

# SUMOylation alterations are associated with multidrug resistance in hepatocellular carcinoma

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Received May 31, 2013; Accepted December 12, 2013

DOI: 10.3892/mmr.2014.1882

**Abstract.** The development of multidrug resistance (MDR) in hepatocellular carcinoma (HCC) may markedly reduce the efficacy of its chemotherapeutic treatment. However, the mechanism regulating the development of MDR in these tumors remains unknown. Given the emerging role of small ubiquitin-like modifier (SUMO)ylation in tumorigenesis, the possibility that it may also be involved in MDR development was investigated. The expression of SUMO-1 was first analyzed using immunohistochemistry in 20 cases of HCC. Nuclear SUMO-1 immunostaining was observed to be significantly increased in HCC specimens compared with matched adjacent non-neoplastic controls. To further investigate the potential role of SUMOylation in MDR in HCC, a multidrug-resistant HCC cell line, HepG2/R, was established by exposing HCC cells to gradually increasing concentrations of 5-fluorouracil. Western blot analysis revealed that the total levels of SUMO-1-conjugated proteins were markedly increased in HepG2/R cells compared with parental HepG2 cells. Furthermore, the expression of

ubiquitin-like modifier activating enzyme 2 and sentrin-specific protease 1, important enzymes in the SUMOylation cascade, were markedly upregulated in the HepG2/R cell line. These findings support the hypothesis that SUMOylation is important in the development of MDR in HCC.

## Introduction

Small ubiquitin-like modifier (SUMO)ylation is a post-translational modification that involves the covalent, but reversible, binding of SUMO (1). Four isoforms of SUMO have been identified, SUMO-1, -2, -3 and -4. These proteins are ~12 kDa in size and are structurally similar to ubiquitin (2-5). Similar to ubiquitination, the SUMOylation cascade contains a SUMO-activating enzyme (E1), a SUMO-conjugating enzyme (E2) and different SUMO ligases (E3s). All SUMO isoforms are expressed as immature precursors with a variable C-terminus comprising 2-11 residues, following an essential GG motif. Maturation of SUMO involves the removal of this C-terminal tail by sentrin-specific proteases (SENPs) and exposing the diglycine motif required for conjugation. SUMO is then activated in an ATP-dependent manner by conjugation to the E1 heterodimer, activator of SUMO-1 (Aos1) and ubiquitin-like modifier activating enzyme 2 (Uba2). Activated SUMO is then transferred to the unique E2 enzyme Ubc9, which covalently attaches the modifier to the ε-amino group of a target lysine residue in the presence of an E3 SUMO ligase (1,6,7). Cleavage after the diglycine motif by SENPs then removes the conjugated SUMO moieties from the target protein.

Due to its regulation of protein activity, post-translational modifications may have profound effects on carcinogenesis. SUMOylation has been shown to regulate various cellular pathways, including DNA replication and repair, chromosome packing and dynamics, genome integrity, nuclear transport, signal transduction and cell proliferation. These functions suggest that abnormal SUMOylation may affect cancer progression and metastasis (8-15).

Hepatocellular carcinoma (HCC) is the sixth most common type of malignancy worldwide. It is the fifth most

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*Abbreviations:* Aos1, activator of SUMO-1; HCC, hepatocellular carcinoma; MDR, multidrug resistance; SENP1, sentrin-specific protease 1; SUMO, small ubiquitin-like modifier; Uba2, ubiquitin-like modifier activating enzyme 2

*Key words:* SUMOylation, hepatocellular carcinoma, multidrug resistance, ubiquitin-like modifier activating enzyme 2, sentrin-specific protease 1

common type of malignant disease in males, the eighth most common type of malignant disease in females and the third most common cause of cancer-related mortality following lung and stomach cancer (16). Systemic chemotherapy represents a palliative treatment following resection and/or transplant surgery for inoperable patients. However, intrinsic or acquired multidrug resistance (MDR) may interfere with the efficacy of chemotherapy in HCC. MDR, which results in increased drug efflux from cells, is associated with increased DNA repair and drug metabolism, as well as decreased apoptosis, all of which affect the tumor microenvironment (17-20). However, the role of SUMOylation in MDR or chemotherapy remains largely unknown.

The aim of this study was to analyze the resistance mechanisms of HCC. A novel multidrug resistant-HCC cell line (HepG2/R) was developed based on HepG2 cells, a model for chemosensitive HCC cells. We investigated the levels of SUMOylation in these cells and analyzed the specific role in the development of drug resistance in HCC cells.

