Lysosome-associated protein transmembrane4β is involved in multidrug resistance processes of colorectal cancer

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Abstract. Colorectal cancer (CRC) is one of the most common reasons for cancer-associated mortality worldwide. The present study aimed to investigate the drug resistance mechanism of the oxaliplatin (OXA)-resistant HT-29 cell line (HT-29/L-OHP) and examine the expression of lysosome-associated protein transmembrane 4β (LAPTM4β), a drug resistance-associated gene. In the present study, a drug concentration gradient method was used to establish the drug-resistant HT-29/L-OHP cell line. Cell apoptosis was analyzed by flow cytometry. LAPTM4β mRNA expression was examined by reverse transcription-quantitative polymerase chain reaction analysis and LAPTM4β-35 expression was examined by western blot analysis. Cell morphology of the HT-29/L-OHP drug-resistant cell line was examined. The results indicated that the intercellular space among HT-29 cells was small, with aggregative growth while the intercellular space among HT-29/L-OHP cells was large, with scattered growth. The apoptotic rate in HT-29/L-OHP cells (17.7%) was significantly lower compared with that in HT-29 cells (P<0.05). LAPTM4β mRNA expression in HT-29/L-OHP cells was significantly increased compared with that in HT-29 cells (P<0.05). The relative expression of LAPTM4β-35 protein in HT-29/L-OHP cells was significantly higher compared with that in HT-29 cells (P<0.05). In conclusion, LAPTM4β may be involved in the multidrug resistance processes of CRC. Therefore, LAPTM4β may serve as a novel biomarker for drug resistance of CRC.

Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer-associated mortality worldwide (1,2). CRC has a poor prognosis due to the insidious symptomatology, rapid progression and late clinical presentation, causing a poor 5-year overall survival rate (3,4), which is <10% in advanced stages of CRC (5). Although a number of novel therapeutic strategies targeting epidermal growth factor receptor (EGFR) and vascular endothelial growth have been identified, the most frequently utilized frontline regimen for patients with metastatic CRC is a combination of oxaliplatin (OXA) and fluoro-pyrimidines (6,7). The cytotoxic effects of OXA on cancer cells mainly depend on the formation of platinum-DNA adducts, which may result in replication blockade, DNA damage and the activation of programmed cell death of cancer cells (8). In the clinic, not all patients with CRC are sensitive to OXA therapy due to developing drug resistance, which is the main obstacle for therapeutic effectiveness (5). However, the mechanisms for the OXA-induced drug resistance in CRC cancer cells are elusive.

The lysosome-associated protein transmembrane (LAPTM) protein family includes LAPTM4α, LAPTM4β and LAPTM5 (9). Among these LAPTMs, LAPTM4α and LAPTM4β are ubiquitously expressed, and LAPTM5 is expressed in immune cells (10,11). Previous studies have reported that LAPTM4β mediates multidrug resistance (MDR) in cancer cells via interacting with multidrug resistance protein (12,13). A previous study reported that LAPTM4β is overexpressed in numerous cancer cells and is involved in tumorigenic processes (14). Therefore, it was speculated that LAPTM4β may enhance the proliferation and/or detoxification potential of cancer cells. Recently, Xia et al (15) demonstrated that LAPTM4β-35 was significantly over-expressed in various cancers including hepatocellular carcinoma, breast cancer, cervical carcinoma, gallbladder carcinoma and ovarian carcinoma. Kang et al (16) reported that LAPTM4β-35 overexpression may be an independent factor in CRC prognosis, which may be a critical potential biomarker for CRC.

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The present study attempted to establish OXA drug-resistant CRC cell lines, and detect the expression of LAPTM4β and LAPTM4β-35. Therefore, the present study aimed to investigate the drug resistance mechanism of OXA in CRC cell lines, and identify a specific and sensitive biomarker for CRC.

Materials and methods

Cell culture. The CRC cell line, including 47 strains of Oxaliplatin resistant HT-29 (HT-29/L-OHP) cells and 31 strains of HT-29 cells were obtained from the Shanghai Institute for Biological Sciences, Chinese Academy of Science (Shanghai, China). HT-29 cells were maintained and cultured in RPMI-1640 growth medium (Gibco BRL; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco BRL; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere containing 5% CO₂.

