Cellular Signalling 26 (2014) 868–879

Contents lists available at ScienceDirect

Cellular Signalling

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

Ezrin expression and cell survival regulation in colorectal cancer

Premila D. Leiphrakpam [a,1], Ashwani Rajput [b,1], Michelle Mathiesen [a], Ekta Agarwal [a], Audrey J. Lazenby [c], Chandrakanth Are [d], Michael G. Brattain [a,*], Sanjib Chowdhury [a,**]

[a] Fred & Pamela Buffett Cancer Center, University of Nebraska Medical Center, 985950 Nebraska Medical Center, Omaha, NE 68198-5950, United States
[b] Department of Surgery, University of New Mexico Health Science Center, 1 University of New Mexico, Albuquerque, NM 87131-0001, United States
[c] Department of Pathology and Microbiology, University of Nebraska Medical Center, 983515 Nebraska Medical Center, Omaha, NE 68198-3135, United States
[d] Department of Surgical Oncology, University of Nebraska Medical Center, 984533 Nebraska Medical Center, Omaha, NE 68198-4533, United States

ARTICLE INFO

Article history:
Received 22 December 2013
Received in revised form 9 January 2014
Accepted 13 January 2014
Available online 22 January 2014

Keywords:
Colorectal cancer metastasis
Cell survival signaling
2D-DIGE proteomics
Ezrin
IGF1R signaling
XIAP and survivin

ABSTRACT

Colorectal cancer (CRC) is the second largest cause of cancer deaths in the United States. A key barrier that prevents better outcomes for this type of cancer as well as other solid tumors is the lack of effective therapies against the metastatic disease. Thus there is an urgent need to fill this gap in cancer therapy. We utilized a 2D-DIGE proteomics approach to identify and characterize proteins that are differentially regulated between primary colon tumor and liver metastatic deposits of the IGF1R-dependent GEO human CRC xenograft, orthotopically implanted in athymic nude mice that may serve as potential therapeutic targets against CRC metastasis. We observed increased expression of ezrin in liver metastasis in comparison to the primary colonic tumor. Increased ezrin expression was further confirmed by western blot and microarray analyses. Ezrin, a cytoskeletal protein belonging to Ezrin–Radixin–Moesin (ERM) family plays important roles in cell motility, invasion and metastasis. However, its exact function in colorectal cancer is not well characterized. Establishment of advanced GEO cell lines with enhanced liver-metastasizing ability showed a significant increase in ezrin expression in liver metastasis. Increased phosphorylation of ezrin at the T567 site (termed here as p-ezrin T567) was observed in liver metastasis. IHC studies of human CRC patient specimens showed an increased expression of p-ezrin T567 in liver metastasis compared to the primary tumors of the same patient. Ezrin modulation by siRNA, inhibitors and T567A/D point mutations significantly downregulated inhibitors of apoptosis (IAP) proteins XIAP and survivin that have been linked to increased aberrant cell survival and metastasis and increased cell death. Inhibition of the IGF1R signaling pathway by humanized recombinant IGF1R monoclonal antibody MK-0646 in athymic mouse subcutaneous xenografts resulted in inhibition of p-ezrin T567 indicating ezrin signaling is downstream of the IGF1R signaling pathway. We identified increased expression of p-ezrin T567 in CRC liver metastasis in both orthotopically implanted GEO tumors as well as human patient specimens. We report for the first time that p-ezrin T567 is downstream of the IGF1R signaling and demonstrate that ezrin regulates cell survival through survivin/XIAP modulation.

1. Introduction

Metastasis to distant organs is mainly responsible for the cancer-related death in solid tumors including colorectal cancer (termed as CRC) [1,2]. This is due to the lack of effective therapies against the disseminated disease. Thus, there is an urgent need to fill this gap in cancer therapy. Therefore, understanding the mechanisms associated with metastatic progression is critical for developing anti-metastatic therapies. Various "omics" based analyses have emerged as key players for the identification of potential targets for the development of new therapies against cancers [3–6]. In the postgenome era, proteomics has developed as a powerful tool for the characterization of normal and abnormal cellular functions associated with malignant transformation, for the discovery of critical disease-specific targets, and identification of novel endpoints for the study of chemotherapeutic agents and its associated toxicity [6]. The study of the proteome which is the functional translation of the genome is directly linked to specific phenotypes [7].

