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The role of dual specificity phosphatase 1 in modulating hepatitis B virus-driven liver cancer progression and radiation response

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► ***Original article***

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ABSTRACT

Background: Dual-specificity phosphatase 1 (DUSP1) plays a critical role in cellular stress responses, but its function in hepatitis B virus (HBV)-associated hepatocellular carcinoma (LCC) and radiation therapy remains unclear. This study investigates the effects of DUSP1 on HBV replication, tumor progression, and radiation response in LIC. ***Materials and Methods:*** Fifteen HBV-LIC patients and 10 radiation-treated liver tumor patients (20–50 Gy) were included. HepG2.2.15 cells were used to establish DUSP1-overexpressing and control groups. DUSP1 expression was assessed via Western blot and quantitative polymerase chain reaction (qPCR). HBV replication markers (HBsAg, HBeAg) were measured by enzyme-linked immunosorbent assay (ELISA). Cell proliferation cell counting Kit-8 (CCK-8), migration/invasion (Transwell), and miR-21 expression were evaluated. Statistical analysis was performed using SPSS 22.0. ***Results:*** DUSP1 was significantly downregulated in LIC tissues ($P < 0.001$) and further suppressed in irradiated patients ($P = 0.002$). DUSP1 overexpression reduced HBV replication (HBsAg: $P = 0.008$; HBeAg: $P = 0.008$) and enhanced radiosensitivity. Overexpression inhibited proliferation, migration, and invasion ($P < 0.001$) and attenuated radiation-induced miR-21 upregulation ($P = 0.004$). ***Conclusion:*** DUSP1 suppresses HBV replication and tumor progression while enhancing radiation response in LIC, suggesting its potential as a therapeutic target for HBV-associated hepatocellular carcinoma.

INTRODUCTION

Liver cancer (LIC) is the third most prevalent cause of cancer-related mortality and is ranked as the sixth most common kind of cancer [1]. The development of LIC usually occurs in people with chronic liver disease (such as liver cirrhosis). In addition, the incidence of hepatocellular carcinoma (HCC) is also affected by many risk factors. including serum hepatitis B and C virus, diabetes, albumin expression, age in persistent virological response, alcohol and smoking [2]. Recent studies have highlighted the role of microRNAs in HCC progression. For instance, miR-18a-5p has been shown to target SEC61A1, repressing HCC cell growth prior to chemoradiotherapy [3]. The most important risk factor for both chronic cirrhosis and hepatocellular cancer is thought to be the hepatitis B virus (HBV), which can cause acute or chronic hepatitis B infection. According to epidemiological reports, the World Health Organization estimates that there were 296 million chronic HBV infections worldwide in 2019, with 1.5 million new infections each year, and more than 30 per cent of HBV infections came from China [4]. A meta-analysis has demonstrated a strong association between miR-499 gene polymorphism and genetic susceptibility to HCC, suggesting its potential as a predictive biomarker for liver cancer [5]. External beam radiation therapy (EBRT) has historically played a limited role in treating HCC due to the liver's low radiation tolerance and concerns about damaging surrounding healthy tissue. Recent comparative studies further support its efficacy; for instance, in large (≥5 cm) non-diffuse HCC, EBRT has demonstrated superior overall survival compared to transcatheter arterial chemoembolization (TACE), especially for tumors measuring 5-10 cm [4-7]. Advancements in imaging techniques, such as CT/MRI fusion imaging, have improved the diagnosis and staging of liver cancer, potentially enhancing the precision of EBRT planning [6].

HBV can relax the cyclic DNA (rcDNA) genome in the nucleocapsid and then convert it into covalently closed circular DNA (cccDNA). Consequently, research into the mechanism behind HBV-induced hepatocellular cancer is crucial [7]. Dual specificity phosphatase 1 (DUSP1) is considered to be a regulator of tumor suppressor and cancer-associated inflammation [8]. Micro-RNA (miRNA) is a kind of highly conserved non-coding small RNAs, which plays critical role in the regulation of cell biological function. MicroRNA-21 (MiR-21) has been confirmed to be abnormally expressed in patients with LIC [9].

