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(R)-Rg3 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(R)-Rg3 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

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 Fromega (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)-Rg1 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-butanol 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)-Rg1 abundant subfraction was applied to reversed phase semi-preparative HPLC consisting 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® 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.
2.2. Cell culture and drug treatment

SW480 and SW620 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in a Dulbecco’s modified eagle medium (DMEM, Gibco BRL Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 50 units/mL of penicillin G and 50 μg/mL of streptomycin which were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Cells were maintained at 37 °C under humidified 95% air and 5% CO2 and grown to confluence in culture dishes (150 mm radius) over 2 or 3 days and then trypsinized and used for the experiments. Stock solutions of tested drugs were diluted to the desired concentration and were treated in the presence of DMEM with reduced serum (0.5% FBS).

2.3. Proteomic sample preparation

The FBS-washed pellet of trypsinized cells was lysed with radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1.0% (v/v) Triton X-100, 0.5% (w/v) deoxycholate and 1x protease inhibitor. 50 μg of denatured protein with sample buffer containing 300 mM Tris–HCl, 0.01% (w/v) bromophenol blue, 15% (v/v) glycerol, 6% (w/v) SDS and 1% (v/v) β-mercaptoethanol were separated on 10% Bis–Tris NuPAGE gels. Gels were stained using 0.04% (w/v) Coomassie brilliant blue G in 3.5% (v/v) perchloric acid for 15 min and then destained using deionized water with several changes overnight. Each gel lane was cut into 20 slices, which were chopped into small pieces. Gel pieces were destained with 50% (v/v) acetonitrile (ACN) containing 25 mM ammonium bicarbonate several times, and then were dehydrated in 100% ACN. After being dried under vacuum and then were suspended in 5% (v/v) ACN containing 5% (v/v) formic acid and incubated at room temperature for 30 min 3 times. The extracts were dried under vacuum and then were suspended in 5% (v/v) ACN containing 3% (v/v) formic acid to be subjected to LC-MS/MS.

2.4. Nano-LC and mass spectrometry analysis

The LC-MS/MS system used consisted of an LTQ/Orbitrap-XL mass spectrometer (Thermo Scientific, Rockford, IL, USA) equipped with a NanoAcquity UPLC system (Waters, Milford, MA, USA). Peptides were separated on a reversed phase analytical column (NanoAcquity BEH C18, 1.7 µm, 150 mm, Waters, Milford, MA, USA) combined with trap column (NanoAcquity, Waters, Milford, MA, USA). Good chromatographic separation was observed with a 75 min linear gradient consisting of mobile phases solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in ACN) where the gradient was from 5% B at 0 min to 40% B at 65 min. MS spectra were acquired by data dependent scans consisting of MS/MS scans of the eight most intense ions from the full MS scan with dynamic exclusion of 30 s.

2.5. Database search and data compiling

The Human International Protein Index (IPI) v3.72 FASTA database (86,392 entries) was used within the Bioworks software v.3.3.1 sp1 applying the SEQUEST search algorithm (SRF v.5). Search parameters were as follows: parent mass tolerance of 10 ppm, fragment mass tolerance of 0.5 Da (monoisotopic), variable modification on methionine of 16 Da (oxidation) and maximum missed cleavage of 2 sites assuming the digestion enzyme trypsin. Search results were compiled using Scaffold software v4.0.5 (Proteome Software, Portland, OR, USA) which provided spectral counts for data comparison under the filter criteria of 2 peptides minimum: XCorr scores of greater than 1.9, 2.3, and 3.4 for singly, doubly and triply charged peptides, deltaCn scores of greater than 0.10. Spectral counts from duplicate analysis were compared using the Power Law Global Error Model (PLGEM) in order to identify the significance of the protein changes [18].

2.6. Western blot analysis

25 μg of denatured samples was loaded onto a Bolt™ 4–12% Bis–Tris Plus gel (Invitrogen, Carlsbad, CA, USA) and separated at 165 V for 30 min at room temperature. Proteins were transferred to nitrocellulose membrane at 20 V at room temperature for 1 h using the Bolt™ Mini Blot Module (Invitrogen, Carlsbad, CA). Transfer efficiency was confirmed by Ponceau S staining of the membrane. After blocking with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 1 h at room temperature, membranes were incubated in primary antibody at 1:500–1:2000 dilution in blocking buffer overnight at 4 °C. Blots were washed for 60 min with 3 changes of TBST and then incubated for 1 h at room temperature in the appropriate HRP conjugated secondary antibody diluted 1:10,000 in blocking buffer. Additional washes for 60 min total, with 3 changes of TBST, were followed by chemiluminescent imaging using ECL Prime substrate and detection using the UVP Biospectrum™ 500 Imaging System (Upland, CA, USA).

