Prevention of hepatocellular carcinoma by targeting MYCN-positive liver cancer stem cells with acyclic retinoid

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Hepatocellular carcinoma (HCC) is a highly lethal cancer that has a high rate of recurrence, in part because of cancer stem cell (CSC)-dependent field cancerization. Acyclic retinoid (ACR) is a synthetic vitamin A-like compound capable of preventing the recurrence of HCC. Here, we performed a genome-wide transcriptome screen and showed that ACR selectively suppressed the expression of MYCN, a member of the MYC family of basic helix–loop–helix–zipper transcription factors, in HCC cell cultures, animal models, and liver biopsies obtained from HCC patients. MYCN expression in human HCC was correlated positively with both CSC and Wnt/β-catenin signaling markers but negatively with mature hepatocyte markers. Functional analysis showed repressed cell-cycle progression, proliferation, and colony formation, activated caspase-8, and induced cell death in HCC cells following silencing of MYCN expression. High-content single-cell imaging analysis and flow cytometric analysis identified a MYCN$^+$ CSC subpopulation in the heterogeneous HCC cell cultures and showed that these cells were selectively killed by ACR. Particularly, EpCAM$^+$ cells isolated using a cell-sorting system showed increased MYCN expression and sensitivity to ACR compared with EpCAM$^-$ cells. In a long-term (>10 y) follow-up study of 102 patients with HCC, *MYCN* was expressed at higher levels in the HCC tumor region than in nontumor regions, and there was a positive correlation between *MYCN* expression and recurrence of de novo HCC but not metastatic HCC after curative treatment. In summary, these results suggest that MYCN serves as a prognostic biomarker and therapeutic target of ACR for liver CSCs in de novo HCC.

MYCN | hepatocellular carcinoma | cancer stem cell | transcriptome | acyclic retinoid

Hepatocellular carcinoma (HCC) is the most common type of liver cancer. HCC is highly malignant and causes nearly 600,000 deaths annually (1). Chronic infection with the hepatitis B virus or hepatitis C virus (HCV) contributes to ∼60% of cirrhosis cases and 80% of all HCC cases globally (2). In addition, a growing body of evidence indicates that alcohol consumption or a high-fat diet substantially increases the risk of HCC associated with impaired immune surveillance (3–5).

HCC carries a very poor prognosis. Only ∼10% of patients survive the first 5 y after diagnosis, and this is partly because of its high rate of recurrence (6). The field cancerization concept proposes that all liver tissue is at high risk for liver carcinogenesis in patients with chronic liver disease (7). A specialized subpopulation of highly tumorigenic cells that reside in precancerous tissues has been defined as cancer stem cells (CSCs) or tumor-initiating cells (TICs) and is thought to contribute to the development and recurrence of HCC (8). CSCs can be identified based on their differentiation stage using specific markers such as EpCAM (9, 10) and CD133 (11). Functional analyses have revealed that two critical features of CSCs are their ability to initiate new tumors and their resistance to therapeutic killing, both of which make them potential targets for cancer therapy and prevention strategies (12). These data led to the use of clonal deletion and inhibition therapy, which is aimed at preventing HCC by targeting liver CSCs (13).

Retinoids are natural and synthetic derivatives of vitamin A. Acyclic retinoid (ACR) is capable of preventing the recurrence of HCC in HCV-positive patients who have undergone curative removal of the primary tumors (14). ACR prevents carcinogenesis in rat livers by selectively killing oval-like cells (15). The

Significance

Hepatocellular carcinoma (HCC) is a highly lethal cancer, partly because of its high rate of recurrence, which is caused by the presence of liver cancer stem cells (CSCs). Here, using a selective chemopreventive agent, acyclic retinoid (ACR), as a bioprobe, we identified *MYCN*, which is mostly recognized as an oncogene in neuroblastoma, as a therapeutic target of ACR for HCC through a selective deletion of MYCN$^+$ liver CSCs. We also demonstrated that the expression of MYCN in HCC served as a prognostic biomarker and positively correlated with recurrence of de novo HCC after curative treatment. Our study highlighted MYCN as a biomarker and therapeutic target in drug discovery for screening chemopreventive agents against the recurrence of HCC.

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mechanism by which ACR acts in HCC chemoprevention has been linked to the inhibition of the hyperphosphorylation of retinoid receptors (16) and lipid metabolic reprogramming (17).

Members of the MYC family of basic helix–loop–helix–zipper transcription factors, including *MYC* (c-Myc), *MYCL* (L-Myc), and *MYCN* (N-Myc), are central regulators of growth-promoting signal transduction that maintain stem cell pluripotency (18). The MYC family members play dual roles in regulating normal stem cell-mediated tissue regeneration and CSC-mediated tumorigenesis, which share common molecular pathways in controlling cell growth (19, 20). During repeated liver damage and compensatory regenerations, aberrant stabilization and activation of *c-Myc* have contributed to the development of liver cancers (21). *MYCN* is a well-recognized oncogene in neuroblastoma. Its amplification is detected in ∼20–25% of all neuroblastoma cases and is strongly associated with rapid tumor progression and poor prognoses in neuroblastoma patients (22). Here, we performed genome-wide screening and identified MYCN as an HCC-selective target of ACR and a biomarker of liver CSCs as well as of the prognosis of de novo HCC.

