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Overexpression and Effect on Apoptosis of the 150-ku Oxygen-regulated Protein (ORP150) in Human Hepatocellular Carcinoma*

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Abstract In previous study, the 150-ku oxygen-regulated protein (ORP150) was identified as a candidate glycoprotein related to hepatocellular carcinoma. In order to further validate the expression level of ORP150 in hepatocellular carcinoma, protein expression was determined by Western blot and cell immunochemistry, and messenger RNA (mRNA) expression was detected by quantitative real-time polymerase chain reaction. The effect of ORP150 on apoptosis and invasive potential of hepatocellular carcinoma cells was evaluated using the small interference RNA (siRNA) technique. Both the protein and mRNA expression levels of ORP150 were significantly upregulated in hepatocellular carcinoma cell lines compared with a non-tumor human liver cell line. After transfection with the specific siRNA of ORP150, significantly greater apoptosis of hepatocellular carcinoma cells was induced compared with untransfected cells. However, no significant effect on invasive potential was found. Overexpression of ORP150 was associated with hepatocellular carcinoma, and ORP150 might promote the proliferation of hepatocellular carcinoma cells by inhibiting apoptosis. ORP150 could be a potential therapeutic target for hepatocellular carcinoma.

Key words 150-ku oxygen-regulated protein(ORP150), human hepatocellular carcinoma, apoptosis
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Human hepatocellular carcinoma is a common and aggressive malignancy with especially high prevalence in Asia and Africa[1~3]. It is the third most frequent cause of cancer deaths in men and the seventh most frequent in women, with a very poor prognosis[4]. Surgery remains the main form of treatment, but 5-year recurrence after surgical resection is as high as 70%[5]. Early diagnosis and control of metastasis of hepatocellular carcinoma are the key issues to improve long-term survival. Therefore, exploring the molecular mechanisms of hepatocellular carcinoma pathogenesis and metastasis is pivotal[6]. Although many efforts have been made, so far, their mechanisms are still not clear.

In our previous study[7], we screened key glycoproteins related to hepatocellular carcinoma pathogenesis and metastasis by comparing glycoprotein profiles of a non-tumor human liver cell line (Chang liver) and two human hepatocellular carcinoma cell lines with different metastatic potential (nonmetastatic, Hep3B and highly metastatic, MHCC97H). The glycoprotein profiles were compared by fluorescence-based multiplexed proteomics technology, which included two-dimensional gel electrophoresis followed by fluorescent staining of glycoprotein and mass spectrometry identification. Comparing these profiles, we found some obviously different glycoproteins, including the 150-ku oxygen-regulated protein (ORP150), which was selected for further study because it had different levels

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of expression in these three cell lines. The ORP150 is a newly discovered member of the family of heat-shock proteins. Little is known about the association between ORP150 and hepatocellular carcinoma. The ORP150 functions as a molecular chaperone in the endoplasmic reticulum [8]. It is an integral participant in ischemic cytoprotective pathways [9] and is expressed in human wounds along with vascular endothelial growth factor (VEGF)[10].

In the present study, we aimed to validate expression of ORP150 in hepatocellular carcinoma cell lines and to analyze its roles in the survival and invasion potential of hepatocellular carcinoma cells.

1 Materials and methods

1.1 Reagents

Tris, glycine, urea, sodium dodecyl sulfate, dithiothreitol, and thiourea were obtained from Sigma (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum, and Dulbecco's modified Eagle's medium were obtained from GIBCO Industries (Los Angeles, CA, USA); TRIzol reagent, reverse transcriptase complementary DNA (cDNA) synthesis kit, and Lipofectamine 2000, from Invitrogen Life Technologies (Gaithersburg, MD, USA); matrigel, from BD Biology Science Corporation; mouse antihuman ORP150 antibody, from IBL Company (Naka Fujioka, Japan); mouse antihuman glyceraldehyde 3-phosphate dehydrogenase (GAPDH), from Santa Cruz Biotechnology; goat anti-mouse IgG conjugated with horseradish peroxidase, from Sino-American Biotechnology Company; the quantitative enzyme-linked immunosorbent assay (ELISA) kit for matrix metalloproteinase-9 (MMP-9) and MMP-2, from Shanghai Senxiong Scientific and Technological Industrial; Transwell permeable supports, from Corning Costar Company; ECL Western blotting substrate, from Pierce; and polyvinylidene fluoride membrane, from Millipore. All chemicals were analytical reagent grade.