## Materials and methods

**Chemotherapeutic drugs and antibodies.** The chemotherapeutic drugs 5-fluorouracil (5-FU, catalog no. F6627-1G), paclitaxel (catalog no. O9512-5MG) and oxaliplatin (catalog no. O9512-5MG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse antibodies against SUMO-1 (catalog no. sc-5308), Aox1 (catalog no. sc-271592), Uba2 (catalog no. sc-136359) and SENP1 (catalog no. sc-271360) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Cell lines.** The HepG2 human HCC cell line was provided by Dr H Tang (Tianjin Life Science Research Center and Basic Medical School, Tianjin Medical University, Tianjin, China). The MDR sub-line HepG2/R was established by culturing HepG2 cells in the presence of increasing concentrations of 5-FU (0.1, 0.2, 0.4, 0.8 and 1.6  $\mu\text{g}/\text{ml}$ ). HepG2/R cells were cultured in the presence of 1.6  $\mu\text{g}/\text{ml}$  (12.3 mM) 5-FU to maintain their phenotype. All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100  $\mu\text{g}/\text{ml}$  penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin at 37°C in 5% CO<sub>2</sub>.

**Human samples.** Samples of paraffin-embedded HCC tissues and their adjacent non-neoplastic controls (n=20) were obtained from patients who underwent surgical resection at the Department of Hepatobiliary Surgery, Tianjin Medical University Cancer Institute and Hospital. HCC in these samples was confirmed by pathological diagnosis. This study was approved by the Committee for the Protection of Human Subjects of Tianjin Medical University Cancer Institute and Hospital and informed consent was obtained from all patients.

**Immunohistochemical analysis.** The expression of SUMO-1 was demonstrated by immunohistochemistry (IHC) according to the manufacturer's instructions (pv-6000; ZSGB-BIO, Beijing, China). Sections (4 $\mu\text{m}$ -thick) were deparaffinized in xylene and endogenous peroxidase activity was blocked

with 3% hydrogen peroxide in 50% methanol for 10 min at room temperature. The slides were rehydrated in a series of alcohol washes, and washed with phosphate-buffered saline (PBS) and pretreated with citrate buffer for 20 min at 95°C. Following blocking of nonspecific binding sites with 10% normal goat serum in PBS for 20 min at 37°C, the sections were incubated overnight at 4°C with anti-SUMO-1 (dilution, 1:200). Sections were rinsed with PBS and incubated with HRP-goat anti-mouse immunoglobulin G (IgG) for 20 min at 37°C. Subsequently, the slides were incubated with 3,3'-diaminobenzidine chromogen for 5-10 min at room temperature and washed with distilled water. Finally, sections were counterstained with hematoxylin for 1 min, dehydrated and mounted on coverslips. PBS without the primary antibody was used as a negative control.

**Western blot analysis.** Protein lysate was separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked in TBST (50 mM Tris pH 7.5, 0.15 M sodium chloride, 0.5% Tween-20) containing 5% non-fat milk. Immunoblotting was performed using indicated antibodies diluted in TBST. The membranes were washed and incubated with goat anti-mouse IgG-horseradish peroxidase conjugate (Bio-Rad, Hercules, CA, USA). Specific complexes were visualized using an enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, UK).

**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.** MTT assays were used to evaluate rates of cell growth (Cell Growth Determination kit; Sigma-Aldrich, catalog no. CGD1). Cells were trypsinized into a single-cell suspension and seeded into 96-well plates at a density of 1x10<sup>3</sup> cells/well. At the indicated time points, 10% of the culture volume (20  $\mu\text{l}$ ) MTT was added to each well and incubated for 4 h at 37°C. Cultures were removed from the incubator and the resultant MTT formazan crystals were dissolved in 180  $\mu\text{l}$  MTT solvent (0.1 N HCl in anhydrous isopropanol). Absorbance values were measured using a microplate absorbance reader (Bio-Rad Laboratories, Richmond, CA, USA) at a wavelength of 570 nm. To measure the effect of the chemotherapeutic agents on growth, the inhibitory rate was calculated as follows: Cell growth inhibition (%) =  $[A_{570}(\text{control}) - A_{570}(\text{experiment})] / A_{570}(\text{control}) \times 100$ .