Results

Transcriptome Analysis Identified MYCN as a Target of ACR. An important characteristic of ACR is that it selectively suppresses the growth of HCC cells (23). We initiated a genome-wide screen with a next-generation sequencing-based Cap Analysis Gene Expression (CAGE) analysis to identify HCC-specific targets of ACR using human HCC cells (JHH7) and normal hepatic cells (Hc) (Dataset S1). By comparing the transcriptional profiles of cells treated with a low-dose of a natural metabolite of vitamin A, all-*trans* retinoic acid (atRA), which was used as the control, the top 10 genes that were differentially up-regulated or down-regulated in ACR-treated JHH7 cells but not in ACR-treated Hc cells were identified (Fig. 1*A*). *MYCN* was expressed at higher levels in JHH7 cells than in Hc cells (Fig. S1*A*), and ACR significantly inhibited *MYCN* expression in JHH7 cells at both the protein (Fig. 1*B*) and gene (Fig. S1*B*) level, while *c-MYC* expression was not affected in an HCC-specific manner following ACR treatment (Fig. 1*C*). A similar inhibitory effect of ACR on *MYCN* gene expression was also observed in at least two other HCC cell lines (Fig. S1*B*). Ester analogs of ACR (Fig. 1*D*) did not suppress growth (Fig. 1*E*) or inhibit MYCN expression in JHH7 cells (Fig. 1*F*). In contrast, the vitamin K2 analog SVK30 with an ACR-like structure containing three isoprene residues in its C-terminal side chain, exhibited HCC-selective cell-killing activity (24) and inhibited *MYCN* expression in JHH7 cells (Fig. S1*C*). *MYCN* is a well-recognized oncogene in neuroblastoma (22). ACR significantly inhibited *MYCN* expression in NB9 neuroblastoma cells, which express high levels of *MYCN* and are highly sensitive to ACR compared with NB69 cells, which express low levels of *MYCN* (Fig. S1 *D–F*). These in vitro data indicated that MYCN acts as a molecular target for ACR during HCC cell killing and in neuroblastoma cells. In a mouse model of atherogenic and high-fat (Ath+HF) diet-induced nonalcoholic steatohepatitis (NASH) (25), a high incidence of liver tumors was observed after 60 wk on an Ath+HF diet, which was completely inhibited in mice supplied with ACR (Fig. 1*G*). These data are in accordance with the preventive effect that ACR exerts against diethylnitrosamine-induced liver tumorigenesis in obese and diabetic mice (26). Along these lines, ACR markedly inhibited the increase in *Mycn* expression that was normally observed in the livers of mice fed an Ath+HF diet for 60 wk (Fig. 1*H*). Of interest, four of the five mice showing increased MYCN gene expression were suffering from liver tumor (Fig. 1*H*).

MYCN Knockdown Represses Cell-Cycle Progression and Induces Cell Death. Next, we characterized the functional role played by MYCN in HCC cell proliferation and survival. Fluorescence

