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ORIGINAL ARTICLE

Epidermal growth factor receptor/heme oxygenase-1 axis is involved in chemoresistance to cisplatin and pirarubicin in HepG2 cell lines and hepatoblastoma specimens

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Abstract

Purpose To investigate the possibility that the antioxidant stress protein Heme oxygenase-1 (HO-1) is involved in the acquisition of chemoresistance in cisplatin and pirarubicin (CITA) therapy.

Methods Human hepatoblastoma-derived cell line (HepG2) was used to generate a knockdown cell line of HO-1 by small interfering RNA (siRNA). Expression of HO-1, epidermal growth factor receptor (EGFR), Akt, and extracellular signal-regulated kinase1/2 (ERK1/2) was examined by Western blot. The cytotoxic effect of cisplatin, pirarubicin, and EGFR inhibitor was examined by trypan blue staining. In human hepatoblastoma specimens ($n=5$), changes of HO-1 expression were examined immunohistochemically before and after CITA therapy.

Results HO-1 expression in HepG2 cells was increased by the treatment of cisplatin (CDDP) and pirarubicin (THP) dose-dependently. In HO-1 knockdown HepG2 cells, the HO-1 was not expressed and the percentage of trypan blue-positive cells (dead cells) was significantly increased after treatment of CDDP and THP. The EGFR inhibitor decreased the levels of HO-1, phospho-Akt and phospho-ERK1/2 in HepG2 cells. Combination treatment of EGFR inhibitor with CDDP and THP increased the cytotoxic effect in HepG2 cells. In human hepatoblastoma specimens, 4 of the 5 patients (80%) showed HO-1 expression changed much stronger in the viable tumor cells after CITA therapy.

Conclusion The cytotoxic effects of CDDP and THP were both enhanced under HO-1 knockdown conditions as well as under conditions that inhibit the activation pathway of HO-1 by EGFR inhibitors. EGFR/HO-1 axis may be involved in acquiring chemoresistance in HepG2 cell lines as well as in human hepatoblastoma.

Keywords Hepatoblastoma · Cisplatin · Pirarubicin · Heme oxygenase-1 · Epidermal growth factor receptor

Introduction

Hepatoblastoma is the most common pediatric liver malignancy accounting for approximately 90% of liver cancers in children under 3 years of age [1, 2]. Complete resection of the tumor plays a key role in successful treatment for this disease. Overall, outcomes have greatly improved over the past 4 decades because of advances in chemotherapeutic agents and administration protocols as well as innovations of surgical approach including liver transplantation [3, 4]. Recent clinical trial studies have suggested that cisplatin-based neoadjuvant chemotherapy is relatively effective for the treatment of hepatoblastoma. The North America-based Children's Oncology Group (COG; P6945 trial) reported that the combination treatment of cisplatin, 5-fluorouracil and vincristine improved the 4-year event-free survival (EFS) of

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patients at stages I/II and III to 84% and 63%, respectively [5]. The European-based International Childhood Liver Tumors Strategy Group (SIOPEL-4 trial) reported that the 3-year EFS was 76% in hepatoblastoma patients sequentially treated with neoadjuvant cisplatin and doxorubicin, surgical treatment and postoperative chemotherapy of carboplatin and doxorubicin [6]. The JPLT-2 trial reported that the combination of cisplatin and 4′-*o*-tetrahydro-pyranyl-adriamycin (pirarubicin; CITA therapy) with surgical treatment resulted in a 5-year event-free survival (EFS) of 62.4% [7]. Given these findings, CITA therapy has been the main chemotherapy regimen for the treatment of advanced hepatoblastoma in Japan. However, in severely advanced cases, such as those with PRETEXT (Pretreatment evaluation of tumor extent) IV or with distant metastasis, current chemotherapeutic regimens are less effective and the outcomes remain poor [7] [8]. Furthermore, dose escalation of these chemotherapeutic agents is impractical, as cisplatin-based chemotherapy results in moderate-to-severe toxicity in half of the treated hepatoblastoma patients [6, 7]. Understanding the mechanism underlying chemoresistance in these lesions might help create new approach to improve the prognosis of children with advanced hepatoblastoma.

Chemotherapy is generally accepted to cause oxidative stress, which leads to the induction of antioxidant enzymes and chemoresistance. Heme oxygenase 1 (HO-1) is an essential antioxidant involved in the NF-E2-related factor 2 (Nrf2)-mediated defense mechanism against oxidative stress. HO-1 is the inducible form of heme oxygenase, the first rate-limiting enzyme in the degradation of heme into biliverdin/bilirubin, carbon monoxide (CO), and ferritin induced by free iron release. Many studies have shown that HO-1 plays a crucial role on the acquisition of chemoresistance in different types of cancer cells and its inhibition is able to sensitize cancer cells to death [9]. However, its contribution in hepatoblastoma is less clear, especially chemoresistance to CITA therapy. Therefore, we investigated the mechanistic role of HO-1 using human hepatoblastoma-derived cell line (HepG2) and examined whether or not any agents might attenuate the HO-1-mediated chemoresistance. Furthermore, to determine the clinical significance of HO-1 in CITA-treated hepatoblastoma, we examined the HO-1 expression in hepatoblastoma patients treated with CITA as neoadjuvant chemotherapy.

