ACTA ABP BIOCHIMICA POLONICA

Vol. 64, No 2/2017
315–322
https://doi.org/10.18388/abp.2016_1421

Regular paper

Suppression of *ID1* expression in colon cancer cells increases sensitivity to 5-fluorouracil

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Adjuvant chemotherapy with 5-fluorouracil remains the basic treatment for patients with advanced colorectal carcinoma. The major obstacle in successful treatment is the ability of CRC cells to acquire chemoresistance. Here we examined the impact of *ID1* silencing on the sensitivity of CRC cells to 5-FU. To suppress *ID1* expression in HT-29 and HCT-116 cells the cells were transduced with a lentiviral vector carrying the *ID1* silencing sequence. Cells with silenced *ID1* showed altered expression of epithelial and mesenchymal markers and exhibited increased proliferation rate compared to the parental cells. HCT-116 cells with suppressed *ID1* became sensitized to 5-FU and this was not observed in HT-29 cells. Silencing *ID1* resulted in altered expression of genes encoding enzymes metabolizing 5-FU. HT-29 cells with suppressed *ID1* had significantly reduced mRNA level for *thymidine phosphorylase*, *uridine-cytydine kinase 2* and *dihydropyrimidine dehydrogenase*. *ID1* suppression in HCT-116 cells resulted in an increase of mRNA level for *thymidine phosphorylase*, *thymidine kinase* and *uridine-cytydine kinase 2* with concurrent drop of *dihydropyrimidine dehydrogenase* and *thymidylate synthetase* mRNA levels. In conclusion, *ID1* expression impacts the sensitivity of colon cancer cells to 5-FU and may be considered as a potential predictive marker in CRC treatment.

Key words: colon cancer, *ID1*, 5-fluorouracil, TYMP, TK, UCK2, DYPD, TYMS.

Received: 07 September, 2016; revised: 06 February, 2017; accepted: 21 February, 2017; available on-line: 17 May, 2017

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Abbreviations: 5-FDHU, 5-fluoro-5,6-dihydrouracil; 5-FdUTP, 5-fluoro-2′-deoxyuridine-5′-triphosphate; 5-FU, 5-fluorouracil; 5-FUTP, 5-fluorouridine-5′-triphosphate; 7-AAD, 7-aminoaxtinomycin D; CDH1, E-cadherin; CDH2, N-cadherin; CRC, colorectal carcinoma; CTNNβ1, beta-catenin; DMSO, dimethyl sulfoxide; DPYD, dihydropyrimidine dehydrogenase; DT, doubling time; dTMP, 2′-deoxythymidine-5′-monophosphate; EMT, epithelial to mesenchymal transition; F-BAL, fluoro-β-alanine; FBS, fetal bovine serum; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FdUR, 5′-deoxy-5-fluorouridine; FGF, fibroblast growth factor; FN1, fibronectin1; FUMP, 5-fluorouridine-5′-monophosphate; FUR, 5-fluorouridine; GFP, green fluorescent protein; HLH, helix-loop-helix; ID1, inhibitor of differentiation 1; LIF, leukemia inhibitory factor; MET, mesenchymal epithelial transition; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TCF3, transcription factor 3; TK1, thymidine kinase 1; TP53, tumor protein p53; TWIST1, twist family, bHLH transcription factor 1; TYMP, thymidine phosphorylase; TYMS, thymidylate synthase; UCK2, uridine-cytydine kinase 2; UMPS, uridine monophosphate synthetase; UPP1, uridine phosphorylase 1; VIM, vimentin

INTRODUCTION

Colorectal carcinoma (CRC) is diagnosed in about 1 million people every year. It is the most frequent gastrointestinal neoplasm (cancer) and one of the most fatal cancers in general (Kelder *et al*., 2006). In most cases surgery remains the basic and sufficient treatment of patients with CRC at stage I and II, however, for patients with more advanced cancer (stage III and IV) the standard treatment following resection includes adjuvant chemotherapy. Implementation of such a treatment prolongs progression-free survival and overall survival (Lombardi *et al*., 2010). Adjuvant chemotherapy was also shown to highly improve 5-year survival in stage IV patients with isolated liver or lung metastases (Cassidy & Graham, 2012). 5-fluorouracil (5-FU) is one of the earliest chemotherapeutic agents and it has been used successively in CRC treatment for over 50 years (Hammond *et al*., 2016). The drug operates through mechanisms involving inhibition of DNA replication and cell death. 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), the primary metabolite of 5-FU inhibits thymidylate synthase (TYMS), an enzyme producing 2'-deoxythymidine-5'-monophosphate (dTMP). Insufficient dTMP bioavailability disrupts DNA synthesis in the dividing cells. The alternative 5-FU metabolites, 5-fluorouridine-5'-triphosphate (5-FUTP) and 5-fluoro-2'-deoxyuridine-5'-triphosphate (5-FdUTP), undergo inclusion into DNA, which results in impaired translation and subsequent cell death (Cassidy & Graham, 2012; Hammond *et al*., 2016).

The major obstacle in successful treatment with 5-FU based adjuvant chemotherapy is the ability of CRC cells to acquire resistance (Hammond *et al*., 2016). Several mechanisms have been proposed to be responsible for the decrease in cancer cells sensitivity to 5-FU including: variable number of tandem repeats, microsatellite instability, single nucleotide polymorphisms occurring within genes encoding enzymes metabolizing 5-FU, and altered expression of 5-FU metabolizing enzymes (Hammond *et al*., 2016). Improvement of 5-FU adjuvant treatment efficacy remains one of the most important challenges in CRC management. One of the main approaches is identification of predictive biomarkers associated with specific response to chemotherapy, which firstly would allow to determine a group of patients benefiting from 5-FU treatment and could exclude the non-responsive ones. Secondly, it may be useful in development and application of novel therapies combining 5-FU and selective agents capable of targeting specific signaling pathways in tumor. It was initially shown that targeting vascular endothelial growth factor in addition to standard chemotherapy improved outcomes of patients with metastatic CRC. However, recent clinical trials demonstrated that patients with CRC stage III did not benefit from such combined regimen. Thus, understanding the tumor biology through identification of biomarkers, which determine the success or failure of treatment at specific

CRC stage, seems to be crucial step in developing a new, more effective treatment for CRC patients (Oyan, 2012).