2.7. Gene knockdown using siRNA transfection

Monolayer SW620 cells were trypsinized and suspended in the culture medium. Then, 1 mL of a single cell suspension containing 1 × 10^5 cells/mL was seeded into each well of a 12-well culture plate. siRNA complexes containing either fatty acid synthase or histone H4 oligonucleotides combined with RNAiMax transfection reagent were added at 5 pmol per well (Invitrogen, Carlsbad, CA). Control samples included transfection reagent alone or no treatment at all. After incubation for 72 h, cells were trypsinized, then were subjected to the cell migration assay.

2.8. Cell migration assay

Cell migration assays were performed to evaluate anti-metastatic activity of the drugs tested. QCM™ Chemotaxis 96-well Cell Migration Assay Kit (Millipore, Bedford, MA, USA) was used for the assay by the manufacturer's protocol. In brief, the cell pellets from the trypsin detachment of cell monolayers were resuspended with serum free DMEM with/without the test
drugs at a concentration of $1.0 \times 10^6$ cells/mL. 100 μL of cell suspensions was transferred to each well of a 96-well plate and absorbance was measured on a Synergy FL microplate reader with 480/520 nm filter set (BioTek, Winooski, VT, USA). The assay was performed in triplicate for each treatment group.

### 2.9. Immunohistochemistry staining of cells and tissues

For immunocytochemistry staining, cells were seeded onto poly-lysine coverslips and incubated for 24 h prior to drug treatment. After 48 h further incubation with or without drugs, cells were prepared from 4% paraformaldehyde fixation for staining. The rest of the procedure was performed using R.T.U. Vectastain ABC Kit by the manufacturer’s protocol. Briefly, after blocking of endogenous peroxidase with BLOXALL blocking solution, coverslips were incubated with primary antibody at the dilution of 1:100 overnight. Secondary incubation was followed using biotinylated secondary antibody for 30 min and then coverslips were incubated with R.T.U. Vectastain ABC reagent for 30 min. After developing with ImmPACT™ DAB, coverslips were rinsed with water, then were dehydrated, cleared and mounted with resin matrix.

For immunohistochemistry, colon cancer tissue microarray slides were stained. Slides were prepared by de-paraffinization with xylene and hydration using alcohol and deionized water. The rest of the procedure was performed using the Autostainer Plus. After blocking of endogenous peroxidase with 3% (v/v) hydrogen peroxide, tissue slides were incubated with primary antibody at the dilution of 1:100. Secondary antibody was followed by peroxidase-conjugated streptavidin for 10 min and 3'-diaminobenzidine for 5 min. Tissue slides were rinsed with water and counterstained with hematoxylin, dehydrated, cleared and mounted with resin matrix. Cells and tissue slides were visualized using an Olympus BX51 microscope equipped with an Olympus DP70 camera and DP controller imaging software (Olympus Corporation, Tokyo, Japan).

### 3. Results and discussion

#### 3.1. Metastasis biomarker candidates

Metastatic cancer biomarkers were characterized by the comparative analysis of two isogenic colorectal adenocarcinoma cell lines. SW480 and SW620 colon carcinoma cell lines were derived from primary and secondary tumors from a single patient. In the current study, the proteomic profile of SW620 was compared to that of SW480 by proteomic analysis and knowledgebase pathway analysis to identify potential markers which affect cancer metastasis. Subsequently, changes in expression of biomarker candidates after treatment with six chemotherapeutics or anti-tumor natural compounds were observed (Fig. 1). Three chemotherapeutics, namely oxaliplatin, sorafenib and 5-FU were treated at concentrations of 10 μM, 0.15 μM and 10 μM, and herbal dietary components, namely ginsenoside R0(S)-Rg3, curcumin and luteolin were also treated at 10 μM, 20 μM and 50 μM respectively based on cytotoxicity assay (MTT assay) results (Supplementary Fig. 2).

