Original Paper

A Direct Interaction Between P53-Binding Protein 1 and Minichromosome Maintenance Complex in Hepg2 Cells

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Key Words

Carcinoma • Hepatocellular • Chromatin • Minichromosome Maintenance Proteins • Tumor Suppressor Protein p53

Abstract

Background/Aims: Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide. DNA damage repair in cancer cells is a promising approach for the treatment of cancers. We aimed to explore the potential interaction between p53-binding protein 1 (53BP1) and minichromosome maintenance (MCMs) proteins during DNA damage in human hepatoma HepG2 cells. **Methods:** The recombinant vectors of 53BP1 and MCMs with tags were constructed and transfected into HepG2 cells. Immunoprecipitation (IP) and mass spectrometry (MS) were performed to identify the possible interactions between 53BP1 and MCMs, and glutathione S-transferase (GST) pull-down assay was carried out to detect the direct interaction. Moreover, the expressions of MCM2 and MCM6 were suppressed by specific short hairpin RNAs (shRNAs), and then the chromatin fraction and foci formation of 53BP1 were examined under the condition of DNA damage. **Results:** The results showed that MCM2/3/5/6 was immunoprecipitated against the hemagglutinin (HA)-tagged 53BP1 in HepG2 cell nuclei. GST results revealed that there was a direct interaction between 53BP1 and MCMs complex. Moreover, the non-chromatin level of 53BP1 was significantly increased by down-regulation of MCM2 or MCM6, but was statistically decreased the chromatin level. Furthermore, we observed that knockdown of MCM2 or MCM6 could statistically inhibit the foci formation of 53BP1 in HepG2 cell nuclei upon bleomycin-induced DNA damage ($P < 0.01$). **Conclusion:** Our results suggest that there is a direct interaction between 53BP1 and MCMs, which is essential for 53BP1 chromatin fraction and foci formation in hepatoma HepG2 cells.

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer (PLC), accounting for about 90% of PLC cases. The incidence rate of HCC has been increasing around the world [1]. It has been estimated that approximately 782500 newly diagnosed cases and more than 700000 HCC-related deaths were reported in 2012 [2]. HCC is ranked as the second most common cause of cancer-related mortality worldwide. Many factors contribute to the occurrence of HCC [3-6]. Although substantial progress has been achieved in both prevention and treatment of HCC in recent years, effective therapeutic alternatives for advanced HCC are limited [7, 8]. An increasing number of evidences demonstrate that the development of HCC is associated with perturbed DNA damage response and repair pathway [9, 10]. Therefore, targeting the components of DNA damage response might be a potential effective treatment for HCC.

Recently, p53-binding protein 1 (53BP1) and its roles in maintaining genomic integrity have been paid a great attention. 53BP1 belongs to a family of evolutionarily conserved DNA damage response (DDR) proteins with breast cancer 1, early onset (BRCA1) C-terminus domains [11, 12]. It has been well demonstrated that 53BP1 is a conserved nuclear protein that rapidly localizes to the sites of DNA double-strand breaks (DSB) and activates p53, as well as other kinases, which is involved in the DNA damage response and apoptosis [13-17]. 53BP1 also plays an essential role in DSB repair pathway in G1 and S/G2 cell-cycle phases [14]. Previous studies have found that 53BP1 presents diffuse nuclear expression in untreated primary cells, and while when it is exposed to radiation, it is localized at the sites of DSBs and forms discrete nuclear foci [18-20]. 53BP1 functions as a molecular scaffold that recruits additional DSB-responsive proteins to the damaged chromatin and repairs the proteins. Besides, 53BP1 stimulates ataxia-telangiectasia mutated (ATM)-dependent checkpoint signaling and promotes the synopsis of distal DNA ends during non-homologous end-joining (NHEJ). Deficiency of 53BP1 has been reported to be responsible for genomic instability and cancer progression [21]. The minichromosome maintenance (MCM) complex are critical replicative helicases in archaea and eukarya [22, 23], composing six related subunits (MCM2 to MCM7). Emerging evidence suggests that MCM complex are necessary for chromosome DNA replication and is required for DNA damage repair [24, 25]. Additionally, it has been reported that MCM complex are direct targets of the ATM and ATR checkpoint kinases which are important regulators of DNA damage signaling and cancer [26, 27].