**Statistical analysis.** All experiments were performed at least in triplicate. Statistical significance was evaluated using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Establishment and characterization of the HCC MDR cell line HepG2/R.** To obtain multidrug resistant-cells, HepG2 cells were treated with increasing concentrations of 5-FU between 0.1 and 1.6  $\mu\text{g}/\text{ml}$ . The drug-resistant subclone HepG2/R was established almost one year following initiation of treatment. HepG2/R cells were morphologically distinct from their parental cell line in that they demonstrated increased pseudopodia formation (Fig. 1A). Cell proliferation did not

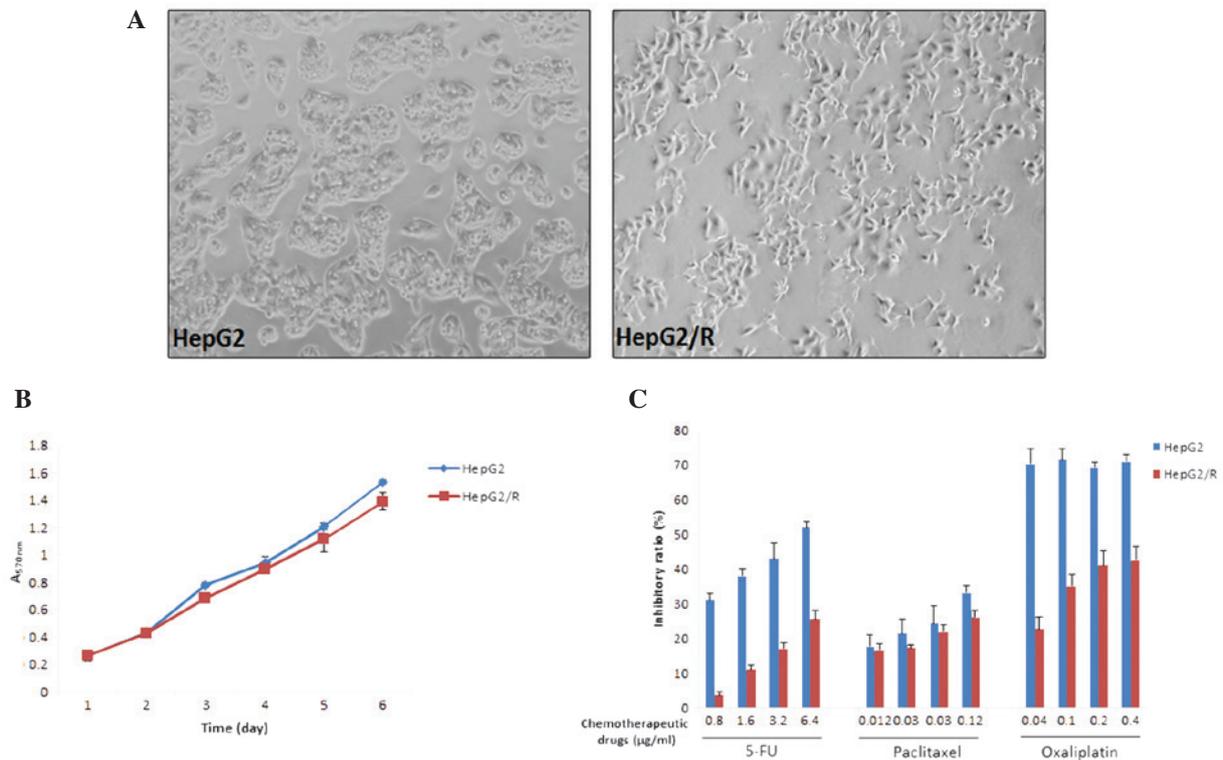


Figure 1. Characterization of the hepatocellular carcinoma multidrug resistance cell line, HepG2/R. (A) HepG2/R cells are morphologically distinct from their parental HepG2 cells, showing increased pseudopodia formation. (B) The MTT assay was used to measure cell growth over a six-day period. Proliferation rates of the HepG2 cells are similar to those of the HepG2/R cells. The results are expressed as the mean  $\pm$  SD of three assays. (C) Cells were treated with various concentrations of chemotherapeutic drugs, 5-FU, paclitaxel and oxaliplatin, and inhibitory ratios were measured using the MTT assay. Results are expressed as the mean  $\pm$  SD from three assays. SD, standard deviation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 5-FU, 5-fluorouracil. Magnification, x200.