A total of 3634 proteins were identified from the duplicated runs of comparative SW620 and SW480 samples. PLGEM analysis of the data provided a list of proteins significantly overexpressed in SW620 cells. Table 1 shows the top-30 up-regulated proteins in SW620 compared to SW480. Among the up-regulated proteins, fatty acid synthase demonstrated the highest signal-to-noise ratio (STN) value. Fatty acid synthase has been recognized as an effective chemotherapeutic target in colon cancer [19]. Fatty acid synthase is an anabolic enzyme involved in de novo biosynthesis of fatty acid that plays a partial role in the formation of phospholipid structure [14]. Neoplastic lipogenesis is known to be mainly responsible for cancer cell growth and survival, including rapid proliferation. Fatty acid synthase is usually found within most cancers, especially breast cancer and prostate cancer, which exhibit high levels of fatty acid synthase. In normal tissues, fatty acid synthase has a low level of expression due to tight regulation by diet, hormones and growth factors. In cancer, high levels of fatty acid synthase expression have been correlated with the failure of clinical treatment, which implies that fatty acid synthase is at least partly responsible for the aggressiveness of these cancers [20]. Metabolic enzymes such as lactate dehydrogenase and pyruvate kinase were found to be overexpressed in metastatic SW620 cells, which also explain elevated function of fatty acid synthase in SW620 cells since pyruvate kinase is involved in Krebs cycle, generating ATP used for the production of long chain fatty acids from acetyl-CoA. Also of particular interest, histone H4 was observed to be highly up-regulated along with histone H1.2 and H2 (not shown in Table 1), which are known to be chief proteins in maintaining chromatin structure and to be involved in chromatin condensation and gene regulation.

The overexpression of target molecules was visualized by immunohistochemical staining of colorectal cancer tissue microarray slides as shown in Fig. 2. Slides were stained with fatty acid synthase and histone H4 antibodies, which demonstrated high level of expression in metastatic cancer tissues (C1–C6, F1–F6), while little expression of the target molecules was observed in primary colon cancer tissues (B1–B6, E1–E6). The expression profiles of target candidates in metastatic tissue correlate with Western blot data (Fig. 4).

#### 3.2. Proteomic alteration of SW620 by the treatment of tested compounds

As shown in Table 1, most of the overexpressed markers were changed significantly by the 48 h incubation in the presence of the six tested compounds. Over 4100 proteins were identified in total from the six treatments of SW620, with 2010 common proteins (Fig. 1). Fatty acid synthase was observed to be down-regulated by the treatment of oxaliplatin, sorafenib and curcumin. Especially, curcumin was revealed to be the most potent compound suppressing fatty acid synthase effectively.
In the previous studies, curcumin has been reported to be one of the most effective anti-cancer compounds derived from herbal dietary supplements [21–24]. Curcumin is one of the major components of the rhizome of *Curcuma longa* and has been known to possess potent anti-cancer, anti-inflammatory and anti-oxidant activities [25]. It has been shown to suppress tumor initiation, promotion, and metastasis by the suppression of proliferation, down-regulation of transcription factors such as NF-kB, AP-1 and Egr-1, down-regulation of COX-2, LOX, and NOS, down-regulation of growth factor receptors such as EGFR and HER2 and inhibition of the activity of c-Jun N-terminal kinase, protein tyrosine kinases and protein serine/threonine kinases [26]. Also curcumin has been reported to suppress fatty acid synthase in several experimental systems with various cancer cell lines such as HEP-G2, HT-29 and Caco-2 cells [27,28]. In the current study, treatment of SW620 cancer cells with curcumin suppressed expression of fatty acid synthase as well as other previously overexpressed proteins such as elongation factor, histone H4 and mRNA splicing factor.

Currently, oxaliplatin and 5-FU are considered to be first line chemotherapeutics in the clinical treatment of colon cancer. In our study oxaliplatin treatment resulted in the suppression of various metastatic biomarker candidates such as fatty acid synthase and histone H4, although 5-FU did not affect protein alterations significantly.

On the other hand, histone H4 up-regulation in SW620 was reversed by treatment with either oxaliplatin or ginsenoside 20(S)-Rg3. Ginsenoside 20(S)-Rg3 is a dammarane-type saponin glycoside, which is a unique component of *P. ginseng* root, a popular oriental traditional medicine. Ginsenoside 20(S)-Rg3 has been reported to show a broad range of biological activity, including anti-cancer activity. It is well known that ginsenoside 20(S)-Rg3 inhibits proliferation and induces apoptosis of cancerous cells [29–32]. The current study is suggestive that ginsenoside 20(S)-Rg3 suppresses SW620 cell proliferation by down-regulation of the histone family proteins, and thus may possibly affect metastatic progression of SW620.