Establishment of drug-resistant cell lines. The OXA-resistant cell line was established in General Surgery laboratory of the Second Affiliated Hospital of Harbin Medical University (Harbin, China) over a period of 12 months by continuous exposure of the HT-29 cell line to gradually increasing concentrations of OXA (4-15 µmol/l) according to a previous study (17). The established OXA-resistant cell line was termed HT-29/L-OHP. The HT-29 cell line was passaged three times at each drug concentration and the cell vials were frozen at each increase in drug concentration. Prior to the following experiments, the HT-29 cells were maintained in no-drug RPMI-1640 medium for at least 7 days. The established HT-29/L-OHP cells were cultured in RPMI-1640 medium with 4 µmol/IOXA solution (final concentration) for subsequent experiments.

Cell morphology observation. The cells were stained using the 10% Giemsa's staining solution at room temperature for 15 min. The cell morphology of the HT-29/L-OHP and HT-29 cells in the logarithmic growth phase were observed and captured under an inverted fluorescence microscope, as previously described (17).

Flow cytometry. The HT-29 cells were harvested by scraping the cells and centrifuging at the speed of 500 x g for 5 min at room temperature. Subsequently, the cells were seeded on 6-well plates at a density of 1x10⁶ cells/well. Cell apoptosis was evaluated by flow cytometry, which monitors annexin V-fluorescein isothiocyanate (FITC) binding (Trevig, Inc., Gaithersburg, MD, USA) and propidium iodide (PI; Trevig, Inc,) uptake simultaneously. Subsequent to culturing for 24 h at 37°C, the HT-29 cells were harvested by scraping the cells and centrifuging at the speed of 500 x g for 5 min at room temperature. Subsequently, the cells were resuspended in annexin V-FITC (at a concentration of 1X) and PI (at a concentration of 5 µg/ml) in the dark at room temperature for 15 min. Subsequently, the cell samples were examined by FACScan flow cytometry (BD Bioscences, Franklin Lakes, NJ, USA). The produced annexin V-FITC fluorescence was monitored via the 530/30-nm band filter (FL-1), while the produced PI fluorescence was monitored via the 585/42-nm band filter (FL-2).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In order to examine the mRNA expression of LAPTM4β, a RT-qPCR assay was performed. Primers sequences are presented in Table I. β-actin was used as the internal control. Total RNA was extracted with the RNA simple Total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. The integrity of RNA was checked by 2% agarose gel electrophoresis and visualized using the ethidium bromide. The concentration of the obtained RNA was examined with an ultraviolet spectrophotometer (DU800; Beckman Coulter, Inc., Brea, CA, USA) according to the manufacturer's protocol. RNA (~2 µg) was reverse transcribed following the protocol of the PrimeScript™ II 1ststrand cDNA Synthesis kit (catalog no., 6210A; Takara Bio, Inc., Otsu, Japan). The obtained complementary DNAs were amplified by using the Sybgreen qPCR kit (Tiangen Biotech Co., Ltd.) in a volume of 20 µl under the following amplification conditions: 95°C for 3 min, 95°C for 10 sec and 60°C for 30 sec, for 40 cycles. The temperature was then successively increased between 70 and 90°C (intervals of 0.5°C every 5 sec). The melting curve assay was employed to demonstrate the purity of the PCR products, as described previously study (17). The experiments were performed in at least three wells and repeated at least three times. Subsequent to electrophoresis on 1.4% agarose gels and visualized using the ethidium bromide, the images were digitally captured with an achage coupled device camera. The captured images were analyzed by NIH Imager beta (version 2.0; Matrix Science, Inc., Boston, MA, USA). The relative levels of target genes were calculated using the 2⁻ΔΔCq method (18).