The inhibitor of differentiation 1 (ID1) belongs to the family of helix-loop-helix (HLH) transcription regulators. These proteins achieve transcriptional activity by forming heterodimers with other members of the family. ID1, however, lacks the ability to bind DNA and plays a role of dominant negative regulator of its dimerization partners (Ling *et al*., 2014). ID1 was shown to promote chemoresistance of esophageal and pancreatic cancer cells (Li *et al*., 2014). On the other hand, it was reported that ID1 is a favorable predictor for surgically treated non-small-cell lung cancer patients undergoing chemotherapy (Cheng *et al*., 2014). Research on the role of ID1 in breast and gastric cancer and glioma revealed that it is also involved in epithelial to mesenchymal transition (EMT) which is considered one of the mechanisms promoting chemoresistance of cancer cells (Tobin *et al*., 2011; Peng *et al*., 2014; Sánchez-Tilló *et al*., 2014). Still, the direct mechanisms of ID1 related chemoresistance in cancer cells are not known, moreover, not much attention in this context has been paid regarding colon cancer. The aim of this work was to examine the relation between the ID1 expression level and the sensitivity of colon cancer cells to 5-FU.

MATERIALS AND METHODS

Cell cultures. All experiments were conducted on colon cancer cell lines HT-29 and HCT-116 (ATCC, Rockville, MD, USA). Cells were cultured in DMEM enriched with 10% fetal bovine serum (FBS), penicillin (10 U/ml) and streptomycin (100 μg/ml) under standard conditions (37ºC, 5% CO_2). The culture medium was replaced twice a week and cells were passaged at 70% confluence.

Transduction procedure. *ID1* silencing shRNA sequence CTCTACGACATTTCAAGAGAATGTCGTAGAGCAGCACGTCTTTTTC (Oligos, Warsaw, Poland) was subcloned into lentiviral vector pLL3.7 (Addgene, Cambridge, MA, USA) to generate pLL3.7-sh*ID1* construct. Scrambled oligonucleotide GGTTTATGCGCTCGATCTCTA (Oligos, Warsaw, Poland) was used to obtain pLL3.7-scramble plasmid, which served as a negative control. The packaging cells HEK-293T (ATCC, Rockville, MD, USA) were transfected with envelope plasmid pMD2.G, and a packaging plasmid psPAX2 (Adgene, Cambridge, MA, USA) together with pLL3.7-sh*ID1* or pLL3.7-scramble. X-treme Gene 9 (Roche, Mannheim, Germany) was used as a transfection agent. Transfected HEK-293T cells were then incubated for 48 hours to allow generation and replication of the virus. HEK-293T medium containing viral particles was subsequently used to transduce HT-29 and HCT-116 cells. Negative control virus generated control (parental) cells, while vector expressing shRNA targeting *ID1* produced cells with suppressed *ID1* expression (HT-29sh*ID1* and HCT-116sh*ID1*). Successfully transduced cells expressed green fluorescent protein (GFP) and number of GFP positive cells was counted under fluorescent microscope (Leica, Wetzlar, Germany). When at least 80% of cancer cells exhibited green fluorescence, the transduction procedure was finished.

MTT assay. Cells viability in appropriate experiments was estimated on the basis of mitochondrial dehydrogenase activity. In living cells 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Poznan, Poland) is converted by the enzyme to insoluble formazan and the extent of conversion reflects the total number of viable cells (Berridge *et al*., 1996). After the end of particular experiment the cells were incubated for two hours in fresh DMEM containing 0.5 mg/ml MTT at 37ºC. Next, the medium was removed and formazan crystals were dissolved in 0.2 ml of acidic isopropanol. Finally, the absorbance of formazan was measured in a microplate reader (1420 multilabel Counter VICTOR3, PerkinElmer, Waltham, MA, USA) at 570 nm and at 690 nm (background subtraction).

Proliferation assays. Cells were seeded onto 24-well plates at a density of 1.5×10^4/well and cultured in complete medium or medium lacking FBS. Medium was replaced every day. Measurement of cell viability with MTT test was conducted after 24 hours and repeated every day for 124-hour period. The growth curves were generated and population doubling times (DT) were calculated on the basis of exponential growth periods (see Statistical analysis).

5-FU dose response assays. Cancer cells were seeded onto 24-well plates at a density of 1.5×10^4/well and left for 24 hours to attach to plate bottom. Next day the medium was replaced with fresh one containing 5-FU (Sigma-Aldrich, St. Louis, MO, USA) at appropriate concentrations. The control cells were cultured in medium with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) at the concentration used to dissolve 5-FU. After 48 hours of incubation the cell culture medium was removed and cell viability was evaluated with MTT test. Concentration at which 50% reduction of cell viability occurred (IC_{50}) was determined (see Statistical analysis).