In addition, from the proteomic dataset, ginsenoside 20(S)-Rg3 was observed to inhibit fatty acid synthase expression. Recently, ginsenoside 20(S)-Rg3 was reported to show an inhibitory effect on lipid metabolism, especially by the interference with arachidonic acid metabolism during the course of COX-2 generation [33]. Since COX-2 has been recently presented as a chemical cancer marker, it is suggestive that anti-cancer activity...
Table 1 – List of top 30 up-regulated proteins in SW620 acquired from the comparative proteomic profiling of SW480 and SW620, primary and metastatic colorectal cancer cells respectively. Most of the proteins were altered significantly by the treatment of the following three of chemotherapeutics and three of natural compounds.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Description</th>
<th>SW480 vs SW620</th>
<th>Oxaliplatin</th>
<th>Sorafenib</th>
<th>5-Fluorouracil</th>
<th>Ginsenoside 20(S)-Rg3</th>
<th>Curcumin</th>
<th>Luteolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPI00026781</td>
<td>Fatty acid synthase</td>
<td>22.91 (0.0001)</td>
<td>-3.79 (0.0006)</td>
<td>-4.17 (0.0014)</td>
<td>-0.42 (0.1605)</td>
<td>-2.83 (0.0041)</td>
<td>-11.21 (0.0000)</td>
<td>-3.41 (0.0001)</td>
</tr>
<tr>
<td>IPI00219217</td>
<td>L-lactate dehydrogenase B chain</td>
<td>21.90 (0.0001)</td>
<td>-3.89 (0.0006)</td>
<td>1.26 (0.0297)</td>
<td>-0.05 (0.3664)</td>
<td>0.97 (0.0298)</td>
<td>0.70 (0.1034)</td>
<td>-3.42 (0.0001)</td>
</tr>
<tr>
<td>IPI00014424</td>
<td>Elongation factor 1-alpha 2</td>
<td>21.87 (0.0001)</td>
<td>0.94 (0.0516)</td>
<td>-4.04 (0.0014)</td>
<td>7.93 (0.0000)</td>
<td>4.59 (0.0003)</td>
<td>-10.55 (0.0000)</td>
<td>-1.16 (0.0325)</td>
</tr>
<tr>
<td>IPI00220644</td>
<td>Isoform M1 of pyruvate kinase isozymes M1/M2</td>
<td>20.06 (0.0001)</td>
<td>-3.77 (0.0006)</td>
<td>4.98 (0.0019)</td>
<td>2.92 (0.0017)</td>
<td>5.23 (0.0002)</td>
<td>-5.22 (0.0001)</td>
<td>-3.25 (0.0004)</td>
</tr>
<tr>
<td>IPI000453473</td>
<td>Histone H4</td>
<td>19.57 (0.0001)</td>
<td>-8.22 (0.0001)</td>
<td>0.69 (0.0726)</td>
<td>1.21 (0.0140)</td>
<td>-10.30 (0.0000)</td>
<td>-3.66 (0.0004)</td>
<td>-1.99 (0.0036)</td>
</tr>
<tr>
<td>IPI000176692</td>
<td>Elongation factor 1-alpha 2</td>
<td>18.37 (0.0001)</td>
<td>-1.26 (0.0208)</td>
<td>0.19 (0.2374)</td>
<td>1.01 (0.0211)</td>
<td>-0.32 (0.1571)</td>
<td>-4.98 (0.0002)</td>
<td>-2.94 (0.0006)</td>
</tr>
<tr>
<td>IPI00030371</td>
<td>Elongation factor 1-alpha 2</td>
<td>17.63 (0.0001)</td>
<td>1.74 (0.0065)</td>
<td>-1.65 (0.0087)</td>
<td>-0.05 (0.3664)</td>
<td>-1.14 (0.0349)</td>
<td>-4.44 (0.0003)</td>
<td>0.91 (0.0489)</td>
</tr>
<tr>
<td>IPI00217966</td>
<td>Elongation factor 1-alpha 2</td>
<td>16.89 (0.0001)</td>
<td>-3.68 (0.0009)</td>
<td>-2.34 (0.0040)</td>
<td>-0.68 (0.0836)</td>
<td>1.95 (0.0053)</td>
<td>-1.44 (0.0174)</td>
<td>-3.98 (0.0001)</td>
</tr>
</tbody>
</table>

* STN: signal-to-noise ratio acquired from PLGEM analysis.
of ginsenoside 20(S)-Rg3 is related to its competitive/inhibitory action on lipid metabolism. Fatty acid synthase is a key member of the saturated fatty acid metabolism pathway, thus, down-regulated fatty acid synthase may be an evidence of suppressive action of ginsenoside 20(S)-Rg3.

