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ORIGINAL ARTICLE

CircFMN2 boosts sorafenib resistance in hepatocellular carcinoma cells via up-regulating CNBP by restraining ubiquitination

Chen Fan[1], Xiaoli Zhu[2], Qi Zhou[1], Weidong Wang[1]

[1]Department of Intervention, Wuxi People's hospital, Nanjing Medical University, Wuxi 214023, China. [2]Department of Intervention, The First Affiliated Hospital of Soochow University, Suzhou 215006, China.

Summary

Purpose: *Non-coding RNAs exert critical biological effects in hepatocellular carcinoma. CircFMN2, a newly discovered functional molecule in prostate cancer and colorectal cancer was first investigated in sorafenib resistance in hepatocellular carcinoma cells.*

Methods: *The level of CircFMN2 was assessed via quantitative real-time PCR (qRT-PCR). The cell proliferation was detected via CCK-8 and colony formation assay. The cell apoptosis was measured via Tunel assay and flow cytometry analysis. Western blot assay was conducted for detection of CNBP level and ubiquitination. RNA pull-down assay and RNA immunoprecipitation were carried out to explore the interaction between circFMN2 and CNBP.*

Results: *CircFMN2 was highly expressed in multidrug resistant (MDR) cells. CircFMN2 overexpression exerted pro-proliferation effects in sorafenib treated liver carcinoma cells, while depletion of circFMN2 displayed mitigative effect in sorafenib treated MDR cells. Moreover, CNBP was verified as the binding protein of circFMN2. CNBP was upregulated in MDR cells, which was achieved via ubiquitination inhibition by circFMN2. Besides, CNBP overexpression was found to boost sorafenib resistance in hepatocellular carcinoma cells.*

Conclusions: *Our data disclosed that circFMN2, aberrant expressed in resistant cells, contributes to sorafenib resistance in hepatocellular carcinoma cells via upregulating CNBP by restraining ubiquitination.*

Key words: *CircFMN2, hepatocellular carcinoma, CNBP, sorafenib resistance, ubiquitination*

Introduction

Hepatic carcinoma has caused a tremendous global health and economic burden on global patients. Of all the cancers, liver cancer is the second most fatal cancer with a death rate of over 90% and its prevalence is still trending up worldwide [1,2]. It was disclosed by the World Health Organization in the annual projections that deaths from liver cancer will be over one million in 2030 [3]. A growing concern is needed for the prevalence of hepatic carcinoma. Although, considerable progress has been achieved in the treatment of hepatic carcinoma, such as microwave ablation, radiofrequency, liver resection, chemotherapy and liver transplantation, there are still many intractable obstacles including low diagnosis rate, high postoperative recurrence, drug resistance and poor survival rates [4-11]. It is imperative to find the effective therapeutic targets, resistance factors and efficient diagnostic markers.

Due to the inconspicuous symptoms of early liver cancer, patients are often confirmed as liver cancer at advanced stage, missing the opportunity of surgical treatment and ablation. Hence, systemic targeted therapy has raised considerable concern. Sorafenib is a first-line FDA approved systemic tar-

Corresponding author: Weidong Wang, PhD. Department of Intervention, Wuxi People's hospital, Nanjing Medical University, No.299 Qingyang Rd, Wuxi 214023, China.
Tel: +86 051085350102; Email: docwdwang@163.com
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geted therapeutic drug, exerting crucial therapeutically effect in hepatocellular carcinoma at later stage [12,13]. In clinical practice, survival benefits after sorafenib therapy have been fully validated [14-16]. However, attributing to the prevalence of liver cancer, therapeutic breakthroughs on sorafenib resistance and existing treatment efficiency are still pressing.

CircRNAs with covalently closed circular structure is a class of stable functional molecules and has been confirmed as vital regulator in diagnosis, treatment and drug resistance in hepatic carcinoma. Circ_100395 exerts anti-cancer effects in liver cancer via regulating epithelial-mesenchymal transition (EMT), apoptosis and proliferation [17]. CircRNA_10156 functions as a hepatoma contributor, which is considered as a prospective biomarker and therapeutic target [18]. Circ_0003418 improves cisplatin chemoresistance via suppression of wnt/β-catenin pathway in hepatic carcinoma [19]. CircUHRF1 contributes to anti-PD1 therapy resistance via disturbing NK cell function in liver cancer [20]. CircFoxo3 drives adriamycin resistance via modulating miR-199a-5p/ABCC1 axis in hepatoma [21]. The effects of many circRNAs in hepatic carcinoma remains unknown and so far, as we know, there are few reports on the circRNAs regulating sorafenib resistance. CircFMN2 is a newly discovered circRNA. CircFMN2 is only reported to participate in prostate cancer and colorectal cancer [22,23], and its functions in sorafenib resistance in hepatocellular carcinoma remains undefined. Therefore, in this report, we decided to explore the role of circFMN2 in sorafenib resistance and its underlying mechanism in hepatoma.

