Irinotecan Resistance – In Search of New Markers in CRC.

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Research Article

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Abstract

Colorectal cancer (CRC) is one of the most prominent causes of cancer death worldwide. Chemotherapeutic regimens consisting of different drugs combinations such as 5-fluorouracil, and oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) have been proven successful in the treatment of CRC. However, chemotherapy often leads to the acquisition of cancer drug resistance followed by metastasis and in the aftermath therapeutic failure. The molecular mechanism responsible for drug resistance is still unclear. The systemic search for new biomarkers of this phenomenon may identify new genes and pathways. To understand the drug resistance mechanism in CRC, the in vitro study based on the molecular analysis of drug-sensitive cells lines vs drug-resistant cells lines has been used. In our study to bridge the gap between in vitro and in vivo study, we compared the expression profiles of cell lines and patient samples from the publicly available database to select the new candidate genes for irinotecan resistance. Using The Gene Expression Omnibus (GEO) database of CRC cell lines (HT29, HTC116, LoVo, and their respective irinotecan-resistant variants) and patient samples (GSE42387, GSE62080, and GSE18105) we compared the changes in the mRNA expression profile of the main genes involved in irinotecan body's processing, such as transport out of the cells and metabolism. Furthermore, using a protein-protein interaction network of differently expressed genes between FOLFIRI resistant and sensitive CRC patients, we have selected top networking proteins (upregulated: NDUFA2, SDHD, LSM5, DCAF4, and COX10, downregulated: RBM8A, TIMP1, QKI, TGOLN2, and PTGS2). Our analysis provided several potential irinotecan resistance markers, previously not described as such.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer death [1]. Currently, the main therapeutic approaches to treat CRC are surgery, chemotherapy, irradiation, and targeted therapy [2]. The first-line chemotherapy strategy is usually a combination of 5-fluorouracil, leucovorin, and either oxaliplatin (FOLFOX) and irinotecan (FOLFIRI) [3].

Irinotecan (also known as Camptothecin-11, CPT-11) is a semisynthetic analog of plant alkaloid, camptothecin (CPT), [4, 5] and was first approved for the treatment of metastatic CRC refractory to 5-fluorouracil (5-FU) in the United States in 1996 [6]. Irinotecan is a pro-drug that is metabolically activated in the body to 7-ethyl-10-hydroxycamptothecin (SN-38) which inhibits topoisomerase-I (Topo 1) activity, an enzyme involved in DNA replication, and can affect the proliferation of tumor cells. The central role of Topo1 in the mechanism of irinotecan action has been largely demonstrated, however, its Topo1-independent activities were reported. Irinotecan inhibits binding of the oncoprotein, FUBP11 (Far Upstream Element (FUSE) Binding Protein 1), to its target sequence FUSE on single-strand DNA. FUBP1 is required for hepatocellular carcinoma tumorigenesis, and its downregulation sensitizes hepatocellular carcinoma cells for apoptosis-inducing chemotherapeutic drugs [7]. Irinotecan was also proven to interact directly with the E3 ubiquitin ligase MDM2 and the anti-apoptotic protein Bcl-xL [8]. Those findings may demonstrate the new mechanism of action for irinotecan as a selective protein binding inhibitor.

After more than two decades of clinical usage, irinotecan turned out to be a versatile chemotherapeutic agent which combines well both with other cytotoxic agents like 5-fluorouracil and oxaliplatin and with monoclonal antibodies, such as cetuximab and bevacizumab. Experimental and clinical studies also indicated that irinotecan can be combined with kinase inhibitors, such as fruquintinib, apatinib, dasatinib, regorafenib, and sunitinib or with cell-cycle checkpoint inhibitors [9, 10]. Although irinotecan still represents the backbone of CRC chemotherapy several serious disadvantages of its administration such as poor bioavailability, lack of tumor specificity, and most of all the susceptibility to multidrug resistance (MDR) have been revealed [11]. To date, the mechanism of irinotecan resistance is still inconclusive and requires further investigation. The several possible mechanisms that may lead to irinotecan
resistance, were suggested: 1) modification of structure or changes in expression level of Topo 1, 2) activation of anti-apoptotic signaling pathway, 3) mutations in enzymes which process irinotecan, and 4) reduction intracellular drug accumulation by active drug efflux. Two latter mechanisms are strictly connected to the complicated metabolism of irinotecan which can be described in three major steps, (1) the conversion into its active metabolite, SN38, (2) the detoxification of SN38 to generate the inactive metabolite SN38-glucuronide, (3) the reactivation of SN38-G in the intestine by bacterial β-glucuronidase (βG) [12]. These three metabolic steps and irinotecan transport have been widely exploited to identify biomarkers of therapeutic effectiveness.