In this present study, we aimed to investigate the potential mechanism about 53BP1 and MCMs during DNA damage in human hepatoma HepG2 cells. Immunoprecipitation (IP) and mass spectrometry (MS) and *in vitro* glutathione S-transferase (GST) pull-down assay were performed to disclose the potential interaction between 53BP1 and MCMs in HepG2 cell nuclei. Furthermore, the expression of MCM2 and MCM6 was knocked down, and then the chromatin fraction and foci formation of 53BP1 were observed under the condition of DNA damage. Our study might provide a new insight into understand the function of 53BP1 and potential treatment of HCC.

Materials and Methods

Cell culture

Human hepatoma HepG2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Gibco; Thermo Fisher Scientific, Inc., USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 100 U/ml penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

Plasmid construction

Standard polymerase chain reaction (PCR) was performed to amplify complementary DNA (cDNAs) encoding MCM2, MCM3, MCM5 and MCM6. Identity of PCR products was then confirmed by sequencing. The

hemagglutinin (HA) and Flag tag sequences were added to the 3' end of 53BP1 and MCMs (MCM2, MCM3, MCM5 and MCM6) by PCR. Thereafter, the fusion sequences were inserted into the pcDNA3.1 vectors (Invitrogen, Carlsbad, CA). The expression plasmids were pcDNA3.1-HA-53BP1, pcDNA3.1-Flag-MCM2, pcDNA3.1-Flag-MCM3 or pcDNA3.1-HA-MCM3, pcDNA3.1-Flag-MCM5, and pcDNA3.1-Flag-MCM6. These plasmids were purified by Plasmid Purification Kit (Merck Sharpe & Dohme, Shanghai, China) according to the manufacturer's protocol.

Cell transfection and treatment

The HepG2 cells were transfected or co-transfected with the above plasmids in serum-free DMEM by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following to the manufacturer's protocol. After the cells were adjusted to a confluency of 70-80% in 24-well plates, the plasmids (1 µg) were added to each well and incubated for 48 h. After incubation, the cells were harvested for further analysis. In addition, specific short hairpin RNA (shRNA) for MCM2 and MCM6 or negative controls was transfected into the HepG2 cells. The shRNA plasmids and negative controls were designed and purchased from GeneChem inc (GeneChem, Shanghai). Thereafter, bleomycin (2 µM) was added and incubated for 1 h in HepG2 cells to induce DNA damage. The supernatants were then collected after transfection for further analysis.

Chromatin fractionation

Cell fractionation was performed as described [28]. Briefly, HepG2 cells stably expressing HA-tagged 53BP1 were lysed in 100 µL of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 mM sucrose, and 10% glycerol) supplemented with 0.2% Triton X-100, 1 mM dithiothreitol (DTT), and Protease Inhibitor Cocktail (Roche Diagnostics, Meylan, France) for 8 min and centrifuged. Thereafter, the supernatant containing the cytoplasmic fraction was prepared. The pellet was then resuspended in 100 µL of buffer B containing 3 mM ethylene diamine tetraacetic acid (EDTA), 0.2 mM ethylene glycol tetraacetic acid (EGTA), 1 mM DTT, and Protease Inhibitor Cocktail on ice for half an hour and centrifuged. The supernatant was collected again and the supernatant was mixed with the cytoplasmic fraction and non-chromatin fraction. Next, the pellet was re-suspended in 150 µL of buffer C which contains 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1% NP-40, 1 mM DTT, and Protease Inhibitor Cocktail, sonicated, centrifuged, and collected. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to quantify the amount of proteins.