The proteomic background of CRC has been extensively characterized under different conditions using different proteomic approaches (including gel-based separation methods, mass spectrometry and array-based methods) to identify several proteins involved in the initiation and progression of CRC [7]. Additionally, proteomic studies have helped provide significant insight into the understanding of cellular functions associated with normal and diseased conditions [5–8]. Ying-Taoa et al. (2005) utilized a two dimensional electrophoresis (2-DE) method to compare metastatic LS174T and non-metastatic SW480 CRC cell lines to explore their differences in protein expression that might be linked to CRC metastasis. The proteins, platelet-derived

* Corresponding author. Tel.: +1 402 559 4902; fax: +1 402 559 4651.
** Corresponding author. Tel.: +1 402 559 5570; fax: +1 402 559 4651.
E-mail addresses: mbrattain@unmc.edu (M.G. Brattain), schowdhury@unmc.edu (S. Chowdhury).
[1] These authors contributed equally.

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http://dx.doi.org/10.1016/j.cellsig.2014.01.014

endothelial cell growth factor (PDECGF), septin and cell division cycle 2 (cdc2) were observed to be highly expressed in the metastatic cell lines [9]. Shi-Yong Li et al. (2010) used a 2D-DIGE (2-dimensional difference in-gel electrophoresis) approach to investigate the differential expression of proteins in human CRC patients with liver metastasis to evaluate its clinical diagnostic potential [10]. They compared protein expression between normal mucosa, primary colonic carcinoma and liver metastasis specimens. It was reported that zinc finger protein 64 homolog (Zfp64), guanine nucleotide exchange factor 4 (GEF4), human arginase, glutathione S-transferases (GSTs) A3, and tumor necrosis factor α (TNF-α)-induced protein 9 are upregulated in human CRC liver metastasis [10]. Recently, Sugihara et al. (2012) identified APC-binding protein EB1 as a candidate of novel tissue biomarker and therapeutic target for colorectal cancer using 2D-DIGE/MS (mass spectrometry) approach [7].

In this study, 2D-DIGE/MS method was used to identify potential molecular targets associated with liver metastasis originating from primary colon carcinoma generated by the orthotopic mouse metastasis model developed in our laboratory [11–19]. Highly metastatic IGF1R-dependent GEO human CRC xenograft tumors were implanted orthotopically in the colon of athymic nude mice to generate spontaneous liver metastasis [14,20]. We compared the global protein expression of primary colon carcinoma and liver metastases of GEO cells and identified an increased expression of ezrin and its phosphorylation at T567 (termed here as p-ezrin T567) in liver metastasis compared to the primary tumor. Previous studies have shown that ezrin plays a critical role in tumorigenesis and has been implicated in metastasis of several types of cancers including CRC [21–25]. We performed *in vitro* characterization of ezrin in CRC cells and identified IGF1R-p-ezrin T567-XIAP axis as a potential cell survival pathway target in IGF1R-dependent subsets of CRC cells. Ezrin and p-ezrin T567 appear to have a key role in the regulation of IGF1R-dependent CRC cell survival properties that may ultimately be utilized for the identification of novel anti-metastatic therapies.

2. Material and methods

2.1. Cell lines

GEO, GEORI, CBS, HCT166 and HCT166b CRC cell lines were originally developed from primary CRC tumors and have been extensively characterized in our laboratory [26–29]. Cells were maintained at 37 °C in humidified atmosphere in a chemically defined serum free medium consisting of McCoy's 5A medium (Sigma-Aldrich) supplemented with amino acids, pyruvate, vitamins, antibiotics, and growth factors transferrin (4 μg/mL; Sigma-Aldrich), insulin (20 μg/mL; Sigma-Aldrich), and EGF (10 ng/mL; R&D Systems). Supplemented McCoy's medium ("SM") is McCoy's 5A medium supplemented with antibiotics and nutrients but lacking any growth factors.