Bioactivation of irinotecan occurs rapidly after injection in the plasma and the liver. Irinotecan is subject of enzymatic cleavage (hydrolyzation) performed by two isoforms of carboxylesterases (CES1 and CES2) and butyrylcholinesterase (hBChE) that removes bulky side chain, forming SN-38 that presents 100 to 1000 fold greater activity than irinotecan itself, CES1 and CES2 are predominantly active in liver, colon, kidney and blood cells, whereas the activity of hBChE is mainly localized in blood plasma [13]. SN-38 may be further conversed by uridine diphosphate-glucuronosyl transferases such as UGT1A1 and UGT1A9 in the liver (or UGT1A1 UGT1A7 and UGT1A10 – extrahepatic in bile) into inactive SN-38 glucuronide (SN-38G), which in turn may be further processed by intestinal bacterial β-glucuronidase back into active SN-38 [14, 15]. Irinotecan, as most of xenobiotics, is oxidized by CYP3A4 and CYP3A5 – members of cytochrome P450 superfamily into inactive aminopentane carboxylic acid (7-ethyl-10[4-N-(5-aminopentanoicacid)-1-piperidino] carbonyloxycamptothecin) - APC and NPC (7-ethyl-10[4-amino-1-piperidino]carbonyloxycamptothecin). NPC is a weak inhibitor of cell growth however contrary to APC, NPC can be further reprocesssed into active SN-38 by CES1 and CES2[16, 17].

Irinotecan metabolism is fairly complex and takes place in several different organs, thus its transport, and transport of its metabolites is a very crucial step executed by transporters belonging to the ATP-binding cassette (ABC) proteins superfamily. They are active transporters with a broad range of substrate spectra and several members of ABCB, ABCC, and ABCG proteins are involved in the elimination of harmful xenobiotics including anticancer drugs and their metabolites [18]. Irinotecan or/and SN-38 are transported into blood or bile from hepatocytes by ABCB1, ABCC1, ABCC2, and ABCG2, whereas OATP transporter (SLCO1B1) enable its influx from blood ABCB1. ABCC2 and ABCG2 are present on the bile canalicular membrane [19, 20], contrary to ABCC1, which is strongly expressed on parenchymal cells, resulting in directed transport from the tissue into the blood [21]. The active efflux of irinotecan and its metabolites by ABC proteins is also one of the mechanisms responsible for acquired MDR resistance. ABC proteins, such as ABCB1, ABCB5, ABCC1, ABCC2, ABCC4, ABCC5, ABCG2, were identified to be involved in irinotecan/SN-38 transport out of the cytosol, to reduce its intracellular concentration and efficacy in cancer cells [22, 23].

Irinotecan resistance due to the complicated nature of its metabolism and transport is very composed. This study used a microarray gene expression profile from an open database to identify potential biomarkers for irinotecan resistance acquired by colorectal cancer cells. We aimed to investigate mRNA profile differences between several CRC cell lines, commonly used in vitro study on irinotecan resistance, such as HT29, LoVo, and HTC116, and their irinotecan (SN-38) resistant variants and patients with metastatic colon cancer sensitive or resistant to first-line treatment of FOLFIRI.

2. Materials And Methods

2.1 Microarray data processing and analysis
The Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) gene expression profile with accession numbers: GSE42387, GSE62080, and GSE18105 were downloaded. GSE42387 - HCT116, HT29, and LoVo cells were exposed in vitro to gradually increasing SN-38 concentrations for about nine months, generating sub-cell lines with acquired resistance. Gene expression profiles of the parental and resistant cell lines were obtained after 2-3 weeks cultured in drug-free medium (each in triplicate) using GPL16297 Agilent-014850 Whole Human Genome Microarray 4x44K G4112F platform (Agilent Systematic Name, collapsed probe, version). GSE62080 database is composed of 21 patients with advanced colorectal cancer treated using FOLFIRI scheme and classified according to the WHO criteria as the responders (sensitive S) and non-responders (resistant R) group. Gene expression profile was obtained using Human Genome GeneChip arrays U133. GSE18105 database is composed of mRNA profiles of normal tissue and CRC patients primary tumors with and without distant metastasis. 111 microarray datasets (77 for LCM samples, and 17 pairs for homogenized samples from tumor and adjacent tissues) were normalized using a robust multi-array average (RMA) method under R 2.6.2 statistical software together with BioConductor package. Next, the gene expression levels were log2-transformed. For our analysis, we have excluded samples named: “metastatic recurrence” obtained from homogenized normal tissue. All data were processed using the GEO2R online analytical tool which uses the R language as described in (https://doi.org/10.1371/journal.pone.0180616).

