Hepatology

Clinical significance of *CHD1L* in hepatocellular carcinoma and therapeutic potentials of virus-mediated *CHD1L* depletion

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ABSTRACT

Background Hepatocellular carcinoma (HCC) is among the most lethal of human malignancies. It is difficult to detect early, has a high recurrence rate and is refractory to chemotherapies. Amplification of 1q21 is one of the most frequent genetic alterations in HCC. *CHD1L* is a newly identified oncogene responsible for 1q21 amplification. This study aims to investigate the role of *CHD1L* in predicting prognosis and chemotherapy response of patients with HCC, its chemoresistant mechanism and whether virus-mediated CHD1L silencing has therapeutic potentials for HCC treatment.

Methods The clinical significance of *CHD1L* in a cohort of 109 HCC cases including 50 cases who received transarterial chemoembolisation treatment was assessed by clinical correlation and Kaplan–Meier analyses. A CHD1L-overexpressing cell model was generated and the mechanism of chemoresistance involving CHD1L was investigated. An adenovirus-mediated silencing method was used to knockdown *CHD1L*, and its effects on tumorigenicity and chemoresistance were investigated in vivo and in vitro.

Results Overexpression of CHD1L was significantly associated with tumour microsatellite formation (p=0.045), advanced tumour stage (p=0.018), overall survival time (p=0.002), overall survival time of patients who received transarterial chemoembolisation treatment (p=0.028) and chemoresistance (p=0.020) in HCC. Interestingly, CHD1L could inhibit apoptosis induced by 5-fluorourail (5-FU) but not doxorubicin. The mechanistic study revealed that the involvement of the Nur77-mediated pathway in chemotherapeutic agent-induced apoptosis can dictate if CHD1L could confer resistance to chemotherapy. Furthermore, an adenoviral vector containing short hairpin RNAs against *CHD1L* (*CHD1L*-shRNAs) could suppress cell growth, clonogenicity and chemoresistance to 5-FU. An in vivo study found that *CHD1L*-shRNAs could inhibit xenograft tumour growth and increase the sensitivity of tumour cells to 5-FU in nude mice.

Conclusions This study highlighted for the first time the prognostic value of *CHD1L* in HCC and the potential application of virus-mediated CHD1L silencing in HCC treatment.

Significance of this study

What is already known about this subject?
► Amplification of 1q21 is detected in 58–78% of human HCCs, and a target oncogene *CHD1L* responsible for this amplification event has been identified.
► CHD1L inhibits cell apoptosis by blocking the Nur77-mediated apoptotic pathway.
► The clinical significance of *CHD1L* and the therapeutic potentials of *CHD1L* are still unknown.

What are the new findings?
► CHD1L overexpression in HCC correlates with the unfavourable clinical features, chemotherapy responsiveness and survival of patients with HCC.
► CHD1L inhibits chemotherapeutic agent-induced apoptosis via the Nur77/cytochrome *c*/caspase 9 pathway, providing a novel insight into the underlying mechanism of chemoresistance in HCC.
► The involvement of the Nur77-mediated pathway in drug-induced apoptosis can dictate if CHD1L could confer resistance to chemotherapy.
► Virus-mediated *CHD1L* depletion can abolish the malignant phenotypes of tumour cells and increase chemosensitivity to chemotherapeutic agent.

How might it impact on clinical practice in the foreseeable future?
► CHD1L expression can be used to predict prognosis and chemotherapeutic response of patients with HCC.
► Virus-mediated CHD1L silencing might serve as promising novel therapeutic options for patients with HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer morbidity and mortality in Southeast Asia, China and the USA, where its incidence has increased by >90% over the past three decades.[1][2] Curative surgery offers the hope for long-term survival to patients suffering from HCC; however, patients are commonly diagnosed at the advanced stage and this means that liver resection is only suitable for a minority of patients. For the majority of patients, the median survival is ∼6 months.[3] Chemotherapy is one of the mainstay treatments for patients with inoperable disease. HCC is a chemoresistant cancer, and the efficacy of chemotherapeutic drugs is generally believed to

depend on their ability to trigger apoptosis.[4] [5] Actually, HCC is one of the well identified cancers that are most resistant to systemic chemotherapy, with the reported response rate varying from 0% to 20% and a median survival of only 4 months.[6] The overall poor outcome thus underlines the urgent need for reliable biomarkers in the diagnosis and prognosis of HCC and improved molecular targets for the development of more efficient treatments.

It is believed that HCC, like many other solid tumours, develops from the accumulation of alterations of cancer-related genes critical to processes such as cell proliferation, apoptosis and other functions. Amplification of chromosome 1q is the most frequent genetic alteration in HCC, which was detected in 58–78% of primary HCC cases by comparative genomic hybridisation.[7–9] The minimal amplified region has been narrowed down to 1q21, and a novel oncogene, *CHD1L* (also named *ALC1*), has been identified.[10] The oncogenic function of *CHD1L* in HCC development and progression has been demonstrated by both in vitro and in vivo studies.[10–13] In the present study, the association between the CHD1L over-expression and clinicopathological features in HCC was investigated. As described previously, CHD1L is able to bind to Nur77 and inhibits the latter's nuclear to mitochondrial translocation, and the subsequent Nur77-mediated caspase activation and apoptosis, thereby strongly extending HCC cell survival by inhibiting Nur77-mediated apoptosis.[12] In light of this, the effect of CHD1L on chemotherapy and the mechanism underlying the chemoresistance of HCC were also investigated. More interestingly, the viral delivery of small interfering RNA (siRNA) targeting *CHD1L* as cancer therapeutics was further investigated and is discussed in this study.

MATERIALS AND METHODS

Patients and clinical specimens

Between June 2001 and June 2008, a retrospective cohort of 109 patients with HCC who underwent hepatectomy at Sun Yat-Sen University Cancer Center (Guangzhou, China) were enrolled into this study. All patients were diagnosed with primary HCC, and none had received prior radio- or chemo-therapy before surgery. Of the 109 patients, 59 have developed recurrent HCCs within 4 years. Fifty patients had prechemo-therapy biopsies and then received about three cycles of trans-arterial chemoembolisation (TACE) treatment (figure 1). The studies using human tissue were reviewed and approved by the Committees for Ethical Review of Research involving Human Subjects at University of Hong Kong and Cancer Center of Sun Yat-Sen University, China.

TACE treatment

Cisplatin (CDDP), 5-fluorourail (5-FU) and an emulsion consisting of doxorubicin (DOX) and lipiodol were injected in turn into the arteries that fed the tumours. The median dose of lipiodol was 10 mg (5–20 mg). The doses of the chemotherapy agents were: DOX 60 mg/m^2, CDDP 120 mg/m^2 and 5-FU 600 mg/m^2 every month for up to three cycles.