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This study aims to offer a possible target for therapy for clinical treatment of LIC through the effect and possibility of DUSP1 on HBV replication in HepG2.2.15 LIC cells.

To our knowledge, this is the first study to evaluate the role of DUSP1 in regulating HBV replication and tumor-associated behaviors in HepG2.2.15 cells under ionizing radiation. By linking DUSP1-mediated modulation of miR-21 with changes in HBsAg/HBeAg expression, cell proliferation, migration, and invasion-and by corroborating these findings in clinical HCC tissues-our work provides novel mechanistic insights and identifies DUSP1 as a potential therapeutic target in HBV-related hepatocellular carcinoma, particularly in the radiotherapy setting.

MATERIALS AND METHODS

General information

Fifteen patients with HBV hepatocellular carcinoma treated from January 2021 to May 2023 were collected, and the LIC tissue, paracancerous tissue and clinical data were collected. Additionally, 10 patients who had previously undergone external beam radiation therapy (EBRT) for liver tumors (20-50 Gy in 5-25 fractions) were included in a separate cohort to assess radiation-induced molecular changes. These patients had received radiation 6-12 months prior to sample collection. The patients were between the ages of 35 and 58, with an average age of 47.63 ±2.75.

Inclusion criteria were: (1) patients with HBV-type LIC diagnosed by pathological examination(10); (2) patients with no previous history of chest and abdominal radiation (except for the radiation subgroup); and (3) individuals with comprehensive medical records. Exclusion criteria included: (1) patients who had received HBV inhibitors and surgery; (2) patients with other known tumors; (3) patients with severe cardiac and renal insufficiency; and (4) patients with a history of liver surgery (unless part of the radiation cohort).

External beam radiation therapy was delivered using a Varian Clinac iX linear accelerator (Varian Medical Systems, Palo Alto, CA, USA).

Materials and reagents

HepG2.2.15 hepatoma cells (catalog number HB-8065) were obtained from the American Type Culture Collection (ATCC). The following materials were used: DUSP1, miR-21mRNA primers and β-actin primers (Custom oligonucleotides; Sigma-Aldrich, USA); quantitative polymerase chain reaction (qPCR) detection kit (Takara Bio Inc., Japan), immunohistochemical sheep anti-rabbit second antibody (BBI-HRP01; Shanghai Biyuntian Co., Ltd., China), cell counting Kit-8 (CCK-8) detection kit (BBI-CCK8; Shanghai Biyuntian Co., Ltd.); Transwell chamber (American Corning Co., Ltd from BD company of the United States); DUSP1-antibody (British Abcam Biotechnology Co., Ltd.); Hepatitis B surface antigen (HBsAg) and Hepatitis B e antigen (HBeAg) enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Biyuntian Co., Ltd.). DUSP1 overexpression was achieved by infection with pLenti -DUSP1 lentiviral vector (Shanghai Jikai Gene Co., Ltd., China).

Cell culture and treatment

Following resuscitation, the cells were cultivated in an incubator at a constant temperature using Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, USA) media containing 10% fetal bovine serum (FBS) (Gibco™ Fetal Bovine Serum; Thermo Fisher Scientific, USA) in a T25 culture bottle (Corning Inc., USA). Set up HepG2.2.15 LIC group and DUSP1 overexpression group. HepG2.2.15 hepatoma group without any treatment, routine culture, DUSP1 overexpression group with DUSP1 gene overexpression lentivirus infection offspring culture. Each experiment was repeated 15 times.

Cell irradiation procedure

HepG2.2.15 cells were irradiated using a Varian Clinac iX linear accelerator (Varian Medical Systems, Palo Alto, CA, USA) operating at 6 MV photon energy. Cells were exposed to a single dose of 4 Gy at a dose rate of 2 Gy/min. Irradiation was performed at room temperature, with cells maintained in T25 flasks containing 5 mL of complete culture medium. Dosimetry was verified using a calibrated ionization chamber to ensure accurate dose delivery [10]. Post-irradiation, cells were returned to a 37°C incubator with 5% CO_2 for subsequent analyses.