Differently Expressed Genes - DEGs were further calculated and visualized using JASP 0.14.1.0 software (https://jasp-stats.org/) using Normality test (Shapiro-Wilk’s) followed by Mann-Whitney U test (for not normally distributed data) or T-test (for normally distributed data). Additionally, in the case of small n size, we have used JASP 0.14.1.0 software (https://jasp-stats.org/) to perform a Bayesian Mann-Whitney U test based on a data augmentation algorithm with 5 chains of 1000 iterations to further verify our data.

2.2 Hierarchical clustering analysis

After extracting the expression values from the gene expression profile, a bidirectional hierarchical clustering heatmap was constructed using an open-source machine learning and data visualization platform Orange (https://orangedatamining.com/).

2.3 Construction of PPI network

Protein-protein interaction (PPI) network of differently expressed genes (DEGs) was created using STRING version 11.0 online software (https://string-db.org/) and an open-source software platform for visualizing complex networks – Cytoscape (https://cytoscape.org/). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways analysis was performed using DAVID online tool (https://david.ncifcrf.gov/) and KEGG PATHWAY Database (https://www.kegg.jp/kegg/pathway.html).

3 Results

We analyzed the mRNA profile of 3 CRC cell lines, HT29, LoVo, and HTC116, and their respective SN-38 resistant variants using the GSE42387 database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42387). Gene expression profiles of the parental and resistant cell lines were obtained from cells cultured 2-3 weeks in a drug-free medium (each in triplicate). In our analysis first, we have focused on ABC proteins, as their expression level is considered to have a high impact on overall SN-38 intracellular concentration, and their expression level is mostly analyzed in CRC cells lines to establish the mechanism of acquired irinotecan resistance [24, 25] Fig. 1A. Since our previous studies showed that the expression of ABC transporters in CRC was correlated to cell phenotype and shifts during ongoing EMT[22], and considering that many other reports showed that irinotecan resistance is often related to advanced EMT, we additionally compared the expression level of the major EMT markers, such as E-cadherin
(CDH1), N-cadherin (CDH2) and vimentin (VIM) in SN-38 cell lines vs parental [26] Fig. 1C. Then, we analyzed the expression level of proteins involved in irinotecan/SN-38 metabolism Fig. 1B.

Our analysis proved the high heterogeneity among all tested cell lines. In general, all tested SN38 resistant variants of CRC cell lines presented the elevated expression of several ABC proteins, however, their profiles were different among all cell lines, Fig. 1A. The expression of prominent SN38/irinotecan transporters, i.e. ABCB1, ABCC1, and ABCC2 was significantly upregulated in the majority of SN-38 resistant variants except for HCT116 cells. We noticed an interesting correlation between ABCC4 and ABCG2 expression. In HT29 and LoVo SN38 resistant variants mRNA level of ABCG2 is significantly upregulated (compared to parental cell lines), whereas in HCT116 mRNA of ABCC4 is upregulated. Our previous studies showed, that in CRC ABCG2/ABCC4 expression shift is correlated with EMT and epithelial HT29 cells presents a high ABCG2 expression level that is switched for ABCC4 in HT29 cells that acquired mesenchymal-like phenotype through Snail overexpression [22]. In this study LoVo SN38 resistant cells, which present a more mesenchymal phenotype than the parental cell line (vimentin and CDH2 upregulation), exhibit higher mRNA expression of ABCG2. Interestingly, HT29 SN38 resistant cells show the upregulation of ABCC5 – that bears a strong resemblance in structure and substrates specificity to ABCC4 [27]. Regarding the expression profile of main enzymes involved in irinotecan processing, we noticed that HT29 SN38 resistant variants demonstrate an increase in CES1 expression and a decrease in CES2 expression. Surprisingly, HCT116 SN38 resistant variants cells present upregulated mRNA level of CES2 and downregulated mRNA level of CYP3A5 that metabolizes SN-38 into NPC. The mRNA level of UGT1 enzymes that inactivate SN38 into SN38G also presents ambiguous results. UGT1A6 is upregulated in HT29 and LoVo SN38 resistant variants, UGT1A8 is upregulated in HT29 SN38 but downregulated in LoVo SN38 cells.