Drug-induced reduction in the expression of metastasis markers was also identified using immunocytochemical staining of SW620 cells. Fig. 3 shows the reduced expression of fatty acid synthase and histone H4 expression as a result of the drug treatments. Immunocytochemistry of SW620 cells demonstrates that fatty acid synthase was down-regulated by treatment with sorafenib and curcumin; and histone H4 was down-regulated by oxaliplatin and ginsenosides 20(S)-Rg3. These results are in a good agreement with the proteomic dataset and Western blot images, and therefore confirmed higher expression of the marker proteins in SW620 cells.

Luteolin did not show inhibitory activity on specific proteins presented in the list. Luteolin has been described as one of the promising anti-cancer agents originating from herbal sources. However, in the current study no significant biological modifications of the SW620 proteome were observed as a result of luteolin treatment, even at the higher dose of 50 μM.

### 3.3. Knowledgebase pathway analysis and validation of proteomic datasets

While PLGEM was developed using a normalized spectral abundance factor (NSAF) as input, its performance with a limited number of replicates has been shown to improve when raw spectral count rather than NSAF is used. Therefore raw spectral count was used as input in our PLGEM analysis. False discovery rates for PLGEM generated significance lists were estimated using the Benjamini–Hochberg estimator [34]. The list of proteins given by PLGEM analysis with STN and p-value was filtered by the degree of change and the significance. The overexpressed proteins with high significance (p < 0.01) from the proteomic data of SW480 versus SW620 were chosen, which were then uploaded into knowledgebase pathway analysis to characterize the biological functions of important identified proteins.

MetaCore™ (Thomson Reuters, MN, USA) was employed for pathway analysis, which provided highly relevant and significant biological pathways. Supplementary Fig. 2A shows the top-10 pathway maps, disease biomarker networks and process networks, which give implications regarding potential biomarker candidates affecting metastasis of cancer. Since SW620, like
other metastatic cell lines, demonstrated dramatic differences in the expression of structural proteins (e.g. cytokeratin, intermediate filaments), pathways involved in cellular structure showed high relevancy overall. From the enrichment analysis, pathway maps and process networks related to cytoskeletal remodeling, cell to cell junction and adhesion were highlighted, this describes well a number of events known to occur during the course of metastatic progression of cancer cells. Pathway maps employing fatty acid synthase and histone H4 as major objects did not appear in top-10 networks and pathways, since the significance of a pathway is based on the number of pathway objects. However, within the top-10 disease biomarker networks, the most significant networks such as the colonic neoplasm, breast neoplasm and colorectal neoplasm networks contained histones and fatty acid synthase as key. This is evidence of the high relevance between the proteins identified from the proteomic analysis and target diseases. Supplementary Fig. 2B represents the results of gene ontology (GO) analysis. GO molecular function analysis suggested that protein binding, nucleotide binding and nucleoside phosphate binding are major molecular functions of SW620 proteins. Especially, from GO processes analysis, several cellular metabolic processes were suggested to be the major events occurring in these cells as determined by proteins up-regulated in SW620. The top-10 GO processes employ histones and fatty acid synthase as critical proteins playing pivotal roles in the cellular metabolic process and cellular components of biogenesis as well.

Proteomic datasets acquired from the analysis of SW620 with treatments were validated using immunoblot assay. Western blot images of representative proteins differentially expressed were compared to their normalized spectral counts directly. Fig. 4 shows that the tested proteins, namely, fatty acid synthase, profilin-1, histone H4, histone H1, heat shock protein-60 kDa (HSP-60) and 90 kDa (HSP-90), β-actin and AHNAK show positive correlation with their spectral count changes overall, which may also confirm the validity of the proteomic datasets.