1.2 Cell culture and total protein preparation of cells

The Chang liver cell line and Hep3B cell line were from Cornell Uuniversity (Ithaca, NY, USA) and cultured in RPMI-1640 medium containing 10% fetal bovine serum. The MHCC97H cell line was established by our institution. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and high glucose. The cells (1×10^7) were lysed with 400 μl lysis buffer containing 40 mmol/L Tris-base, 4% CHAPS detergent, 200 mmol/L dithiothreitol, and 0.5 μl phenylmethylsulphonyl fluoride (400 mmol/L) for 45 min at 4℃, with occasional vibration. The mixture was centrifuged (20 000 *g*) for 45 min. The supernatant was collected and stored at −80℃.

1.3 Detection of ORP150 messenger RNA(mRNA) level in cell lines

Total cell RNA was extracted with TRIzol reagent by standard isopropanol and chloroform precipitation as described in the manufacturer's instructions. The cDNA was synthesized from 2 μg cell RNA using a reverse transcriptase cDNA synthesis kit according to manufacturer's instructions on a Bio-Rad PCR iCycler. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed for messenger RNA (mRNA) expression. ORP150 cDNA was amplified with the primers (forward: 5′ GTGGA-GTTCACGAGGGAGGT 3′; reverse: 5′ ACTTTGCG-TTGAGGGTAGGG 3′). The qRT-PCR was performed with a DNA Master SYBR Green Ⅰ on the iQ5 Multicolor Real-time PCR detection system (Bio-Rad). Amplification of qRT-PCR involved an initial 10-min step at 95℃ for denaturation, followed by up to 45 cycles of a 94℃ denaturation for 10 s, annealing at 52℃ to 58℃ for 20 s, and 72℃ for an appropriate extension time (25 s). Reactions for qRT-PCR amplification were performed in triplicate on material from at least two independent reverse transcription reactions. To serve as the internal control, GAPDH mRNA was detected at the same time. Quantification data were analyzed by the delta C_t method.[6]

1.4 Western blot

The total protein of cells was loaded in equal amounts for sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% separation gel and 5% stacking gel. The proteins isolated were transferred onto a polyvinylidene fluoride membrane. After treating with 5% bovine serum albumin in 1 × TBST (25 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, and 0.05% Tween-20) for 1 h at room temperature, the membranes were incubated with mouse anti-human ORP150 (1 mg/L) for 2 h at room temperature, followed by horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The GAPDH was used as an internal positive control. Target proteins were detected by enhanced chemiluminescence.

1.5 Immunofluorescence detection of ORP150

Cultures of cells were washed in phosphate-buffered saline (pH 7.4) and fixed in methanol for 5 min. The cells were incubated with mouse anti-human ORP150 primary antibodies (5 mg/L) for 30 min at 37℃, and with Cy3-conjugated anti-mouse IgG secondary antibody (1∶300 dilution, Sigma-Aldrich Corporation, Germany) for 30 min at room temperature. The negative control was performed without primary antibody. The cells were washed in phosphate-buffered saline three times each for 3 min. The slides were sealed in glycerin, and ORP150 expression and distribution pattern were observed by fluorescent microscopy (Zeiss, Oberkochen, Germany)[5].

1.6 Transfection of small interference RNA (siRNA) against ORP150 into Hep3B cells and the cell apoptosis assay

1.6.1 Transfection of siRNA. Small interference RNA (siRNA) transfection were performed as described previously [11]. Briefly, the specific ORP150 siRNA sequence was screened from three designed siRNA sequences by qRT-PCR. The validated specific ORP 150 siRNA sequences were sense 5′ GACAUU-GAAGCUAAGAUGAtt 3′ and antisense 5′ UCAU-CUUAGCUUCAAUGUCtt 3′. The cell lines were transfected with a specific ORP150 siRNA. For transfection, 4 μl Lipofectamine reagent was mixed with 100 μl OptiMEM (Invitrogen, SanDiego, CA, USA) and allowed to stand at room temperature for 10 min. The original siRNA preparation 6 μl was mixed with OptiMEM 100 μl and incubated at room temperature for 20 min to form the transfection mixture. Before transfection, the cells were incubated in serum-free media for 24 h. At transfection time, the cells were washed with Dulbecco's modified Eagle's medium and incubated in media containing serum. The transfection mixture was added to all cultures, and the cells were incubated at 37℃ for 72 h.

