Experimental Cell Research xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Oxaliplatin and irinotecan induce heterogenous changes in the EMT markers of metastasizing colorectal carcinoma cells

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ARTICLE INFO

Abbreviations:
CRCColorectal cancer
ITIrinotecan
OPTOxaliplatin
EMTEpithelial-mesenchymal transition
ABCATP binding cassette
MRPMultidrug resistance-associated protein

Keywords:
Primary cell lines
Metastatic colon cancer
Oxaliplatin
Irinotecan
Drug resistance
Epithelial-mesenchymal transition

ABSTRACT

In patients with advanced colorectal cancer (CRC), surgery is complemented with systemic therapy – chemotherapy and radiochemotherapy. Although the patients' overall survival has been significantly improved, tumor resistance is still a frequent cause of chemotherapy failure. Several factors contribute to chemoresistance of tumor cells including changes related with epithelial-mesenchymal transition (EMT). The present study was designed to verify the presence of EMT markers in paired CRC primary cell lines obtained from primary tumor sites and lymph node metastases of three patients and to investigate the effect of irinotecan and oxaliplatin treatment on these markers as well. The samples of the higher stage of CRC and positive for angioinvasion were selected and qPCR, western blot analysis, migration assay, cytotoxicity testing was performed.

Results confirmed the increased expression of several markers characteristic of EMT and invasiveness in lymph node metastatic cells, with a significant variability between individual samples. Irinotecan and oxaliplatin decreased migration activity of the cells and to the varying degree affected the expression of EMT and invasiveness markers. In conclusion, in CRC EMT is present in metastatic cells over a phenotypic continuum whose expression is altered heterogeneously upon irinotecan and oxaliplatin treatment.

1. Introduction

Colorectal cancer (CRC) continues to be amongst the most frequently diagnosed malignant conditions in Western countries and although mortality from CRC is generally decreasing, it is still contributing to the significant morbidity and premature mortality in patients. Despite our advancing knowledge of pathological mechanisms contributing to the origin and development of CRC as well as improved diagnostics and treatment strategies (i.e. surgical resections alone or combined with systemic regimens – chemotherapy or radio-chemotherapy), up to 30% of patients present synchronous metastases and 50–60% will develop metastases that require aggressive chemotherapy. This may include use of both traditional as well as newer targeted drugs including but not limited to 5-fluorouracil 5-FU/LV, capecitabine, irinotecan (IT), oxaliplatin (OPT), bevacizumab, cetuximab, and panitumumab either in combination or as single agents [1].

The mechanisms underlying the appearance of malignant phenotypes characterized by aggressive biological behavior as well as the development of chemoresistance continue to be a target of intensive scientific investigation in many solid tumors including CRC [2,3].

Recently, investigations have demonstrated a convincing association between epithelial-mesenchymal transition (EMT) and CRC progression as well as therapeutic resistance [4]. In broader terms, EMT represents a cellular trans-differentiation continuum occurring under physiological (embryogenesis, tissue healing) and pathological conditions (endometrial adhesion) [5]. During EMT epithelial cells gradually lose their typical morphological features (polarity, membrane adhesion, cell-to-cell contacts) and develop mesenchymal spindle morphology. Thus transformed cells further show increased motility, matrix remodeling activity and invasiveness. These phenotypic changes are concomitant with the up-regulation of mesenchymal markers (i.e. N-cadherin or vimentin) and extracellular matrix components (i.e. specific collagens) as well as the down-regulation of epithelial cell surface markers and cytoskeleton components (i.e. E-cadherin, cytokeratins and others) [6]. Moreover, extensive changes in the expression and cellular localization of specific transcription factors (i.e. Snail, Slug,

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https://doi.org/10.1016/j.yexcr.2018.05.032
Received 24 January 2018; Received in revised form 25 May 2018; Accepted 26 May 2018
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ZEB1/2, Twist1/2) have now been well documented [7,8]. EMT is a critical early event involved in invasion and metastasis of CRC [9]. Reduced expression of E-cadherin was shown as a negative prognostic factor in several studies of colon adenocarcinoma [10–12]. More specifically, the relationship between the expression of cadherins and its clinical significance in CRC has been also described. The lower level of E-cadherin and higher level of N-cadherin in tumor tissue correlated significantly with local infiltration depth, tumor stage, vascular invasion, tumor grade and CA19-9 blood level [13]. Moreover, N-cadherin overexpression was associated with advanced TNM stage, lymph nodes metastasis and distant metastasis in CRC patients [14].

Chemoresistance of malignant tumor cells is a recognized fact, with multiple contributing factors that could be grouped into several classes traditionally viewed as instrinsic (drug-independent) and acquired (drug-dependent) [3]. Drug-dependent mechanisms comprise overexpression of ATP-binding cassette (ABC) transmembrane transporters such as P-glycoprotein, organic cation transporters 1,2,3, copper efflux transporters, P-type ATPases – ATP7A and ATP7B and multidrug resistance-associated protein (MRP), which actively transport chemotherapy agents out of the cell [15,16]. Several published studies further brought evidence of chemotherapy resistance in cancer cells with upregulated EMT markers in gastrointestinal malignancies [17,18]. In CRC cells, chronic OPT exposure resulted in resistant cells that displayed the phenotypic alterations associated with EMT such as loss of polarity, spindle shape, and increased mobility [18]. The OPT-resistant cells also demonstrated decreased E-Cadherin expression, as well as increased Snail and Vimentin expression, hallmark molecular changes associated with EMT [18]. Forced Snail expression in colon cancer cells enhanced the OPT resistance, thus demonstrating that EMT mediators are directly involved in therapeutic resistance [19].