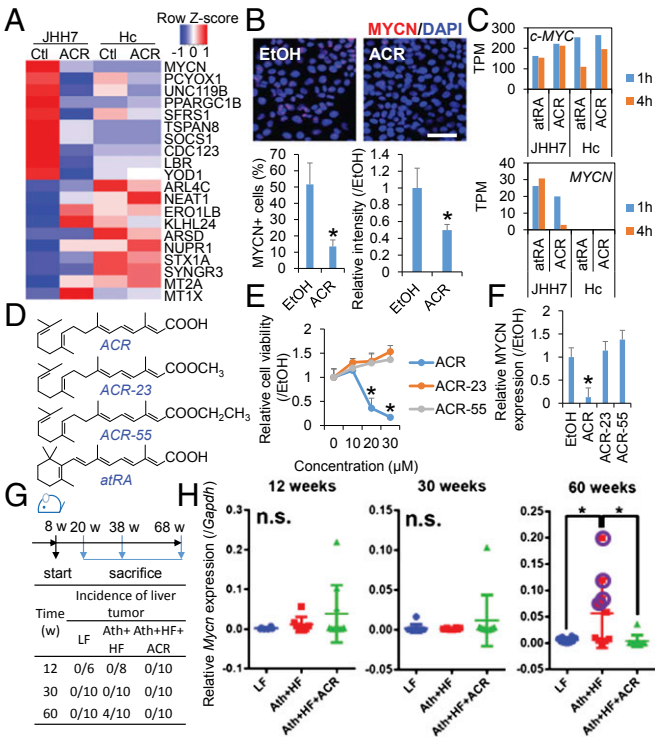

Fig. 1. Identification of MYCN as a molecular target of ACR. (*A*) Heatmap of the top 10 up-regulated and down-regulated genes that were differentially expressed in JHH7 cells and Hc cells 1 and 4 h after starting treatment with 1 μM atRA (control, Ctl) or 10 μM ACR as assessed by CAGE analysis. The genes were ranked by the fold change when ACR treatment was compared with low-dose atRA treatment. (*B*, *Upper*) Immunofluorescence staining for MYCN in JHH7 cell cultures treated with 0.05% ethanol (EtOH, vehicle) or 10 μM ACR for 24 h ($n = 3$). (Scale bar, 50 μm.) (*Lower Left*) Percentages of $MYCN^+$ cells among the total number of JHH7 cell cultures counted. (*Lower Right*) The relative fluorescent intensity of MYCN protein vs. EtOH. (*C*) *c-MYC* (*Upper*) and *MYCN* (*Lower*) gene expression as assessed by CAGE analysis. TPM, tags per million mapped reads. (*D*) Chemical structures of ACR, its ester analogs, and atRA. (*E* and *F*) The effects of ACR and its ester analogs on cell viability 24 h after treatment (*E*) and *MYCN* gene expression levels 4 h after treatment (10 μM each) (*F*) in JHH7 cell cultures ($n = 3$). (*G* and *H*) Schematic overview of experimental procedures and time-dependent incidence of liver tumor (*G*) and *MYCN* gene expression in the livers (*H*) of a diet-induced mouse model of NASH ($n = 6$–10 mice per group). Ath+HF, atherogenic and high fat diet; LF, low fat diet. Purple circles indicate the mice suffering from liver tumor. The data are presented as the mean ± SD; $^*P < 0.05$, Student's *t* test. n.s., not significant.

staining of JHH7 cells showed MYCN was localized predominately in cells with higher DNA intensity shown by DAPI staining (Fig. S2*A*). A high-content, single-cell imaging analysis showed a strong positive correlation between the integrated nuclear intensity of DAPI and MYCN staining in JHH7 cells (Fig. S2*B*). Cell-cycle staging of individual cells based on their DNA content (Fig. S2 *C* and *D*) showed that MYCN was expressed at higher levels in JHH7 cells in the S and G2 phases than in those in the G0/1 phases (Fig. S2*E*). To provide direct evidence, cells in G1 and G2 phases were obtained through flow cytometric cell sorting of Hoechst 33342-stained JHH7 cells. *Cyclin B* (Fig. 2*A*) and *MYCN* (Fig. 2*B*) were expressed at significantly higher levels in cells in the G2 phase than in the G1 phase. As functional evidence, transfecting JHH7 cells with a pool of three target-specific siRNAs against *MYCN* (Fig. 2*C* and Fig. S2 *F–I*) inhibited cell proliferation (Fig. S2*J*) and repressed cell-cycle progression by reducing the number of cells in the G2 phase and concomitantly increasing the number of cells in the G0/1 phases (Fig. 2*D* and Fig. S2*K*). Both a decrease in the