Western blot analysis. The HT-29 cellular lysates were harvested by 0.25% trypsin/EDTA in PBS solution, pelleted by short centrifugation at speed of 500 x g for 5 min at room temperature, and suspended in lysis buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to extract the total proteins. The concentration of the extracted proteins was examined with a bicinchoninic acid protein quantification kit (Beyotime Institute of Biotechnology, Haimen, China). Cell lysates were separated by 15% SDS-PAGE (loading, 50 µg/well) and electrotransferred to polyvinylidene fluoride membranes. Subsequently, membranes were blocked with 5% defatted milk

| Gene               | Primers                                           |
|--------------------|--------------------------------------------------|
| LAPTM4β Forward    | 5'-GGAGACGACAGACCTT-3'                           |
| LAPTM4β Reserve    | 5'-TTATTCGATCTCAACACCT-3'                        |
| β-actin Forward    | 5'-CCTGTGGCATCAGAATT-3'                         |
| β-actin Reserve    | 5'-GAAGCATTTCGGGACG-3'                          |

Table I. Primers for the LAPTM4β and β-actin genes.
for 1 h at room temperature in PBS-Tween-20 solution (PBST; PBS adjusted to pH 7.6, containing 0.05% Tween-20). The membranes were incubated with rabbit anti-human LAPTM4β polyclonal antibody (catalog no., ab82810; dilution, 1:2,000; Abcam, Cambridge, UK) and mouse anti-human β-actin monoclonal antibody (catalog no., sc-130300; dilution, 1:3,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in PBST at 4°C overnight. The membranes were continuously washed with PBST three times, for 10 min each time. The reactive signals were visualized using an enhanced chemiluminescence luminescence kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The immunoblot was scanned with GE Typhoon TM FLA 7000 (GE Healthcare Life Sciences, Uppsala, Sweden) and images were captured. The quantitative analysis for the immunoblot images was performed using Image J software (version 2.0; National Institutes of Health, Bethesda, MD, USA).

Figure 1. Cell morphology of the drug-resistant HT-29/L-OHP cell line and normal HT-29 cells. (A) HT-29 cells; (B) HT-29/L-OHP cells. HT-29/L-OHP, oxaliplatin-resistant HT-29 cell line. The cells were stained using the Giemsa's staining method and the cell morphology was observed using an inverted fluorescence microscope (magnification, x200).

Figure 2. Evaluation of cell apoptosis by annexin V/FITC/propidium iodide double staining. (A) Flow cytometric assay of HT-29/L-OHP cells and HT-29 cells. (B) Statistical analysis of the cell apoptosis. P<0.05 represents the significant difference in cell apoptotic rate in HT-29/L-OHP cells compared with that in HT-29 cells. Q, quadrant; A, annexin V; UL, upper left; UR, upper right; LL, lower left; LR, lower right; HT-29/L-OHP, oxaliplatin-resistant HT-29 cell line; FITC, fluorescein isothiocyanate; PI, propidium iodide.
Statistical analysis. All data are presented as the mean ± standard deviation. Statistical analysis was performed with SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The differences between the groups were analyzed by a paired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Cell morphology of HT-29/L-OHP drug-resistant cell line. The cell morphology of the HT-29/L-OHP and HT-29 cells in the logarithmic growth phase was observed and captured under an inverted fluorescence microscope. The results indicated that the intercellular space among the HT-29 cells was small, with aggregative growth (Fig. 1A). However, the intercellular space among the HT-29/L-OHP cells was large, with scattered growth (Fig. 1B).

Apoptotic rate is inhibited in HT-29/L-OHP cells. The HT-29/L-OHP cell apoptotic rate was observed by the annexin V-FITC/PI double staining method. The apoptotic rate was calculated as the early apoptosis [quadrant (Q)4-upper left] plus the late apoptosis (Q4-upper right). The results demonstrated that the apoptotic rate in the HT-29/L-OHP cells (11.7%) was significantly lower compared with that in the HT-29 cells (17.7%) (P<0.05; Fig. 2). This result suggests that the HT-29/L-OHP cells were resistant to OXA application.

LAPTM4β mRNA expression is enhanced in HT-29/L-OHP cells. LAPTM4β mRNA was evaluated in 47 strains of HT-29/L-OHP cells and 31 strains of HT-29 cells by qPCR assay. The melting curve demonstrated that the melt peak is homogeneous; therefore, the PCR product was purified (Fig. 3). The results indicated that the LAPTM4β mRNA expression in HT-29/L-OHP cells was significantly increased compared with that in the HT-29 cells (P<0.05; Table II; Fig. 4).

