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Acid ceramidase inhibition sensitizes human colon cancer cells to oxaliplatin through downregulation of transglutaminase 2 and β1 integrin/FAK–mediated signalling

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ABSTRACT

Acid ceramidase (ASAH1) has been implicated in the progression and chemoresistance in different cancers. Its role in colon cancer biology and response to standard chemotherapy has been poorly addressed so far. Here, we have investigated ASAH1 expression at the protein level in human colon cancer cell lines and tissues from colon cancer patients, and have examined *in vitro* the possible link between ASAH1 expression and functional activity of p53 protein whose inactivation is associated with the progression from adenoma to malignant tumour in colon cancer. Finally, we have explored the role of ASAH1 in response and resistance mechanisms to oxaliplatin (OXA) in HCT 116 colon cancer cells. We have demonstrated that human colon cancer cells and colorectal adenocarcinoma tissues constitutively express ASAH1, and that its expression is higher in tumour tissues than in normal colonic mucosa. Furthermore, we found an inverse correlation between ASAH1 expression and p53 functional activity. Obtained data revealed that ASAH1 was involved in HCT 116 cell response to OXA and that anti-proliferative, pro-apoptotic, anti-migratory and anti-clonogenic effects of OXA could be significantly increased by combination treatment with ASAH1 inhibitor carmofur. Increased OXA sensitivity was associated with downregulation of signalling involved in acquired resistance to OXA in colon cancer, in particular transglutaminase 2 and β1 integrin/FAK, which resulted in the suppression of NF-κB and Akt. Thus, combination of OXA with ASAH1 inhibitors could be a promising strategy to counter chemoresistance and improve treatment outcome in advanced colon cancer.

1. Introduction

Colorectal cancer (CRC) represents the global public health burden being the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths worldwide. Oxaliplatin (OXA), a third-generation platinum compound, has shown high activity in patients with metastatic CRC when combined with 5-fluorouracil and leucovorin. However, despite initial sensitivity to OXA, most cancer cells ultimately acquire drug resistance, which necessitates the development of novel combination strategies to increase OXA efficiency.

Acid ceramidase (ASAH1), a lysosomal lipid hydrolase that catalyses the degradation of pro-apoptotic ceramide into sphingosine and free fatty acid, has been implicated in the progression and resistance to therapy in different cancers including melanoma [1], glioblastoma multiforme [2], acute myeloid leukaemia [3], prostate [4], head and neck [5], breast [6] and liver cancer [7]. Its enzymatic activity and expression at the mRNA level were previously detected in several human colorectal adenocarcinoma cell lines [1], although little is known about the regulatory mechanisms controlling ASAH1 expression in colorectal carcinogenesis. Pharmacological inhibition of ASAH1 activity was shown to induce apoptosis and inhibit growth of human colon cancer cells *in vitro* and *in vivo* [8,9]. Furthermore, the addition of highly potent ASAH1 inhibitors to 5-FU potentiates anti-proliferative effects of 5-FU in human colon carcinoma cells *in vitro* in a synergistic manner [9]. These findings suggest that ASAH1 plays a crucial role in regulating colon cancer

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cell survival and response to chemotherapy.

We set out to investigate ASAH1 expression at the protein level in human colon cancer cell lines and tissues from CRC patients, and to examine *in vitro* the possible link between ASAH1 expression and functional activity of p53, whose inactivation is associated with the progression from adenoma to malignant tumour in CRC. Finally, we explored the possibility of targeting ASAH1 activity to increase the sensitivity of human colon cancer cells to oxaliplatin *in vitro*.

2. Materials and methods

2.1. Drugs

Oxaliplatin (OXA), piftirin-α (PFT-α) and carmofur (C) were purchased from Pliva, Enzo Life Sciences, Inc. and Sigma-Aldrich, respectively.

2.2. Patient information and tissue specimens

Ten patients diagnosed at the Department of Pathology, Clinical Hospital Centre Rijeka, with paraffin-embedded colorectal adenocarcinoma tissues were included in this pilot analysis. The study was approved by the Clinical Hospital Centre Rijeka Ethics Committee and the University of Rijeka Faculty of Medicine Ethics Committee for biomedical research. Eight of ten selected paraffin blocks for immunohistochemical analysis contained normal colonic mucosa and tissue of low grade colorectal adenocarcinomas type.

2.3. Cell culture

Human colon cancer cell lines HT-29, HCT 116, RKO, SW480 and SW620 (American Type Culture Collection) were cultured in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 2 mM L-glutamine (GIBCO, Invitrogen). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Cell viability assay

Cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay as previously described [10].