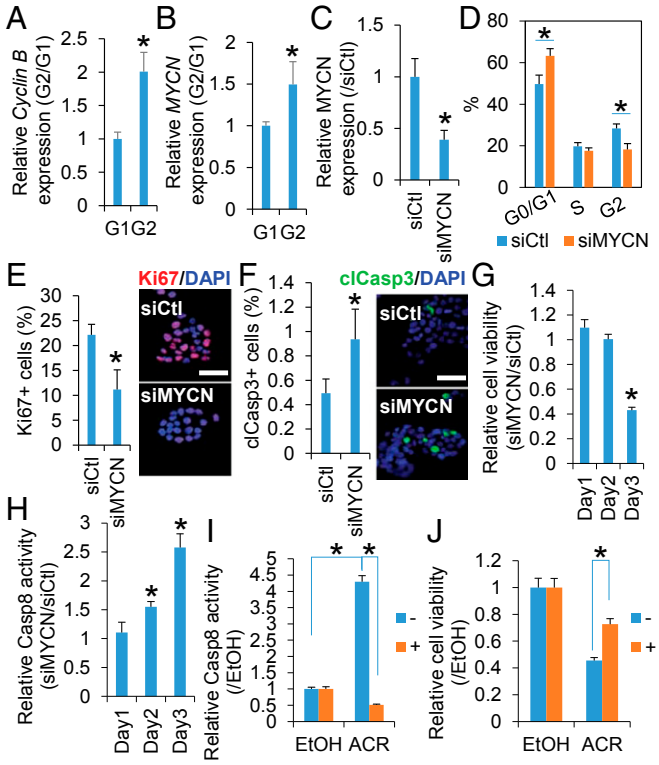

Fig. 2. Loss-of-function analysis of MYCN in JHH7 cells. Cells in the G1 and G2 phases were obtained through flow cytometric cell sorting of Hoechst 33342-stained JHH7 cells. (*A* and *B*) *Cyclin B* (*A*) and *MYCN* (*B*) gene expression in sorted cells. (*C*) *MYCN* gene expression in JHH7 cells transfected with either control siRNA (siCtl) or siRNA to MYCN (siMYCN, three target-specific siRNAs targeting human *MYCN*) for 72 h. (*D*–*F*)The cells were reseeded in 96-well plates, and 72 h later the cell-cycle stages (*D*) and percentages of Ki67$^+$ (*E*) or clCasp3$^+$ (*F*) cells in the JHH7 cell cultures were examined using imaging analysis. (*G* and *H*) The time-dependent effect of MYCN knockdown on cell viability (*G*) and Casp8 activity (*H*) in JHH7 cell cultures. (*I* and *J*) Casp8 activity (*I*) and cell viability (*J*) in JHH7 cell cultures that were treated with either 0.05% EtOH or 10 μM ACR for 24 h in the absence (–) or presence (+) of 20 μM z-IETD-FMK, a Casp8 inhibitor. (Scale bars, 50 μm.) The data are presented as the mean ± SD (n = 3–4). *P < 0.05, Student's t test. siCtl, control siRNA.

percentage of proliferating cells labeled with the cellular proliferation marker Ki67 (Fig. 2*E*) and an increase in the percentage of apoptotic cells labeled with the apoptosis marker cleaved caspase-3 (clCasp3) (Fig. 2*F*) were observed in MYCN-knockdown JHH7 cells. Since the complete inactivation of Casp8, a key enzyme at the top of the apoptotic cascade, was observed almost exclusively in *MYCN*-amplified neuroblastomas (27), we investigated whether Casp8 activity was affected following MYCN knockdown in JHH7 cells. In addition to suppressing cell growth (Fig. 2*G*), knocking down MYCN increased Casp8 activity in JHH7 cells in a time-dependent manner (Fig. 2*H*). Consistently, Casp8 was markedly activated in ACR-treated JHH7 cells and was completely blocked in cells grown in the presence of the Casp8 inhibitor z-IETD-FMK (Fig. 2*I*). Importantly, z-IETD-FMK significantly interfered with the ACR-induced suppression of the growth in JHH7 cells (Fig. 2*J*), suggesting MYCN/Casp8-dependent pathways in apoptosis-targeted therapies for HCC.

MYCN Expression Is Correlated with CSC Markers in Human HCC. CSCs are thought to be responsible for the development and recurrence of HCC (8). Therefore, we sought to determine whether the expression of MYCN is correlated with CSC-related markers. A human HCC microarray dataset (GSE25097) (28) revealed that *MYCN* is expressed at significantly higher levels in liver tumors than in healthy livers, cirrhotic livers, or adjacent nontumor liver tissues (Fig. S3*A*). Correlation analysis revealed that in human HCC, *MYCN* expression was positively correlated with liver CSC markers including *AFP*, *EpCAM*, *CD133*, *DLK1*, and *GPC3* and with Wnt/β-catenin signaling markers including *β-catenin*, *DKK1*, *BAMBI*, and *CCND1* (Fig. S3 *B* and *C*). There was no correlation between the expression levels of *MYCN* and c-*MYC* or of the biliary epithelial markers *KRT19* (CK19) and *KRT7* (CK7) (Fig. S3*B*). *MYCN* expression was negatively correlated with the expression of mature hepatocyte markers, such as *CYP3A4* and *UGT2B7* (Fig. S3*D*), suggesting that *MYCN* expression is restricted to hepatic stem cell-like HCC (HpSC-HCC) that is accompanied by activated Wnt/β-catenin signaling but is not seen in bile duct epithelium-like HCC (BDE-HCC) or mature hepatocyte-like HCC (HP-HCC) (Fig. S3*E*). The correlations between MYCN and the liver CSC markers AFP, EpCAM, and CD133 were further validated in three HCC cell lines—JHH7, Huh7, and FLC5—by double-staining flow cytometric analysis (Fig. S4).