The present study was designed to (1) verify the presence of EMT markers in paired CRC primary cell lines obtained from primary tumor site and lymph node metastases of three patients and to (2) investigate the effect of IT and OPT treatment on these markers. In addition, their comparison with well described CRC model, immortalized cell lines SW480 and SW620, was also evaluated.

2. Material and methods

2.1. Cell lines

Human epithelial colorectal adenocarcinoma line SW480 and SW620 were purchased from ATCC (LGC Standards, Poland). Cells were multiplied in three passages, frozen in aliquots and stored in liquid nitrogen. The absence of mycoplasma in all cell lines used in the laboratory was periodically checked. For every set of experiments (lasted 3–9 weeks) new storage cells were resuscitated. Both, SW480 and SW620 cells were cultured in DMEM supplemented with 10% FBS and 0.5% penicillin/streptomycin. Cells were grown in T-75 cm2 culture flasks in a humidified atmosphere containing 5% CO2 at 37 °C.

2.2. Clinical samples

All clinical samples were obtained from patients, who underwent surgery for colorectal carcinoma at Faculty Hospital in Hradec Králové . The study was approved by local ethics committee (Reference No. 201604S O3P – attached as a supplementary file) and patients gave their written consent. Subjects with different stage and grade of the disease were included into the study. The amount and quality of material sampled for cell line derivation varied based on the features of surgical specimen. 3 cases were chosen (samples No. 42, 44 and 54) for cultivation and analysis of both cell lines derived from primary tumor and lymph node metastasis. Cell isolation from tumor samples and further handling with cells were described previously [20].

2.3. Cytotoxicity assay

Cytotoxicity of chemotherapeutics (irinotecan, oxaliplatin) in colorectal carcinoma primary cells and immortilized cancer cells was evaluated by WST-1 which is a colorimetric assay based on the cleavage of the tetrazolium salt to colored formazan by mitochondrial dehydrogenases in viable cells. This assay quantifies cell proliferation and viability by measuring activity of mitochondrial enzymes. Colorectal carcinoma cells in 96-well microtiter plates were exposed to tested chemotherapeutics at various concentrations in RPMI for up to 48 h. At the end of each interval, cells were rinsed with PBS, 100 μl of WST-1 solution (1:20 final dilution) was added and cultures were further incubated for 2 h. The absorbance was recorded at 450 nm with 650 nm of reference wavelength by Tecan Infinite M200 spectrophotometer (Tecan, Switzerland).

2.4. Western blot analysis

Confluent cells were washed with PBS and harvested in ice-cold lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, β-glycerolphosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 200 μM sodium orthovanadate, 2 mM DTT). The lysates were resuspended and the amount of protein in supernatant was determined by BCA assay. The whole cell lysates were boiled for 5 min/95 °C in SDS sample buffer (Tris–HCl pH 6.8, 40% glycerol, 6% SDS, 0.2 M DTT, 0.1 g bromphenol blue) and thereafter 30 μg of sample were loaded onto SDS/polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane (100 V, 90 min) and incubated at 25 °C for 1.5 h with a solution containing 5% nonfat dry milk, 10 mM Tris–HCl (pH 8.0), 150 mM sodium chloride, and 0.1% Tween 20 (TBST). Membranes were incubated with primary antibody (polyclonal rabbit anti E-cadherin 1:2 000; polyclonal rabbit anti-N cadherin, 1: 1 500 – Cell signaling technology; polyclonal rabbit anti-vimentin, 1: 2 000; monoclonal mouse anti-MDR1, 1:2 000 – Cell signaling technology; monoclonal mouse anti- β actin, 1: 10,000 - Sigma Aldrich) at 4 °C overnight followed by six 5 min washes in TBST. Next, the membranes were incubated with secondary peroxidase-conjugated antibodies (1:20,000, 2 h, 25 °C), washed with TBST and the signal was developed with a chemiluminescence ECL Prime Western Blotting Detection Reagent (Amersham, GE Healthcare Life Science). Quantity of chemiluminescence was detected using Imaging System (Gel Logic 2200 Pro).

2.5. RNA extraction and cDNA synthesis

Total RNA was isolated from treated and untreated cells using TriReagent (Sigma Aldrich, Czech Republic) according to manufacturer's instructions (Biotech, Czech Republic). RNA yields and purity were determined measuring the absorbance at 260 and 280 nm using NanoDrop ND-1000 UV–Vis Spectrophotometer (Thermo Scientific, Czech Republic). All samples had absorption ratio A260/A280 greater than 1.8. The quality of RNA was checked by agarose gel electrophoresis. First strand cDNA was synthesized from 1 μg total RNA ProtoScript II reverse transcriptase (NEB, USA) and random hexamers following the manufacturer protocol. After initial heat denaturation of 1 μg of total RNA (65 °C for 5 min), the reactions (20 μl) were incubated for 10 min at 25 °C, for 50 min at 42 °C and for 5 min at 80 °C. Obtained cDNA was diluted (5×) prior to qPCR. For the cDNA synthesis of miRs the reaction mixture included mix of Stem-Loop Oligos specific for each miR tested. First strand synthesis was carried out using ProtoScript II reverse transcriptase. After initial heat denaturation of total RNA (65 °C for 5 min), the reactions (10 μl) were incubated for 30 min at 16 °C, for 30 min at 42 °C and for 5 min at 80 °C. Obtained cDNA was diluted (50×) prior to qPCR. All cDNAs were stored at −20 °C until qPCR assay.

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