Methods

Cell culture and treatment

Hepatocellular carcinoma (HCC) cells (BEL-7402) and multidrug resistant HCC cells (BEL-7402/5-Fu) were purchased from Wuhan chundo Biotechnology Co. LTD (Wuhan, China). The BEL-7402 and BEL-7402/5-Fu cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibico, Rockville, MD, USA) containing 100 μg/ml streptomycin, 100 IU/ml penicillin and 10% fetal bovine serum (FBS) (Gibico, Rockville, MD, USA) at 37°C in an incubator with 5% CO_2. For the role of circFMN2 and CNBP on sorafenib (SOR) resistance, the cells were treated with sorafenib (6.5 μmol/L) for 24 h.

Cell transfection

After sorafenib treatment, the BEL-7402 cells were transfected with pcDNA3.1-CircFMN2 vector (BlueGene Biotech, Shanghai, China), pcDNA3.1-CNBP vector and their corresponding negative controls. BEL-7402/5-Fu cells were transfected with si-circFMN2 (BlueGene Biotech, Shanghai, China) and its negative control. The transfection was completed via using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) on the basis of manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Isolation of total RNAs was conducted via using RNAprep Pure cell kit (TianGen Biotech, Beijing, China). HiFiScript complementary DNA (cDNA) Kit (CWBIO, Beijing, China) was used to synthesize cDNA. Quantity analysis was carried out on StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). U6 served as internal reference. The quantitative calculation was progressed using $2^{-\Delta\Delta Ct}$ methods.

Cell counting kit-8 (CCK-8) assay

The cell viability was conducted using CCK-8 (GlpBio, Shanghai, China) in line with the instructions of the manufacturer. Briefly, the cells were seeded in 96-well plates and after sorafenib treatment and transfection, each well of the cells was incubated with CCK-8 solution (10 μL) for 2 h. The cell viability was determined by measuring the absorbance at 450 nm.

Colony formation assay

The cells after sorafenib treatment and transfection were seeded in 6-well plates. Fourteen days later, 4% paraformaldehyde fixation was carried out, followed by crystal violet staining. The colonies were counted and observed on a microscope (Olympus, Tokyo, Japan).

Flow cytometry assay

The cell apoptosis was evaluated using Annexin V-FITC/PI Apoptosis Detection Kit (YEASEN, Shanghai, China) as described in the instructions of the manufacturer. Briefly, after sorafenib treatment and transfection the cells were digested with trypsin; following centrifugation at 4°C, the cells were resuspended in the binding buffer (100 μL). Then, 5 μL Annexin V-FITC and 10 μL PI staining solution were incubated with the cells in the dark at room temperature for 15 min. The analysis of cell apoptosis was carried out on FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Tunel assay

The cells apoptosis was measured using Colorimetric Tunel apoptosis assay kit (Beyotime, Shanghai, China) in accordance with the instructions of the manufacturer. Briefly, the cells after treatment in the study groups were washed with phosphate buffered saline (PBS) followed by 4% paraformaldehyde fixation. After rinse, the cells were incubated with 3% Triton X-100 at room temperature for 5 min, followed by a rinse with PBS. Then the cells were incubated in the PBS containing 0.3% H_2O_2 for 20 min. Then the cells were reacted with biotin-labeled solution which is prepared as described in the instructions for one h at 37°C. Subsequently, streptavidin-HRP working solution was added into the cells. After diaminobenzidine staining and hematoxylin counterstain, the cell apoptosis was analyzed on a microscope (Olympus, Tokyo, Japan).