The MYCN-Positive CSC Subpopulation Is Selectively Targeted by ACR. Data mining in the Cancer Cell Line Encyclopedia (CCLE) database (29) revealed a strong correlation between the gene expression of MYCN and EpCAM, one of the most commonly described surface markers in liver CSCs (10), in a total of 25 HCC cell lines (Fig. 3*A*). Immunofluorescence double staining demonstrated that MYCN expression was significantly correlated with that of EpCAM in heterogeneous JHH7 cells (Fig. 3*B*). Blocking MYCN expression using siRNA attenuated the colony-forming capacity of JHH7 cells in a limiting dilution assay (Fig. 3*C*), along with a decrease in the frequency of EpCAM$^+$ cells among whole JHH7 cell cultures (Fig. 3*D*). The MYCN$^+$EpCAM$^+$ subpopulation of JHH7 cells was selectively killed by ACR in a dose-dependent manner (Fig. 3*E*). Consistent with the immunofluorescence data, flow cytometric analysis showed an increase in the presence of EpCAM$^-$ cells in JHH7 cells treated with ACR (Fig. 3*F*). To provide further direct evidence, EpCAM$^+$ and EpCAM$^-$ subpopulations were obtained from JHH7 cells using FACS (Fig. 3*G*). The purity of sorted cells was confirmed in both flow cytometric (Fig. 3*H*) and immunofluorescence (Fig. 3*I*) analysis. EpCAM$^-$ cells with a decrease in cell–cell adhesion showed notable phenotypic differences compared with EpCAM$^+$ cells (Fig. 3*J*). In accordance with the correlation between MYCN and EpCAM described above, the gene (Fig. 3*K*) and protein (Fig. 3*L*) expression of MYCN was dramatically upregulated in the subset of EpCAM$^+$ cells compared with EpCAM$^-$ cells. Furthermore, increased cell killing by ACR was observed in EpCAM$^+$ cells, suggesting a differential sensitivity to ACR in EpCAM$^+$ and EpCAM$^-$ cells (Fig. 3*M*). ACR also induced cell death in EpCAM$^-$ cells, but to a much lesser extent than in EpCAM$^+$ cells, suggesting that ACR targets other liver CSC populations in addition to EpCAM$^+$ cells. Consistent with the above microarray data, immunofluorescence double staining also demonstrated a strong correlation between MYCN and another liver CSC marker, CD133 (11), and identified a MYCN$^+$ CD133$^+$ subpopulation in the heterogeneous JHH7 cells (Fig. S5*A*). Flow cytometric analysis indicated that CD133 is expressed in the cytoplasm of JHH7 cells (Fig. S5*B*). In a rat model of liver carcinogenesis, precancerous oval cells, especially the CD133$^+$ CD44$^+$ subpopulation, have been reported as targets of ACR in studies aimed at preventing de novo HCC (15, 30). In agreement with these studies, our imaging analysis showed that the MYCN$^+$ CD133$^+$ cells were selectively killed following MYCN knockdown (Fig. S5*C*) as well as by ACR treatment (Fig. S5*D*). In addition, it has been shown that cell density can affect stemness gene expression and contribute to cancer drug resistance (31, 32). JHH7 cell cultures seeded at a high density contained low numbers of Ki67$^+$ proliferating cells and did not respond to ACR (Fig. S6 *A* and *B*). They expressed much lower levels of both