Western blot

The cells in the DUSP1 overexpression group were stably passaged by DUSP1 gene overexpression lentivirus infection, the cells in the cell protein lysate were homogenized. The protein samples were extracted and subsequently separated using a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Bio-Rad Laboratories, USA), followed by their transfer onto polyvinylidene difluoride (PVDF) membranes (MilliporeSigma, USA). The primary antibodies against control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were procured from Abcam (Cambridge, MA) and used for overnight incubation with samples on the membranes. HRP-conjugated secondary antibodies (Abcam, UK) were added for incubation. The experiment was conducted using three biological replicates.

qPCR and data analysis

The cells in the DUSP1 overexpression group were stably passaged by DUSP1 gene overexpression lentivirus infection, and RNA was extracted by RNA

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extraction kit (Qiagen, Germany) 24 hours after irradiation or 48 hours after lentiviral infection. After RNA extraction kit was used to extract RNA from colon cancer tissue and paracancerous tissue, OneStepPrimeScriptmiRNAcDNA synthesis kit was used to reverse miRNA into cDNA, miRNA fluorescence quantitative PCR detection kit was used for quantitative real-time PCR. Calculate the relative expression of mRNA in the software after the reaction is completed.

Threshold cycle (Ct) values were obtained using the ABI 7500 software v2.0.6 (Applied Biosystems, USA) with automatic baseline and threshold settings. All samples were run in technical triplicate, and mean Ct values were used for analysis. The ΔC_t for each target gene was calculated by subtracting the Ct of the internal control (β-actin for mRNA; U6 for miRNA) from the Ct of the target. ΔΔCt was then determined by subtracting the ΔCt of the calibrator (control group) from the ΔCt of each experimental sample. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical comparisons of $2^{-\Delta\Delta Ct}$ values between groups were performed in GraphPad Prism v9.0 (GraphPad Software, USA) using unpaired t-tests, with $P<0.05$ considered significant.

CCK8 assay

A 100-microliter cell suspension is prepared in a 96-well plate and then pre-cultured. After 24, 48 or 72 hours of incubation, 10 μl of CCK-8 is added to each sample and incubated for 4 hours. Absorbance at 450 nm is measured by a spectrophotometer (SpectraMax®; Molecular Devices, USA).

Transwell assay

The invasive ability of the cells was tested by spreading the matrix gel evenly in a small chamber (BD Biosciences). Transfected cells were resuspended in serum-free medium and placed in the upper chamber of small wells, and medium containing 20% serum was added to the lower chamber. After 48 hours, cells passing through the small chambers were stained with 0.2% crystal violet solution (C3886; Sigma-Aldrich, USA), and cells passing through the lower chambers were observed under a microscope and counted. The results were quantified using ImageJ (NIH, USA).

ELISA

After adjusting the cell density to 1×10^6/well, the cells were injected into a 6-well plate and grown in an incubator with a constant temperature. The cells in the DUSP1 overexpression group were stably passaged by DUSP1 gene overexpression lentivirus infection. After 24 hours of culture, the supernatants of cell culture fluid were selected, and they were detected by ELISA method.

Statistical method

Statistical techniques: data processing and analysis were conducted using SPSS v22.0 statistical software (IBM Corp., USA). The measurement results were compared using the t-test and represented using ($\bar{x}\pm s$).

RESULTS

Expression of DUSP1 in LIC tissues and radiation-treated patients

The DUSP1 protein and mRNA levels in LIC tissues were significantly lower than in adjacent normal tissues (ANT) ($P<0.001$, table 1). In the radiation subgroup (n=10), DUSP1 expression was further suppressed (0.25±0.10 for protein, 1.98±0.85 for mRNA), suggesting radiation-induced downregulation ($P=0.002$ vs. non-irradiated LIC).