Flow cytometric analysis. Cancer cells were seeded onto 60 mm plates at density of 1.5×10^5 cells/plate and cultured overnight. Next day the medium was replaced with fresh one containing 5-FU at appropriate concentrations. The control cells were cultured in medium with equal amount of DMSO. After 48 hours of incubation the experimental medium was removed and the cell viability was evaluated by flow cytometric analysis. Control and experimental cells were stained with 7-aminoaxtinomycin D (7-AAD) and annexin V-PE from PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA) according to manufacturer protocols. Analysis was conducted on FACScan flow cytometer (BD Biosciences, San Diego, CA, USA). The cells negative for annexin V and 7-AAD staining were considered as viable, while early apoptotic cells were marked by decreased size and staining with annexin V. Late apoptotic cells exhibited diminished diameter and were positive both for annexin V and 7-AAD staining, while necrotic cells were defined by size ranging between normal and increased and presence/emission of 7-AAD signal.

Reverse transcription-quantitative PCR. Total RNA Prep Plus Kit (A&A biotechnology, Gdynia, Poland) was used to isolate RNA from the cells. The expression level of each gene was analyzed with One-Step Real-Time PCR carried out using LightCycler 2.0 (Roche, Basel, CH). Path-ID Multiplex One-Step RT-PCR Kit (ThermoFisher Scientific, Waltham, MA, USA) and appropriate probes from Universal ProbeLibrary (Roche, Basel, CH) were applied to prepare samples. Each transcript level was normalized to *ACTIN BETA* (ACTB) mRNA. Table 1 shows the list of primer sequences, TaqMan probes and cycling conditions.

Protein analysis. Qualitative determination of ID1 level in parental and *ID1* suppressed cancer cells was performed using Western Blot. Protein samples were prepared as described previously (Maciejewska *et al*., 2014) and loaded on gradient (4–20%) SDS-PAGE gel.

Table I. List of primers, TaqMan probes and cycling conditions used for RT-PCR.

Gene*	Primers	TaqMan probe
ID1	CTGGACGAGCAGCAGGTAA CTCCAACTGAAGGTCCCTGA	Universal ProbeLibrary Probe # 6 (Roche)
FN1	TTGCTCTTTTCTAACCATTGTA-ATTCT TATTTCCCTTGCAGGCAATC	Universal ProbeLibrary Probe # 39 (Roche)
VIM	TGGTCTAACGGTTTCCCCTA GACCTCGGAGCGAGAGTG	Universal ProbeLibrary Probe # 56 (Roche)
CDH2	ACGCTCTCCCTCCCTGTT GGACTCGCACCAGGAGTAAT	Universal ProbeLibrary Probe # 17 (Roche)
CDH1	CAGGCTCAAGCTATCCTTGC AGTCATGCGTAGTGGTGCAT	Universal ProbeLibrary Probe # 33 (Roche)
CTNNB1	CCATTTTAAGCCTCTCGGTCT CAGACCTTCCTCCGTCTCC	Universal ProbeLibrary Probe # 74 (Roche)
DPYD	GGGATTGCAAAGCGAACTAC TCACAGCTCTCAAAGCAATAGG	Universal ProbeLibrary Probe # 25 (Roche)
TYMP	CATTCTCAGTAAGAAACTCGTGGA GGCCCCTCCGAACTTAAC	Universal ProbeLibrary Probe # 77 (Roche)
TK1	CAGCTTCTGCACACATGACC CGTCGATGCCTATGACAGC	Universal ProbeLibrary Probe # 79 (Roche)
TYMS	GGGCAGCCCTCTCCTTTA GCAGTTGGTCAACTCCCTGT	Universal ProbeLibrary Probe # 43 (Roche)
UMPS	AGGAAAGAAACAAAGGATTATG-GA TGGTGACAACATCTTCAATGATTA	Universal ProbeLibrary Probe # 2 (Roche)
UPP1	AGAAACTGAGCAAGGCCTGA CACAACAGGGGATTTTGGAC	Universal ProbeLibrary Probe # 62 (Roche)
UCK2	ATCCAGTGGTGCTTGGTTCT CCTAAACACTTGGTCCACACAC	Universal ProbeLibrary Probe # 4 (Roche)
ACTB	CAACCGCGAGAAGATGAC GTCCATCACGATGCCAGT	Universal ProbeLibrary Reference Gene Assay Roche, Human ACTB Gene Assay

Reverse transcription: 48°C (10 min), 95°C (10 min). Amplification: 95°C (10 s), 60°C (45 s). *The human genes are listed according toHUGO Gene Nomenclature Committee

After electrophoresis proteins were transferred to PVDF membranes by electro blotting and blocked by incubation in 3% bovine serum albumin. Afterwards, membranes were incubated overnight with goat ani-ID1 antibody (1:500 dilution) (R&D Systems, MN, USA) and subsequently washed and incubated with alkaline phosphatase-conjugated rabbit anti-goat IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Reference protein ACTIN BETA was detected with mouse anti-ACTB monoclonal antibody (Sigma-Aldrich, Poznan, Poland) and goat ALP-anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Quantitative determination of ID1 protein levels was carried out with ELISA test (#MBS911748) (MyBioSource, Inc., San Diego, CA, USA) according to manufacturer's instruction. Standards curves were obtained with online ElisaAnalysis Platform (elisakit.com, Scoresby, VIC, Australia).

Statistical analysis. GraphPad Prism 5.0 (La Jolla, CA, USA) software was used to generate nonlinear trend lines. In proliferation assays population DTs were calculated from growth curves according to exponential growth model. In 5-FU dose response assays concentration at which 50% reduction of cell viability occurred (IC_{50}) was determined from dose response curves with four-parameter dose response model. In both models differences between control and experimental group were determined with extra-sum-of squares F test. In other cases differences between groups were examined by Student's *t*-test. *P* values below 0.05 were considered significant.