Definition of an objective response

The effects of TACE on the tumour were assessed by dynamic CT within the first month after TACE treatment. The tumour response was evaluated using the Response Evaluation Criteria in Solid Tumors criteria.[14] Patients were categorised into two groups. Those whom did not obtain clinical benefit and developed progressive disease were classified as the 'chemotherapy-resistant'
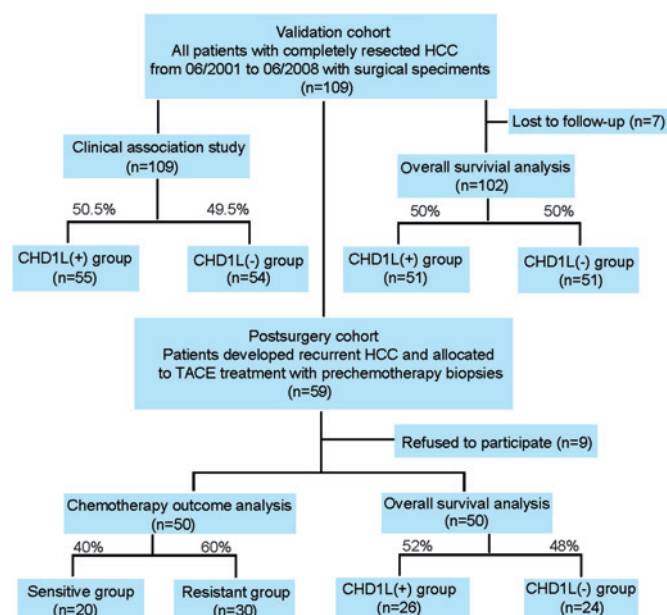

Figure 1 Study flow chart: inclusion criteria, exclusion criteria and outcome. HCC, hepatocellular carcinoma.

group. Those who obtained clinical benefit by achieving complete response, partial response and stable disease were classified as the 'chemotherapy-sensitive' group.

Immunohistochemistry (IHC)

IHC was performed as described previously.[13] CHD1L immu-noreactivity was scored as absent expression (0), weak expres-sion (1), moderate expression (2) and strong expression (3) according to the semi-quantitative scoring method as reported by Ng *et al*.[15] In this study, compared with non-tumour tissue, the higher IHC score of CHD1L in the tumour was defined as 'overexpression of CHD1L in HCC'. For prechemotherapy biopsies, a low (0–1) or high (2–3) IHC score of CHD1L in tumour tissue was defined as 'CHD1L-negative' or 'CHD1L-positive' HCC, respectively. In this study, all histological eval-uations were carried out in a double-blind manner by two pathologists to minimise observational bias.

Cell lines

HCC cell lines QGY-7703 and PLC8024 were obtained from the Institute of Virology, Chinese Academy of Medical Sciences, Beijing, China. The cells were maintained in Dulbecco's modi-fied Eagle's medium (DMEM; Gibco BRL, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL). The cells were incubated at 37°C in a humidified chamber containing 5% CO_2.

XTT proliferation assay

Chemotherapy-induced cytotoxicity was determined by XTT Cell Proliferation Assay (Roche Diagnostics, Indianapolis, Indiana, USA) according to the manufacturer's instructions. The relative number of viable cells as compared with the number of cells without drug treatment was expressed as percentage cell viability using the following formula: cell viability (%)=A_{490} of treated cells/A_{490} of untreated cells. Three independent experi-ments were performed in duplicate.

Confocal microscopy

For visualising Nur77 protein, cells were transiently transfected with a green fluorescent protein (GFP)/Nur77 expression

construct as described previously.[12] For mitochondrial staining, before the fixation, Mitotracker Red (Molecular Probes, Eugene, Oregon, USA; 100 nM) was added to the culture medium and stained cells at 37°C for 15 min. Slides were fixed and mounted with Vectashield Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, California, USA), and images were captured using a Zeiss LSM 510 Meta confocal laser-scanning microscope (Carl Zeiss MicroImaging, Thornwood, New York, USA).

TUNEL assay

Apoptotic cells were determined by the terminal deoxy-ribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labelling (TUNEL) assay. Apoptotic cells were determined using the In Situ Cell Death Detection Kit POD (Roche Diagnostics), which quantitatively determines DNA fragmentation visualised with a fluorescence microscope. Apoptotic indices were obtained by counting the percentage of TUNEL-positive cells (green colour).

In vitro tumorigenic assays

For foci formation assay, 1×10^3 cells were seeded in a 6-well plate. After culture for 7 days, surviving colonies (>50 cells/colony) were stained by Giemsa (Invitrogen, Carlsbad, California, USA) and counted. Anchorage-independent growth was assessed by colony formation ability in soft agar. Briefly, 1×10^4 cells were suspended in 1 ml of soft agar mixture ($2 \times$ DMEM, 20% FBS and 0.7% agarose) and were subsequently overlaid on the agar base. After 2—3 weeks, colonies (≥ 10 cells) were counted under the microscope in 10 fields per well. Three independent experiments were performed in duplicate.

Antibodies and western blotting

Mouse anti-CHD1L and β-actin antibodies were purchased from Abcam (Cambridge, MA, USA). Rabbit anticaspase 3, anticaspase 9 and anti-PARP (poly(ADP-ribose) polymerase) and mouse anticaspase 8 were all purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Mouse anti-cytochrome c (Cyto c) antibody was purchased from Santa Cruz

Figure 2 Overexpression of CHD1L was associated with a poor survival rate in hepatocellular carcinoma (HCC). (A) Representative images of CHD1L expression in a pair of HCC (right) and adjacent non-tumour liver tissue (left) detected by immunohistochemistry (IHC). Nuclear staining of CHD1L (brown) was detected in tumour tissue. The tissue sections were also stained with H&E as a reference. Original magnification, ×200. (B) Representative images of H&E staining (upper left) and IHC staining with an anti-CHD1L antibody (lower left) on serial sections of cirrhotic (Cir) tissue surrounding a HCC (Tu). The boxed regions are magnified and shown in the right panels. (C) H&E staining (left) and IHC analysis of CHD1L (middle) were performed on serial sections of cirrhotic (Cir) tissue surrounding a HCC (Tu). The boxed regions are magnified and shown in the right panel. CHD1L was expressed in the cytoplasm of hepatocytes in cirrhotic tissue. (D) Kaplan—Meier overall survival (OS) curve of patients with HCC in correlation with CHD1L expression. The OS rate was significantly decreased in patients with HCC with CHD1L overexpression (+) compared with patients without CHD1L overexpression (−) (p=0.001). (E) Kaplan—Meier analysis indicated that the OS rate of patients with HCC who received transarterial chemoembolisation (TACE) was significantly decreased in the CHD1L (+) group compared with the CHD1L(−) group (p=0.028).
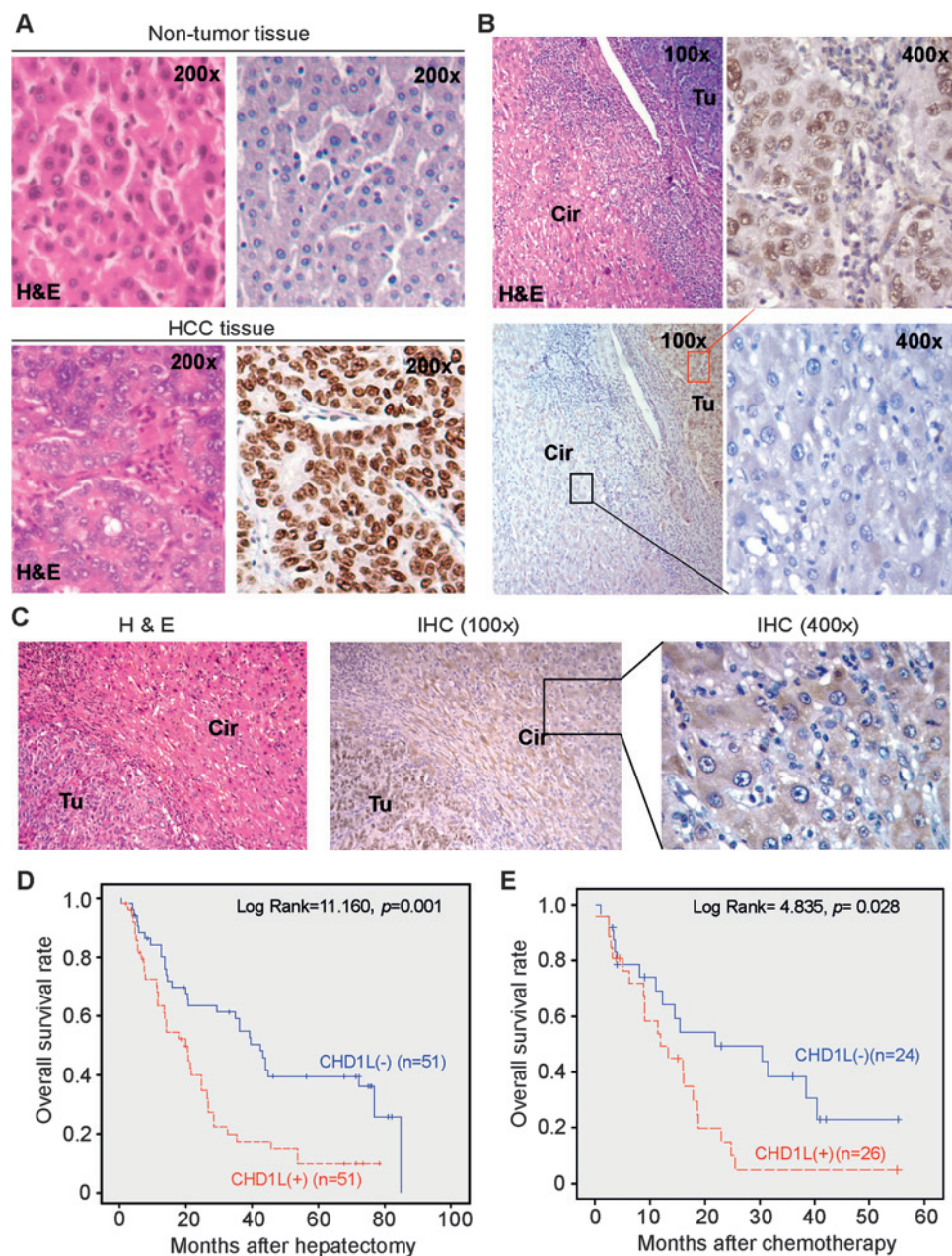
Table 1 Clinicopathological correlation of CHD1L expression in HCC