Materials and methods

Reagents

Cisplatin (Toronto Research Chemicals, Downsview, ON, Canada) was dissolved in 0.9% sodium chloride, and pirarubicin (LKT Laboratories, St. Paul, MN, USA) was dissolved in dimethyl sulfoxide (DMSO). LY294002 (a phosphoinositide 3-kinase/AKT inhibitor; Cell Signaling Technology, Beverly, MA, USA), U0126 (an extracellular signal-regulated kinase1/2 [ERK1/2] inhibitor; Calbiochem, San Diego, CA, USA), AG1478 (an inhibitor of epidermal growth factor receptor [EGFR]; Calbiochem), PD173074 (an inhibitor of basic fibroblast growth factor; Santa Cruz Biotechnology, Santa Cruz, CA, USA), SU11274 (an inhibitor of hepatocyte growth factor receptor; ChemScene, Monmouth Junction, NJ, USA), SB431542 (an inhibitor of Transforming growth factor-β; Abcam, Cambridge, MA, USA), IWP-2 (an inhibitor of Wnt/β-catenin signaling; Santa Cruz Biotechnology), OSI-906 (an inhibitor of insulin-like growth factor-1; LKT Laboratories), rapamycin (an inhibitor of mammalian target of rapamycin; Toronto Research Chemicals) and erlotinib (a molecular-targeting drug of EGFR; LKT Laboratories) were dissolved in DMSO and used at 25 μM, 10 μM, 10 μM, 1 μM, 2.5 μM, 10 μM, 5 μM, 3 μM, 20 nM and 5 μM, respectively. The final concentration of DMSO in the treatment media was set at < 0.1% in all experiments.

For the Western blotting analyses, the following primary antibodies were used: polyclonal rabbit antibodies recognizing cleaved PARP (Asp214), phospho-AKT (p-AKT) (Ser473), phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology) and rabbit monoclonal antibodies against HO-1 (Abgent Incorporation, San Diego, CA, USA), EGFR (Cell Signaling Technology) and phospho-EGFR (Tyr1068) (Cell Signaling Technology). A mouse monoclonal antibody against β-actin was obtained from Sigma Chemical Company (St. Louis, MO, USA).

Cell culture

Human hepatoblastoma-derived cell line, HepG2 cells [10] (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and exposed to cisplatin, pirarubicin or both simultaneously at appropriate concentrations. Pretreatment with chemical agents was carried out 1 h prior to the treatment with chemotherapy agents.

To analyze the cytotoxic effect, cells plated on 96-well plates (0.2×10^5 cells/mL) were exposed to cisplatin or pirarubicin alone or simultaneously. After 24 h of treatment, the percentage of dead cells was determined by trypan blue staining with an automated cell counter TC10 (Bio-Rad, Richmond, CA, USA). Cells treated with anti-cancer agents for 48 h were reacted with colorimetric water-soluble tetrazolium salt (WST) using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan). Absorbance in the WST assay was determined at 450 nm using a Multiscan FC microtiter-plate reader (Thermo Fisher Scientific, Vantaa, Finland).

In all experiments, cells were plated in three wells, and the results were represented as the mean ± standard deviation (SD) of three independent experiments.

RNA interference experiment

A combination of four gene-specific small interfering RNA (siRNA) FlexiTube GeneSolution™ targeting the human HO-1 gene (GS3162 for HMOX1) and negative control siRNA (SI03650318) were obtained from Qiagen (Hilden, Germany). HepG2 cells (1×10^5 cells/mL) were transfected with each of the siRNAs using the HyperFect transfection reagent (Qiagen) according to the manufacturer's instruction. At 48 h after transfection, the cells were exposed to chemotherapy agents for 24–48 h.

Western blotting

Cells were lysed in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) supplemented with a Complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) and a Phospho-Stop cocktail (Roche Diagnostics). Lysed protein samples (10 μg) were heat-denatured in Laemmli sample buffer (Bio-Rad), electrophoresed on 10% SDS-polyacrylamide gels and transferred onto Immobilon-P polyvinylidene difluoride membranes. Samples were reacted with each of the primary antibodies and with corresponding horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using an Enhanced Chemiluminescence Prime Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ, USA). The protein band intensity normalized by the β-actin band was calculated using the Image-J analysis software program (ver. 1.44; NIH, Bethesda, MD, USA).

Patients and tissue sample preparation

From 1999 to 2008, a total of 5 patients (3 males and 2 females; mean age 19 ± 13 months [3 months–3 years]) underwent neoadjuvant CITA therapy as scheduled according to the JPLT-2 protocol [7] at Niigata University Medical and Dental Hospital, Niigata, Japan (Table 1). The patients had no evident distant metastasis at the time of the diagnosis. In the JPLT-s protocol, preoperative CITA was allowed to be substituted by transarterial chemoembolization (TACE). Two of five patients received CITA plus TACE as neoadjuvant chemotherapy. Then, all five patients received curative surgical resection of the tumor and confirmed complete surgical resection histologically. After surgery, all five patients received adjuvant chemotherapy. Two patients again received CITA, two patients received half dose of CITA (low CITA), and one patient received ifomide-pirarubicin-etoposide-carboplatin (ITEC). None died of tumor

Table 1 Clinical features of the five hepatoblastoma cases treated with neoadjuvant CITA therapy

Case	Age (months)	Gen-der	PRE-TEXT stage	Size of largest tumor before NAT (mm)	AFP before NAT (ng/mL)	NAT	Size of largest tumor after NAT (mm)	AFP after NAT (ng/mL)	Surgical treatment	AT	Outcome	Follow-up period (months)
1	3	M	III	115×95	394,549	CITA	55×55	296	Rt. HL	CITA	Alive	117
2	12	M	II	75×70	4684	CITA	60×60	NA	PH	Low CITA	Alive	115
3	12	F	IV	110×90	313,390	CITA + TACE	30×28	96	Lt. HL	ITEC	Alive	72
4	36	F	IV	110×90	307,641	CITA + TACE	25×20	19	Lt. HL + PH	CITA	Alive	72
5	11	M	II	70×60	28,887	CITA	32×30	1112	Rt. HL	Low CITA	Alive	48