Western blot

The extraction of the total proteins was performed via lysing in lysis buffer (50 mM DTT, 0.1% SDS and 1% NP-40) followed by centrifugation at 4°C (10,000×g, 15 min). The supernatants were collected and protein was quantified via using bicinchoninic acid (BCA) protein assay kit (Abcam, Cambridge, MA, USA). Then, resolution of proteins (25 μg) was performed on 15% SDS-PAGE followed by transferring to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After 5% skimmed milk blockage, the membranes were reacted with the primary antibodies against CNBP (cat no. ab272676, Abcam, Cambridge, MA, USA) overnight and then incubated with goat anti-rabbit secondary antibody (cat no. ab216773, Abcam, Cambridge, MA, USA). Odyssey infrared scanner (Li-Cor) was used for detection of the blots.

RNA pull-down assay

The interaction between circFMN2 and CNBP was explored via using RNA pull-down kits (Guangzhou Saicheng Biological Technology Co. LTD, Guangzhou, China) according to the instructions of the manufacturer. Briefly, the cell lysates were prepared via using lysis buffer followed by centrifugation. The probers including biotin-labeled circFMN2, biotin-labeled antisense circFMN2 and biotin-labeled circFMN2 fragments were incubated with streptomycin magnetic beads for 6 h. Then, the magnetic bead-prober complex was obtained and incubated with the cell lysates overnight.