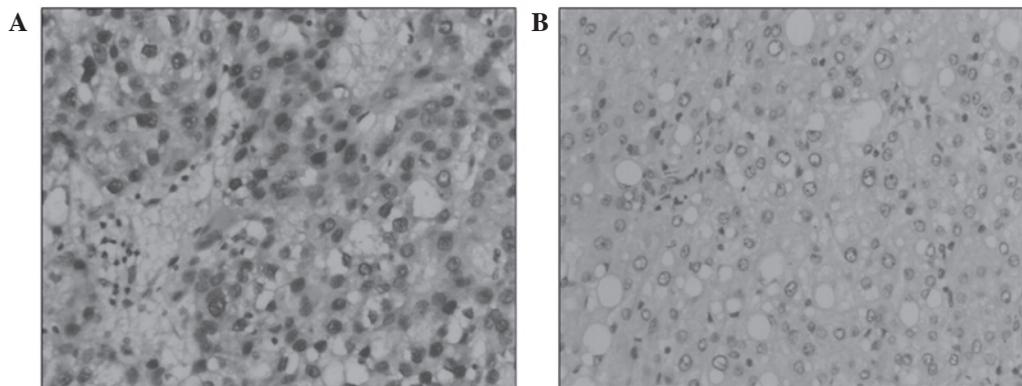


Figure 2. SUMO-1 immunostaining of HCC tissue and the adjacent non-neoplastic liver tissue (magnification, x200). (A) Strong nuclear SUMO-1 staining in HCC tissue. (B) Nuclear SUMO-1 staining was not detected in non-neoplastic liver tissue. Each staining experiment was performed with a negative control. SUMO-1, small ubiquitin-like modifier; HCC, hepatocellular carcinoma. Immunoperoxidase stain; magnification x200.

differ significantly between the HepG2 and HepG2/R cells (Fig. 1B). Next, the sensitivity of the two cell lines to specific concentrations of chemotherapeutic drugs was investigated. Following treatment with 0.8-6.4  $\mu\text{g/ml}$  5-FU, the inhibitory ratios of HepG2/R were markedly lower compared with those of the parental HepG2 cells (Fig. 1C). The cross-resistance to other chemotherapeutic drugs, including paclitaxel and oxaliplatin was also investigated. HepG2/R cells were observed to develop cross-resistance to paclitaxel and oxaliplatin to varying degrees (Fig. 1C).

*SUMO-1 is overexpressed in clinical HCC samples.* Immunostaining was used to evaluate the SUMO-1 expression in tumor cells from HCC tissues and normal cells from the adjacent non-neoplastic liver tissue. Nuclear SUMO-1 expression was detected in HCC neoplastic cells from all samples (100%). However, in the matched non-neoplastic controls, little or no SUMO-1 expression was observed (Fig. 2A and B).

*SUMO-1-conjugated proteins differ between HepG2 and HepG2/R cells.* To investigate the pattern of SUMOylation

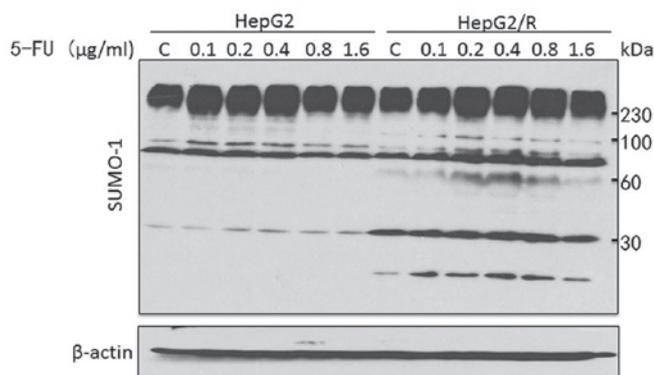


Figure 3. Total levels of SUMO-1-conjugated proteins are increased by different doses of 5-FU in HepG2/R compared with HepG2 cells. A distinct SUMO-1-conjugated protein (60 kDa) and free SUMO-1 protein (12 kDa) were detected in HepG2/R cells. Expression of SUMO-1-conjugated proteins at 30 and 100 kDa were also clearly increased. 5-FU, 5-fluorouracil. SUMO-1, small ubiquitin-like modifier.