Clinicopathological features	Number (n=109)	CHD1L expression		p Value
		Without OE	With OE	
Gender				
Male	95	46	49	
Female	14	8	6	0.580
Age				
≤60	88	46	42	
>60	21	8	13	0.332
HbsAg				
Negative	12	7	5	
Positive	97	47	50	0.556
Serum AFP (ng/ml)				
≤400	55	29	26	
>400	54	25	29	0.567
Tumour size (cm)*				
≤5	23	15	8	
>5	86	39	47	0.103
Cirrhosis†				
Absent	29	13	16	
Present	70	38	32	0.508
Tumour encapsulation†				
Absent	41	21	20	
Present	58	30	28	1.000
Microsatellite formation†				
Absent	68	40	28	
Present	29	10	19	0.045
Tumour stage (AJCC)				
Stage I	24	18	6	
Stage II	63	27	36	
Stage III	22	9	13	0.018

*Tumour size was measured by the length of the largest tumour nodule.
†Partial data are not available, and statistics were based on the available data.
AFP, α-fetoprotein; AJCC, American Joint Committee on Cancer; HBsAg; hepatitis C surface antigen; HCC, hepatocellular carcinoma; OE, overexpression.

Biotechnology (Santa Cruz, California, USA). Western blotting was performed as described previously.[10]

Construction and amplification of recombinant adenovirus vectors

Replication-incompetent adenoviruses expressing short hairpin RNA (shRNA) were constructed using the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen) according to the manufacturer's instructions. We first designed four pairs of siRNAs based on full-length CHD1L (accession no NM_004284). The sequences that could effectively knock down CHD1L were converted to shRNA sequences by adding hairpin sequences. The isolated adenoviral expression clones were then digested to expose the inverted terminal repeat and then transfected into 293A cells (provided with the kit) using Lipofectamine 2000 (Invitrogen) to generate crude adenoviral stocks. Large-scale amplification of adenoviral vectors was conducted in 293A cells and the titres of the purified virus were determined by standard plaque-forming assay according to the manufacturer's instructions.

Adenoviral infection in vitro

PLC8024 cells were grown to ∼80% confluency. After washing with phosphate-buffered saline (PBS), cells were incubated with adenoviral vectors for 2 h in DMEM containing 5% FBS, followed by culturing in complete medium.

Xenograft tumorigenesis and treatment with 5-FU in nude mice

PLC8024 cells were used to generate tumours in nude mouse as described previously.[13] When tumours reached ∼5 mm in diameter, each mouse was then given an intratumoral injection of adenoviral vectors (CHD1L-shRNA or Adv-Lacz) at a dose of 2×10^9 plaque-forming units (pfu) in 20 μl of PBS once per week. After the injection of viral vectors, the mice were treated with 5-FU (40 mg/kg body weight; intraperitoneally) or with 20 μl of PBS (pH 7.4; intraperitoneally). Tumour growth was assessed by the average volume of tumours as described previously.[13]

Statistical analysis

The Statistical Package for the Social Sciences, version 16 (SPSS 16.0, SPSS, Chicago, Illinois, USA) was used for data analysis. CHD1L expression levels in tumour tissue and matched non-tumour tissue were compared using Wilcoxon signed rank test. The clinicopathological features in CHD1L-positive patients and CHD1L-negative patients were compared using the Pearson χ^2 test for categorical variables, and an independent Student t test for continuous data. Kaplan—Meier plots and log rank tests were used for survival analysis. A p value <0.05 was considered statistically significant.

RESULTS
Patient characteristics

Of the 109 patients (supplementary table 1), 87.1% were male; the median age was 46 years (range 21—75 years). Most patients were chronic carriers of hepatitis B virus (89.9%). The median α-fetoprotein value was 71.34 ng/ml (range 0—210 000 ng/ml). A total of 102 patients with follow-up data were eligible for overall survival (OS) analysis (seven patients without follow-up data were excluded). The median follow-up time was 24.8 months (95% CI 17.969 to 31.631). Within 4 years (1.0—45.0 months), 59 patients had developed recurrent HCCs and 50 patients had received about three cycles of TACE treatment (figure 1). These 50 patients were eligible for analyses of OS and the association between CHD1L expression and chemotherapy outcome.