1.6.2 Analysis of apoptotic cells by TUNEL *in situ* detection. Cells were cultured and treated with ORP150 siRNA or non-targeting siRNA for 24 h. Discard media and fix air dried cell samples with a freshly prepared solution (4% paraformaldehyde in PBS, pH 7.4) for 1 h at room temperature and incubate in permeabilisation solution (0.1% Trixon X-100 in 0.1% sodium citrate) for 10 min on ice. Then add 50 μl TUNEL (Roche) reaction mixture on sample and incubate for 60 min at 37℃ in a humidified atmosphere in the dark. Samples were analyzed in a drop of PBS under a fluorescence microscope at this state.

1.6.3 Analysis of apoptotic cells by Flow Cytometry. At 72 h after transfection, the medium was removed, and the cells were harvested for apoptosis analysis by Annexin V-FITC and PI double staining. The cells were detached by trypsin treatment and washed once with phosphate-buffered saline. Cells were suspended in 2 ml citric acid buffer and fixed at room temperature for 30 min. After centrifugation, cell pellets were digested in 1.8 ml solution A (trypsin digestion solution), and digestion was stopped with 1.5 ml solution B (trypsin inhibitor solution). Samples were stained in 1.5 ml solution C (propidium iodide) for at least 15 min. Samples were filtered by 53 mm nylon mesh before running on the flow cytometry.

1.7 Cell invasion assay

The invasive property of tumor cells was measured by a transwell chamber assay *in vitro* as described previously [12]. In the upper compartment, filters coated with matrigel were covered with 100 μl diluted cells transfected with specific ORP150 siRNA, and in the lower compartment, the chambers were filled with 600 μl conditioned culture media. After incubating with 5% CO_2 for 36 h at 37℃, the cells that did not invade the basement membrane were removed gently with wet cotton. Cells that had invaded the membrane were fixed in formaldehyde for 15 min at room temperature and stained with Giemsa for 30 min. The invading cells were counted under light microscopy, and the average number was obtained from three independent experiments.

1.8 Matrix metalloproteinases (MMPs) in cell culture medium detected by avidin biotin complex ELISA

The amounts of MMP-2 and MMP-9 in serum-free media were estimated by ELISA after Hep3B cells were transfected with specific ORP150 siRNA for 72 h. The ELISA assays were performed following the manufacturer's protocols.

2 Results

2.1 Upregulation of ORP150 gene in hepatocellular carcinoma cell lines

As shown in Figure 1, the expression of ORP150 gene was significantly upregulated in the two hepatocellular carcinoma cell lines (Hep3B and MHCC97H) compared with the non-tumor Chang liver cell line ($P < 0.05$). The expression level of ORP150 gene in Chang liver cells was only approximately 6%

of that in Hep3B or MHCC97H cells. Gene expression of ORP150 in the MHCC97H cell line was slightly higher than that in the Hep3B cell line; however, this difference was not statistically significant ($P > 0.05$).

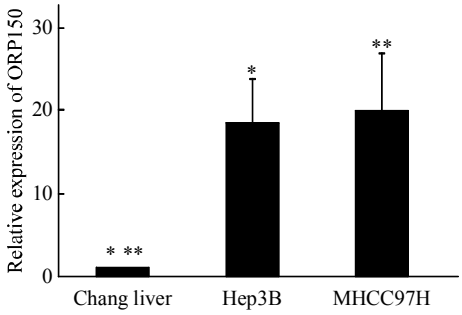

Fig. 1 Expression levels of the ORP150 gene in cell lines detected by qRT-PCR

The ORP150 gene levels were elevated in both hepatocellular carcinoma cell lines, Hep3B (nonmetastatic) and MHCC97H (highly metastatic), compared with the non-tumor human liver cell line, Chang liver ($P < 0.05$). The ORP150 gene level in the MHCC97H cell line was higher than that in the Hep3B cell line, although the difference was not statistically significant ($P > 0.05$). Values were normalized to GAPDH gene expression and expressed relative to the control sample (Chang liver). Values are given as $\bar{x} \pm s$ deviation ($n = 3$). *, ** $P < 0.05$.