LAPTM4β-35 expression is increased in HT-29/L-OHP cells. The HT-29/L-OHP cell LAPTM4β-35 expression was examined
by western blot analysis. The results demonstrated that the relative expression of LAPTMB35 protein in HT-29/L-OHP cells was significantly higher compared with that in the HT-29 cells (P<0.05; Fig. 5).

Discussion

CRC is one of the most prevalent malignant tumors, the reoccurrence and metastasis of which is usually treated by chemotherapy (19). However, the outcomes are usually poor for patients with CRC. MDR is one of the most important factors leading to the reduction in chemotherapeutic effects in the clinic (20-22). The classical mechanism of the MDR protein mainly results in the overexpression of the adenosine triphosphate-binding cassette family protein, which also interacts with the drug to decrease the drug concentration to sub-lethal levels (23,24).

LAPTMB4 is a novel carcinoma-associated gene, which has been mapped to chromosome 8q22.1, spanning at least 50 kb, is composed of 7 exons and 6 introns (25). The LAPTMB4 gene codes a 35-kDa membrane glycoprotein (25). The LAPTMB4 gene-coded LAPTMB4-35 protein has been shown to be upregulated in numerous cancers, including gastric cancer (26), prostate cancer (27), cervical carcinoma (28) and hepatocellular carcinoma (29), and performs an important role. Lee et al (30) reported that LAPTMB4 upregulation is associated with activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling transduction pathway. Other studies also demonstrated that the PI3K/Akt signaling transduction pathway could regulate and strengthen the MDR (31,32). Therefore, it was speculated that LAPTMB4 may also be associated with CRC.

The establishment of drug-resistant cell lines may provide a strategy for cancer therapy and neoplasm metastasis mechanism (33). In the present study, the drug-resistant CRC cell line HT-29/L-OHP was established, which could stably grow in OXA solution at a concentration of 15 µmol/l. LAPTMB4 mRNA levels and LAPTMB4-35 protein levels were detected by qPCR and western blot analysis, respectively. The results indicated that the mRNA and protein expression levels in HT-29/L-OHP cells were significantly higher compared with those in the HT-29 cells. These results indicate that long-term OXA treatment could enhance LAPTMB4 expression, which may be an important drug-resistant mechanism for CRC therapy.

A previous study revealed that the upregulated LAPTMB4 in drug-resistant HT-29/L-OHP cells could increase the efflux of OXA in tumor cells, which becomes a critical reason for drug resistance in CRC cells (12). Li et al (12) reported that LAPTMB4 induces MDR of cancer cells by promoting drug efflux via the co-localization and interaction with P-glycoprotein (P-gp), and anti-apoptosis by triggering the signaling pathway of PI3K/Akt. Another study (34) also reported that the PI3K/Akt pathway was involved in the modulation of P-gp-mediated MDR in the mouse leukemic L1210/VCR cell line. Furthermore, MDR may reversely affect the effects of LY294002 on vincristine-induced apoptosis in HeLa cells. Li et al (35) also revealed that MDR could increase drug efflux and decrease the drug concentration entering into nucleus by P-gp, and reduce drug-induced DNA injury and drug-caused apoptosis. Tan et al (36) reported that LAPTMB4 may promote EGFR association with the autophagy inhibitor Rubicon, which in turn disassociates Beclin 1 from Rubicon to initiate autophagy. Li et al (37) reported that LAPTMB4 renders the tumor cells resistant to anthracycline by triggering lysosome-mediated cell death. However, the drug-resistant mechanism for OXA remains unknown.

In conclusion, the present study established a stable OXA-resistant CRC cell line. The LAPTMB4 gene and the LAPTMB4-35 protein expression levels in this drug-resistant cell line were significantly increased, compared with those in the normal CRC cell line, which suggests that LAPTMB4 is involved in the MDR processes of CRC. Therefore, LAPTMB4 may become a novel biomarker for drug resistance of CRC.

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