Overexpression of CHD1L is associated with microsatellite tumour formation, advanced stage and poor prognosis in HCC

To determine the prevalence and clinical significance of CHD1L in HCC, the correlation between CHD1L overexpression and the clinicopathological features was investigated in a retrospective cohort of 109 HCC cases by IHC. In the 109 cases examined, CHD1L overexpression was detected in 50.5% (55/109) of HCC tissues (figure 2A and supplementary table 2). Expression of CHD1L was significantly stronger in tumour tissues than adjacent non-tumour tissues (p<0.001; Wilcoxon signed rank test). Furthermore, liver cirrhosis was obviously observed at the peritumoral area in 56.9% (62/109) of HCC specimens. In the

Table 2 The association between CHD1L overexpression and tumour response to chemotherapy in 50 patients with HCC

Parameters	Number (N=50)	CHD1L negative (N=24) (%)	CHD1L positive (N=26) (%)	p Value
Response*				
Complete response	0	0	0	
Partial response (PR)	11	8	3	
Stable disease (SD)	9	6	3	
Progressive disease (PD)	30	10	20	0.020
2-year survival rate†		9/24 (37.5%)	2/26 (7.7%)	
3-year survival rate†		7/24 (29.2%)	1/26 (3.9%)	
Median OS (months)†		21.9 (1.0—44.1)	11.9 (5.4—18.4)	0.028

*The tumour response was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) described in the Materials and methods section.
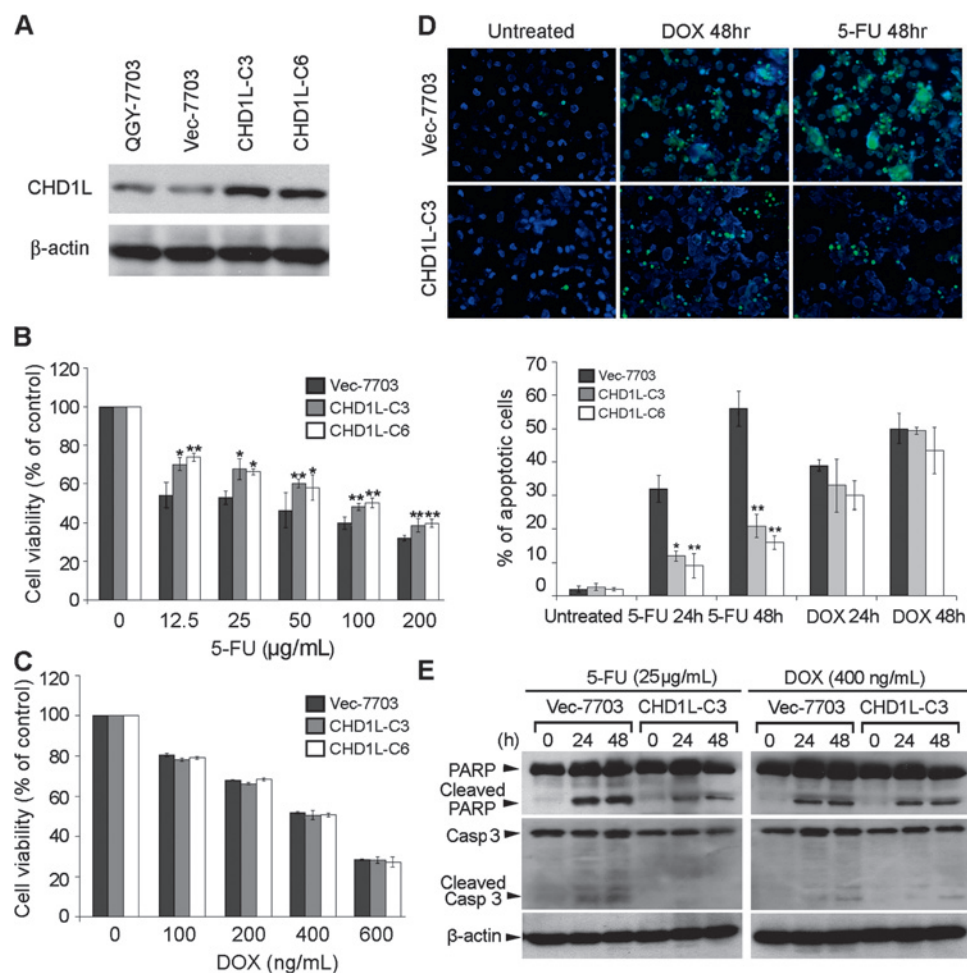†Overall survival (OS), 2-year and 3-year survival rate were calculated from the data of the first treatment, using the Kaplan—Meier method.

peritumoral cirrhotic tissues, absent, weak or moderate expression of CHD1L was observed in 37.1% (23/62), 43.5% (27/62) or 19.4% (12/62) of HCC cases, respectively (figure 2B and supplementary table 3). Expression of CHD1L was significantly higher in tumour tissues than in the corresponding peritumoral cirrhotic tissues (p<0.001; Wilcoxon signed rank test). Interestingly, CHD1L was expressed in the cytoplasm of hepatocytes in cirrhotic tissues (figure 2C). The correlation analysis (Peason χ^2 test) showed that CHD1L overexpression in HCC tumours was significantly associated with tumour microsatellite tumour formation (p=0.045) and advanced tumour stage (p=0.018, table 1). The prognostic significance of CHD1L overexpression was also studied in this cohort of 102 patients with valid follow-up data. The results showed that CHD1L overexpression was significantly associated with poorer OS of patients (log rank=11.160, p=0.001; figure 2B). The 5-year survival rate dropped from 31.4% (16/51) in patients without CHD1L ovexpression (defined as 'CHD1L negative') to 7.8% (4/51) in patients with CHD1L overexpression (defined as 'CHD1L positive'). The median OS times in CHD1L-negative (n=51) and CHD1L-positive (n=51) subgroups of HCC patients were 42.6 months (95% CI 35.1 to 50.1) and 19.8 months (95% CI 12.6 to 27.0), respectively. These data indicated that CHD1L could be a potential prognostic marker to predict the OS of patients with HCC.

Overexpression of CHD1L correlates with the poorer outcome of chemotherapy

The effect of CHD1L overexpression on chemotherapy was further studied in 50 patients with recurrent HCC in this cohort. They had received TACE treatment with the chemotherapeutic drugs 5-FU in combination with DOX and CDDP. Tumour response to chemotherapy and OS time were calculated from the date of the first treatment. Among these patients with HCC, 30/50 (60%) of them developed progressive disease during chemotherapy and were classified as the 'chemoresistant' group. In the remaining 20 patients, partial response and stable diseases were observed in 11 and 9 cases, respectively, and they were defined as the 'chemosensitive' group (table 2). IHC results showed that the frequency of CHD1L overexpression was significantly higher in the 'chemoresistant' group (20/30, 66.7%) than that in the 'chemosensitive' group (6/20, 30%, p=0.02, Pearson χ^2 test, table 2). As shown in figure 2C, overexpression of CHD1L was also significantly correlated with OS time of patients with HCC who received chemotherapy (log rank=4.835, p=0.028). The 2-year and 3-year survival rate dropped from 37.5% and 29.2% in patients with CHD1L-negative HCC to 7.7% and 3.9% in those with CHD1L-positive HCC, respectively (table 2). In addition, the median OS time in CHD1L-negative (n=24) and CHD1L-positive (n=26) subgroups of patients with HCC were 21.9 months (95% CI 1.0 to 44.1)

Figure 3 The inhibitory effect of CHD1L on 5-fluorouracil (5-FU)-induced apoptosis. (A) The expression of CHD1L was detected by western blot analysis in CHD1L-transfected (*CHD1L*-C3 and *CHD1L*-C6), parental (QGY-7703) and empty vector-transfected cells (Vec-7703). (B, C) Cell viabilities of *CHD1L*-C3, *CHD1L*-C6 and Vec-7703 cells were detected by XTT proliferation assay after treatment with 5-FU (B) or doxorubicin (DOX) (C) at the indicated concentrations for 48 h. Data represent the mean±SD derived from three independent experiments with triplicate wells (*p<0.05; **p<0.01). (D) After treatment with 5-FU (25 μg/ml) or DOX (400 ng/ml) for 48 h, the apoptotic index was determined by TUNEL (deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labelling) assay. The upper panel shows the representative images of the TUNEL assay. The apoptotic cells were stained with fluorescein isothiocyanate (FITC)-labelled DNA strand breaks (green signals). The nuclei were counterstained by 4′,6-diamidino-2-phenylindole (DAPI). The apoptotic index was calculated and is summarised in the bar chart (lower panel). Data represent the mean±SD derived from three independent experiments in triplicate (*p<0.01; **p<0.001). (E) After treatment with 5-FU or DOX for the indicated times, the cleavages of caspase 3 and PARP (poly(ADP-ribose) polymerase) was detected in *CHD1L*-C3 and Vec-7703 cells by western blot analysis. β-Actin was used as a loading control.

and 11.9 (5.4–18.4) months (95% CI 9.2 to 30.4), respectively (table 2).