2.5. Apoptosis detection

Detection and quantification of apoptosis were carried out by Annexin-V-FLUOS Staining Kit (Roche Applied Science) according to the manufacturer's instructions. Briefly, 2×10^4 cells/well were seeded into 8-well Nunc Lab-Tek II Chamber Slide system (Thermo Fisher Scientific) and treated for 48 h. Slides were analysed by fluorescence microscopy (Zeiss Axio Observer Z1 Inverted Phase Contrast Fluorescent Microscope).

2.6. Wound healing and clonogenic assays

For cell migration and colony formation assays, cells were seeded in 6-well plates at density of 3×10^5 and 500 cells/well, respectively. Assays were preformed following protocols previously described [10].

2.7. Western blot analysis

After indicated treatment period, 3×10^5 cells were lysed using RIPA buffer (Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitor cocktails (Roche Applied Science). Western blot analysis and signal visualization were performed as previously described [10]. Blots were probed with primary antibodies raised against ASAH1, *p*-FAK and β1-integrin purchased from Abcam; *p*-Akt, transglutaminase-2, p-p53 and p-p65-NF-κB from Cell Signalling Technology; and α-tubulin (Sigma Aldrich) at 4 °C overnight followed by 1 h incubation with secondary antibody (Dako).

2.8. Immunohistochemical analysis and evaluation of staining

Immunohistochemical analysis was performed on 4-μm thick paraffin sections using anti-acid ceramidase/ASAH1 mouse monoclonal antibody (clone 2C9, LS-C104888, LSBio, LifeSpan BioSciences, Inc.) as primary antibody. According to the manufacturer's instructions, for ASAH1 staining we employed antigen retrieval using PT Link 20 min at 97 °C, in target retrieval solution high pH (3 in 1). Standard immunohistochemistry procedure was performed in Dako Autostainer Plus (DakoCytomation Colorado, Fort Collins, CO, USA) according to the manufacturer's protocol using Envision procedure (DAKO EnVision FLEX, High pH KIT K801021, Glostrup, Denmark). Samples were immunohistochemically stained with anti-ASAH1 for 30 min, dilution 1:100. The semi-quantitative scoring system was used to assess the staining intensity and area extent, as immunoreactivity scoring system (IRS). Each specimen was assigned a score according to the intensity of the cytoplasmic staining: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining; and extent or percentage of stained tumour cells: 0, 0%; 1, 1–24%; 2, 25–49%; 3, 50–74%; and 4, 75–100%.

2.9. Statistical analysis

Data are presented as the means ± SD. Statistical analyses were performed using Student's t-test ($p < 0.05$) and ANOVA ($p < 0.05$) in Statistica (TIBCO Software Inc.).

3. Results

3.1. ASAH1 is constitutively expressed in human colon cancer cell lines and colorectal adenocarcinoma tissues

ASAH1 expression was analysed in colon cancer cell lines derived from primary tumour (HT-29, HCT 116, RKO and SW480) and lymph node metastasis (SW620) using Western blot method (Fig. 1a). ASAH1 was detected in all colon cancer cell lines albeit in variable levels. SW620 cells exhibited profoundly diminished level of ASAH1 in comparison with other tumour cell lines, which suggests that loss of ASAH1 expression could be associated with metastatic phenotype of colon cancer. Immunohistochemical analysis of colorectal adenocarcinoma tissues and adjacent normal colonic mucosa (Fig. 1b) showed that the expression of ASAH1 was heterogeneous in normal colonic mucosa, from tumours that were completely negative to those that showed some positivity, generally the positivity in normal mucosa was markedly lower than in adenocarcinoma tissues (Supplementary Table 1). In the normal colonic mucosa, ASAH1 positivity was mostly observed in the upper part of the gland and surface epithelium, while the lower parts of the glands were usually negative. The positivity for ASAH1 was granular and in most cases present in the epithelial cells toward luminal surface. The positivity was also observed in stromal cells (Fig. 1b, A and B). In colon adenocarcinomas, the heterogeneous expression was more present compared to normal colonic mucosa. In 1 out of 10 cases, tumour cells were completely negative while other adenocarcinomas show low, moderate or high tumoral and stromal cell expression in different areas of tumour (Fig. 1b, C and D). The pattern of positivity was granular with different localization within the tumoral cells as presented in Fig. 1b, E and F. These

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