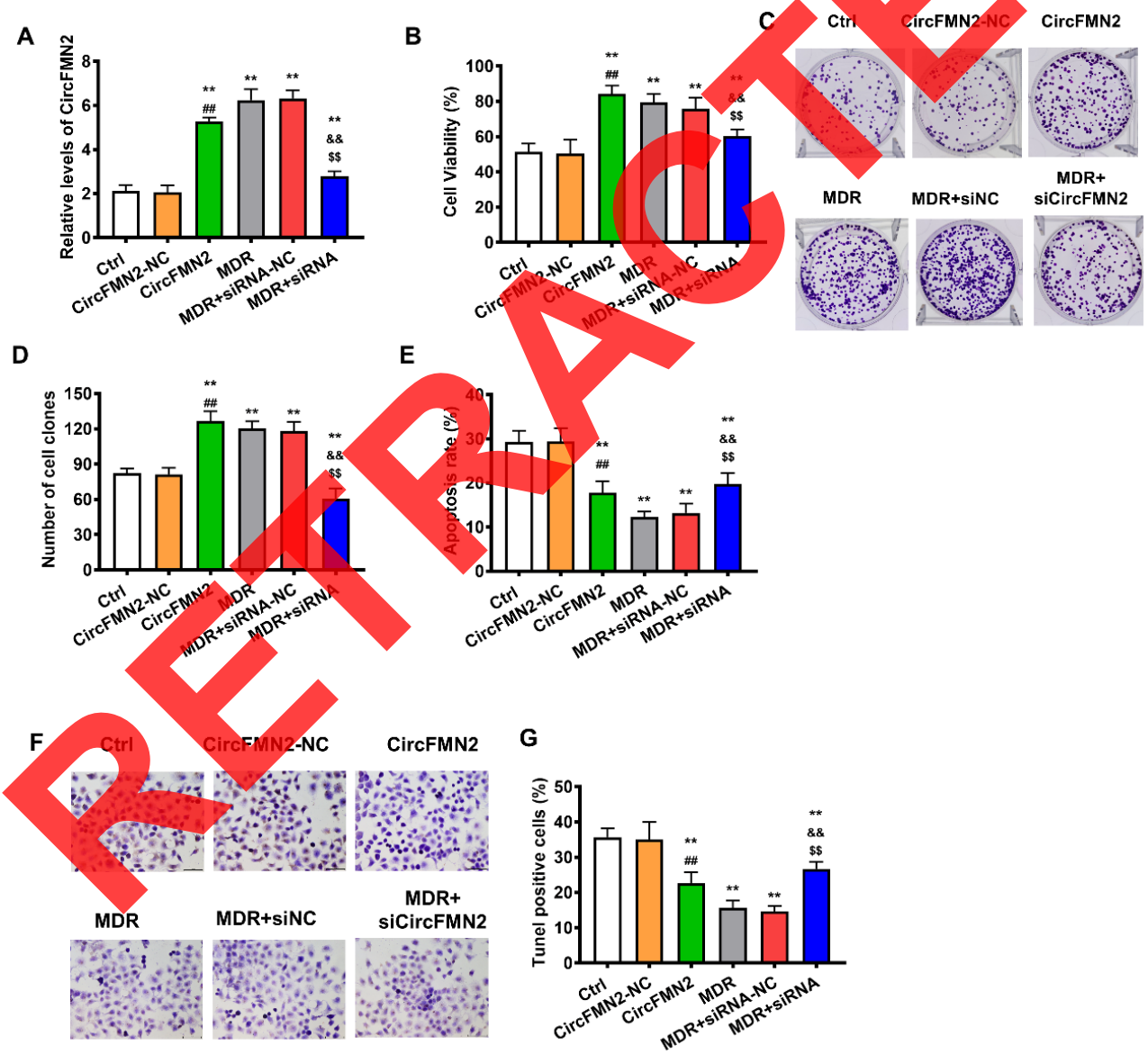

Figure 1. CircFMN2 was upregulated in MDR cells. The influence of circFMN2 on cell proliferation and apoptosis in multidrug resistant cells and nonresistant cells. The level of circFMN2 assessed via RT-qPCR **(A)**; cell proliferation measured by CCK-8 assay **(B)**; colony formation ability **(C, D)**; cell apoptosis analyzed by flow cytometry **(E)** and Tunel assay **(F, G)** in the study groups. ***p<0.001, **p<0.01 vs. control group; ##p<0.01, ###p<0.001 vs. circFMN2-NC group; &p<0.05, &&p<0.01, &&&p<0.001 vs. MDR group; $p<0.05, $$p<0.01, $$$p<0.001 vs. MDR + siRNA-NC group.

The target protein was eluted and detected via Western blot assay.

RNA immunoprecipitation

The interaction of circFMN2 and CNBP was further examined through RNA immunoprecipitation assay via using Imprint® RNA Immunoprecipitation (RIP) Kit (Sigma-Aldrich, St. Louis, MO, USA) in line with the protocol of the manufacturer. Briefly, after cell lysis, the supernatants were collected and incubated with magnetic bead anti-CNBP antibody complex or magnetic bead-IgG complex, respectively. After purification of the immunoprecipitated RNA, the gene level was quantitatively assessed via RT-PCR assay.

Ubiquitination assay

The cells were transfected with pcDNA3.1-CircFMN2 vector or its negative control and 5 μmol/ml MG132 were added. After transfection for 48 h, the cells were lysed and the supernatant was collected. Then, immunoprecipitation was performed via using anti-CNBP antibody and IgG. The immunoprecipitated protein was analyzed through Western blot assay via using anti-ubiquitin antibody (Cell Signaling Technology, Danvers, MA, USA).

Statistics

SPSS 19.0 statistical package (IBM, Armonk, NY, USA) was applied for data analysis and the data analysis result were exported as mean ± standard deviation. Statistical significance was assessed via using one-way analysis of variance (ANOVA). P value under 0.05 suggested significant difference.

Results

CircFMN2 was highly expressed in multidrug resistance (MDR) cells, displaying crucial role in sorafenib resistance in hepatoma cells

In order to examine the underlying role of circFMN2 in BEL-7402 and BEL-7402/5-Fu cells, BEL-7402 and BEL-7402/5-Fu cells were transfected with pcDNA3.1-CircFMN2 vector and si-circFMN2, respectively. As revealed by the results of PCR assay, the level of circFMN2 was elevated after circFMN2 transfection versus control and decreased obviously by si-circFMN2 transfection in comparison with MDR, suggesting that the overexpression and silence of circFMN2 were realized successfully (Figure 1A). Besides, a significant increase of circFMN2 level was found in MDR group versus control, demonstrating that circFMN2 may act as a crucial player in multidrug resistant cells. Cell apoptosis and cell proliferation were crucial parameters for sorafenib resistance. Herein, we further examined the effects of circFMN2 on cell proliferation and apoptosis after sorafenib treatment. As shown by the results from CCK-8 assay (Figure 1B) and colony formation assay (Figure 1C and 1D), circFMN2 overexpression significantly elevated the cell viability and colony formation ability of BEL-7402 cells after sorafenib treatment, verifying the accelerative effect of circFMN2 overexpression on sorafenib resistance. Moreover, silence of circFMN2 decreased cell proliferation in sorafenib treated MDR cells, indicating that knockdown of circFMN2 may be an efficient avenue in improving sorafenib resistance. The impact of circFMN2 on cell apoptosis after sorafenib treatment was assessed by flow cytometry and Tunel assay. The apoptotic cells indicated by flow cytometry (Figure 1E) and Tunel assay (Figure 1F and 1G), were increased significantly in sorafenib treated MDR cells by circFMN2 depletion, further uncovering the improving effects of circFMN2 depletion on sorafenib resistance in MDR cells. On the other hand, this result also indicated that circFMN2 is a crucial sorafenib resistant target in hepatoma.

