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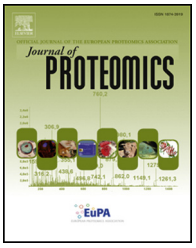

Identification of anti-metastatic drug and natural compound targets in isogenic colorectal cancer cells

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ABSTRACT

Therapeutic strategies for cancer treatment often remain challenging due to the cumulative risk derived from metastasis, which has been described as an aggressive state of cancer cell proliferation often resulting in failure of clinical therapy. In the current study, anti-metastatic properties of three chemotherapeutic drugs and three compounds from natural sources were investigated by comparative proteomic analysis. Proteomic profile comparison of the isogenic primary and metastatic colon cancer cell lines SW480 and SW620 identified two potential metastasis related molecular targets: fatty acid synthase and histone H4. To demonstrate their biological roles in cancer metastasis, the expression of these target genes was suppressed by siRNA transfection. Subsequent cell migration assays demonstrated reduced migratory effects. SW620 cells were treated with six anti-cancerous components. Through comprehensive proteomic analysis, three of the tested compounds, oxaliplatin, ginsenoside 20(S)-Rg_3 and curcumin, were revealed to have a suppressive effect on FASN and histone H4 expression. SW620 cells treated with these drugs showed significantly reduced migratory activity, which suggests that drug-induced targeted suppression of these genes may affect cell migration. The validity of the proteomic datasets was verified by knowledgebase pathway analysis and immunoblotting assays. The anti-metastatic components revealed by the current proteomic analysis represent promising chemotherapeutic candidates for the treatment of colorectal adenocarcinoma.

Biological significance
The current study demonstrates anti-metastatic activity of chemotherapeutics and natural components by the suppression of target molecules, fatty acid synthase and histone H4 identified by a comparative proteomic analysis employing the isogenic primary and metastatic colon cancer cell lines, SW480 and SW620. Three tested drugs, namely, oxaliplatin, ginsenoside 20(S)-Rg_3 and curcumin were revealed to possess suppressive effects on fatty acid synthase and histone H4 and reduce metastasis as determined by cell migration assay. Data were confirmed by the correlation between spectral counts from proteomic data and Western blot analysis, which were in good agreement with immunohistochemistry.

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1. Introduction

Cancer metastasis is considered as a key barrier for clinical treatment that makes therapeutic strategies complicated and leads to poor clinical outcomes due to its unpredictable and aggressive nature [1]. It is also widely recognized that metastasis is frequently accompanied by decreased responsiveness to chemotherapeutics and radiotherapy, which limits cancer treatment options [2,3].

Metastasis is a multistep process including the loss of cell-to-cell adhesion, which promotes cell motility and migration/invasion into surrounding tissues as well as transport through the blood stream [4]. Changes in expression of the proteins cytokeratin and integrin are commonly known to contribute to the metastatic process [5,6]. Since metastasis progression is related to both cell motility and morphology, comparative proteomic analysis of primary versus metastatic cancer cells often reveals a list of proteins involved in the maintenance of cellular structure. However, significant changes in specific proteins may vary depending on cancer type [7–10].

The combination of SW480 and SW620 has been recognized as an effective in vitro model since these lines are isogenic colorectal cancer (CRC) cells with different metastatic potentials. They have been utilized for a number of comparative studies to identify and investigate molecular targets involved in metastasis [11]. One recent study on the key molecules involved in CRC metastasis reported that significant changes in the expression of actin filaments, intermediate filaments and microtubule associated proteins were observed by iTRAQ quantitative proteomic profiling of SW480 and SW620, primary and metastatic cells [12]. Those proteins were described as products of changes in the processes of cellular adhesion, assembly and organization.

In addition, 5-fluorouracil (5-FU) and oxaliplatin are currently used for the treatment of CRC. However, metastatic progression carries resistance against anticancer drug treatments. SW620 has been reported to show lower responsiveness to 5-FU treatment compared to SW480 [13].

In this study, comparative biological and proteomic research profiling SW480 and SW620 cells provided several putative molecular targets. Among the reported molecules, fatty acid synthase has been previously shown to be overexpressed in various human cancer cells and to be involved in cancer metastasis, thus representing a promising molecular target for the treatment of cancer [14–16].