2.2 Upregulated protein expression of ORP150 in hepatocellular carcinoma cell lines

As shown in Figure 2, protein expression of ORP150 was elevated in both hepatocellular carcinoma cell lines (Hep3B and MHCC97H), compared with the Chang liver cell line, and the Hep3B cells had the highest ORP150 protein expression level among the three cell lines. The immunofluorescence assay also demonstrated that both the MHCC97H and Hep3B cell lines had higher expression of ORP150 protein, compared with the Chang liver cell line (Figure 3).

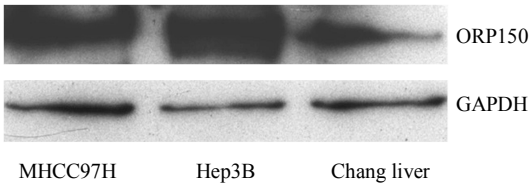

Fig. 2 Western blot analysis for ORP150 protein expression in Chang liver, Hep3B, and MHCC97H cell lines

The target bands displayed higher density in the MHCC97H and Hep3B lanes, compared with the Chang liver lane. The Hep2B lane displayed the highest density.

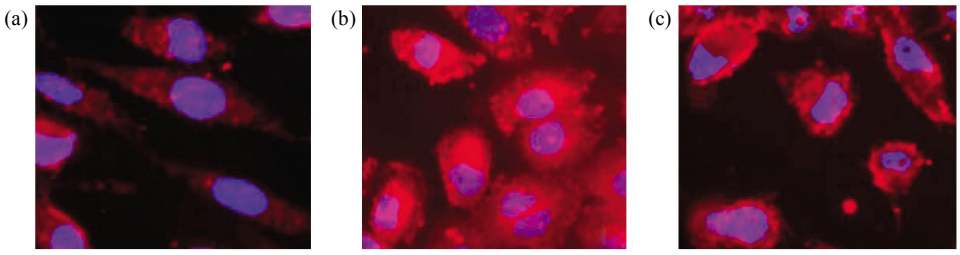

Fig. 3 Immunofluorescence detection of ORP150 in Chang liver, Hep3B, and MHCC97H cell lines

Immunofluorescent microscopy (200×) depicts weak red staining in the cytoplasm of Chang liver cells (a), compared with intense red staining in the cytoplasm of Hep3B cells (b) and MHCC97H cells (c).

2.3 Detection of specific ORP150 small interference RNA (siRNA) transfection efficiency

The transfection of cells with ORP150-specific siRNA resulted in significantly decreased gene expression of ORP150 at 48 h. Protein expression of ORP150 was significantly reduced at 72 h after transfection, as shown in Figure 4. Low intensity of the target band in lane *1* (cells transfected with specific ORP150 siRNA) indicated that the expression of ORP150 was successfully inhibited.

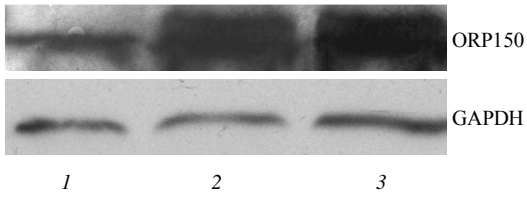

Fig. 4 Assessment of siRNA transfection efficiency by Western blot

1: Cells transfected with specific ORP150 siRNA; *2*: Cells treated with non-targeting siRNA; *3*: Non-transfected cells. Low intensity of the target band in lane *1* indicated that the expression of ORP150 was successfully inhibited.