IP analysis

To analyze 53BP1 and its potential interactive proteins, IP analysis was performed in both nucleoprotein and the whole cell extracts (WCE) of HepG2 cells stably expressing HA-tagged 53BP1. EpiQuik Nuclear Extraction Kit (Epigentek Group Inc, New York, NY, USA) was used to extract nucleoprotein samples. IP was carried out by using Co-IP Kit (Thermo scientific Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, HepG2 cells were washed with phosphate-buffered saline (PBS), lysed in cold IP lysate buffer, and then centrifuged. Next, the cell lysates were immunoprecipitated with anti-HA antibody (Rabbit mAb #3724, Cell Signaling Technology, Beverly, MA) and incubated overnight at 4°C. SDS-PAGE was then conducted to examine the protein levels.

GST pull-down assay

The interaction between 53BP1 and MCMs was analyzed by *in vitro* GST pull-down assay. GST pull-down assay was performed as previously described [29]. GST fusion proteins were manufactured by inserting 53BP1 cDNA into the Escherichia coli strain BL21 site of the vector pGEX4T1 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). His-tagged 53BP1 protein was purified by nickel-nitrilotriacetic (Ni-NTA) affinity chromatography (Qiagen, Hilden, Germany) as per the manufacturer's instruction. GST-mediated pull-down assays were conducted using GST Protein Interaction Pull-Down Kit (Pierce, Rockford, IL) according to the manufacturer's instruction. GST protein was used as a negative control. Western blot was then performed to test the pull-down productions.

Immunofluorescence microscopy

Immunofluorescence was performed to examine the foci forming of 53BP1 in HepG2 cell nuclei. The cells were transfected with MCM2 or MCM6 shRNA, incubated with bleomycin, fixed with 4% paraformaldehyde

for 10 min, permeabilized in 0.2% Triton X-100 for 5 min, and maintained in specific primary antibody against 53BP1 (#4937, Cell Signaling Technology, Beverly, MA) for 2 h at room temperature. The cells were then washed with PBS, and incubated with appropriate secondary antibodies (Cell Signaling Technology, Beverly, MA) for 2 h at room temperature. Thereafter, the nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Stained nuclear was analyzed by a fluorescence microscope (Olympus IX51, Tokyo, Japan) and analyzed by NIH ImageJ software (National Institutes of Health, Bethesda, MD).

MS assay

HepG2 cells stably expressing HA-53BP1 was fractionated into cytoplasmic and nuclear compartments. 53BP1-interacting proteins were denatured with 8 M urea, decreased with 10 mM tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP; Pierce), alkylated with 55 mM iodoacetamide (IAM; Promega, Madison, WI, USA), and followed by trypsin digestion (Sigma-Aldrich). Thereafter, the protein samples were desalted, eluted by linear ion trap (LTQ) MS (Thermo Scientific), and chromatographed by Agilent 1200 High Performance Liquid Chromatography (HPLC; Agilent Technologies, Santa Clara, CA). Raw spectral data were processed with RawXtract 1.9.9 [30].

Western blot

Total protein was extracted from HepG2 cells after transfection by using Protein Extraction Reagent supplemented with a protease inhibitor cocktail (Thermo Fischer Scientific, Waltham, MA). The protein samples were then subjected to SDS-PAGE and transferred to nitrocellulose (NC) blotting membranes (Merck Millipore, Billerica, MA, USA). After washing with PBS, the membranes were incubated with the following antibodies overnight at 4°C: anti-MCM2 antibody (ab4461, Abcam, Cambridge, UK), anti-MCM6 antibody (ab201683, Abcam), anti-origin recognition complex 2 (Orc2) antibody (ab68348, Abcam), anti-phospholipase C gamma 1 (PLCγ-1) antibody (Abcam), anti-Flag tag antibody (ab1257, Abcam), anti-His antibody (ab18184, Abcam). Next, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam) for 2 h at room temperature. GAPDH was used as a loading control. The immunoreactive proteins were detected using enhanced chemiluminescence (ECL; Pierce). Expressions of proteins were quantified using Bio-Rad Image Lab™ 3.0 version software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Each experiment was carried out at least four times and the data were expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare comparisons between groups using Statistic Package for Social Science version 19.0 (SPSS; SPSS Inc., Chicago, IL, USA) statistical software. $P < 0.05$ was considered as statistically significant differences.