Our preliminary study on the proteomic differences between SW480 and SW620 demonstrated highly elevated fatty acid synthase expression, including chromatin components which may play pivotal roles in cellular membrane formation and cell proliferation. In the current study, the potential role in metastatic progression of top-ranked proteins with overexpression in SW620 was investigated. Target genes were suppressed by silencer RNA transfection of SW620 cells, which were then subjected to cell migration assays to demonstrate that target genes play an important role in CRC metastasis. Subsequently, six chemical agents, including three chemotherapeutics and three natural components known to possess anticancer activity, were used to treat SW620 cells to evaluate anti-migratory activity and perform comprehensive proteomic profiling.

2. Material and methods

2.1. Chemicals and reagents

The mobile phase system for LC/MS analysis consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (ACN) purchased from EMD (Gibbstown, NJ, USA). SDS-PAGE gels (10% Bis–Tris NuPAGE) for protein separation were obtained from Life Technologies (Gaithersburg, MD, USA) and sequencing grade modified trypsin was from Promega (Madison, WI, USA). Complete™ protease inhibitor cocktail tablets were obtained from Roche (Mannheim, Germany). Oxaliplatin, 5-FU and sorafenib were from Selleckchem (Houston, TX, USA) and curcumin and luteolin were from Sigma (St. Louis, MO, USA). Ginsenoside 20(S)-Rg_3 was isolated from steamed *Panax ginseng* root by the method reported previously [17]. Briefly, methanolic extract of steamed and dried root of *P. ginseng* was provided by the reflux extraction with methanol, which was then evaporated for dryness. After resuspension of dried residue with water, ginsenoside rich fraction was afforded by the liquid–liquid extraction with n-butanol (Supplementary Fig. 1A). A portion of evaporated n-butanolic fraction was subjected to silica-gel column chromatography eluting with a chloroform/methanol stepwise gradient (40:1 → 10:1) yielding 10 subfractions (Supplementary Fig. 1B). Among them, the most ginsenoside 20(S)-Rg_3 abundant subfraction was applied to reversed phase semi-preparative HPLC consisted of two L-7100 pumps coupled with L-4000 UV detector (Hitachi, Japan) repetitively for isolation and purification. The purity as assessed by reversed phase HPLC coupled with SEDEX evaporative light scattering detector (Sedere, France) was >95.0%, and its chemical structure was elucidated by the direct comparison of its spectral data from fast-atom bombardment (FAB) MS and nuclear magnetic resonance (NMR) analysis with those of authentic compound isolated previously by our group (Supplementary Fig. 1C–D).

Ammonium bicarbonate, ammonium acetate, DTT, iodoacetamide, Tris–HCl, bromophenol blue, β-mercaptoethanol, Tween 20, formic acid and SDS were obtained from Sigma (St. Louis, MO, USA). Glycerol was from Life Technologies (Gaithersburg, MD, USA). All buffers and solutions were prepared using deionized water by Milli-Q, Millipore (Bedford, MA, USA). Antibodies against human fatty acid synthase, histone H1, plectin-1, heat shock protein 60 kDa, β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and AHNAK were purchased from Santa Cruz (Dallas, TX, USA). Human histone H4 antibody was from Abcam (Cambridge, MA, USA) and human profilin-1 antibody was from OriGene Technologies (Rockville, MD, USA). The antibody against human heat shock protein 90 kDa was from BD Biosciences (Franklin Lakes, NJ, USA). Proteins were visualized using Amersham ECL Prime Western blotting detection reagent from GE Healthcare (Fairfield, CT, USA). For immunohistochemistry staining, cells were fixed onto 12 mm poly-lysine coated round coverslip obtained from BD Biosciences (Franklin Lakes, NJ, USA), and stained using R.T.U. Vectastain® Universal Elite® ABC Kit, ImmPACT™ DAB peroxidase substrate and BLOXALL blocking solution purchased from Vector Laboratories (Burlingame, CA, USA). For further validation, immunohistochemical staining was performed using colorectal cancer tissue microarray slides purchased from OriGene Technologies, Inc.

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