2.4 Effect of ORP150 on hepatocellular carcinoma cell apoptosis

Through TUNEL *in situ* detection, we did not find typital apoptotic bodies or morphological change after 24 h treatment with siRNA against ORP150 in Hep 3B cells compared with control groups(Figure 5a). In addition to TUNEL-staining, we attempted to reconfirm the apoptosis ratio of ORP150-RNAi Hep 3B with Flow Cytometry by Annexin V-FITC and PI double staining(Figure 5b, 5c). It could be seen that apoptosis in the Hep3B hepatocellular carcinoma cells transfected with ORP150-specific siRNA was significantly increased compared with untransfected hepatocellular carcinoma cells ($P < 0.01$), which indicated that ORP150 effectively inhibited the apoptosis of cultured hepatocellular carcinoma cells.

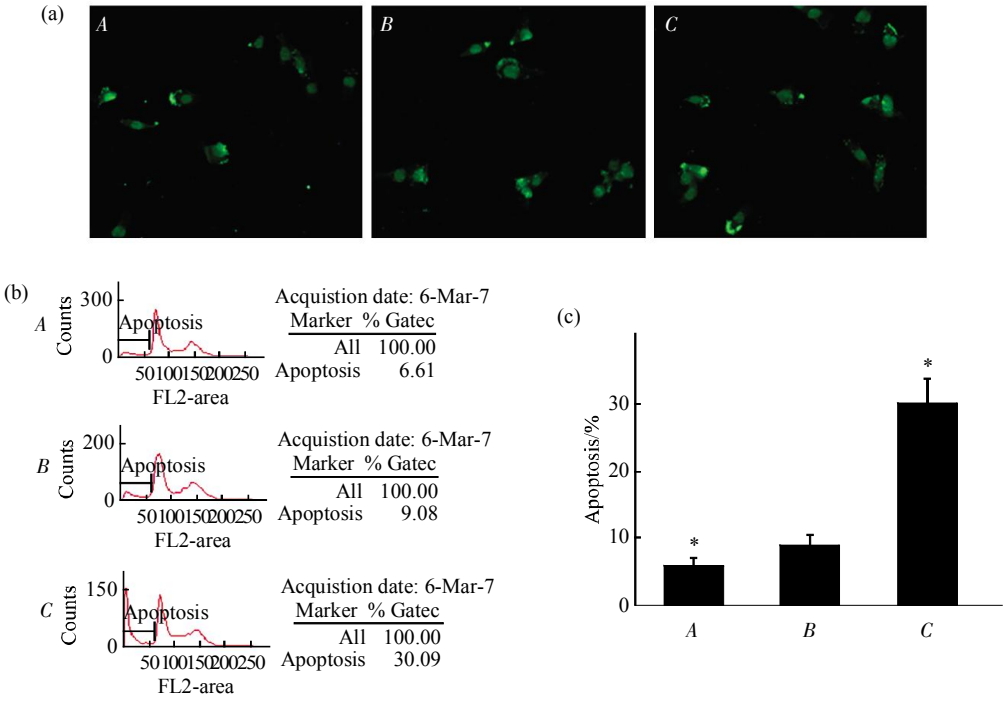

Fig. 5 The effects of ORP150 on cell apoptosis in Hep3B cells

Hep3B cells apoptosis were detected by TUNEL technology *in situ* (a, 200×), and the apoptotic rate were confirmed by Flow Cytometry (Annexin V-FITC and PI double staining) (b, c). The non-transfected Hep3B cells were designated as *A*; the Hep3B cells transfected with non-targeting siRNA were designated as *B*; and the Hep3B cells transfected with ORP150-specific siRNA were designated as *C*. Values are shown as $\bar{x} \pm s$ (n=6). * $P < 0.01$.

2.5 Effect of ORP150 on hepatocellular carcinoma cell invasion potential

As shown in Table 1, levels of MMP-2 and MMP-9 secreted by the transfected Hep3B hepatocellular carcinoma cells in serum-free media were not statistically different compared with levels in the control group (untransfected hepatocellular carcinoma cells). After ORP150 expression in hepatocellular carcinoma cells was inhibited, the number of cells invading through the transwell membrane did not differ significantly compared with the control group (Figure 6, $P > 0.05$).