PRETEXT, pretreatment evaluation of tumor extent; NAT, neoadjuvant therapy; AT, adjuvant therapy; F, female; M, male; CITA, cisplatin-pirarubicin; TACE, transcatheter arterial chemoembolization; low CITA, half-dose cisplatin-based CITA; ITEC, ifomide–pirarubicin–etoposide–carboplatin; NA, not available; Rt. HL, right hepatic lobectomy; Lt. HL, left hepatic lobectomy; PH, partial hepatectomy

progression and all five were alive without recurrence of tumor during the period of observation after surgical treatment (48–117 months; median 72 months). Tissue samples obtained after receiving informed consent were fixed in 10% buffered formalin, embedded in paraffin, and processed for immunohistochemical analyses under the surveillance of the Regional Ethics Committee of Niigata University Medical School (#2016-0056).

Immunohistochemistry

Deparaffinized tissue sections were processed for antigen retrieval using microwave heating in 0.01 M sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by immersing in 3% hydrogen peroxide with methanol for 30 min. Immunostaining was performed by reaction with a rabbit monoclonal antibody against HO-1 (1:100; Abgent) overnight at 4 °C using a Vector Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) with 3,3-diaminobenzidine. Counterstaining was done by hematoxylin staining. Negative control immunohistochemical staining was performed using normal rabbit immunoglobulin.

The degree of immunohistochemical staining was examined by two independent observers. As previously reported [11], the immunostaining score of HO-1 was calculated by multiplying the staining intensity score (0, none; 1, weak; 2, moderate; 3, intense) with the score for the density of stained cells (0, < 5.0%; 1, 5–25%; 2, 26–50%; 3, > 51%).

Statistical analyses

All in vitro experiments were performed in triplicate. Statistical analyses were performed with mean ± SD values using Student's *t* test and a two-way analysis of variance (ANOVA) with Bonferroni's correction. p values of < 0.05 were considered significant.

Results

HO-1 expression is induced by anti-cancer agents in HepG2 cells

Western blotting analyses showed that the treatment of anti-cancer agents dose-dependently increased the HO-1 expression in HepG2 cells, from 1.6- to 2.4-fold by cisplatin (10 and 50 μg/mL, respectively) and from 1.4- to 1.8-fold by pirarubicin (1 and 5 μg/mL, respectively) over control cells (Fig. 1a). When cells were treated with both anti-cancer agents simultaneously, the HO-1 expression was significantly increased from 3.4- to 4.8-fold (cisplatin 10 μg/mL + pirarubicin 1 μg/mL and cisplatin 50 μg/mL + pirarubicin 5 μg/mL, respectively) over control cells (Fig. 1a).

HO-1 contributes to chemoresistance in HepG2 cells

When control siRNA-transfected cells were treated with low levels of cisplatin (2 μg/mL) or pirarubicin (0.5 μg/mL), protein bands of cleaved PARP were invisible by Western blotting. In contrast, in cells transfected HO-1 siRNA, cleaved PARP was clearly detected by the same treatment (Fig. 1b). The percentage of trypan blue-positive cells was increased in HO-1 siRNA-transfected cells treated with cisplatin (control siRNA vs. HO-1 siRNA; 14% ± 2% vs. 23% ± 3%, $p < 0.05$) and with pirarubicin (control siRNA vs. HO-1 siRNA; 7% ± 2% vs. 18% ± 3%, $p < 0.05$). A synergic cell-killing effect of HO-1 gene knockdown was also observed in cells treated with cisplatin and pirarubicin simultaneously (control siRNA vs. HO-1 siRNA; 19% ± 3% vs. 38% ± 6%, $p < 0.05$) (Fig. 1c). The WST assay showed that the knockdown of the HO-1 gene expression effectively enhanced the decrease in the cell proliferation of cisplatin-treated cells (control siRNA vs. HO-1 siRNA; relative absorbance 71% ± 6% vs. ± 52% ± 4%, $p < 0.05$) and in pirarubicin-treated cells (control siRNA vs. HO-1 siRNA; relative absorbance 81% ± 3% vs. 58% ± 6%, $p < 0.05$) (Fig. 1d). Cell proliferation was also suppressed in cells treated with cisplatin and pirarubicin simultaneously by HO-1 siRNA transfection (control siRNA vs. HO-1 siRNA; relative absorbance 63% ± 4% vs. 28% ± 5%, $p < 0.05$).

EGFR signaling regulates the HO-1 expression in HepG2 cells

To examine the regulatory mechanism of HO-1, cells were treated with different types of chemical inhibitors. Among the agents examined, only AG1478 decreased the HO-1 expression (0.3-fold of control, $p < 0.05$) (Fig. 2a). AG1478-induced HO-1 suppression was also observed in cells treated with cisplatin or pirarubicin (Fig. 2b). Western blot analyses showed that the levels of p-EGFR were increased by cisplatin (10 μg/mL and 50 μg/mL; 2.4- and 2.7-fold of control, both $p < 0.05$) and pirarubicin (1 μg/mL and 5 μg/mL; 3.8- and 3.6-fold of control, both $p < 0.05$) (Fig. 2c).