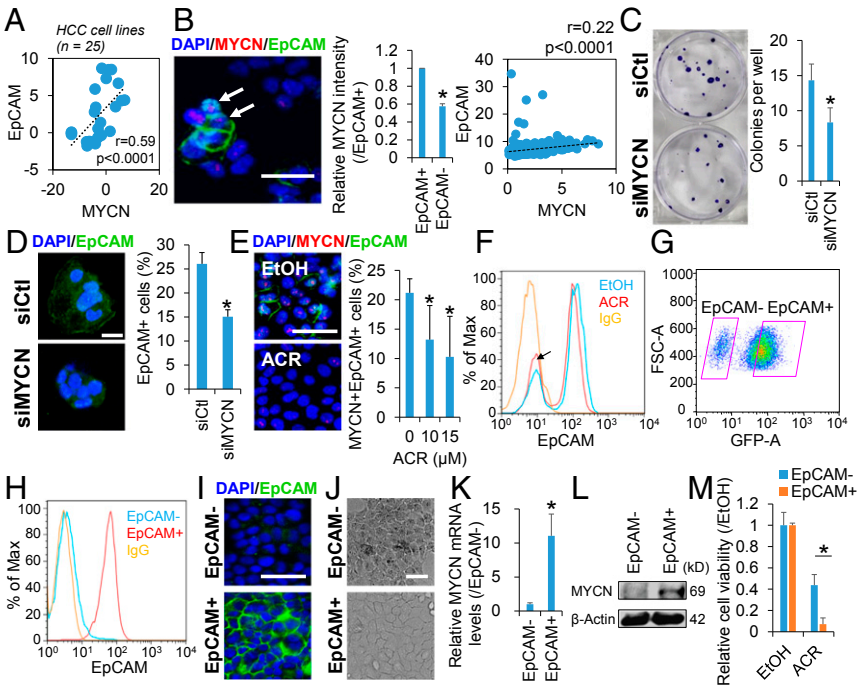

Fig. 3. The MYCN-positive CSC subpopulation of JHH7 cells is selectively targeted by ACR. (*A*) Correlation between gene expression of MYCN and EpCAM in a total of 25 HCC cell lines in the CCLE database. The data are presented as a robust multiarray average (RMA). (*B*, *Left*) Immunofluorescence triple staining of DAPI (blue), MYCN (red), and a liver CSC marker EpCAM (green) in JHH7 cell cultures. (Scale bar, 20 μm.) (*Center*) MYCN intensity in EpCAM$^+$ and EpCAM$^-$ cells. (*Right*) The correlation between the intensities of MYCN and EpCAM in individual cells was assessed using the Pearson's correlation coefficient. (*C* and *D*) The inhibitory effect of MYCN knockdown on the colony-forming ability of JHH7 cells was evaluated in a limiting dilution assay (*C*) and by the percentage of EpCAM$^+$ cells in JHH7 cell cultures (*D*). (Scale bar, 20 μm.) (*E*) A dose-dependent inhibitory effect of ACR treatment for 24 h on the percentage of MYCN$^+$EpCAM$^+$ cells in JHH7 cell cultures. (Scale bar, 50 μm.) (*F*) Flow cytometric analysis of EpCAM expression in JHH7 cells treated with 15 μM ACR for 24 h. Dead cells were excluded by propidium iodide (PI) staining. The arrow indicates the increased presence of EpCAM$^-$ cells following ACR treatment. (*G*) EpCAM$^+$ and EpCAM$^-$ cells were obtained through flow cytometric cell sorting of EpCAM-Alexa 488–stained JHH7 cells. (*H–J*) The sorted cells were reanalyzed in flow cytometric analysis (*H*) and were characterized in immunofluorescence (*I*) and brightfield microscopic (*J*) analysis. (Scale bars, 50 μm.) (*K* and *L*) Gene (*K*) and protein (*L*) expression of MYCN. (*M*) Effect of ACR treatment at 10 μM for 48 h on the cell viability in the sorted cells. The data are presented as the mean ± SD ($n = 3$ or 4). *$P < 0.05$, Student's t test.

MYCN and CD133 than the same cultures seeded at a low density (Fig. S6 *C* and *D*). Taking these findings together, we propose that ACR is a promising drug for the chemoprevention of HCC because it selectively eliminates liver CSCs, especially the MYCN$^+$ subpopulation, but not differentiated tumor cells (Fig. S6*E*).

MYCN Is a Prognostic Factor for the Recurrence of de Novo HCC. Finally, we explored the prognostic implications of MYCN expression in human HCC. In 12 patients whose HCC had been eradicated through curative resection or ablation, liver biopsies were obtained before and 8 wk after ACR treatment. Four of six HCC patients (66.7%) who had received ACR at a dosage of 600 mg/d for 8 wk showed decreased *MYCN* expression (<0.5-fold) in their liver biopsies; this effect was not seen in six patients treated with 300 mg/d of ACR (Fig. 4*A*). These results are in accordance with a clinical study demonstrating a decreased risk of HCC recurrence in patients administered ACR at 600 mg/d but not in patients administered ACR at 300 mg/d (33). In addition, a gene-expression pattern analysis performed using liver tissues obtained before and after ACR administration also demonstrated little or no change in the expression of recurrence-related genes, such as tumor suppressor-related genes and hepatocyte differentiation genes, when patients were treated with ACR at 300 mg/d or 600 mg/d (34). Immunohistochemical and immunofluorescence staining of human liver biopsy specimens showed a stronger nuclear MYCN expression in HCC than in normal adjacent tissues (Fig. 4*B* and Fig. S7*A*). Overexpressed nuclear MYCN in HCC liver tissue was observed in 2 of 10 HCC patients. CAGE analysis in a European cohort study (35) demonstrated that *MYCN* gene expression was significantly more abundant in HCC tumor tissues ($n = 50$) than in the matched nontumor adjacent tissues ($n = 50$) or in normal liver tissues ($n = 5$) (Fig. 4*C*). In a Japanese cohort study of 102 HCC patients followed up more than 10 y (Fig. S7*B*) (36), significantly higher expression of *MYCN* was also observed in surgical HCC tissues than in matched normal adjacent tissues (Fig. 4*D*), suggesting a potential role of MYCN in hepatic tumorigenesis in different ethnic groups. Furthermore, higher expression of *MYCN* was observed in both HCC and normal adjacent tissues of patients with HCC recurrence than in the corresponding tissues of patients without HCC recurrence (Fig. 4*E*), suggesting a potential role of MYCN in HCC recurrence. Consistent with this, Kaplan–Meier estimates of the proportion of patients who were free of HCC recurrence over time revealed that high *MYCN* expression in HCC tumors was correlated with significantly higher recurrence rates in patients of group 1 without intrahepatic metastasis ($n = 74$) (Fig. 4*F*) but not in patients of group 2 with multiple intrahepatic metastases ($n = 28$) at the time of curative treatment or in all 102 patients of both group 1 and 2 (Fig. S7 *C* and *D*). These data suggest that MYCN is involved in the de novo carcinogenesis of HCC. Finally, the clinical significance of MYCN overexpression in HCC was analyzed using The Cancer Genome Atlas (TCGA) RNA-sequencing (RNA-seq) mRNA data (37). Among the 371 HCC patients, 10 patients (2.7%) had up-regulated MYCN mRNA expression with a selection threshold of a Z score greater than 2 (Fig. S8*A*). Remarkably, these patients with MYCN overexpression suffered a dramatically worse prognosis than the other patients (Fig. S8 *B* and *C*).