Table 1 Invading cells per field in the cell invasion assay and quantification of MMP-2 and MMP-9 levels in hepatocellular carcinoma cell culture medium by ELISA

Cell lines	Invading cells	ρ(MMP2)/(ng•L^{-1})	ρ(MMP9)/(ng•L^{-1})
Hep3B	4.25 ± 1.98	10.27 ± 4.58	7.06 ± 3.59
Hep3B/unspecific siRNA	4.00 ± 1.80	11.31 ± 3.79	8.83 ± 3.85
Hep3B/ORP150 siRNA	5.13 ± 2.45	11.49 ± 3.64	9.28 ± 3.71

The group Hep3B represents non-transfected Hep3B cells; Hep3B/unspecific siRNA represents Hep3B cells transfected with non-targeting siRNA; and Hep3B/ORP150 siRNA represents Hep3B cells transfected with ORP150-specific siRNA. The number of invading cells did not differ significantly among the three groups (one-way analysis of variance, $P > 0.05$). The levels of MMP-2 and MMP-9 were not significantly different among the three groups (one-way analysis of variance, $P > 0.05$).

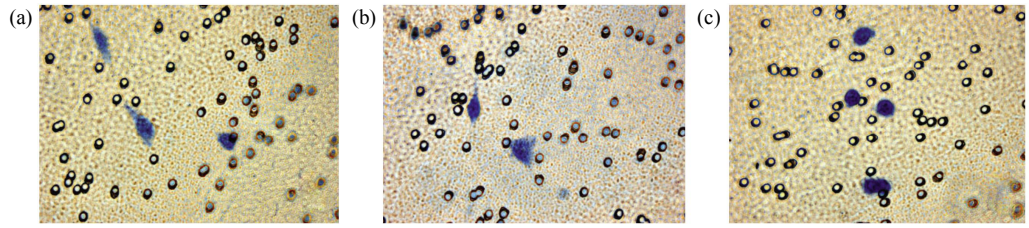

Fig. 6 The effects of ORP150 on the invasive potential of Hep3B hepatocellular carcinoma cells

The invasive ability of hepatocellular carcinoma cells did not differ significantly after ORP150-specific siRNA transfection. (a) Non-transfected cells. (b) Cells transfected with non-targeting siRNA. (c) Cells transfected with ORP150-specific siRNA. Original magnification ×200.

3 Discussion

Glycoproteins participate in a plethora of biological processes, including viral entry, signal transduction, inflammation, cell-cell interactions, bacteria-host interactions, fertility, and development [13~16]. In a previous study, we compared glycoproteins from a non-tumor human liver cell line (Chang liver) and two human hepatocellular carcinoma cell lines with different metastatic potential (Hep3B and MHCC97H), and we identified 32 distinctly different glycoproteins[7]. The ORP150, a differently expressed glycoprotein with high glycosylation in hepatocellular carcinoma cell lines but not in the non-tumor human liver cell line, drew our interest for further functional exploration. To the best of our knowledge, expression of ORP150 among the three cell lines was first reported in the present study.

In this investigation, we confirmed that gene and protein expression of ORP150 was elevated in hepatocellular carcinoma cell lines compared with the non-tumor human liver cell line. In addition, ORP150 significantly inhibited the apoptosis of cultured hepatocellular carcinoma cells. However, it did not influence the invasive potential of hepatocellular carcinoma cells.

Through Flow Cytometry analysis, we found that apoptotic ratio of HCC cells increased significantly after inhibition of ORP150 expression. The data suggested an important role of up-regulated ORP150 in inhibiting HCC cells apoptosis. But we did not find typital apoptotic bodies or morphological change during early stage. It is well known that morphologic criteria are considered as the most reliable evidence of apoptosis. However, demonstration of complete apoptotic morphology by a sinle method is difficult. Kerr[17] think that the use of morphologic criteria has some inherent limitations. The exact sequence of morphological changes may vary in different cell types. As has been shown for annexin V reagents *in vitro*, annexin V radionuclide imaging can provide a tool which can directly assess for early stages of programmed cell death, before membrane vesicle formation and DNA degradation particularly as measured by the TUNEL method [18, 19]. We consider that Flow Cytometry was preciser than morphology or TUNEL.