CHD1L inhibits cell apoptosis in HCC cells induced by 5-FU, but not DOX

Since overexpression of CHD1L was significantly associated with the poor outcomes of chemotherapy in patients with HCC, we further investigated the inhibitory effect of CHD1L on two conventional chemotherapeutic agents, 5-FU and DOX. For this purpose, *CHD1L* expression plasmid or empty vector was stably transfected into a HCC cell line QGY-7703 as described previously.[12] Elevated expression of CHD1L was confirmed in two CHD1L transfectants (*CHD1L*-C3 and *CHD1L*-C6) by western blot analysis (figure 3A). *CHD1L*-7703- and empty vector-transfected (Vec-7703) cells were then exposed for 72 h to varying concentrations of DOX (0, 100, 200, 400, 500 and 600 ng/ml; IC_{50} at 400 ng/ml) and 5-FU (0, 12.5, 25, 50, 100 and 200 μg/ml; IC_{50} at 25 μg/ml). The XTT proliferation assay showed that *CHD1L*-7703 (C3 and C6) cells were more viable compared with Vec-7703 cells after 5-FU treatment (figure 3B). However, no obvious difference in cell viability was observed between *CHD1L*-7703 and Vec-7703 cells when cells were treated with DOX (figure 3C).

Next, we studied whether the preferential survival of *CHD1L* transfectants after exposure to 5-FU was attributed to low rates of apoptosis. After the treatment with 5-FU, the apoptotic cells were detected by TUNEL assay. The apoptotic index, obtained by counting the percentage of TUNEL-positive cells, was compared between *CHD1L*-7703 and Vec-7703 cells. The apoptotic index of Vec-7703 cells increased rapidly after the treatment with 5-FU, reaching >57% after 48 h of exposure to 5-FU (figure 3D). In contrast, the apoptotic index of *CHD1L*-7703 cells was significantly lower at every time point in the presence of 5-FU. However, after DOX treatment, there was no dramatic difference in the apoptotic index between *CHD1L*-7703 and Vec-7703 cells (figure 3D). To confirm this finding, activation of caspase 3 and PARP, which are two typical characteristics of cell apoptosis, was investigated by western blot analysis in Vec-7703 and *CHD1L*-C3 cells. As seen in figure 3E, the cleaved caspase 3 and PARP were dramatically decreased in *CHD1L*-C3 cells compared with Vec-7703 cells after 5-FU treatment. However, no notable difference was detected between *CHD1L*-C3 and Vec-7703 cells when cells were treated with DOX. These data suggest that *CHD1L* could inhibit the apoptotic progression of HCC cells upon 5-FU stimulation, but not DOX stimulation.

CHD1L confers chemoresistance by inhibiting the Nur77/Cyto c/caspase 9 pathway

Our previous study demonstrated that CHD1L is able to block nuclear to mitochondrial translocation of Nur77 via their interaction, and subsequently to inhibit Cyto *c* release into the cytoplasm and the following activation of caspase 9 and caspase 3, finally inhibiting apoptosis.[12] Based on these findings, we investigated whether the inhibitory effect of CHD1L on 5-FU is
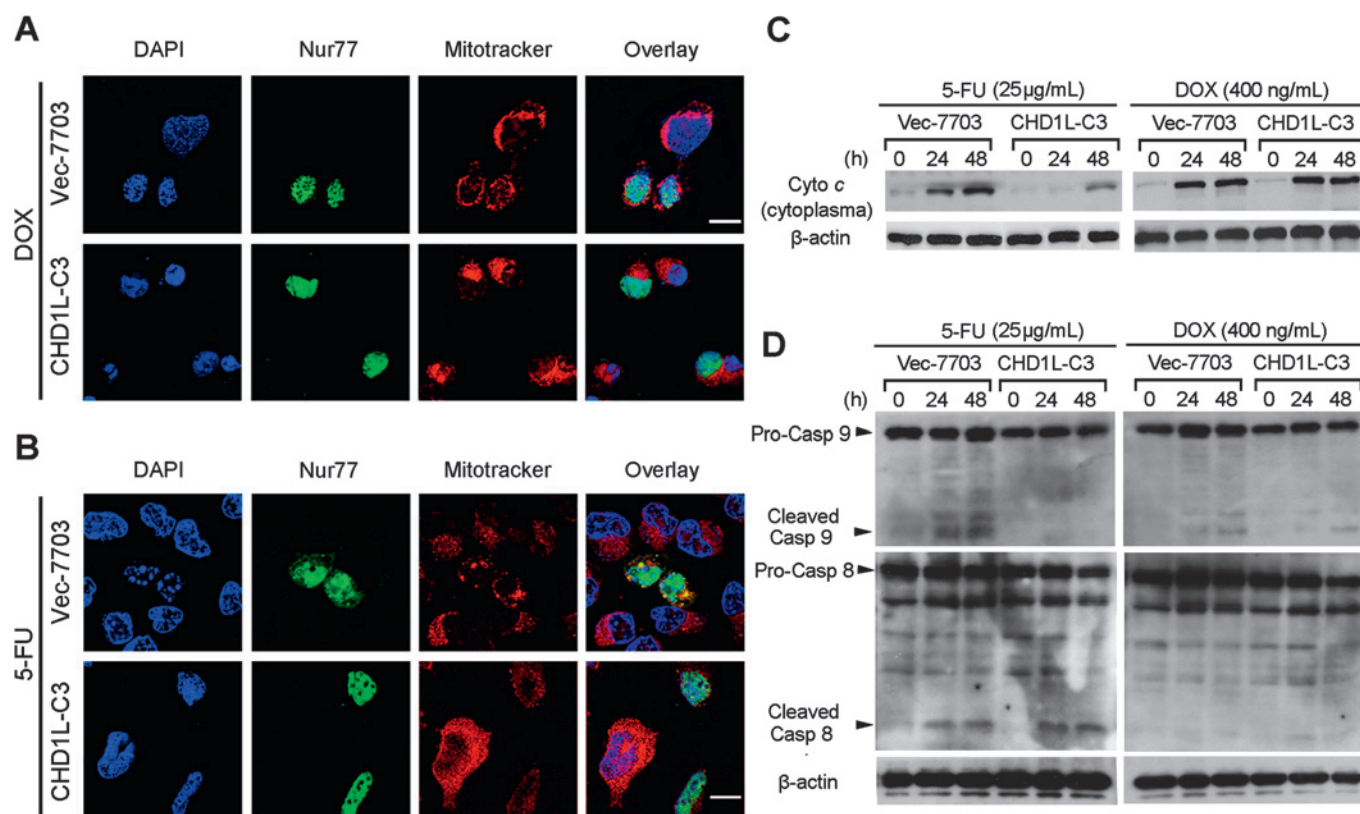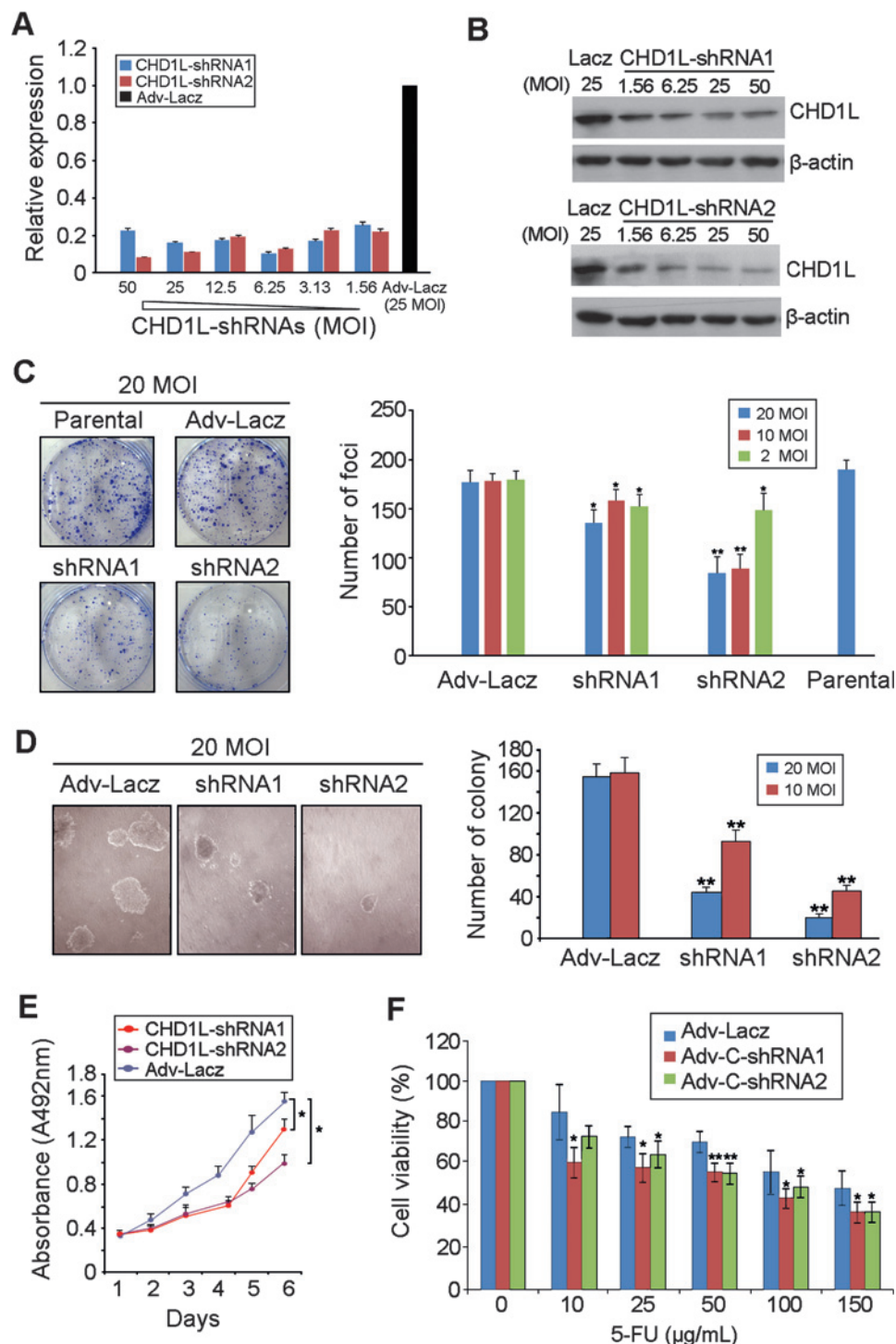