EGFR inhibitor suppresses HO-1 by inhibiting Akt and ERK1/2 signaling

To address whether or not the MAPK/ERK signal is involved in EGFR-mediated HO-1 expression, cells were treated with chemical inhibitor of Akt and ERK. Western blot analyses showed that the HO-1 expression was decreased by both LY294002 and U0126 treatment (0.1- and 0.3-fold of control, respectively, both $p < 0.05$) (Fig. 3a). Protein bands of p-Akt and p-ERK1/2 were invisible or faint in control cells but stronger after short-term exposure to cisplatin and

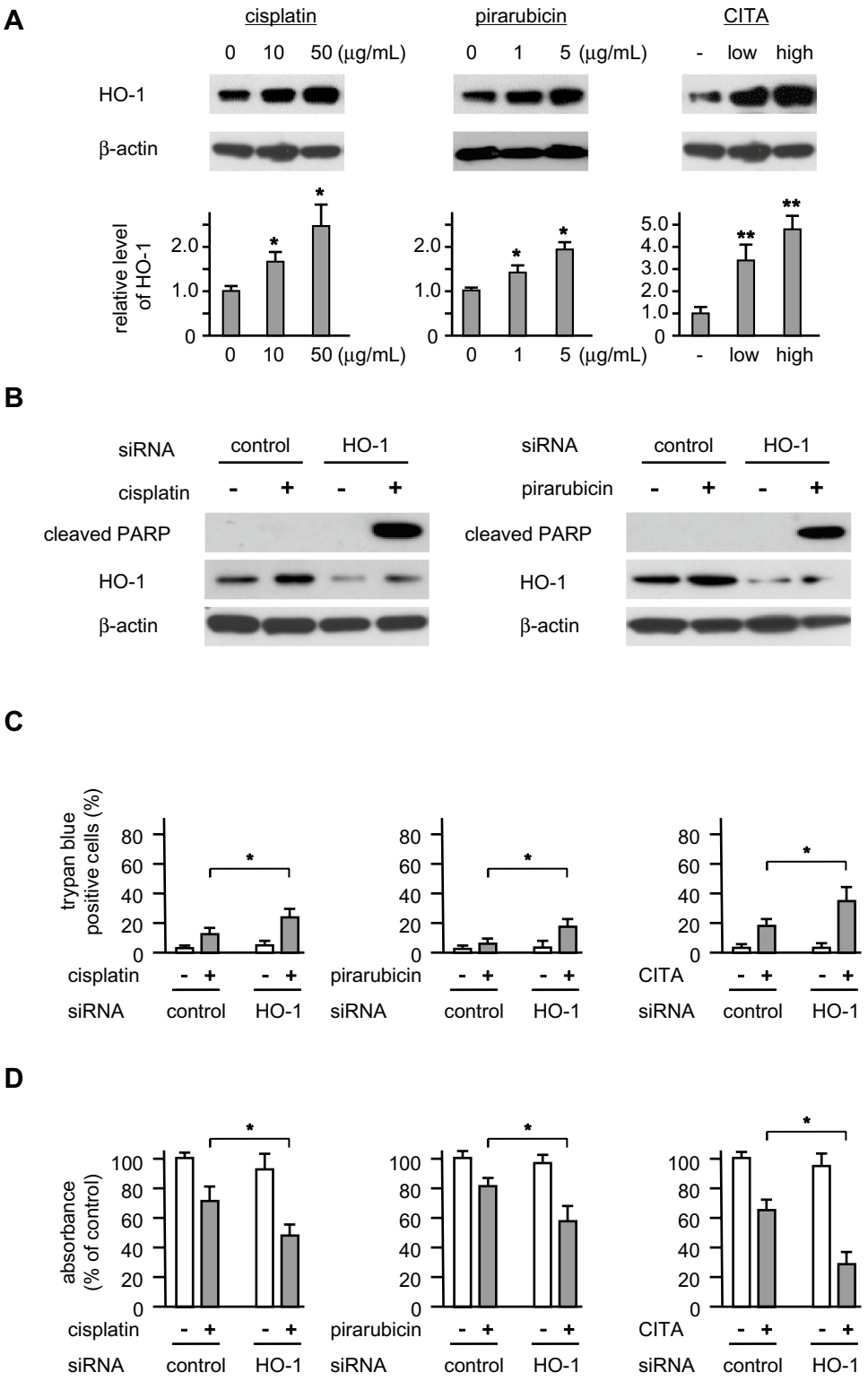

Fig. 1 HO-1 enhances chemoresistance in HepG2 cells. **a** Cells were treated with cisplatin (10 and 50 μg/mL), pirarubicin (1 and 5 μg/mL) or both simultaneously (CITA-low; cisplatin 10 μg/mL plus pirarubicin 1 μg/mL, CITA-high; cisplatin 10 μg/mL plus pirarubicin 1 μg/mL) for 20 h. Columns represent the relative fold-value of band intensities of control cells normalized against β-actin bands (*$p < 0.05$). **b** Western blot analyses of cleaved PARP in HO-1 siRNA-transfected cells treated with low-dose cisplatin (2 μg/mL) or pirarubicin (0.5 μg/mL) for 20 h. **c** Trypan blue assays of HO-1 siRNA-transfected cells treated with cisplatin (10 μg/mL), pirarubicin (1 μg/mL) or both simultaneously (CITA; cisplatin 10 μg/mL plus pirarubicin 1 μg/mL) for 24 h. **d** WST assays of HO-1 siRNA-transfected cells treated with cisplatin (10 μg/mL), pirarubicin (1 μg/mL) or both simultaneously (CITA; cisplatin 10 μg/mL plus pirarubicin 1 μg/mL) for 48 h. White column, non-treated cells. Grey column, anti-cancer drug-treated cells (*$p < 0.05$)

pirarubicin (Fig. 3b). However, this effect was suppressed when cells were pretreated with AG1478 before exposure to cisplatin or pirarubicin (Fig. 3c).