RESULTS

The impact of *ID1* gene suppression on ID1 protein content in the cell

Transduction procedure of colon cancer cells resulted in a significant reduction of *ID1* expression (Fig. 1). The *ID1* transcript level in parental HT-29 cells was 0.262±0.011 compared to 0.005±0.001 observed in HT-29sh*ID1* cells (53-fold decrease). In HCT-116sh*ID1* cells the relative *ID1* transcript level was reduced 68 fold compared to parental HCT116 cells (from 0.674±0.025 to 0.010±0.001) (Fig. 1B). Western blot analysis showed that the ID1 protein content decreased in transduced HT-29 and HCT-116 cell lines (Fig. 1C). Quantitative analysis of ID1 protein in parental HT-29 and HCT-116 cell lines yielded 2.61±0.30 and 2.81±0.26 pg of ID1 protein/10^6 cells, respectively. In HT-29sh*ID1* and HCT116sh*ID1* cells the ID1 protein levels averaged 1.62±0.13 pg/10^6 cells and 1.03±0.10 pg/10^6 cells, respectively (Fig. 1D). The efficiency of *ID1* suppression both at mRNA and protein level was higher in HCT-116sh*ID1* cells and resulted in significantly lower amount of ID1 protein compared to HT-29sh*ID1*cells.

Altered expression of epithelial and mesenchymal markers in cells with silenced *ID1*

To examine the impact of *ID1* suppression on possible epithelial to mesenchymal transition (EMT) we analyzed expression of selected epithelial and mesenchymal markers. We observed that in cells with silenced *ID1* the expression of epithelial markers i.e. *E-cadherin* (*CDH1*) and *beta-catenin* (*CTNNB1*) was altered differently (Fig. 2). The transcript level of *CDH1* increased in both cell lines, however, the change was significant only in HCT-116sh*ID1* cells. *ID1* suppression resulted in significant elevation of *CTNNB1* transcript levels in both cell lines, although greater degree of change was observed in HT-29sh*ID1* compared to HCT-116sh*ID1* cells (6.5-fold *versus* 1.3-fold, respectively). We noted that suppression of *ID1* resulted in changes in expression of mesenchymal markers (*N-cadherin*, *vimentin* and *fibronectin*) in examined cells. The expression of *N-cadherin* (*CDH2*) decreased 4.9-fold, and increased 3.4-fold in HT-29sh*ID1* and HCT-116sh*ID1* cells, respectively (Fig. 2). The expression level of *vimentin* (*VIM*) changed differently in HT-29 and HCT-116 cells after *ID1* silencing. HT-29sh*ID1* cells showed 17-fold increase in *VIM* mRNA level compared to control cells, however, it should be pointed that the transcript level was very low before as well as after *ID1* knockdown ($1.0\times10^{-8}\pm10.0\times10^{-10}$ and $2.4\times10^{-7}\pm1.0\times10^{-8}$, respectively). Low *VIM* transcript level was also observed in parental HCT-116 ($7.0\times10^{-8}\pm2.0\times10^{-8}$), and silencing *ID1* expression resulted in a drop of this mRNA levels below the detection limit. The *fibronectin1* (*FN1*) expression was undetectable in both parental HT-29 and