CircFMN2 elevated CNBP level via restraining its ubiquitination degradation

The downstream mechanism of circFMN2 in sorafenib resistance was further examined herein. CNBP was predicted as the binding protein of circFMN2 via using bioinformatics online tools (StarBase and RNA interactome Database website). The CNBP level was measured via Western blot assay. CNBP was up-regulated by circFMN2 overexpression in BEL-7402 cells in contrast to control (Figure 2A and 2B), revealing that circFMN2 was an up-regulator of CNBP. The level of CNBP was higher in MDR cells than in BEL-7402 cells, suggesting that CNBP may be another drug resistance factor in MDR cells. The interaction between circFMN2 and CNBP was further validated through RNA-pull down and RNA immunoprecipitation assay. The results of RNA-pull down assay revealed that the enrichment of CNBP was found in the circFMN2 with positive-sense strand group (Figure 2C). Moreover, it was observed in the results of RNA immunoprecipitation assay that circFMN2 level was enriched in the CNBP immunoprecipitation group and in contrast, very small amounts of circFMN2 were found in other groups (Figure 2D). These findings substantiate the binding relationship between circFMN2 and CNBP. The binding sites of circFMN2 was further explored via deletion-mapping analysis and the results manifested that the CNBP was pulled down by circFMN2 fragments 306-458nt and 459-612nt (Figure 2E and 2F). The regulatory mechanism of circFMN2 on CNBP was also examined via ubiquitination assay and, as reflected by the results, the ubiquitination level of CNBP was weakened transparently by

circFMN2 overexpression (Figure 2G), disclosing that circFMN2 overexpression elevates CNBP level via inhibiting its ubiquitination.

CircFMN2 boosts sorafenib resistance in hepatocellular carcinoma cells by upregulating CNBP

As stated in the aforementioned results, CNBP was upregulated in resistant cells and may exert an underlying role in drug resistance. So, we further examined the effects of CNBP on sorafenib efficacy in hepatoma cells. We found that CNBP was decreased obviously by sorafenib in hepatoma cells when compared with control and CNBP overexpression reversed this effect (Figure 3A and 3B). The cell viability and colony formation capacity were all reduced significantly by sorafenib in hepatoma cells (Figure 3C-3E). Moreover, the cell apoptosis assessed by flow cytometry and positive cells in Tunel staining were all increased by sorafenib in hepatoma cells (Figure 3F-3H). These results were consistent with previous findings [24,25], demonstrating an anti-proliferation and pro-apoptosis