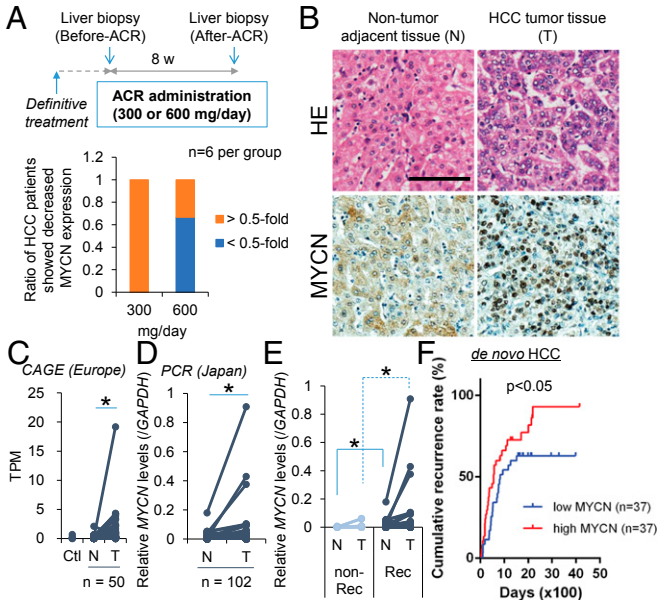

Fig. 4. MYCN is a prognostic factor for the recurrence of de novo HCC. (*A*, *Upper*) Schematic overview of a clinical study that enrolled 12 HCC patients who received 8 wk of ACR administration (300 mg/d or 600 mg/d) after definitive treatment ($n = 6$ per group). (*Lower*) The ratio of HCC patients who showed decreased *MYCN* expression in their liver biopsies (<0.5-fold) after ACR treatment. (*B*) Representative images of H&E staining and immunohistochemical staining of MYCN in liver sections of nontumor adjacent (N) and HCC (T) tissues. (Scale bar, 100 μm.) (*C*) *MYCN* gene expression in normal liver tissues collected at a distance from a liver metastasis of colon cancer (Ctl, $n = 5$) and matched nontumor adjacent (N, $n = 50$) and HCC (T, $n = 50$) liver tissues as assessed by CAGE analysis in a European cohort. (*D* and *E*) *MYCN* gene-expression levels in surgical matched nontumor adjacent (N) and HCC (T) liver tissues in all the enrolled HCC patients ($n = 102$) (*D*) or in subpopulations of patients without HCC recurrence (non-Rec, $n = 24$) or with HCC recurrence (Rec, $n = 78$) (*E*) during a long-term follow-up (>10 y) after curative treatment in a Japanese cohort. (*F*) Prognostic significance of MYCN expression in human HCC assessed using the Kaplan–Meier method and log-rank test in subpopulations of patients without intrahepatic metastasis ($n = 74$) at the time of curative treatment. Low MYCN, HCC patients with MYCN expression lower than the median expression value. High MYCN, HCC patients with MYCN expression equal to or higher than the median expression value. $^*P < 0.05$, Student's *t* test.

Discussion

Recurrence in HCC involves metastatic or de novo mechanisms. Early recurrence within 1 y is likely to be related to intrahepatic metastasis, whereas de novo recurrence tends to occur at least 1–2 y after resection (38, 39). It therefore is very likely that the high long-term recurrence rate (>70% at 5 y) of HCC can be attributed mainly to de novo carcinogenesis (40). Consistent with this proposal, administering ACR at 600 mg/d reduced HCC recurrence at 2 y after curative treatment (41). Importantly, a unique characteristic of ACR is that it selectively suppresses the growth of HCC cells but not of normal Hcs (23). Our data suggested that these phenomena could be explained at least in part by the restricted expression of MYCN in liver CSCs but not in normal hepatocytes and mature HCC cells.

In precancerous tissues, liver CSCs/TICs are thought to contribute to the de novo recurrence of HCC (13). Here, we found that in human HCC, *MYCN* expression was positively correlated with a wide range of HpSC-HCC markers but not with BDE-HCC and HP-HCC markers. Notably, it was previously reported that patients suffering from HpSC-HCC had poorer prognoses than those with BDE-HCC and HP-HCC (9). This finding was in agreement with the finding in this study that patients with high MYCN expression in de novo HCC had a poor prognosis. Wnt/β-catenin signaling is critical for maintaining stem cell pluripotency (42). The correlation between the expression levels of *MYCN* and Wnt/β-catenin signaling markers in human HCC suggested that the role played by MYCN in liver CSCs is probably related to its regulation of Wnt/β-catenin signaling. A genomic analysis revealed that coexpressed genes associated with EpCAM, which is a direct transcriptional target of Wnt/β-catenin signaling (10), are functionally linked with MYCN in human HCC (9). This is consistent with the current results that the expression of MYCN is positively correlated with the expression of EpCAM in HCC cells. Further studies that inactivate MYCN using CRISPR might be more effective in investigating the biological function of MYCN in regulating hepatic tumorigenesis.