Figure 4 CHD1L inhibits Nur77-mediated apoptosis induced by 5-fluorouracil (5-FU), but not by doxorubicin (DOX). (A and B) After treatment with DOX (400 ng/ml) (A) or 5-FU (25 μg/ml) (B) for 24 h, GFP/Nur77 (green), mitochondria (red) and nuclei (blue) were captured using confocal microscopy. Both Vec-7703 and *CHD1L*-C3 cells showed an exclusively nuclear localisation of Nur77 after treatment with DOX. However, after treatment with 5-FU, Nur77 in Vec-7703 cells, but not in *CHD1L*-C3 cells, began to migrate from the nucleus to the mitochondria (bar=10 μm). (C and D) The level of cytochrome *c* (Cyto *c*) in the cytosolic fraction (C), and expression of caspase 9 and 8 (D) in Vec-7703 and *CHD1L*-C3 cells were detected by western blotting after 5-FU or DOX treatment at the indicated time points. β-Actin was used as a loading control. DAPI, 4′,6-diamidino-2-phenylindole.

associated with the Nur77/Cyto c/caspase 9 pathway. For this purpose, the GFP/Nur77 expression plasmid was transiently transfected into Vec-7703 and CHD1L-C3 cells, which were then treated with 5-FU or DOX. The subcellular location of Nur77 was detected by confocal microscopy. When treated with DOX for 24 h, apoptosis (characterised as nuclear condensation and fragmentation) occurred in Vec-7703 and CHD1L-7703 cells transfected with GFP/Nur77 (figure 4A). In both Vec-7703 and CHD1L-C3 cells, GFP/Nur77 was exclusively localised in the nucleus after DOX treatment, suggesting that apoptosis induced by DOX was not through the Nur77-mediated apoptotic pathway. However, the nuclear to mitochondrial translocation

of Nur77 was observed in Vec-7703 cells after 5-FU treatment (figure 4B), suggesting that the proapoptotic effect of 5-FU was via the Nur77-mediated apoptotic pathway. As expected, CHD1L could inhibit nuclear to mitochondrial translocation of Nur77 and the translocation was not observed in CHD1L-C3 cells after 5-FU treatment (figure 4B). The effects of CHD1L on Cyto c release into the cytoplasm and the activation of caspase 9 were then studied by western blot analysis. Compared with Vec-7703 cells, the levels of Cyto c in the cytoplasm and of cleaved caspase 9 were obviously decreased in CHD1L-C3 cells after 5-FU treatment (figure 4C,D). However, CHD1L had no effect on Cyto c release and the activation of caspase 9

Figure 5 Adenoviral CHD1L-shRNAs (short hairpin RNAs) inhibits tumorigencity and chemoresistance in vitro. (A and B) Knockdown efficiency of CHD1L-shRNAs (CHD1L-shRNA1 and CHD1L-shRNA2) was detected by quantitiative PCR (A) or western blot analysis (B) in PLC8024 cells. Cells were infected with CHD1L-shRNAs at the indicated concentrations or with the negative control (Adv-Lacz). For quantitative PCR, three independent experiments were performed in triplicate. β-Actin was used as a loading control for western blot analysis. (C) Representative images of the inhibitory effect of CHD1L-shRNAs on foci formation in PLC8024 cells (left panel) compared with parental cells and Adv-Lacz-infected cells. Surviving colonies (>50 cells/colony) were counted and are shown in a bar chart (right panel). Data represent the mean±SD derived from three independent experiments with triplicate wells (*p<0.01; **p<0.001). (D) Representative images of colony formation in soft agar (left panel). The numbers of colonies (≥10 cells) were counted and are summarised in a bar chart (right panel). Data represent the mean±SD derived from three independent experiments with triplicate wells (**p<0.001). (E) Effects of CHD1L-shRNAs on the growth rate in PLC8024 cells. Growth curves of cells infected by adenoviral vectors (at a multiplicity of infection (MOI) of 2) were detected for 6 days by XTT proliferation assay. Data represent the mean±SD derived from three individual experiments with triplicate wells (*p<0.001). (F) The inhibitory effect of CHD1L-shRNAs on chemoresistance to 5-fluorouracil (5-FU). After CHD1L-shRNA (MOI=10) infection, PLC8024 cells were treated with 5-FU for 48 h at the indicated concentration. The cell viabilities were detected by XTT assay. Compared with Adv-Lacz, CHD1L-shRNAs could significantly inhibit the chemoresistance to 5-FU (*p<0.05; **p<0.01). Three independent experiments were performed in triplicate.