Results

53BP1 interacts with MCMs

To better understand the functional role of 53BP1 in HepG2 cells, specific 53BP1-interacting proteins in the nucleus were identified. The transfected cells were immunoprecipitated with the HA-53BP1, and then subjected to MS analysis. As shown in Table 1, the results revealed that the peptide recovery of 53BP1 was 13%, 16%, 18%, and 8%, respectively. MS analysis showed that not all the MCMs were identified in IP of BRCA1, only MCM2, MCM3, MCM5 and MCM6 were identified, indicating that MCM2, MCM3, MCM5 and MCM6 might interact with 53BP1 in HepG2 cells. Western blot was then performed to confirm the results. The HepG2 cells overexpressing HA-tagged 53BP1 and Flag-tagged MCM2, MCM3, MCM5 and MCM6 were incubated with antibodies against HA and Flag respectively to identify these proteins. As shown in Fig. 1 A-D, HA-tagged 53BP1 or Flag-tagged MCM2, MCM3, MCM5 and MCM6 could be identified in WCE of HepG2 cells, demonstrating that these proteins were successfully overexpressed. In addition, we found that Flag-tagged MCMs could not be identified in cell nuclei but only were detected on fusion protein. These results suggested that 53BP1 interacted with the MCM2, MCM3, MCM5 and MCM6 in HepG2 cell nuclei.

Fig. 1. Western blot results for IP. HA-tagged 53BP1 and Flag-tagged MCM2/3/5/6 were overexpressed in HepG cells, and then Western blot was carried out to detect 53BP1 or MCM2 (1 A), MCM3 (1 B), MCM5 (1 C), and MCM6 (1 D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. MCM, minichromosome maintenance protein; 53BP1, p53-binding protein 1; HA, hemagglutinin; IP, immunoprecipitation; WCE, whole cellular extract.

Although the IP results showed that 53BP1 interacted with MCMs, it was not clear that if 53BP1 directly interacted with MCMs or 53BP1 interacted with MCMs via association of other proteins. Therefore, *in vitro* GST pull-down assay were performed using fusion proteins. As demonstrated in Fig. 2, the results revealed that GST-tagged 53BP1 could pull down MCM2/3/5/6 in input and the pull-down samples. None of these MCMs proteins could be identified in GST negative control. Hence, the GST pull-down assay confirmed that 53BP1 might directly interact with MCM2/3/5/6.

Fig. 2. Western blot results for GST pull-down assay. GST pull-down was conducted to assess the direct interaction *in vitro* between GST-tagged 53BP1 and His-tagged MCM2, MCM3, MCM5 or MCM6. Thereafter, Western blot was carried out with input samples as positive controls. ** $P < 0.01$. MCM, minichromosome maintenance protein; 53BP1, p53-binding protein 1; GST, glutathione S-transferase.

Down-regulation of MCMs reduces 53BP1 chromatin fraction

According to the above results, we assumed that MCMs might be involved in the function of 53BP1. To confirm the assumptions, we knocked down the expressions of MCM2

Fig. 3. Downregulation of MCMs reduces 53BP1 chromatin fraction HepG cells were transfected with MCM2 shRNA or MCM6 shRNA, and then the protein levels of 53BP1 in non-chromatin and chromatin fractions were measured. Orc2 and PLC γ -1 were used as marker proteins for chromatin and non-chromatin fractions, respectively. * P or # P < 0.05, *** P or ### P < 0.001. MCM, minichromosome maintenance protein; 53BP1, p53-binding protein 1; Orc2, Origin recognition complex 2; PLC γ -1, phospholipase C gamma 1; shRNA, short hairpin RNA.