It has been widely hypothesized that CSCs constitute only a rare subpopulation of the cells in untreated tumors (43). Flow cytometry analysis showed small percentages (1.4% and 5.2%, respectively) of $EpCAM^+$ HCC cells in two cases of HCC clinical specimens (10). The low frequency of MYCN overexpression in HCC based on the TCGA database is partly consistent with the notion that MYCN marks a small population of CSC-like cells in HCC. Early evidence in animal models demonstrated that retinoids have potent anticancer activity in the prevention of carcinogen-induced and spontaneous transgenic cancers but have limited effect in the treatment of transplantable cancers (44). Together with the inverse relationship between MYCN expression and the prognosis of de novo (but not metastatic) HCC, our study highlights MYCN as a biomarker and a therapeutic target of ACR in preventing HCC at an early stage through a selective deletion of $MYCN^+$ liver CSCs, but not in preventing progressive HCC.

We previously reported that the prevention of hepatic tumorigenesis by ACR accompanies the blocking of lipogenesis acceleration, especially linoleic acid metabolism (17). Intriguingly, in animal models of NASH, the accumulation of linoleic acid in hepatocytes caused the selective loss of intrahepatic $CD4^+$ T lymphocytes (5), suggesting that they are a link between lipid dysregulation and liver carcinogenesis that involves the impairment of antitumor surveillance (4). Notably, lipid biosynthesis has specifically been shown to be critical for MYCN-derived tumors, whereas inhibitors of fatty acid synthesis are toxic to cells expressing high levels of MYCN (45). Further experiments are needed to determine the role played by MYCN in the regulation of lipid metabolic reprogramming in liver carcinogenesis.

Since the CAGE analysis was developed by counting the number of capped RNAs at a particular transcriptional start site (46), this means that ACR regulates the transcription rather than the protein stabilization of MYCN in HCC cells. Developing drugs that affect MYC proteins is challenging because they have no apparent surface structures to which small molecules can bind (47). Therapeutic strategies are currently focused on aurora kinase A inhibitors, which mediate the proteolytic degradation of MYCN protein (47). We previously reported that ACR induced nuclear translocation of a cross-linking enzyme transglutaminase 2 (TG2) by accelerating the formation of a trimeric complex with importin-α/β (48). Subsequently, accumulated TG2 in nucleus resulted in the cross-linking and inactivation of Sp1 transcription factor, thereby reducing expression of the Sp1-responsive genes such as epidermal growth factor receptor (*EGFR*) and leading to apoptosis of HCC cells (49). In contrast, suppression of TG2 partially restored these phenomena (23). Notably, Sp1 has been reported to cooperate with E2F in activating the MYCN promoter (50), suggesting a TG2/Sp1-dependent pathway by which ACR reduces the expression of *MYCN* in HCC cells.

In summary, using ACR as a bioprobe, we provided evidence that MYCN serves as a prognostic biomarker and therapeutic target of ACR for liver CSCs in de novo HCC. Because clinical microarray data analysis showed a correlation between MYCN and Wnt/β-catenin signaling markers in human HCC, MYCN might be a pan marker of liver CSC/TIC-like cells with tumorigenic capacity.

Materials and Methods

Ethics Statement. The animal experiments were performed in accordance with protocols approved by the Institutional Committee of Animal Experiment of Kanazawa University and RIKEN and adhered to the guidelines in the Institutional Regulation for Animal Experiments and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The human clinical study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committees of Kanazawa University Hospital, Tottori University Hospital, Kyoto University Hospital, and the RIKEN Institute Research Ethics Committee. The investigator or the subinvestigator evaluated patients who had undergone topical medical therapy or surgical resection and obtained informed consent in writing from each patient deemed eligible to participate in this study (34).

Clinical Studies. Formalin-fixed, paraffin-embedded liver tumor tissues and normal adjacent tissues of HCC patients were obtained from Gifu University or were purchased from ProteoGenex (20). cDNA samples were synthesized from liver biopsies that were obtained from 12 HCV-positive patients whose HCC had been eradicated through curative resection or ablation and who underwent a liver biopsy at baseline and during week 8 of treatment with a daily dose of either 300 or 600 mg ACR at Kanazawa University Hospital (Kanazawa, Japan) (34). For the Japanese cohort, RNA samples were isolated from HCC and adjacent liver tissues that were obtained from 102 patients who underwent curative surgical resection at Kyoto University Hospital (Kyoto, Japan) and Tottori University Hospital (Yonago, Japan) (36). For the European cohort, liver tissues were collected from 50 patients resected for HCC and five patients resected for metastatic liver colon cancer at INSERM (Villejuif, France) (35).

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