when Vec-7703 and CHD1L-7703 cells were treated with DOX (figure 4C,D). Western blot analysis of 5-FU- or DOX-induced caspase 8 activation indicated that the antiapoptotic effect of CHD1L on 5-FU or DOX treatment was not associated with the death signalling pathway. All these data suggested that CHD1L could inhibit apoptosis induced by 5-FU, rather than DOX, via the inhibition of the Nur77-mediated apoptotic pathway and confer chemoresistance to 5-FU.

Virus-mediated CHD1L silencing suppresses the tumorigenic ability of CHD1L and chemoresistance to 5-FU

To study further the tumorigenic and chemoresistant effects of CHD1L in HCC, two shRNAs against CHD1L were cloned into the pENTR/U6 vector for adenovirus vector construction (CHD1L-shRNA1 and CHD1L-shRNA2), and Adv-Lacz was included as a negative control. To test the efficiency of inhibition by CHD1L-shRNAs on the expression of CHD1L in PLC8024 cells, cells were infected with various concentrations of virus ranging from a multiplicity of infection (MOI) of 1.5 to 50. Real-time quantitative PCR and western blot analyses showed that CHD1L-shRNAs could efficiently silence CHD1L expression at both the mRNA and protein levels (figure 5A,B). In vitro assays were then used to investigate the inhibitory effects of CHD1L-shRNAs on tumorigenicity in the HCC cell line PLC8024.

Compared with Adv-Lacz-infected cells, CHD1L-shRNA-infected cells showed a lower efficiency of foci formation ($p < 0.01$, independent Student t test, figure 5C). Similarly, the colony formation in soft agar was significantly reduced in CHD1L-shRNA-infected cells compared with Adv-Lacz-infected cells ($p < 0.001$, independent Student t test, figure 5D). Next, XTT proliferation assays showed that after viral infection, the growth rate of PLC8024 cells was significantly inhibited by CHD1L-shRNAs ($p < 0.001$, independent Student t test; figure 5E). Importantly, after viral infection followed by treatment with 5-FU at different concentrations (0, 10, 25, 50, 100 and 150 μg/ml; IC_{50} at 75 μg/ml) for 72 h, the cell viability was significantly inhibited by CHD1L-shRNA-treated cells when compared with Adv-Lacz-infected cells ($p < 0.05$, independent Student t test; figure 5F).

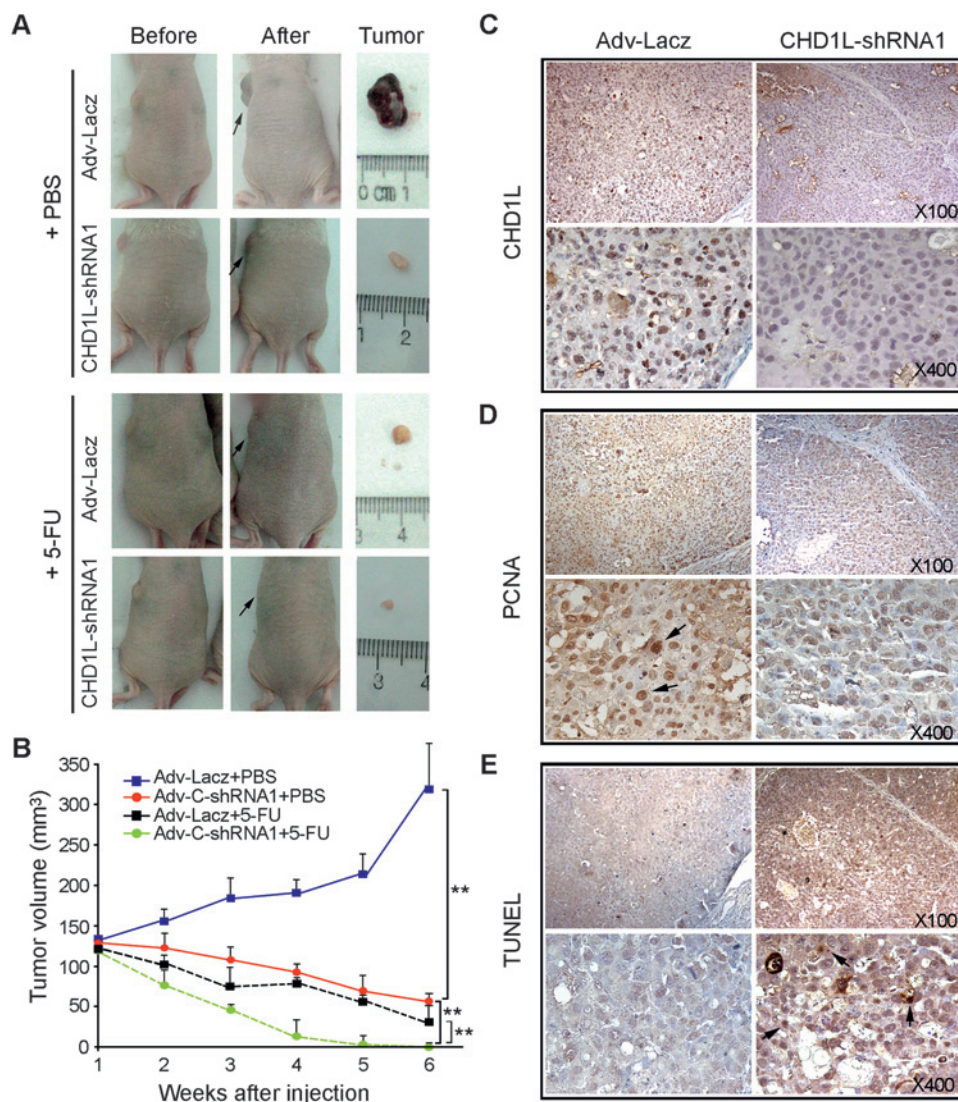### Virus-mediated CHD1L silencing inhibits tumorigenesis and increases chemosensitivity to 5-FU in nude mice

To explore the effect of CHD1L-shRNA in vivo, xenograft tumours were generated by injecting PLC8024 cells subcutaneously into nude mice. When the xenograft tumours reached ∼5 mm^3, each group of mice (5 mice per group) was given CHD1L-shRNA1 or Adv-Lacz (intratumoral injection, 2×10^9 plaque-forming units (pfu), once a week for 6 weeks), combined