EGFR inhibitor confers HO-1-mediated chemoresistance in HepG2 cells

Trypan blue staining of HepG2 cells showed that the treatment with either AG1478 or erlotinib caused no significant

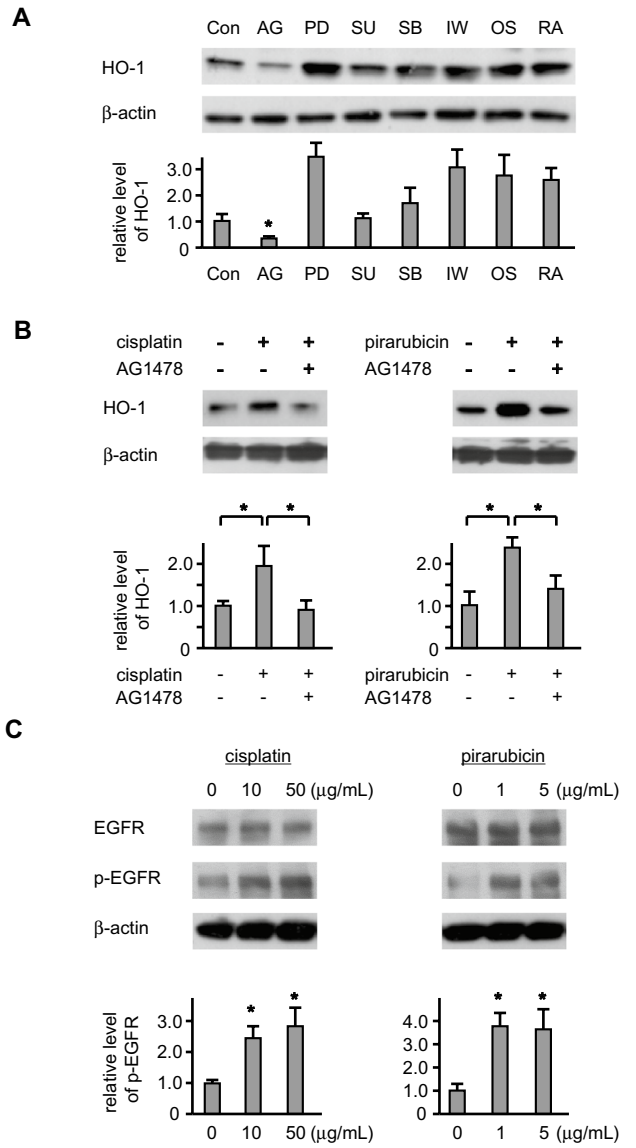

Fig. 2 HO-1 expression is induced by EGFR signaling in HepG2 cells**. a** Western blot analyses of HO-1 in cells treated with different types of chemical inhibitors for 20 h. Con: control, AG: AG1478, PD: PD173074, SU: SU11274, SB: SB431542, IW: IWP-2, OS: OSI-906 and RA: rapamycin. **b** Western blot analyses of HO-1 in cells treated with cisplatin (50 μg/mL) or pirarubicin (5 μg/mL) for 20 h with or without pre-treatment of AG1478. **c** Western blot analyses of EGFR and p-EGFR in cells treated with cisplatin (10 and 50 μg/mL) or pirarubicin (1 and 5 μg/mL) for 2 h. Columns represent the relative fold-values of band intensities normalized against β-actin bands (*$p < 0.05$)

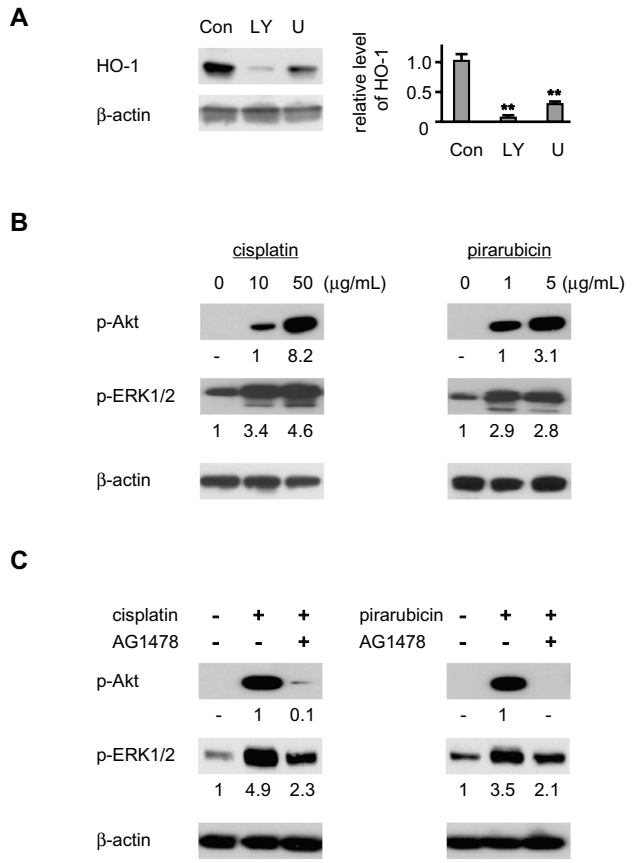

Fig. 3 EGFR/HO-1 axis is mediated by Akt and ERK signaling**. a** Western blot analyses of HO-1 in cells treated MAPK/ERK inhibitors for 20 h. Con, control; LY, LY294002 and U, U0126. Columns represent the relative fold-values of band intensities normalized against β-actin bands of control cells (**$p < 0.01$). (**b**) Western blot analyses of p-Akt and p-ERK1/2 in cells treated with cisplatin (10 and 50 μg/mL) or pirarubicin (1 and 5 μg/mL) for 2 h. **c** Western blot analyses of p-Akt and p-ERK1/2 in cells treated with cisplatin (50 μg/mL) or pirarubicin (5 μg/mL) for 2 h with or without pre-treatment of AG1478. The numbers under the p-ERK1/2 protein bands indicate the relative fold-values of band intensities normalized against β-actin bands of control cells, and those under p-Akt indicate the relative fold-values of cells treated with low-dose anti-cancer agents (cisplatin 10 μg/mL or pirarubicin 1 μg/mL)