In addition to our findings of overexpression of ORP150 in hepatocellular carcinoma, expression of ORP150 was elevated in breast, thyroid, pancreatic, and bladder cancer[20~23]. The ORP150 is a novel endoplasmic reticulum-associated polypeptide in the heat-shock protein family that functions as an ATP-dependent chaperone, and is probably involved in the folding and assembly of polypeptides, as well as in the insertion of polypeptides into microsomal membranes[24]. Tumor cells subjected to environmental stress, for example, oxygen deprivation, redirect their biosynthetic pathways to express heat-shock proteins. During carcinogenesis, expression of heat-shock proteins changes. Among members of the heat-shock protein family, HSP-27 and HSP-70 affect the malignant potential of tumor cells[24~26]. Here, ORP150, a newly discovered member of the heat-shock protein family, promoted the survival of hepatocellular carcinoma cells by resisting their apoptosis. In previous reports, ORP150 protected cells from ischemia-induced cell death[9], and the cytoprotective role of ORP150 under hypoxic conditions was, in part, due to the suppression of apoptosis[27], consistent with our results. The resistance of malignant cells to apoptosis favors their survival. Therefore, we suggest that ORP150 promotes hepatocellular carcinoma tumorigenesis in part by suppressing the apoptosis of

malignant cells. We speculate that, in the early stage of hepatocellular carcinoma development, since liver fibrosis that usually exists in hepatocellular carcinoma patients results in poor blood supply and local hypoxia, ORP150 is highly induced in such livers, and further, ORP150 may be involved in hepatocellular carcinoma tumorigenesis by promoting the survival of malignant liver cells. Furthermore, we have detected the target genes associated with apoptosis pathway, and have found the upregulation of caspase-3 and caspase-6 induced by ORP150, which may have some effects on the NF-κB pathway (We will publish those results in another paper soon).

In the present study, protein expression of ORP150 in Hep3B was higher than that in MHCC97H; in contrast, gene expression of ORP150 in Hep3B was lower than that in MHCC97H. The reason for such dissociation between the protein and mRNA levels of ORP150 in hepatocellular carcinoma cell lines is not clear, but it may be related to posttranscriptional or posttranslational regulation of the proteins, the methods used for quantifying proteins, and the property of antibodies used[28].

The ORP150 expression differed between nonmetastatic and highly metastatic hepatocellular carcinoma cell lines, which implied that ORP150 might be associated with hepatocellular carcinoma invasion or metastasis. In present study, the invasive potential of hepatocellular carcinoma cells did not change after inhibition of ORP150. However, the cells used in this study were cultured cell lines, and the functional analysis may be more reflective of the culture conditions rather than the original *in vivo* status of the protein; therefore, their functions remain to be further validated. Especially since our previous results showed that ORP150 had different glycosylation status between nonmetastatic and highly metastatic hepatocellular carcinoma cells, further investigations as to whether altering the glycosylation of ORP150 influences the invasive and metastatic potential of hepatocellular carcinoma cells will be valuable.

4 Concluding remarks

In summary, ORP150 was overexpressed in hepatocellular carcinoma cell lines and may be involved in tumorigenesis by promoting the survival of malignant cells. On the other hand, ORP150 does not affect the invasive potential of hepatocellular carcinoma cells. Results of the present study provide a better understanding of the association between ORP150 and hepatocellular carcinoma, but also help to provide a potential molecular target for therapy and a biomarker for diagnosis of hepatocellular carcinoma.

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氧调节蛋白 150 在人肝细胞癌中的过度表达及其对凋亡的作用 *

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摘要 在前期研究中发现，氧调节蛋白 150(ORP150)是与肝细胞癌相关的糖蛋白．进一步研究了 ORP150 的表达水平与肝细胞癌的相关性．免疫印迹、细胞免疫化学和定量 PCR 分别在蛋白质水平和 mRNA 水平检测了 ORP150 的表达．运用 RNA 干扰技术检测了其对凋亡和肝细胞癌侵袭性的影响．发现：无论是蛋白质水平还是 mRNA 水平，与正常肝细胞相比，ORP150 在肝细胞癌中表达明显上调；经 RNA 干扰后，肝细胞癌的凋亡明显增加，但肿瘤细胞的侵袭性无改变．肝细胞癌中，ORP150 表达上调，它可能抑制肿瘤细胞的凋亡而促进其生长．ORP150 有可能成为肝细胞癌的治疗靶点．

关键词 氧调节蛋白 150，人肝细胞癌，凋亡
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