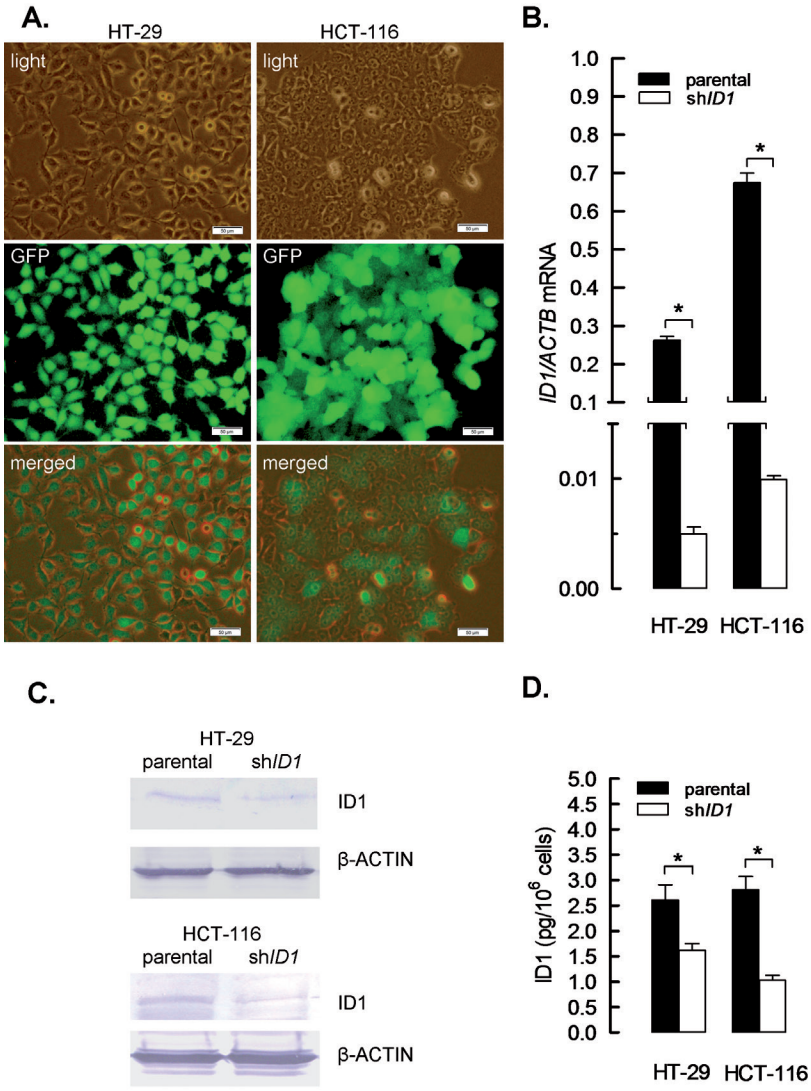


Figure 1. Efficiency of *ID1* suppression in HT-29 and HCT-116 cells.
Transduction with lentiviral pLL3.7 scrambled vector generated control (parental) cells while introduction of pLL3.7-sh*ID1* vector encoding the *ID1* silencing sequence (sh*ID1*) produced cells with suppressed *ID1* expression (HT-29sh*ID1* and HCT-116sh*ID1*). (**A**) Presentation of HT-29sh*ID1* and HCT-116sh*ID1* cells in normal light and under a fluorescent microscope (GFP). Efficient transduction resulted in GFP expression. Merged pictures are shown at the bottom. (**B**) Relative *ID1* transcript levels determined by quantitative PCR. Results were normalized to *ACTB* mRNA level and are presented as fold of change between parental and sh*ID1* cells. *Indicates significant difference compared to control cells ($P<0.05$). (**C**) Qualitative examination of protein content by Western Blot. ACTIN BETA was used as a reference protein. (**D**) An enzyme-linked immunosorbent assay (ELISA) was used to quantitatively determine ID1 protein level in control and pLL3.7-sh*ID1* transduced HT-29 and HCT-116 cells. *$P<0.05$ *vs* control.

HCT-116 cells, however, suppression of *ID1* resulted in a rise of this transcript level up to $1.3\times10^{-3}\pm1.6\times10^{-4}$ and $2.6\times10^{-3}\pm1.0\times10^{-4}$ in HT-29sh*ID1* and HCT-116sh*ID1* cell lines respectively (Fig. 2).

Suppression of *ID1* expression affects cell proliferation

In the next step of our investigation we examined the impact of *ID1* suppression on cells proliferation rate. Comparison of both parental cell lines showed that HT-29 cells exhibited higher proliferation rate (DT= 27 h; 95%CI: 21-24) compared to parental HCT-116 (DT=33 h; 95%CI: 29-40) (Fig. 3). Suppression of *ID1* enhanced proliferation of investigated cells, the doubling time dropped to 23 h (95%CI: 21-24) and 30 h (95%CI: 27-33) in HT-29sh*ID1* and HCT-116sh*ID1* cell lines respectively. HT-29 and HCT-116 cell lines, independently of *ID1* expression, showed rather similar progression of growth curves. Lag phase lasted approximately 48 h and afterwards cells approached phase of exponential growth. However, only HT-29 cells, both parental and transduced, reached the stationary step, which occurred after 120 h. FBS is a source of growth factors boosting cellular proliferation. To examine whether *ID1* suppression alters the proliferation rates of investigated cells irrespectively of exogenous growth stimuli we evaluated growth of cell cultures under serum-starvation conditions. In HT-29 line a peak of density occurred at 120th and 96th hour for parental and transduced cells respectively and was followed by constant decline. During the last three days of measurement parental HT-29 cells showed higher proliferation rate compared to HT-29sh*ID1* cells (Fig. 3). Parental HCT116 cells as well as HCT-116sh*ID1* cells were unable to grow without FBS supplementation. Nevertheless, none of the examined cell lines reached the exponential growth phase; thus, the DTs remained undetermined under serum-starvation conditions.

Effect of *ID1* suppression on the cells sensitivity to 5-fluorouracil

We examined whether suppression of *ID1* affected the colon cancer cell lines response to 5-FU. The cells underwent treatment with increasing concentrations of 5-FU ranging from 0.01 to 100 μM, and subsequently the concentration at which 50% of growth inhibition occurred (IC_{50}) was calculated. Parental and transduced HT-29 cells did not show significant differences in response to 5-FU and shared IC_{50} value was 6.2 μM (95%CI: 4.0–9.9) (Fig. 4). We observed that *ID1* expression level modulated the response of HCT-116 cell line to 5-FU. 50% growth inhibition of parental HCT-116 cells was achieved at concentration of 12.4 μM (95%CI: 7.5–20.6), whereas IC_{50} for HCT-116sh*ID1* averaged 1.5 μM (95%CI: 0.8–2.7) (Fig. 4).

Examination of HT-29 cell line by flow cytometry showed that under normal culture conditions 92.53±0.78% of parental HT-29 cells were viable while the majority of dead cells was at the stage of late apoptosis (Table 2). *ID1* suppression significantly increased proportions of late apoptotic and necrotic fractions resulting in diminished viability of HT-29sh*ID1* cells (76.40±5.00%). Viability of parental HCT-116

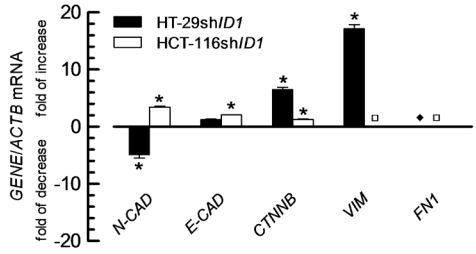
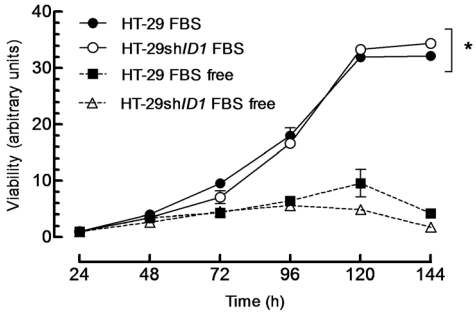


Figure 2. Impact of *ID1* suppression on the expression of epithelial and mesenchymal markers.
Determination (quantitative PCR) of relative transcript levels of the epithelial markers: *CDH1* (*E-cadherin*) and *CTNNB1* (*beta catenin*); and the mesenchymal markers: *FN1* (*fibronectin*), *VIM* (*vimentin*), *CDH2* (*N-cadherin*). Results were normalized to *ACTB* mRNA level and are presented as fold of change between transduced and parental cells. * indicates significant difference compared to control cells ($P<0.05$). (♦, □) Indicates that relative transcript level in parental or pLL3.7-sh*ID1* transduced cells was below the detection limit in HT-29 or HCT-116 lines respectively.