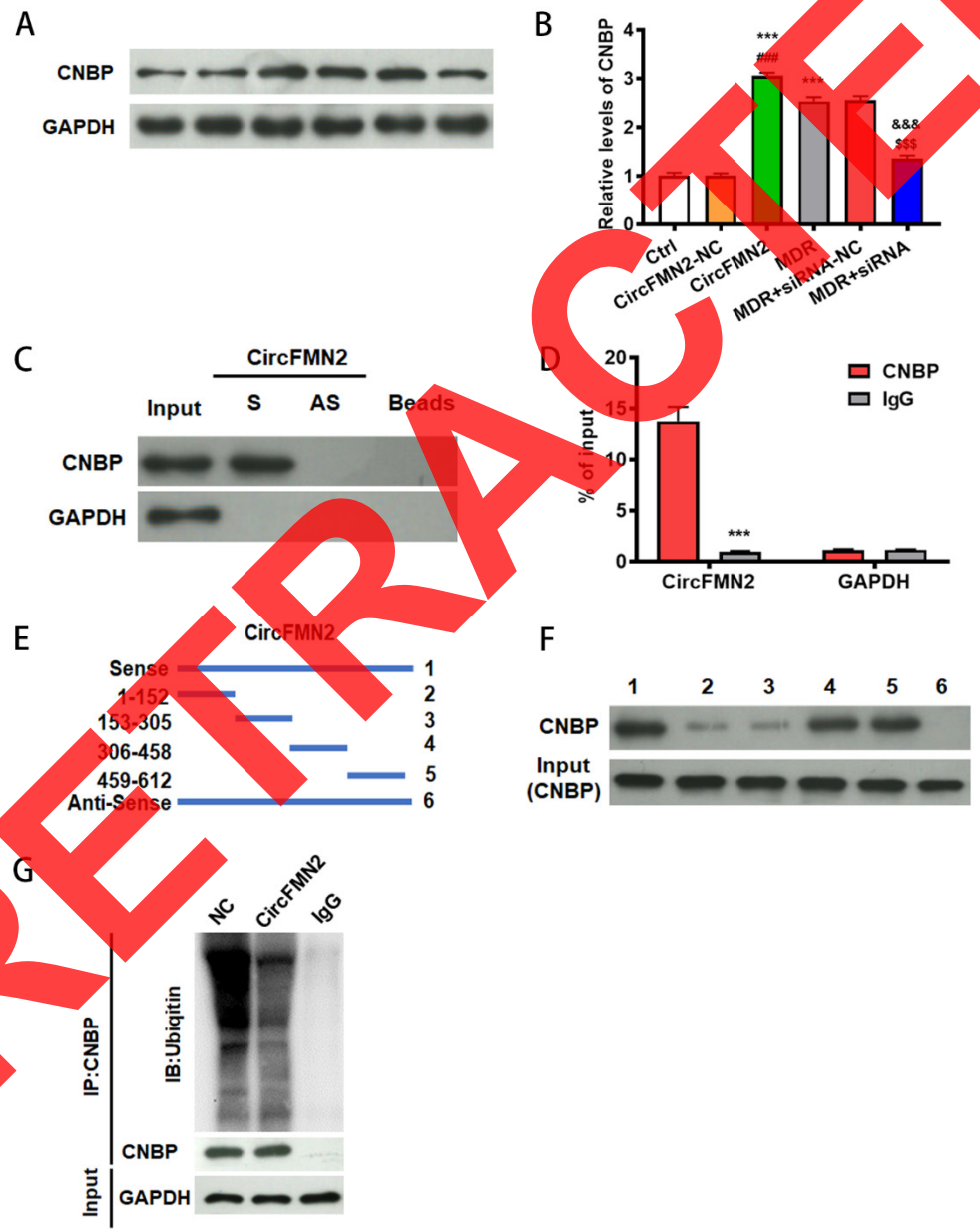

Figure 2. CNBP was confirmed as the binding protein of circFMN2. CNBP level was measured via Western blot **(A, B)**; Western blot analysis of CNBP level after RNA pull-down assay **(C)**; circFMN2 level detected by RT-qPCR following RNA immunoprecipitation assay **(D)**; The deletion fragments, sense strand and anti-sense strand of circFMN2 in deletion mapping analysis **(E)**; Western analysis of CNBP level following RNA pull-down assay with different circFMN2 constructs in deletion mapping analysis **(F)**; ubiquitination level detected by Western blot assay **(G)**. ***p<0.001 vs. control group; ###p<0.001 vs. circFMN2-NC group; &&&p<0.001 vs. MDR group; $$$p<0.001 vs. MDR + siRNA-NC group.