Fig. 4. Downregulation of MCMs reduces 53BP1 foci formation The expression of MCM2 and MCM6 was knockdown by specific shRNAs, and bleomycin was added to induce DNA damage. Thereafter, the effects of MCM2 or MCM6 downregulation on foci formation of 53BP1 were analyzed. A, Western blot was performed to conform the knockdown expression of MCM2 or MCM6; B and C, the foci formation of 53BP1 in HepG cell nuclei was identified by immunofluorescence. * P < 0.05, ** P < 0.01, *** P < 0.001. MCM, minichromosome maintenance protein; 53BP1, p53-binding protein 1; shRNA, short hairpin RNA; DAPI, 4', 6-diamidino-2-phenylindole.

and MCM6 by specific shRNAs, and then we analyzed the chromatin fraction of 53BP1. As shown in Fig. 3, the results showed that transfection with shRNAs successfully induced the knockdown expressions of MCM2 or MCM6 in both non-chromatin and chromatin fractions of HepG2 cells. Moreover, we observed that knockdown expression of MCM2 or MCM6 could significantly raise the non-chromatin level of 53BP1, but reduce the chromatin level of 53BP1. These results suggested that down-regulation of MCMs could reduce chromatin fraction of 53BP1.

Down-regulation of MCMs decreases 53BP1 foci formation

Furthermore, we analyzed the effects of MCM2 or MCM6 knockdown on the foci formation of 53BP1 under the situation of DNA damage which was induced by bleomycin. The results in Fig. 4A revealed that MCM2 and MCM6 were both effectively downregulated in HepG2 cells after MCM2 shRNA and MCM6 shRNA transfection. Fig. 4B and 4C showed that bleomycin significantly increased foci formation of 53BP1 compared to the control group (P < 0.01), and while knockdown of MCM2 or MCM6 reversed the results (both P < 0.01). These results indicated that down-regulation of MCMs decreased 53BP1 foci formation in response to DNA damage.

Discussion

In the present study, we find that there is a direct interaction between 53BP1 and MCM complex in HepG2 cells. 53BP1 directly interacts with MCM2, MCM3, MCM5, and MCM6 in HepG2 cell nucleus. Down-regulation of MCM2 and MCM6 could reduce the chromatin fraction of 53BP1, as well as the foci formation of 53BP1 in response to DNA damage. Our results indicate that the interaction between 53BP1 and MCM complex is essential for 53BP1 chromatin fraction and foci formation in hepatoma HepG2 cells.

53BP1 was first identified as one of the binding partner of the tumor suppressor protein p53 [31, 32], and also enhanced transactivation of p53 in a transient transfection assay [33, 34]. Inactivation of 53BP1 is reported to be associated with carcinoma progression and deficient of 53BP1 is cancer-prone in mice [15, 35]. Therefore, 53BP1 is regarded as a tumor suppressor. Previous study found that the expression of 53BP1 was correlated with cell proliferation, cell apoptosis, and prognosis in colorectal cancer [36]. Recently, it has been suggested that 53BP1 also plays an important role in maintaining cell genomic stability and inhibiting tumor development by means of participating DNA damage. 53BP1 is at the intersection of NHEJ repair pathway and homology-directed repair (HDR) repair pathway, and it can integrate cellular inputs to guarantee the execution in the appropriate cellular contexts [37]. Although new data has shown how 53BP1 is loaded onto chromatin and how 53BP1 tracks the formation of subnuclear foci at damaged chromatin, the exact mechanism still remain unclear. Since proteins show their functions through interaction with other proteins, 53BP1 is no exception. Previous studies have suggested that 53BP1 could be interacted with numerous proteins. For example, the interaction between 53BP1 and histone variant H2AX (' γ H2AX') was found in the DNA damage response [38]. 53BP1 has been suggested to be related with replication protein A (RPA) and is essential for RPA2 hyperphosphorylation following DNA damage [39]. Emerging evidence demonstrated that MCM complex is required for chromosome DNA replication and this complex might be responsible for DNA damage repair [40, 41]. Therefore, we assumed that there might be a potential interaction between 53BP1 and MCM complex.