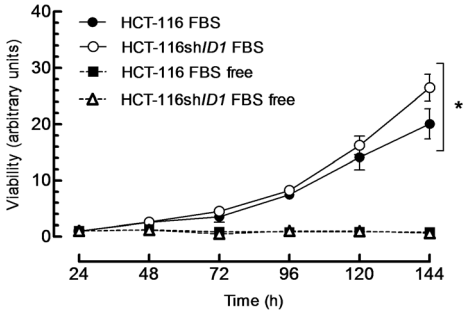
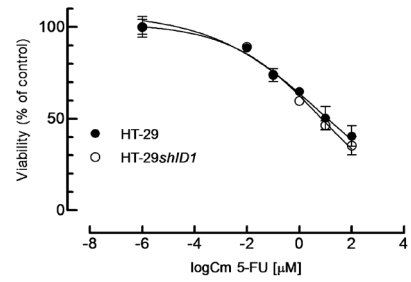


Figure 3. The effect of *ID1* suppression on the proliferation of colon cancer HT-29 and HCT-116 cells.
The cells were plated onto 24-well plates at a density of $1.5x10^4$/ml/well and were cultured in the presence or absence of 10% fetal bovine serum (FBS) for time indicated, afterwards the cell viability was determined by MTT test. *Indicates significant difference in population doubling time (DT) values between control and *shID1* cells ($P<0.05$).

cells cultivated under standard conditions remained at 86.97±0.64% which was significantly less in comparison with parental HT-29 cells (Table 2). Decreased content of ID1 in HCT-116sh*ID1* cells was associated with a decrease of cell viability (65.42±2.22%). In both parental and transduced HCT-116 cells the second largest fraction beside viable cells consisted of early apoptotic cells. In the parental HCT-116 cell line early apoptotic cells averaged 9.67±1.80% and rose to 29.09±5.10% in HCT-116sh*ID1* cells. Inclusion of 1 μM 5-FU to the cell culture resulted in a significant reduction of viable cells in each cell line and major enrichment was observed in



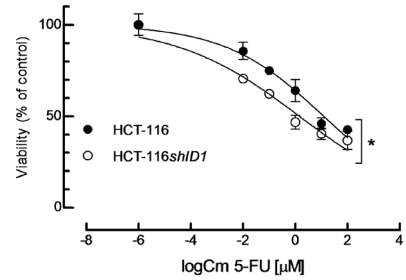


Figure 4. The effect of *ID1* suppression on the sensitivity of colon cancer HT-29 and HCT-116 cells to 5-fluorouracil (5-FU).
Parental and sh*ID1* cells were cultured for 48 hours with addition of 5-FU at concentrations indicated. The cells viability was determined with MTT test. Untreated cells were maintained both in normal medium and in DMEM supplemented with an equal amount of solvent (0.01% DMSO) used to dissolve 5-FU. The results are expressed as the percentage viability of 5-FU treated cells in comparison to untreated cells. *Indicates significant difference in 5-FU concentrations at which 50% reduction of cell viability occurred (IC_{50}) between control and sh*ID1* cells ($P<0.05$).

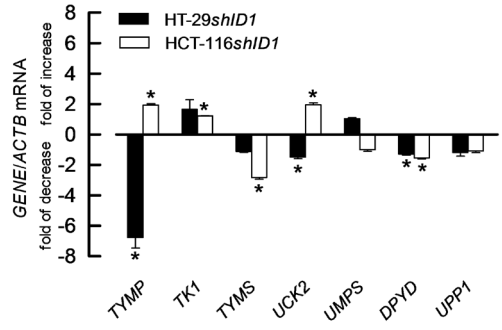


Figure 5. The Effect of *ID1* suppression on the expression levels of genes encoding enzymes metabolizing 5-FU.
Determination of relative expression levels of genes encoding dihydropyrimidine dehydrogenase (DPYD), thymidine phosphorylase (TYMP), thymidine kinase 1 (TK1), thymidylate synthase (TYMS), uridine monophosphate synthetase (UMPS), uridine-cytidine kinase 2 (UCK2), and uridine phosphorylase 1 (UPP1) was performed using quantitative PCR. Results were normalized to *ACTB* mRNA level and are presented as fold of change between sh*ID1* and parental cells. *Indicates significant difference compared to control cells ($P<0.05$).

fraction of early apoptotic cells. This was accompanied by a decreased fraction of late apoptotic and necrotic cells (Table 2).

Impact of *ID1* suppression on the expression level of genes encoding enzymes metabolizing 5-fluorouracil

Activity of 5-FU depends on its enzymatic conversion to metabolically active forms. Thus, we examined the expression of genes encoding major 5-FU metabolizing enzymes in order to assess possible impact of *ID1* sup-

Table 2. Impact of *ID1* suppression on 5-FU-induced cell death. *Indicates significant difference ($P<0.05$) between parental and transduced cells under particular conditions.