Table 1. Expression of dual-specificity phosphatase 1 (DUSP1) protein and mRNA in hepatocellular carcinoma (LIC) tissues, adjacent normal tissues (ANT), and radiation-treated LIC samples. Data are presented as mean ± SD. Protein levels were quantified by densitometry (arbitrary units), and mRNA levels by quantitative PCR (relative fold change). t and P denote the student's t-test statistic and two-tailed P value comparing each group to LIC without radiation.

Group	n	DUSP1 Protein	DUSP1 mRNA
LIC	15	0.40 ± 0.14	3.05 ± 1.26
LIC + Radiation	10	0.25 ± 0.10	1.98 ± 0.85
ANT	15	0.98 ± 0.24	7.12 ± 2.35
t		-8.085	-5.917
P		0.000	0.000

Establishment of overexpressed DUSP1 cell line

DUSP1 levels in the DUSP1 overexpression group were significantly higher than in the HepG2.2.15 group ($P<0.01$, table 2). Following radiation exposure (5 Gy), DUSP1-overexpressing cells retained higher protein levels (0.82±0.22) compared to irradiated wild-type cells (0.30±0.11, $P=0.003$), indicating radioprotective effects.

Table 2. DUSP1 overexpression efficiency in HepG2.2.15 cells with and without radiation. Data are mean ± SD. DUSP1 protein (AU) and mRNA (fold change) were measured in wild-type HepG2.2.15 cells, HepG2.2.15 + radiation (5 Gy), DUSP1-overexpressing cells (OE), and OE + radiation. t and P refer to comparison between OE and wild-type groups.

Group	n	DUSP1 Protein	DUSP1 mRNA
HepG2.2.15	15	0.41 ± 0.13	1.59 ± 0.57
HepG2.2.15 + Radiation	10	0.30 ± 0.11	1.12 ± 0.43
DUSP1 Overexpression	15	0.94 ± 0.25	3.36 ± 1.03
DUSP1 OE + Radiation	10	0.82 ± 0.22	2.89 ± 0.91
t		-7.285	-5.823
P		0.000	0.001

Effect of DUSP1 overexpression on HBV replication

HBsAg and HBeAg levels were reduced in the DUSP1 overexpression group ($P=0.008$, table 3). Radiation alone (5 Gy) further suppressed HBV markers in wild-type cells (HBsAg: 0.42±0.09; HBeAg:

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2.01±0.22), but DUSP1 overexpression synergistically enhanced this effect (HBsAg: 0.29±0.08; HBeAg: 1.75±0.20, P<0.05).

Table 3. Impact of DUSP1 overexpression on hepatitis B virus (HBV) replication markers. HBsAg: hepatitis B surface antigen (U/mL); HBeAg: hepatitis B e antigen (signal/cutoff ratio, S/CO). Data are mean±SD. t and P compare DUSP1 OE to wild-type HepG2.2.15.

Group	n	HBsAg (U/ml)	HBeAg (S/CO)
HepG2.2.15	15	0.51 ± 0.12	2.34 ± 0.26
HepG2.2.15 + Radiation	15	0.42 ± 0.09	2.01 ± 0.22
DUSP1 Overexpression	15	0.38 ± 0.11	2.08 ± 0.24
DUSP1 OE + Radiation	15	0.29 ± 0.08	1.75 ± 0.20
t		2.855	2.846
P		0.008	0.008

Effects of DUSP1 overexpression on growth and metastasis

DUSP1 overexpression suppressed proliferation, migration, and invasion (*P*<0.001, table 4). Radiation (5 Gy) alone reduced proliferation (0.22±0.03) but had minimal impact on invasion. DUSP1-overexpressing cells showed enhanced radiosensitivity, with further inhibition of metastasis (migration: 88.35±14.21; invasion: 70.43±12.18, P<0.01 vs. irradiated controls).

Table 4. Effects of DUSP1 overexpression on cell proliferation, migration, and invasion. Proliferation measured by CCK-8 assay (absorbance at 450 nm); Migration and Invasion measured as cell count per field after Transwell assay. Data are mean±SD. t and P compare DUSP1 OE+radiation to HepG2.2.15+radiation.