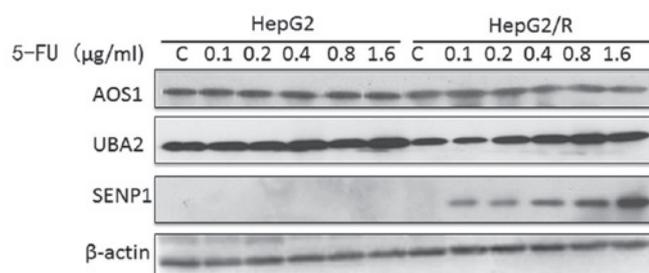


Figure 4. Expression levels of SUMOylation enzymes in HepG2 and HepG2/R cells treated with different doses of 5-FU. Aos1 expression was unaffected by 5-FU treatment in HepG2 or HepG2/R cells. Uba2 levels were markedly increased in a dose-dependent manner in HepG2/R cells compared with HepG2 cells. SENP1 was not detected in HepG2 cells, but was expressed in a dose-dependent manner in HepG2/R cells. 5-FU, 5-fluorouracil; SUMO-1, small ubiquitin-like modifier; Aos1, activator of SUMO-1; Uba2, ubiquitin-like modifier activating enzyme 2; SENP1, sentrin-specific protease 1.

in HepG2 and HepG2/R cells treated with 5-FU at different concentrations, whole-cell lysates were probed with a SUMO-1 antibody. The total levels of SUMOylation were significantly enhanced in HepG2/R cells. As shown in Fig. 3, distinct SUMO-1-conjugated and free SUMO-1 proteins were detected in HepG2/R cells at ~60 and 12 kDa, respectively. The levels of SUMO-1-conjugated proteins at 30 and 100 kDa were also markedly increased.

*Uba2 and SENP1 are upregulated in the HepG2/R cell line.* In addition, the levels of SUMOylation enzymes in the HepG2 and HepG2/R cells were compared to determine whether the SUMOylation pathway differed. Although Aos1 levels were unaffected by the 5-FU treatment, Uba2 and SENP1 levels increased in a dose-dependent manner in HepG2/R cells (Fig. 4).

## Discussion

Drug resistance is a major obstacle for the successful chemotherapeutic treatment of HCC. Often following chemotherapy,

tumors fail to reduce in size or the cancer recurs following an initial response. MDR is particularly problematic as it involves the simultaneous resistance to numerous chemotherapeutic drugs of different classes. However, the mechanism of MDR remains unclear. To elucidate the molecular mechanisms in HCC a multidrug-resistant cell model is required. 5-FU has been used alone or in combination with other chemotherapeutic drugs for HCC and other malignant tumors (21,22). Thus, an MDR HCC cell line, HepG2/R, was established by exposing HepG2 cells to increasing concentrations of 5-FU over one year. HepG2/R cells exhibited a strong resistance to 5-FU and also demonstrated cross-resistance to multiple, structurally diverse chemotherapeutic agents, including paclitaxel and oxaliplatin.

SUMOylation is essential for the maintenance of protein stability, transcriptional regulation and modification of particular transcription factors. SUMOylation is also involved in cellular processes, including mitosis, differentiation, senescence and apoptosis (8,23-26). Guo *et al* (27) reported that SUMO-1 was overexpressed in all HCC cell lines and clinical HCC samples that were tested. In agreement with this observation, the current study showed an increase in the expression of SUMO-1 in clinical HCC samples compared with the adjacent non-neoplastic liver tissue controls. This suggests that SUMOylation may be important in HCC. In addition, total levels of SUMO-1-conjugated proteins are increased in HepG2/R cells compared with parental HepG2 cells. This demonstrates that higher levels of SUMOylation are associated with MDR in HCC, possibly by affecting pathways that induce MDR.

SUMOylation is a dynamic process involving the conjugation (Aos1/Uba2, Ubc9 and different E3 ligases) and de-conjugation machinery (SENPs) (1). Notably, these enzymes have also been observed to affect tumorigenesis. Inactivation of Uba2 leads to mitotic catastrophe and cell death following *Myc* hyperactivation. Uba2 inhibition switches a transcriptional subprogram of *Myc* from activated to repressed (28). SENP1 is highly expressed in prostate cancer and correlates with hypoxia-inducing factor 1 (HIF-1) expression, which is associated with an increase in P-glycoprotein expression and the occurrence of MDR in tumor cells (29,30). In the current study, high concentrations of 5-FU were found to increase Uba2 expression in HCC MDR cells. This increase may affect cellular processes regulated by oncogenic *Myc*. 5-FU treatment also increased SENP1 expression in a dose-dependent manner, potentially implicating HIF-1 in the development of MDR in HCC.

In conclusion, the development of MDR in HCC was observed to be associated with increased SUMOylation, likely due to the increased expression of Uba2 and SENP1. These results suggest that the SUMOylation pathway may be a novel therapeutic target for preventing the development of MDR in HCC, thus, increasing the efficacy of chemotherapy for this widespread disease.

## Acknowledgements

This study was supported by scientific research grants from Tianjin Medical University (grant nos. 2010ky04 and 2011ky42).

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