:759-772.
43. Xu MZ, Yao TJ, Lee NP, et al. Yes-associated protein is an independent prognostic marker in hepatocellular carcinoma. *Cancer*. 2009;115:4576-4585.
44. Zender L, Spector MS, Xue W, et al. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell*. 2006;125:1253-1267.
45. Dong J, Feldmann G, Huang J, et al. Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell*. 2007;130:1120-1133.
46. Zhao B, Wei X, Li W, et al. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev*. 2007;21:2747-2761.
47. Steinhardt AA, Gayyed MF, Klein AP, et al. Expression of Yes-associated protein in common solid tumors. *Hum Pathol*. 2008;39:1582-1589.
48. Zhou D, Conrad C, Xia F, et al. Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Yap1 oncogene. *Cancer Cell*. 2009;16:425-438.
49. Couzens AL, Knight JD, Kean MJ, et al. Protein interaction network of the mammalian Hippo pathway reveals mechanisms of kinase-phosphatase interactions. *Sci Signal*. 2013;6:rs15.
50. Chiba K, Lorbeer FK, Shain AH, et al. Mutations in the promoter of the telomerase gene TERT contribute to tumorigenesis by a two-step mechanism. *Science*. 2017;357:1416-1420.
51. Kim H, Yoo JE, Cho JY, et al. Telomere length, TERT and shelterin complex proteins in hepatocellular carcinomas expressing "stemness"-related markers. *J Hepatol*. 2013;59:746-752.
52. Oh BK, Kim H, Park YN, et al. High telomerase activity and long telomeres in advanced hepatocellular carcinomas with poor prognosis. *Lab Invest*. 2008;88:144-152.
53. Li L, Davie JR. The role of Sp1 and Sp3 in normal and cancer cell biology. *Ann Anat*. 2010;192:275-283.

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