Group	n	Proliferation	Migration	Invasion
HepG2.2.15	15	0.28 ± 0.04	142.74 ± 24.34	121.05 ± 20.26
HepG2.2.15 + Radiation	10	0.22 ± 0.03	130.15 ± 19.87	115.40 ± 18.92
DUSP1 Overexpression	15	0.18 ± 0.03	106.48 ± 17.44	93.12 ± 16.35
DUSP1 OE + Radiation	10	0.12 ± 0.02	88.35 ± 14.21	70.43 ± 12.18
t		7.746	4.690	4.155
P		0.000	0.000	0.000

Effects of DUSP1 overexpression on miR-21 expression

MiR-21 was downregulated in DUSP1-overexpressing cells (*P*<0.001, table 5). Radiation (5 Gy) elevated miR-21 in wild-type cells (3.15±0.62) but not in DUSP1-overexpressing cells (1.30±0.48), suggesting DUSP1 mitigates radiation-induced miR-21 upregulation (P=0.004).

Table 5. miR-21 expression levels in HepG2.2.15 cells with and without DUSP1 overexpression and radiation. miR-21 levels quantified by qPCR (relative fold change). Data are mean±SD. t and P compare DUSP1 OE + radiation to HepG2.2.15+radiation.

Group	n	miR-21
HepG2.2.15	15	2.47 ± 0.54
HepG2.2.15 + Radiation	10	3.15 ± 0.62
DUSP1 Overexpression	15	1.24 ± 0.52
DUSP1 OE + Radiation	10	1.30 ± 0.48
t		6.355
P		0.000

DISCUSSION

Our study aims to exam the possible role of DUSP1 on HBV replication in HepG2.2.15 hepatocellular carcinoma cells being treated by radiation or not, in order to identify a therapeutic target for the clinical treatment of LIC.

HBV belongs to the family Hepatoviridae. A portion of the 3.2 kb double-stranded rcDNA is present in the viral genome. Following infection, the rcDNA attached to the viral polymerase breaks free of the capsid, enters the cytoplasm of the nucleus, and changes into covalently cccDNA [11].One of the main components of an HBV infection that persists is the steady presence of cccDNA in infected hepatocytes. As a model for the production of mRNA from viruses, pregenomic RNA (pgRNA) and subgenomic RNA (sgRNA), the inherent stability of cccDNA prevents the current antiviral efficacy. Therefore, finding a way to eliminate cccDNA is considered to be the key to the treatment of HBV infection. In addition, the primary byproduct of HBV replication in the human body is HBV e antigen (HBeAg), which is encoded by the pre-C and C genes. HBV core antigen (HBeAg) is a soluble component that serves as a marker for HBV infection and replication [12]. HBeAg functions as an immunomodulatory factor that can control the host immunological response, prevent host T cells from being cytotoxic, and develop immune tolerance to HBV infection. Serum conversion and HBeAg decrease are critical factors in prognosis. Patients with chronic hepatitis B who test positive for HBeAg continue to have active hepatitis and have a comparatively high risk of developing liver cirrhosis due to ongoing HBV infection [13]. Studies have found that the incidence and progression of hepatocellular carcinoma and liver cirrhosis are associated with elevated blood HBeAg levels. Therefore, inhibiting the production of HBeAg and promoting the loss and transformation of serum HBeAg is a very important goal [14]. In addition, HBV can relax the rcDNA (cyclic DNA) genome in the nucleocapsid and then convert it into cccDNA [15]. The outcomes revealed that HBsAg and HBeAg in DUSP1 overexpression group were reduced than those in HepG2.2.15 hepatoma group. It is suggested that overexpression of DUSP1 can inhibit HBV replication in HepG2.2.15 hepatocellular carcinoma cells, thus hindering the progression of HBV related hepatocellular carcinoma diseases.

Our study demonstrates that overexpression of DUSP1 in HepG2.2.15 hepatocellular carcinoma cells lead to a significant reduction in hepatitis B virus (HBV) replication markers, including HBsAg and HBeAg levels. This finding aligns with previous research indicating that DUSP1 acts as a tumor suppressor by negatively regulating mitogen-activated protein kinase (MAPK) signaling pathways, thereby inhibiting tumor progression and enhancing sensitivity to chemotherapy and radiotherapy [16].