Environment	Cell Line	Viable cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)	Necrotic cells (%)
Control	HT-29	92.53±0.78%	0.52±0.11%	5.8±1.23%	1.14±0.57%
	HT-29 shID1	76.4±5.0%*	0.93±0.06%*	16.25±1.28%*	6.42±0.42%*
1 μM 5-FU	HT-29	71.66±3.19%	21.69±1.13%	6.58±1.24%	0.07±0.01%
	HT-29 shID1	57.85±2.42%*	28.46±1.87%*	12.57±0.99%*	1.12±0.07%*

Environment	Cell Line	Viable cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)	Necrotic cells (%)
Control	HCT 116	86.97±0.64%	9.67±1.80%	3.08±0.20%	0.38±0.19%
	HCT 116 shID1	65.42±2.22%*	29.09±5.10%*	4.99±0.62%	0.2±0.03%
1 μM 5-FU	HCT 116	74.86±1.15%	18.91±0.66%	6.68±0.41%	0.22±0.05%
	HCT 116 shID1	43.8±0.36%*	50.12±3.44%*	5.96±0.64%	0.11±0.01%

pression on biotransformation of the drug. We observed that the mRNA content of *thymidine phosphorylase* (*TYMP*) was significantly decreased (6.8-fold) in HT-29sh*ID1* cells compared to parental line (Fig. 5). Some decrease in the expression level of genes encoding uridine-cytydine kinase 2 (UCK2) and dihydropyrimidine dehydrogenase (DPYD) in HT-29sh*ID1* cells was also evident. Suppression of *ID1* in HT-29 cells did not affect the expression levels of genes encoding thymidine kinase 1 (TK1), thymidylate synthetase (TYMS), uridine monophosphate synthetase (UMPS), and uridine phosphorylase 1 (UPP1). The transcriptional response to *ID1* knock-down in HCT-116 cells was more variable compared to HT-29 line. HCT-116sh*ID1* cells showed significant increase in mRNA levels of *TYMP* (1.9-fold), *TK1* (1.2-fold) and *UCK2* (2.0-fold) compared to parental cells. On the other hand, decline in *ID1* expression significantly reduced transcript levels of *TYMS* (2.8-fold) and *DPYD* (1.5-fold). HCT-116sh*ID1* cells did not exhibit altered expression of *UMPS* and *UPP1* compared to parental line (Fig. 5).

DISCUSSION

Resistance of colon cancer cells to 5-FU chemotherapeutic is a major obstacle in treatment of people suffering from CRC. In order to include or exclude patients for particular therapeutic regimen predictive biomarkers are used for identification of responsive and nonresponsive individuals. Here, we showed that expression of ID1 belonging to HLH protein family impacts the sensitivity of colon cancer cell lines to 5-FU; thus, making it a candidate for further investigation on potential predictive value.

Knockdown of *ID1* resulted in altered expression of mesenchymal and epithelial markers, however, the pattern of changes did not resemble "classical" mesenchymal-epithelial transition (MET). Decreased level of ID1 in HT-29 cells led to a significant reduction of *CDH2* expression but did not affect transcription of *CDH1*. On the other hand, both genes were upregulated significantly after *ID1* suppression in HCT-116 cells. Those observations suggest that depletion of ID1 did not induce cadherin switch, a basic hallmark of MET (Thiery *et al.*, 2009). These results indicate that *ID1* expression alone is insufficient to determine an evident shift between the mesenchymal and epithelial phenotype. In number of research devoted to cancer cells direct or indirect suppression of *ID1* was shown to correlate with downregulation of EMT markers (Gumireddy *et al.*, 2009; Tobin *et al.*, 2011; Sánchez-Tilló *et al.*, 2014). It was recently revealed, however, that overexpressed ID1 exerts antagonistic effects (Stankic *et al.*, 2013). Upregulation of *ID1* generates breast cancer cells with properties of stem cells capable of invasion. It is established that stem cell-like characteristic and mesenchymal phenotype closely overlap with each other (Thiery *et al.*, 2009; Stankic *et al.*, 2013). On the other hand, ID1 promotes MET in metastatic breast cancer cells, which have previously undergone EMT (Stankic *et al.*, 2013). This dual mode of ID1 action depends on local expression of twist family bHLH transcription factor 1 (TWIST1) and snail family transcriptional repressor 1. Those transcriptional inducers and regulators of EMT differentially modulate ID1 function (Stankic *et al.*, 2013). Moreover, the present knowledge on the transition between epithelial and mesenchymal phenotypes suggests that it is not a binary process and many tumor cells acquire an intermediate E/M states rather than evident shifts between two phenotypes. These transitional forms are called hybrid epithelial/mesenchymal phenotype (Jolly *et al.*, 2015). It was also noted that stable lentiviral *ID1* overexpression producing functional protein in melanoma cells failed to affect transcription/translation of the ID1 target genes suggesting that *ID1* expression alone is insufficient to determine cell fate (Healey *et al.*, 2010). Thus, it may be assumed that specific response triggered by ID1 depends on molecular context in the cancer cell or its environment. Further investigations should be made in order to reveal the role of this protein in signaling cascades and loops governing epithelial-mesenchymal plasticity.