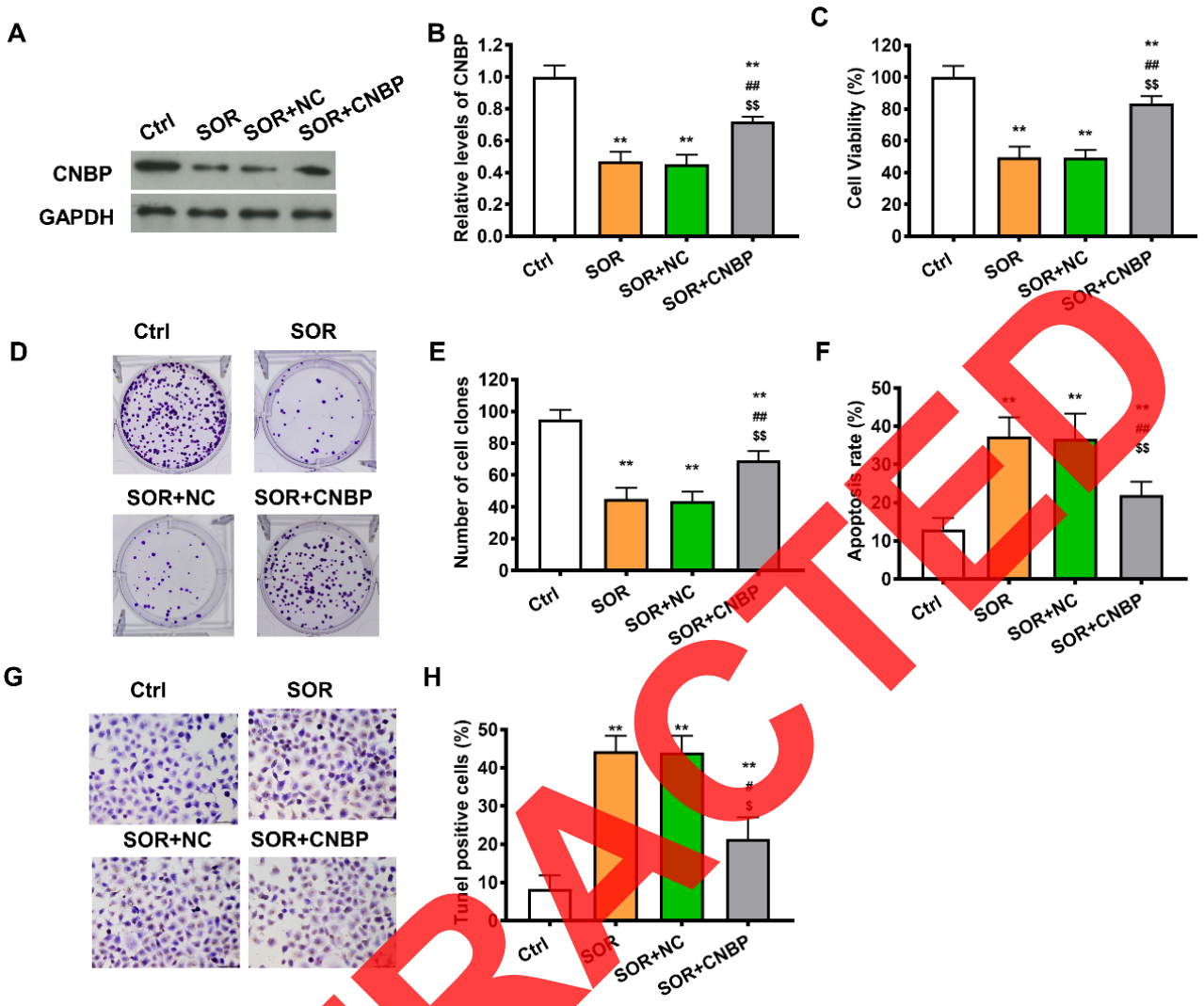

Figure 3. CNBP was upregulated in MDR cells. The influence of CNBP on cell proliferation and apoptosis in hepatoma carcinoma cells. The level of CNBP assessed via Western blot **(A, B)**; cell proliferation measured by CCK-8 assay **(C)**; colony formation ability **(D, E)**, cell apoptosis analyzed by flow cytometry **(F)** and Tunel assay **(G, H)** in the study groups. ***p<0.001 vs. control group; #p<0.05, ##p<0.01 and ###p<0.001 vs. SOR group; $p<0.05, $$p<0.01 and $$$p<0.001 vs. SOR+NC group.

role of sorafenib in hepatoma. CNBP was highly¬expressed in multidrug resistant cells and after CNBP overexpression, the effects of sorafenib on cell apoptosis and proliferation in hepatoma cells were reversed, supporting that CNBP acts as a contributor in sorafenib resistance. Besides, it has been confirmed in the aforementioned results that up-regulation of CNBP was realized via inhibition of ubiquitination by circFMN2. Hence, we concluded that circFMN2 contributes to sorafenib resistance via upregulating CNBP.

Discussion

Sorafenib as a first-line anticancer drug, has become a standardized care of advanced liver cancer, wining a crucial position in hepatocellular carcinoma therapy [26-28]. The sorafenib therapy has yielded a modest survival benefit in patients with advanced HCC [29,30]. Nevertheless, sorafenib treatment still confronts tremendous challenges in sorafenib resistance [31]. The sorafenib resistant mechanism is still not completely understood and its elucidation is imperative. In the present report we found that circFMN2 facilitates sorafenib resistance via up-regulating CNBP through restraining inhibiting ubiquitination.