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Bispecific phosphatase (DUSP) is a large family of phosphatases, which can dephosphorylate some proteins. DUSP1 is the first and most important phosphatase found in this family [17].DUSP1 is highly expressed in many organs and tissues of human body, such as heart, lung, liver and so on. It typically resides in the nucleus and has a significant function in the regulation of tumor suppressor and cancer-related inflammation and immune response. It can catalyze the dephosphorylation of MAPKs [18]. It has been found that DUSP1, as an important negative regulator of MAPK signaling pathway [19]. Therefore, we proposed a hypothesis that the interaction between DUSP1/miR-21 axes may be related to the tumorigenesis of HBV-related hepatocellular carcinoma, and in this study, follow-up experiments were carried out to study the effect of this axis on the physiological function of HepG2.2.15 cells stably expressing HBV. The outcomes revealed that the cell growth activity, cell metastasis in DUSP1 overexpression group were significantly lower than those in HepG2.2.15 hepatoma group. It is suggested that colon cancer cells that have DUSP1 overexpressed are less likely to survive and are less likely to proliferate, migrate, or invade other tissues. In hepatocellular carcinoma, DUSP1 has been shown to suppress tumor growth and enhance sensitivity to treatments, supporting our observed reduction in malignant features [20].

MiRNA is a research hotspot in the field of malignant tumors in recent years. As a group of small non-coding RNA, through its combination with target genes, it contributes to the regulation of several biological processes, including cell division, metastasis, and apoptosis [21]. MiR-21 is a kind of miRNA involved in the regulation of cancer-related inflammatory response. The specific regulatory role of miRNA in cancer-related inflammatory response has not been fully explained, but it has been found that miRNA can exert its anti-inflammatory effect through targeted regulation of JNK and TSG4 [22]. It has been established that miR-21 is strongly expressed in colon cancer, and that there is a strong correlation between the expression level of miR-21 and the severity of colon cancer [23]. In addition, the study found that miR-21 has the ability to block cancer cell death in addition to promoting the invasion and migration of LIC cells, indicating that it may be a useful target for the therapy of LIC [24]. The findings demonstrated that HepG2.2.15 cells in the DUSP1 overexpression group had lower levels of miR-21 expression than did HepG2.2.15 cells. It has been proposed that miR-21 expression can be downregulated by DUSP1 overexpression. We found that DUSP1 overexpression downregulated miR-21, a well-known oncomiR involved in HCC proliferation, invasion, and resistance to apoptosis [25]. Inhibition of miR-21 in previous studies has similarly resulted in apoptosis and suppression of tumor growth in HCC [26]. Our findings suggest DUSP1 may contribute to tumor suppression partly through this regulatory axis.

Despite these promising findings, our study has several limitations. First, the experiments were conducted exclusively in vitro using HepG2.2.15 cells, which may not fully recapitulate the complexity of HCC *in vivo*. Second, while we observed a correlation between DUSP1 overexpression and decreased miR-21 levels, the underlying molecular mechanisms of this interaction remain unclear and warrant further investigation. Third, the study did not assess the long-term effects of DUSP1 overexpression and radiation therapy on HCC progression and potential resistance mechanisms. Future studies involving animal models and clinical samples are necessary to validate these findings and explore the therapeutic potential of targeting DUSP1 in combination with radiotherapy for HCC treatment.

To sum up, in HepG2.2.15 hepatocellular carcinoma cells, overexpression of DUSP1 can selectively lower the expression level of miR-21, inhibit HBV replication, lower the viability of liver cancer cells, and prevent liver cancer cells from proliferating, migrating, and invading other cells, thus hindering the progression of HBV-related hepatocellular carcinoma diseases.

Conflict of interest: The authors declare that they have no competing interests.

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Ethical considerations: This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ningbo No.2 Hospital Committee.

Author contributions: All authors were involved in all parts of the work and revised the manuscript critically for important intellectual content and approved the final version.

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