Figure 6 CHD1L depletion by adenoviral CHD1L-shRNAs (short haipin RNAs) inhibits tumorigenicity and increases the chemosensitivity to 5-fluorouracil (5-FU) in vivo. (A) Representative images of xenograft tumours in nude mice induced by PLC8024 cells. Left column, before treatment; middle column, 6 weeks after treatment with adenoviral vectors (intratumoral injection, 2×10^9 plaque-forming units, once a week for 6 weeks) combined with 5-FU (intraperitoneal injection, 40 mg/kg body weight, once a week for 6 weeks) or phosphate-buffered saline (PBS); right column. representative of tumours excised from mice after 6 weeks of treatment. (B) The tumour growth curve of each group of mice which received the scheduled treatment as described above (n=5, **$p < 0.0001$). (C—E) Immunohistochemical staining of CHD1L (C) and proliferating cell nuclear antigen (PCNA) (D) and TUNEL (deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labelling) assay (E) were performed on serial sections of xenograft tumours excised from mice after 6 weeks of treatment. Arrows indicate the strong nuclear expression of PCNA (D) and TUNEL-positive cells (E).

with 5-FU (intraperitoneal injection, 40 mg/kg body weight, once a week for 6 weeks) or PBS. Compared with the Adv-Lacz/PBS group, tumour growth rates and tumour volumes were significantly inhibited in the three other groups (figure 6A,B). Upon intraperitoneal treatment with PBS, the average tumour volume of the harvested xenograft tumours from the *CHD1L*-shRNA1 group (55 ± 12 mm^3) was significantly smaller than tumours from the Adv-Lacz group (320 ± 65 mm^3, p<0.0001, independent Student t test). Interestingly, the inhibitory effect of *CHD1L*-shRNA1 on tumour growth was similar to that of 5-FU treatment (figure 6B). Furthermore, when mice were treated with the combination of *CHD1L*-shRNA1 and 5-FU, the tumour growth was significantly inhibited compared with mice treated with either *CHD1L*-shRNA1 or 5-FU (p<0.0001; independent Student t test, figure 6B). In 3/5 of mice treated with *CHD1L*-shRNA1 and 5-FU, the tumour had completely disappeared after 6 weeks.

To investigate further the effect of *CHD1L*-shRNA1 on the chemosensitivity to 5-FU in vivo, immunohistochemical staining of proliferating cell nuclear antigen (PCNA) and CHD1L, as well as TUNEL assay were performed to detect the proliferating and apoptotic cells on serial sections of xenograft tumours from 5-FU-injected mice. The results showed that the endogenous expression of CHD1L was apparently decreased in *CHD1L*-shRNA1-treated tumours compared with Adv-Lacz-injected tumours (figure 6C). As expected, fewer PCNA-positive (figure 6D) and more TUNEL-positive cells (figure 6E) were observed in tumours treated with *CHD1L*-shRNA1, compared with Adv-Lacz-treated tumours. All these data suggested that silencing endogenous CHD1L expression by virus-mediated RNA interference (RNAi) might inhibit the tumorigenicity and increase the chemosensitivity to 5-FU.

DISCUSSION

HCC is a major health problem worldwide because of its extremely poor prognosis. The high mortality rate for HCC is due to the limited treatment options. Potential curative treatments including liver resection, transplantation and percutaneous ablation are available for early-stage HCC, which accounts for 30—40% of all HCC cases.[16—18] TACE is the recommended first-line non-curative treatment for patients with large multifocal inoperable HCC[19] because it has been shown to have survival benefits in recent randomised controlled trials.[20 21] Although chemotherapy can improve the survival rate for advanced HCC cases, chemoresistance remains a tremendous therapeutic challenge in treating HCC.[16 22 23] The efficacy of chemotherapeutic drugs is generally believed to depend on their ability to trigger apoptosis.[4 5] Therefore, identification and characterisation of genes correlated with chemoresistance is imperative to understand the molecular mechanism of chemoresistance in HCC and find new therapeutic targets for HCC treatment.

Amplification of 1q21 is an early event in HCC development,[24] implying that the putative oncogene within this region may play an important role in the initiation of HCC pathogenesis. In our previous studies, we isolated a candidate oncogene *CHD1L*[10] and demonstrated that its oncogenic function is associated with its antiapoptotic role.[12] In the present study, HCC tissues showed increased expression of CHD1L compared with non-tumour (including cirrhotic) tissues in all 109 cases examined. Overexpression of CHD1L was significantly associated with tumour microsatellite formation, advanced tumour stage and shorter OS time. In addition, overexpression of CHD1L was also significantly associated with poor response to

chemotherapy in 50 patients with HCC who received the TACE treatment with 5-FU in combination with DOX and CDDP. As reported previously, CHD1L could confer strong resistance to apoptosis by inhibiting Nur77-mediated apoptosis.[12] These findings provide us with new insights into the clinical value of CHD1L and its role in drug resistance in HCC. The role of CHD1L as an indicator and target in chemosensitivity was further investigated in this study. The results showed that CHD1L could confer drug resistance against chemotherapeutic agents, such as 5-FU, that induce apoptosis through the Nur77-mediated pathway. However, CHD1L fails to confer chemoresistance to agents such as DOX, that induce apoptosis through other pathways. Interestingly, it has been reported that the administration of 5-FU does not significantly benefit patients with HCC,[25] and a clinical study indicated that patients with HCC treated with 5-FU had a lower 2-year survival rate than patients treated with DOX.[14] Taken together, these data demonstrate that the overexpression of CHD1L is one of the major mechanisms of chemoresistance to 5-FU in HCC treatment.

Given that CHD1L is frequently overexpressed in HCC, CHD1L may become a useful new therapeutic target in HCC treatment. DNA vector-based shRNA as a means of RNAi is a promising mechanism for the precise disruption of gene expression to achieve a therapeutic effect. However, the clinical usage of shRNA therapeutics in cancer is limited by ineffective delivery into target cancer cells. The viral delivery of shRNAs has been reported to be used for tumour-specific gene therapy to reverse the oncogenic phenotype of cancer cells.[26] Adenoviral vectors can efficiently transduce genes into a broad range of cell types and have been used extensively as gene delivery vehicles.[27] In recent years, adenoviral vectors carrying shRNA expression cassettes have been developed and tested in several studies.[28—30] In this study, we generated Adv-*CHD1L*-shRNAs and tested the therapeutic efficacy of this viral construct in a HCC cell line PLC8024 in vitro and in vivo. We found that this viral construct could significantly inhibit the endogenous expression of CHD1L in vitro. Further studies indicated that Adv-*CHD1L*-shRNAs were able to suppress cell growth, clonogenicity and chemoresistance to 5-FU in vitro. More interestingly, in vivo studies showed that Adv-*CHD1L*-shRNAs could inhibit xenograft tumour growth and increase the sensitivity to 5-FU. The inhibitory efficiency of *CHD1L*-shRNA is similar to 5-FU treatment. The combination of *CHD1L*-shRNA and 5-FU showed a significant inhibitory effect on tumour growth in nude mouse compared with the treatment with either *CHD1L*-shRNA or 5-FU alone. In addition, complete tumour disappearance was observed in 3/5 tested mice. These data strongly suggest that silencing CHD1L expression in HCC by the corresponding shRNA has a great therapeutic potential in HCC treatment, especially to increase the chemosensitivity combined with chemotherapy.

samples, analysed the clinico-pathological features and performed statistical analysis. X-YG and Y-FY supervised the project and provided suggestions. LC and X-YG drafted and revised the manuscript. All authors reviewed, critiqued and offered comments on the text and approved the final version of manuscript.

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Clinical significance of *CHD1L* in hepatocellular carcinoma and therapeutic potentials of virus-mediated *CHD1L* depletion

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