3.4. Evaluation of anti-metastasis activity

Based on the comparative proteomic analysis of SW480 versus SW620, several proteins such as fatty acid synthase and histone H4 were suggested to play major roles in metastatic progression. From the pathway analysis, those proteins were revealed to be participants in the top significant disease biomarker networks. To evaluate their roles in metastasis progression, cell migration assays were performed employing fatty acid synthase- and histone H4 knockdown SW620 cells in this study. As shown in Fig. 5A, the expressions of fatty acid synthase and histone H4 were successfully suppressed by transfection of siRNA/lipofectamine complexes even out to 72 h post-transfection. Normal cells and transfected cells were subjected to Boyden-chamber migration assays. Cells transferred into the migration chamber (upper) migrated through 8 μm-membrane by chemotaxis employing 10% FBS as a chemo-attractant (Fig. 5D). Cells passing through the membrane were lysed with a buffer containing CyQuant fluorescent dye and then absorbance measured by fluorescent plate reader. Fig. 5B shows that migrating cell populations were reduced by the suppression of histone H4 and fatty acid synthase expression. A number of chemical modifications of histones (e.g. acetylation) have been reported to be key modulatory parameters that affect metastasis of colorectal adenocarcinoma [35]. This evidence of the impact of
the interruption of native histone expression is supported in the current study, where the migratory action of SW620 was reduced by the silencing of the histone H4 gene. Fatty acid synthase knockdown in SW620 demonstrated similar results to histone H4 knockdown cells.

SW620 cells were subjected to migration assay in the presence of six test drugs at the same concentration used for proteomic analysis. Judging from MTT assays performed, treatment concentration had no apparent effect on the viability of SW620 cells with 24 h incubation (data not shown). As a result, oxaliplatin, ginsenoside 20(S)-Rg3 and curcumin exhibited potent inhibitory activity, corresponding well to their proteomic data which showed those compounds to have suppressive effects on fatty acid synthase and histone H4. These results support the proteomic data by proving reduced migratory action of cells by silencing target genes.

Additionally, all three of the compounds were individually shown to suppress fatty acid synthase and histone H4 significantly. This suggests that the suppression of either fatty acid synthase, histone H4 or both together could inhibit metastasis, but dual suppression of these two genes may synergize the activity. A few fatty acid synthase inhibitors and histone deacetylase (HDAC) inhibitors are available for chemotherapy of various cancers, however, the suppression of a single target often results in chemoresistance and complicates therapeutic strategies. Thus, chemotherapeutics which target multiple proteins could provide opportunities to improve clinical outcomes.

4. Conclusions
The anti-metastatic activity of three chemotherapeutics and three natural components was investigated. Target biomarkers that related to metastasis were identified by a comparative proteomic analysis approach employing the isogenic primary and metastatic colon cancer cell lines, SW480 and SW620. The biological function of the target proteins was evaluated by Boyden chamber assay with gene knockdown cells. Three tested drugs, namely, oxaliplatin, ginsenoside 20(S)-Rg3 and curcumin showed suppressive effects on fatty acid synthase and histone H4 as determined by their anti-migratory activity in the treated cells. Furthermore, these data corresponded well to the proteomic datasets. The anti-metastatic activities of these agents that were revealed by the current proteomic analysis could represent promising chemotherapeutic possibilities for the treatment of colorectal adenocarcinoma.
Fig. 5  (A) Fatty acid synthase and histone H4 had been suppressed by the siRNA transfection for 72 h and 24 h respectively. Scrambled RNA was used as a negative control (Con (-)). (B) Gene suppression of fatty acid synthase and histone H4 decreased cell migratory action (*p < 0.001). (C) Drug induced suppression of fatty acid synthase and histone H4 expression resulted in reduced cell migration. Degree of anti-migration activity corresponded to signal to noise (STN) ratio appeared in proteomic datasets overall (*p < 0.001). (D) Schematic overview of cell migration assay system based on Boyden chamber assay.

Abbreviations

CRC colorectal cancer
iTRAQ isobaric tag for relative and absolute quantification
5-FU 5-fluorouracil; synthase
GAPDH glyceraldehyde 3-phosphate dehydrogenase
RIPA radio-immunoprecipitation assay
PLGEM Power Law Global Error Model
STN signal to noise ratio
NF-κB nuclear factor-κB
AP-1 the activator protein-1
Egr-1 early growth response protein-1
COX-2 cyclooxygenase-2
LOX lipoxygenase
NOS nitric oxide synthase
EGFR epidermal growth factor receptor
HER2 human epidermal growth factor receptor-2
NSAF normalized spectral abundance factor
GO gene ontology
HSP-60 heat shock protein 60 kDa
HSP-90 heat shock protein 90 kDa
HDAC histone deacetylase
IHC immunohistochemistry

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.10.009.

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