As revealed by accumulative evidence, aberrant expressed non-coding RNAs have become an essential factor in hepatocellular carcinoma therapy and sorafenib resistance [32,33]. Much of the research on the treatment of liver cancer and sorafenib resistance are focused on aberrantly expressed miRNA or lncRNA [33-38]. Nevertheless, the reports of

circRNA participating in sorafenib resistance are summing up. Compared with miRNAs and lncRNAs, circRNAs, owing to its stable circular structure, have more potential to be an excellent treatment and drug resistant biomarker. In the present study, circFMN2, a new-found circRNA in cancer cells, was found to be upregulated in multidrug resistant hepatocellular carcinoma cells, implying an underlying role in drug resistance.

Cell proliferation and apoptosis are critical indicators of drug resistance. β-catenin regulated by Nek2 contributes to sorafenib resistance via regulating cell apoptosis and proliferation [39]. Rage participates in sorafenib resistance via modulating proliferation and apoptosis by AMPK/mTOR pathway [40]. CircFMN2 was reported to exert a carcinogenic effect in colorectal and prostate cancer cells via regulating cell proliferation and apoptosis [22,23]. In this research, we found that after circFMN2 overexpression, the cell viability and colony formation capacity were increased and cell apoptosis was reduced in sorafenib treated cells, hinting that circFMN2 is a critical functional molecule in facilitating sorafenib resistance. As circFMN2 is highly expressed in MDR cells, we further conducted experiments into the effects of circFMN2 depletion on cell apoptosis and proliferation in sorafenib treated MDR cells. Surprisingly, circFMN2 depletion displayed mitigative effects on sorafenib resistance via augmenting cell apoptosis and suppressing cell proliferation in multidrug resistance cells. The outcome disclosed that circFMN2 is an underlying sorafenib resistance target, on the other hand, high level of circFMN2 is the crucial inducer of sorafenib resistance.

CircRNAs as critical regulators, commonly work by regulating their downstream miRNA targets, binding proteins and certain signaling pathways. As reported in previous articles, circRNA-SORE is found to facilitate sorafenib resistance through β-catenin signaling in liver cancer [41]. CircFN1 augments sorafenib resistance via sponging miR-1205 and modulating the expression of e2f1 in hepatocellular carcinoma cells [42]. In the present study, we sought to find more drug resistant via exploring the downstream mechanism of circFMN2.

As disclosed by the bioinformatics tools, CNBP is predicted as the binding protein of circFMN2. CNBP is ubiquitous in various tissues and organs, exerting dual regulator functions at translational and transcriptional level [43,44]. In this report, CNBP has been identified as the binding protein via RNA pull-down and RNA immunoprecipitation assay. As further verified by deletion-mapping analysis, CNBP is capable to bind to circFMN2 fragments 306-458nt and 459-612nt. Besides, circFMN2 upregulated CNBP by ubiquitination inhibition. Since circFMN2 is an underlying multidrug resistant target, we conjectured that CNBP as downstream binding protein may have similar effects. We found that CNBP is also upregulated in MDR cells versus nonresistant cells, which preliminary matches our speculation. Many studies have confirmed that CNBP is a vital regulator of cell apoptosis and proliferation [45-47]. Besides, CNBP is also confirmed to act as a crucial regulator of cell biology via modulating oncogene expression in tumors [48], while the role of CNBP in anticancer drug resistance remains obscure. Herein, we further examined whether CNBP is involved in sorafenib resistance in liver cancer. After sorafenib treatment, the cell apoptosis was increased and cell proliferation was reduced, which was consistent with previous studies [24,25], while CNBP overexpression reduced the effects of sorafenib on cell apoptosis and proliferation, motivating cell resistance to sorafenib, which is consistent with the effects of circFMN2 overexpression. Since CNBP is upregulated by circFMN2 and CNBP overexpression can be realized via circFMN2 overexpression (Figure 2), we concluded that circFMN2 and CNBP functions consistently and circFMN2 is capable of facilitating sorafenib resistance via upregulating CNBP by ubiquitination inhibition.

Conclusions

In this research, circFMN2, a drug resistant target was found in hepatocellular carcinoma cells. We also identified a new sorafenib resistant mechanism that circFMN2 contributes to sorafenib resistance via upregulation of CNBP through ubiquitination inhibition. The findings of this research provide a new solution for sorafenib resistance and extends our thought for sorafenib resistance in hepatocellular carcinoma.

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Conflict of interests

The authors declare no conflict of interests.

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