increase in the percentage of dead cells (Fig. 4a). However, when cells were treated with cisplatin or pirarubicin in the combination of EGFR inhibitors, the percentage of dead cells was increased (cisplatin alone vs. plus AG1478 vs. plus erlotinib; 15% ± 3% vs. 29% ± 5% vs. 28% ± 4%, both $p < 0.05$ vs. cisplatin alone) (pirarubicin alone vs. plus AG1478 vs. plus erlotinib; 8% ± 2% vs. 22% ± 3% vs. 20% ± 3%, both $p < 0.05$ vs. pirarubicin alone) (Fig. 4b). A synergic cytotoxic effect of EGFR inhibitors was also observed in cells treated with cisplatin and pirarubicin simultaneously (cisplatin/pirarubicin vs. plus AG1478 vs. plus erlotinib; 19% ± 3% vs. 42% ± 4% vs. 39% ± 5%, both $p < 0.05$ vs. cisplatin/pirarubicin) (Fig. 4b).

WST assays support the results of trypan blue staining, showing that cell proliferation was inhibited by the combination treatment of EGFR inhibitors with cisplatin (cisplatin alone vs. plus AG1478 vs. plus erlotinib; relative absorbance 72% ± 5% vs. 39% ± 6% vs. 42% ± 4%, both $p < 0.05$ vs. cisplatin alone) and pirarubicin (pirarubicin alone vs. plus AG1478 vs. plus erlotinib; relative absorbance 81% ± 3%

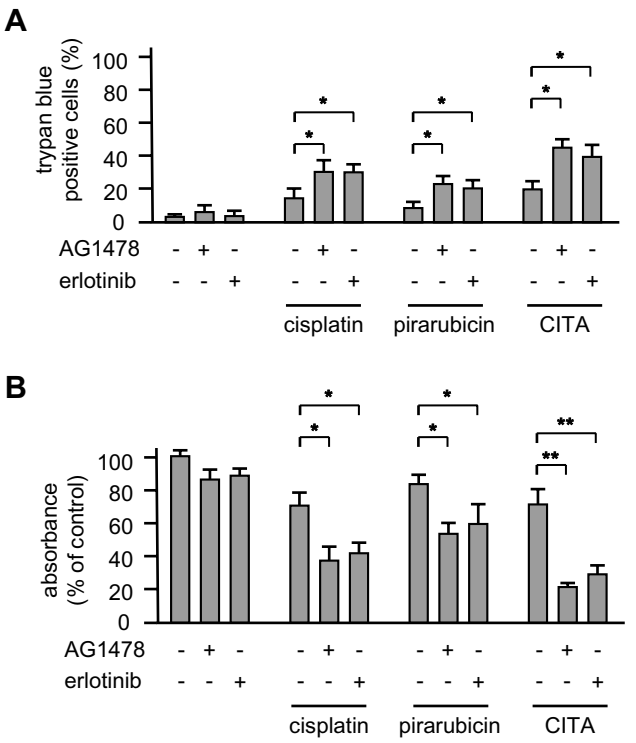

Fig. 4 EGFR inhibitors rescue HO-1-induced chemoresistance in HepG2 cells. **a** Trypan blue assays of cells treated with cisplatin (10 μg/mL), pirarubicin (1 μg/mL) or both (CITA; cisplatin 10 μg/mL plus pirarubicin 1 μg/mL) for 24 h with or without AG1478 or erlotinib (5 μM). **b** WST assays of cells treated with cisplatin (10 μg/mL), pirarubicin (1 μg/mL) or both (CITA; cisplatin 10 μg/mL plus pirarubicin 1 μg/mL) for 48 h with or without AG1478 or erlotinib (5 μM) (*$p < 0.05$, **$p < 0.01$)

vs. 55% ± 4% vs. 60% ± 6%, both $p < 0.05$ vs. pirarubicin alone) (Fig. 4b). The inhibitory effect of EGFR inhibitors on cell proliferation was significant in cells exposed to cisplatin and pirarubicin simultaneously (cisplatin/pirarubicin vs. plus AG1478 vs. plus erlotinib; relative absorbance 72% ± 5% vs. 21% ± 2% vs. 30% ± 4%, both $p < 0.05$ vs. cisplatin/pirarubicin).

HO-1 expression increases in the tumor tissues of CITA-treated patients

HO-1 expression was detected in the nuclear membrane and cytoplasm of the hepatoblastoma cells in all pre- and post- CITA cases. Immunohistochemical staining for HO-1 showed that 5 of 5 tumors expressed HO-1 in chemotherapy-naïve hepatoblastoma patients from faint to strong (immunostaining score 2–9) (Fig. 5) (Table 2). After CITA therapy, the HO-1 expression was increased in 4 of 5 tumors (80%), with an increase in the immunostaining score ranging from 3 to 7. There was no evident correlation of the HO-1 expression between the patient etiologies and tumor characteristics (tumor size and pathology).