We showed that suppression of *ID1* resulted in an increased proliferation rate of both examined cell lines. Available data on the impact of ID1 on cancer cells proliferation varies depending on cancer type and experimental methodology. Stankic's work (Stankic *et al.*, 2013) reported that suppression of *ID1* in breast cancer cells cultivated in a complete medium did not affect proliferation rate. On the contrary, it was shown that *ID1* silencing in salivary gland cancer cells decreased proliferation rate irrespectively of FBS presence (Sumida *et al.*, 2013). In our experiments we utilized MTT method which measures metabolic activity in viable cells and under certain conditions is considered as valuable tool for assessment of proliferation (Berridge *et al.*, 1996). We also calculated

DT values of cells basing exclusively on the phases of exponential growth. Moreover, normalized data sets were used (at t_1 y=1) which allowed comparing only dynamics of growth between examined cell lines. Thus, the lack of universal approach for analysis of cellular proliferation leads to somewhat problematic comparison of different results. Under serum starvation condition *ID1* silencing did not affect the proliferation, moreover, parental HT-29 cells grew faster than transduced counterparts. FBS is the source of growth factors, including fibroblast growth factor (FGF) (Brunner *et al.*, 2010). In studies on calvarias development FGF2 was shown to promote *TWIST1* expression, whereas ID1 antagonized TWIST1 action through blocking dimerization of TWIST1 and transcription factor 3 (TCF3) (Rice *et al.*, 2000; Connerney *et al.*, 2008). T-TCF dimers promote more aggressive phenotype (Gajula *et al.*, 2015) and number of reports showed that TWIST1 enhances proliferation of cancer cells (Hasselblatt *et al.*, 2009; Qian *et al.*, 2013; Qiang *et al.*, 2014; Zhang *et al.*, 2015; Zhu *et al.*, 2015; Li & Wu, 2016). Thus, the observed increase of FBS-dependent cell growth after *ID1* silencing might be the result of enhanced formation of T-TCF heterodimers.

In our study we observed differential response of examined cell lines to 5-FU treatment. Silencing of *ID1* in HT-29 cells did not affect the sensitivity of these cells to 5-FU, whereas HCT-116 cells were significantly sensitized to 5-FU by suppression of *ID1*. It was recently shown that CRC cells acquire chemoresistance through leukemia inhibitory factor (LIF)-MDM2 axis (Yu *et al.*, 2014). LIF protein interacts with signal transducer and activator of transcription 3 and ID1 inducing *MDM2* expression. In turn, MDM2 protein promotes degradation of tumor protein p53 (TP53) leading to a generation of cell which is less prone to apoptosis in response to 5-FU (Yu *et al.*, 2014). HT-29 cell line already contains inactive TP53 protein, owing to mutated *TP53* gene, which may result in nonfunctional and degradation-resistant TP53 protein uncontrolled by LIF-ID1-MDM2 axis. Such a mechanism may explain why *ID1* suppression failed to enhance the sensitivity of HT-29 cells to 5-FU. Impaired *TP53* could also account for differences between HT-29 and HCT-116 cell lines observed in flow cytometric analysis. Under normal conditions as well as in the presence of 5-FU parental and transduced HT-29 cells exhibited higher viability and smaller apoptotic fractions compared to HCT-116 counterparts.

Cellular conversion of 5-FU may lead, through metabolic activation or degradation, to fluoro-β-alanine (F-BAL) (Longley *et al.*, 2003). DPYD catalyses transformation of 5-FU to 5-fluoro-5,6-dihydrouracil (5-FDHU) and this reaction is the primary rate-limiting step in degradation of 5-FU. Responsiveness of patients to 5-FU treatment was shown to depend on the drug catabolism (Etienne *et al.*, 1995). Level of *DPYD* expression is related to sensitivity to 5-FU. Recent study on hepatocellular carcinoma cell lines revealed that indirect modulation of *DPYD* transcription correlated with 5-FU-mediated decrease in cells proliferation (Oie *et al.*, 2007). Suppression of *DPYD* expression in HCT-116 cells produced stronger apoptotic effect of 5-FU (Offer *et al.*, 2014). In our experiment depletion of *ID1* resulted in a similar range of *DPYD* downregulation in both HT-29 and HCT-116 cells. Thus, it may be assumed that differences in the response to 5-FU of these cell lines do not depend on *DPYD* expression level.

In the cell, 5-FU activation proceeds either through synthesis of 5-fluorouridine-5'-monophosphate (FUMP) or generation of FdUMP (Longley *et al.*, 2003). Production of FUMP may be catalyzed in direct manner by UMPS or sequentially by transformation of 5-FU to 5-fluorouridine (FUR) via UPP and subsequent production of FUMP catalyzed by UCK. FdUMP, on the other hand, is produced through conversion of 5-FU to 5'-deoxy-5-fluorouridine (FdUR) catalyzed by TYMP and subsequent TK-dependant modification to FdUMP. Suppression of *ID1* in HT-29 cells resulted in decreased expression of *TYMP* and did not alter transcription of *TK1* and *TYMS*. HCT-116sh*ID1* cells, on the contrary, exhibited increased mRNA levels of *TYMP* and *TK1* concomitant with depletion of *TYMS* transcript. It may, therefore, be suspected that sensitization of HCT-116sh*ID1* cells to 5-FU might be due to enhanced FdUMP production. However, according to current data major pathway of 5-FU anabolism does not lead through generation of FdUMP but rather through production of FUMP (Peters *et al.*, 1986; Peters *et al.*, 1991). *ID1* suppression in both examined cell lines affected only the expression of *UCK2,* although differentially. *UCK2* was upregulated in HCT-116sh*ID1* which suggests that increased response to 5-FU in these cells is related, at least partially, with enhanced activation of drug through FUMP synthesis.

In conclusion, we showed here that the low level of ID1 in colon cancer cells may sensitize these cells to 5-FU cytotoxicity, although the ID1 effect is differentially modified by mutations present in cancer cells. We assume that the determination of *ID1* expression level together with other markers may help more precisely identify patients which may respond positively to 5-FU treatment.

Acknowledgements

This work was partially realized using the resources of the Cellular Function and Pathology Imaging Network of the Medical University of Gdańsk and the University of Gdańsk.

This work was supported by Medical University of Gdansk research grant MN 01-0231/08/249 to TP.

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