To confirm the assumption, IP was performed in the nuclear protein of HepG2 cells followed by MS to find out the potential interacting proteins of 53BP1. Our data showed that MCM2, MCM3, MCM5, and MCM6 were immunoprecipitated by antibodies against the HA-53BP1 in HepG2 cell nuclei. However, not all the MCM complex was immunoprecipitated. MCM2 is able to initiate eukaryotic genome replication and it can be formed a complex with MCM3/5/6, which plays a significant role in mediating the helicase activity of the complex [42-44]. Therefore, there might be a possibility of the interaction between 53BP1 and MCM complex. The possible interaction was further confirmed by GST pull-down assay *in vitro*. From the results, we can basically exclude the connecting of other proteins between 53BP1 and MCM complex. Hence, it can be assumed that 53BP1 directly interacts with MCM2/3/5/6 in HepG2 cell nuclei from the analysis. Thereafter, the expression of MCM2 or MCM6 was suppressed by transfection with specific MCM2 shRNA or MCM6 shRNA in HepG2 cells, respectively. The levels of 53BP1, MCM2, and MCM6 in both non-chromatin and chromatin were examined. We observed that the protein levels of MCM2 and MCM6 in both non-chromatin and chromatin could be statistically inhibited by transfection with specific MCM2 shRNA or MCM6 shRNA alone. These results were similar with a previous study that indicated that specific MCM2 shRNA or MCM6 shRNA alone can inhibit the entire MCM complex [25]. Moreover, we found that suppression of MCM2 or MCM6 obviously increased 53BP1 non-chromatin levels but decreased the chromatin levels. The results indicated that MCM suppression may prevent the expression of MCM complex and its interactive protein 53BP1, possibly because the DNA replication was reduced and DNA-damage-response proteins including 53BP1 was less required in HepG2 cell nuclei. Similar results were also observed in reduced chromatin mobility induced by suppression of 53BP1 [45].

Neoadjuvant-adjuvant therapy in cancer treatment is mainly relied on promoting cancer cell DNA damage and cell cycle retardation and increasing cell apoptosis [46-48].

Therefore, DNA damage repair in cancer cells may distinctly affect the outcome of radiotherapy and chemotherapy on cancers. Down-regulation of 53BP1 resulted in impaired p53 accumulation in a human cancer line [17]. Additionally, previous study suggested that MCM6 was a novel biomarker for HCC patients [49] and adjuvant therapy with sorafenib might be an effective therapeutic strategy for MCM7-positive HCC patients [50]. In the present study, DNA damage in HepG2 cells was induced by administration of bleomycin. The inhibited chromatin fraction and foci formation by knockdown of MCM complex demonstrated the possibility to regulate DNA damage repair in HepG2 cells via suppression of 53BP1 and its potential regulators including MCMs, thus improving the effects of adjuvant radiotherapy and chemotherapy on cancer cells.

Previous studies have confirmed that MCM complexes are direct targets of the ATM and ATR checkpoint kinases that are responsible for DNA damage signaling and cancer [26, 27]. Recently, it has been reported that activation of Notch pathway down-regulates MCM2 and MCM6 expression by interfering with retinoblastoma protein (Rb) phosphorylation, which are involved in cell proliferation [51]. Meantime, Notch1 signaling pathway is mediated by inhibiting mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-hydroxy kinase (PI3K)/Akt pathways. Moreover, extracellular signal-regulated kinase (ERK)/MAPK signaling pathway plays an important role in mediating E2F-dependent MCM expression and DNA replication in vascular smooth muscle cell (VSMC) [52]. Therefore, there might be potential interactions between MCM complexes between the above pathways. However, further studies should be performed to confirm the results.

In conclusion, our results demonstrate that 53BP1 directly interacts with MCM2/3/5/6, and suppression of MCMs reduces the chromatin fraction and foci formation of 53BP1 upon DNA damage in HepG2 cells. The potential interactions might provide a new insight into improve the outcome of radiotherapy and chemotherapy in HCC (Table 1).

Table 1. Results of MS for MCMs in HepG2 cell nuclei.

Abbreviations: MCM, minichromosome maintenance protein; MS, mass spectrometry

Protein	Peptides	Recovery
MCM2	16	13%
MCM3	12	16%
MCM5	12	18%
MCM6	10	8%

Disclosure Statement

The authors declare to have no conflict of interests.

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