2.2. Antibodies

The following primary antibodies were obtained from Cell Signaling Biotechnology (Danvers, MA): IGF1Rβ (catalog#3027), p-IGF1Rβ(Y1135) (catalog#3918), p-ezrin/ERM(T567) (catalog#3149). The following primary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX): Ezrin (catalog#sc-71082) and survivin (catalog#sc-17779). XIAP (catalog#ab28151) was obtained from abcam (Cambridge, MA). β-Actin (catalog#A2066) and GAPDH (catalog#G8795) were obtained from Sigma-Aldrich (St. Louis, MO).

2.3. Pharmacological antagonists

Recombinant humanized IGF1R monoclonal antibody MK-0646 was provided by Merck Oncology and small molecule tyrosine kinase inhibitor OSI-906 was obtained from Chemietek. The ezrin inhibitors NSC668394 and NSC305787 were kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatments and Diagnosis, National Cancer Institute.

2.4. Orthotopic implantation

All the experiments involving animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. The orthotopic implantation methodology has been described in detail in previous studies from Brattain laboratory [11–14,16–19]. Briefly, GEO and CBS cells were transfected with green fluorescence protein (GFP). Exponentially growing GFP-labeled GEO and CBS cells (approximately 7 million cells/mL Serum Free media) were inoculated subcutaneously onto the dorsal surfaces of athymic nude male mice. Once xenografts were established, approximately 500 mm^3 in size, they were excised and minced into 1–2 mm^3 pieces. Two of these pieces were then orthotopically implanted into other athymic nude mice. Approximately 60 days post-implantation, animals were euthanized. Organs were explanted, imaged, washed in chilled 1X PBS and immediately placed in liquid nitrogen and 10% formalin respectively. 15–30 mg of primary and liver metastasis tissues obtained after orthotopic implantation of GEO cells was shipped in dry ice to Applied Biomics, Inc. for proteomic analysis.

2.5. Enhancement of CRC liver-metastatic ability by serial passaging

Previous studies have shown that training of CRC cell lines to be more aggressive through serial passaging of vertical selection of metastatic cells to initiate primary tumors leads to enhancement of metastatic capability [30,31]. In our study, an orthotopic implantation procedure was performed using GEO CRC cells and the metastatic liver tissues were harvested. The tissues were homogenized and the metastatic cells were grown in tissue culture. This was followed by the subcutaneous dorsal injection of the metastatic cells to another nude mouse which was subsequently orthotopically implanted to initiate primary colon tumors. The whole procedure was repeated for 6 cycles of vertical selection of IGF1R dependent GEO liver metastases to initiate primary tumors, and thus the highly aggressive cell lines after 6th passage termed as GEO Met6 was obtained.

2.6. Two-dimensional differential in-gel electrophoresis (2D-DIGE) and mass spectrometry

The 2D-DIGE and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI/TOF MS) was performed by Applied Biomics, Inc. (Hayward, CA) [32,33].

2.7. Microarray analysis

Microarray analysis was performed on the orthotopically implanted GEO CRC primary tumor and liver metastasis using the Illumina platform.

2.8. Tissue and cell lysate preparation

Orthotopically implanted primary tumor and liver metastasis tissues were harvested and snap frozen in liquid nitrogen and stored in −80 °C. Tumor tissues were first washed in cold 5% PBS and collected in lysis buffer [50 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 1% NP40, 2 mmol/L EDTA, 0.1% SDS, 50 mmol/L NaF, 10 mmol/L Na VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/mL β-glycerophosphate, and one protease inhibitor cocktail tablet from Roche]. The CRC cells were washed in cold 5% PBS and collected in lysis buffer. Crude tissue lysates were homogenized using a homogenizer to shear DNA and lysed for 30 min on ice at 4 °C. The crude cell lysates were homogenized using a 21 gauge needle to shear DNA and lysed for 30 min on ice at 4 °C.