Discussion

In the present study we examined whether or not HO-1 interferes with anti-cancer treatment in hepatoblastoma and found that EGFR/Akt/ERK/HO-1 signaling is a leading cause of chemoresistance. Our results showed that HO-1 expression was increased by both cisplatin and pirarubicin treatment in hepatoblastoma-derived HepG2 cells, and the cytotoxic effects of these chemotherapy agents were enhanced by siRNA-mediated HO-1 gene knockdown. Cisplatin-based treatment has recently been recognized as a major chemotherapy regimen for treating hepatoblastoma [5–8]. Combination treatment of cisplatin with pirarubicin, a doxorubicin analog with enhanced anti-tumor activity and low cardiotoxicity [12, 13], has become a standard therapy in Japan, but its clinical efficacy in advanced cases is limited due to acquired resistance. Determining the mechanisms underlying chemoresistance is therefore crucial for improving the survival of patients with hepatoblastoma.

HO-1 is a key stress-inducible antioxidant with cytoprotective properties and has been well documented as a strong inducer of chemoresistance in many types of cancers [9, 14]. Several recent studies have reported that, upon exposure to stimuli such as inflammation or cytotoxicity, phosphatidylinositol-3 kinase (PI3K)/Akt and mitogen-activated protein kinase ERK signaling activate Nrf2 [15, 16], which leads to the antioxidant response element (ARE) sequence-mediated HO-1 gene induction [9]. To address the cell signaling involved in the HO-1 expression in hepatoblastoma cells, we treated HepG2 cells with different types of cell signaling inhibitors. Western blot analyses showed that only AG1478, a specific EGFR tyrosine phosphorylation blocker [17], reduced the level of HO-1 expression, both with and without cisplatin/pirarubicin treatment. Our data also showed that treatment with both cisplatin and pirarubicin increased the levels of phosphorylated EGFR, suggesting that EGFR signaling is a vital inducer of HO-1 in hepatoblastoma cells.

While few studies have explored the functional relationship between EGFR and HO-1, Kuroda et al. [18] recently reported that the EGFR/Akt/HO-1 pathway determines the chemosensitivity of lung cancer cells. Intriguingly, Yoshida et al. [19] reported that cisplatin induced the cleavage of heparin-binding epidermal growth factor-like growth factor (HB-EGF), which binds and activates EGFR. In our study, the HO-1 expression in HepG2 cells was significantly decreased by both Akt and ERK inhibitors. The expressions of phosphorylated Akt and ERK1/2 were significantly inhibited by AG1478 in the presence of cisplatin or pirarubicin. Taken together, these findings support the idea that Akt and

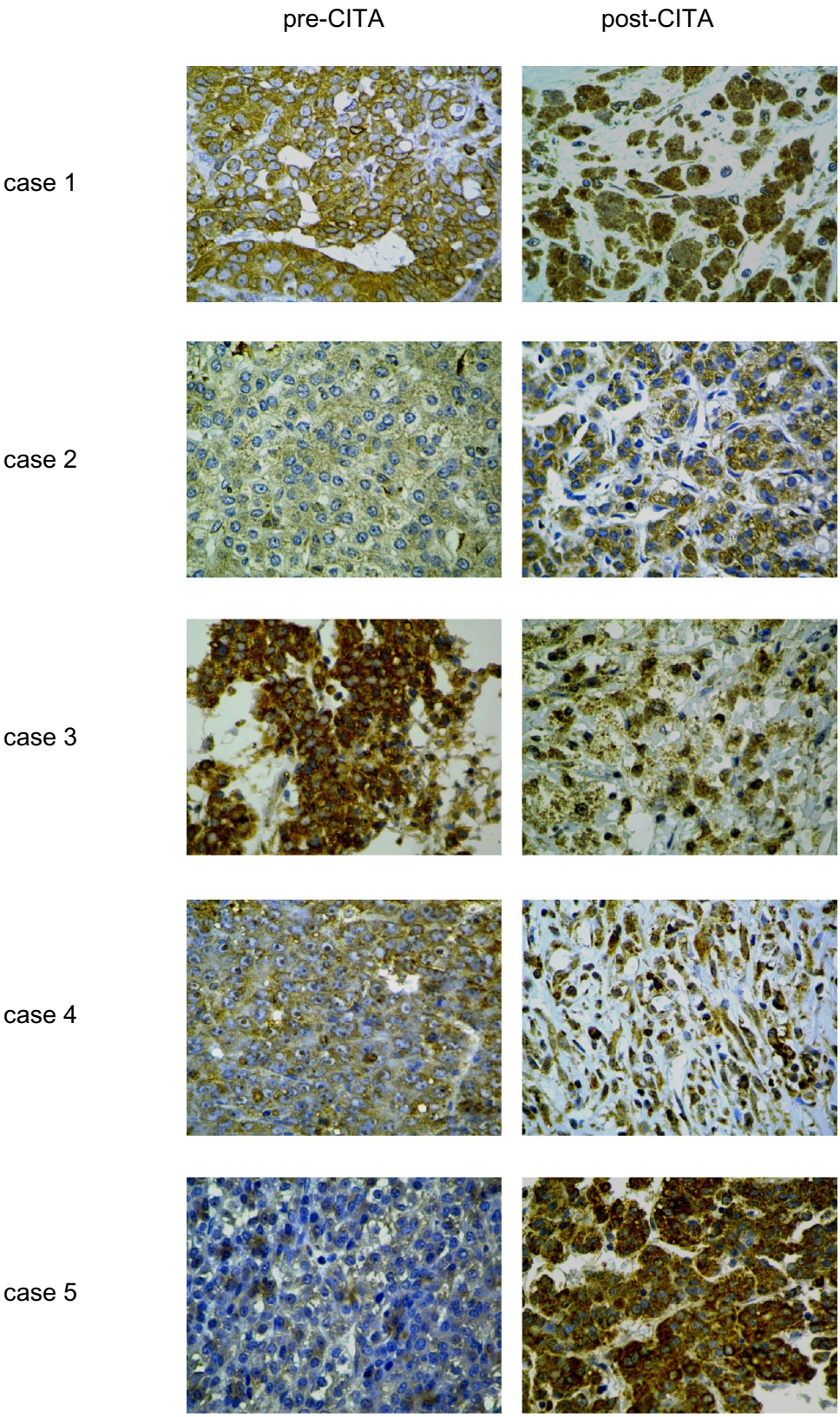

Fig. 5 Immunohistochemical staining of HO-1 in hepatoblastoma patients. Pre-CITA: tumor tissues of the patients (case 1–5) before treated with CITA therapy. Post-CITA: tumors obtained from matched cases treated with CITA therapy (original magnification × 40). In case 1, the HO-1 expression was strong and was observed diffusely in the pre-CITA tumor tissue. After CITA therapy, HO-1 was observed diffusely, and its intensity was slightly decreased in the tumor tissue. In case 2 and 4, HO-1 was moderately and diffusely observed in the pre-CITA tumor tissues. After CITA therapy, the intensity of HO-1 was increased and strong in both tumor tissues. In case 3, HO-1 was weakly and diffusely observed in the pre-CITA tumor tissues. After CITA therapy, the intensity of HO-1 was increased and moderate in the tumor tissue. In case 5, HO-1 was moderately and focally observed in the pre-CITA tumor tissues. After CITA therapy, HO-1 was strongly and diffusely observed in the tumor tissue

Table 2 Pathological data and HO-1 expression in CITA-treated hepatoblastoma

Case	Histology		HO-1 score of pre-CITA			HO-1 score of post-CITA		
	Pre-CITA	Post-CITA	Intensity score	Density score	Multiplied score	Intensity score	Density score	Multiplied score
1	Poorly	Poorly	2	3	6	3	3	9
2	Well	Well	1	3	3	2	3	6
3	Poorly	Poorly	3	3	9	3	2	6
4	Poorly	Poorly	1	3	3	3	3	9
5	Poorly	Poorly	2	1	2	3	3	9

CITA, cisplatin–pirarubicin; HO-1, score immunostaining score of HO-1; poorly, poorly differentiated hepatoblastoma; well, well differentiated hepatoblastoma

MAPK signaling are central mediators of the EGFR/HO-1 pathway in hepatoblastoma cells.

In recent decades, clinical trials have focused on combination therapy of molecular-targeting agents and chemotherapy. For example, a Phase III trial in epidermal growth factor receptor 2 (HER2)-positive advanced gastric cancer showed that a monoclonal antibody against HER2 (trastuzumab) in combination with cisplatin and fluoropyrimidines significantly improved the overall survival [20]. A multicenter randomized phase II trial in hepatocellular carcinoma patients found that the multi-kinase inhibitor sorafenib plus intra-arterial cisplatin infusion achieved a favorable overall survival compared with sorafenib alone [21]. More recently, a phase III trial in advanced ovarian cancer showed that the combination treatment with the angiokinase inhibitor nintedanib and carboplatin and paclitaxel significantly increased the progression-free survival despite causing gastrointestinal adverse events [22]. Although further studies in more diverse populations of cancer patients are needed to clarify the efficacy and safety of target drug-combined chemotherapy, these findings encourage the development of new molecular-based strategies for treating refractory malignant diseases.

We examined whether or not clinically applied EGFR inhibitors strengthen the effect of chemotherapeutic agents in hepatoblastoma. Trypan blue and WST assays showed that the combination of either AG1478 or erlotinib with cisplatin/pirarubicin significantly increased the cytotoxic effect in HepG2 cells. It should be noted, however, that the safety and efficacy of erlotinib-combined chemotherapy are unclear, varying based on the type of cancer and the treatment protocol [23, 24]. Because the numbers of hepatoblastoma patients are small, the pharmaceutical clinical trials are very difficult to perform. A thorough investigation of the molecular status of the tumor tissues is necessary.

There are several limitations in this study. The results of this HepG2 cell line model may not be applied to the actual patients. It is too far to imply the role of the EGFR/HO-1 cascade in the patient of actual hepatoblastoma. Furthermore, the sample size was small ($n=5$) and the quality was inconsistent with some samples coming from well- and poor-differentiated ones. Some cell samples have been treated with TACE. This may affect the analysis. This study focused on the cisplatin-based protocol but in the high-risk patients, the majority will also receive the PLADO protocol and therefore, the findings from this study may have limited clinical application only.

In conclusion, we determined the HO-1 expression in hepatoblastoma tissue samples. Despite the few patients enrolled, immunohistochemical analyses showed that all five tumors expressed HO-1, from faint to strong. Furthermore, four of the five cases showed increased HO-1 expression after CITA therapy, suggesting that regulatory efforts against HO-1, such as administering EGFR inhibitors, might support the anti-tumor effects of chemotherapy in hepatoblastoma patients. No studies have evaluated the trends in the HO-1 expression of hepatoblastoma tissue samples, so further studies in larger populations at different facilities are awaited.

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