To summarize changes between different cell lines and their differences in SN-38 acquired resistance, we performed clustering analysis using mRNA levels of above-analyzed genes Fig. 2. Our results indicated that all SN-38 resistant variants presented significantly different profiles compared to the respective parental cell lines, however, the HT29 cell line and its SN-38 resistant variant are significantly different than other tested cell lines, and clusters separately. Interestingly LoVo cell line is more closely related to the HCT116 and HTC116 SN8 than to the LoVo SN38 variant, thus we can assume that obtained resistance for SN-38 triggered the biggest changes in mRNA expression of tested genes.

Next, we analyzed samples from 21 patients with advanced colorectal tumor (GSE62080 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62080), that at the end of the first-line treatment of FOLFIRI scheme were define to the responders (sensitive S) and non-responders (resistant R) group according to the WHO criteria [25]. As expected, the expression profile related to chemotherapy resistance observed in CRC tissue samples differs substantially from those present in the resistant cell lines. In patient samples no statistically significant changes were observed in mRNA expression levels of neither ABC transporters nor irinotecan processing enzymes UGT1 variants between sensitive and resistant patient group. To better characterized the patients cohort we analyzed the expression of Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5). LGR5 potentiates canonical Wnt/β-catenin signaling and is considered a marker of advance CRC state and unfavorable prognostic marker [28]. Bayesian Manna-Whitney U test BF10 suggests “anecdotal evidence” of mRNA upregulation in resistant group. Surprisingly, mRNA level of vimentin (EMT marker) and TWIST-1 (EMT related transcription factor) were significantly downregulated in resistant patient group (supplementary files). The epithelial phenotype may be related to the 5FU resistance [29] as patients were treated with FOLFIRI that contains 5FU.

Next, we analyzed top 250 differently expressed genes between resistant and sensitive patient groups using protein-protein interaction (PPI) network by STRING version 11.0 (https://string-db.org/) Fig. 3. PPI network was enhanced by
known proteins that fills the gaps in proper networking as described in [30]. Upgraded networking for up and down regulated gens respectively contained of: number of nodes: 198; number of edges: 2141; average node degree: 1.6; avg. local clustering coefficient: 0.64; expected number of edges: 488; PPI enrichment p-value: < 1.0e-16; and: number of nodes: 203; number of edges: 2989; average node degree: 29.4; avg. local clustering coefficient: 0.656; expected number of edges: 756; PPI enrichment p-value: < 1.0e-16. KEEG pathway analysis using KEGG PATHWAY Database (https://www.kegg.jp/kegg/pathway.html), STRING version 11.0 and DAVID online tool indicated several signaling pathways that reached False Discovery Rate (FDR) < 0.05 and p < 0.05 as shown in Table 1. Top 5, respectively, up and down regulated gens with highest networking score are shown in Table 2 and their respective KEGG pathways are presented in Table 3. Even though, chosen genes presented the highest networking score, DCAF4 QKI and TGOLN2 shows no KEGG involvement. On the other hand, NDUAF2, SDHD and PTGS2 are involved in high number of pathways. Interestingly, COX10, one of the highest networking protein places on number 1 spot among all up-regulated genes in resistant patient group compared to sensitive group. Finally, we have investigate chosen DEGs influence on CRC patients survival using The Human Protein Atlas data base (https://www.proteinatlas.org/) [31]. 5 year survival time of colon adenocarcinoma patients expressing high or low level of chosen DEGs were compared to the rectum adenocarcinoma, Table 4. We have noticed that 5 year survival time substantially depend on cancer locations. Importantly, LSM5 and DACF4 upregulated in resistant patient samples seems to be unfavorable factor in colon adenocarcinoma patients leading to higher mortality. On the other hand in rectum adenocarcinoma upregulation of NDUFA2 is highly unfavorable factor presenting 19% 5-year survival rate in comparison to 67% for NDUFA2 low expression. Furthermore, for DEGs downregulated in resistant patients, low expression of PTGS2 is unfavorable factor for both colon and rectum adenocarcinoma whereas QKI and RBM8A only for rectum adenocarcinoma. Surprisingly, NDUFA2, SDHD and COX10 that are upregulated in resistant patients presents positive influence on colon adenocarcinoma patient survival.

Tab.1 KEGG pathway analysis of PPI network. *Visualization of top dysregulated pathways by up and down-regulated genes in resistant patients samples from GSE62080 database.*
| Up-regulated genes                                      | Count In Network | FDR     |
|--------------------------------------------------------|------------------|---------|
| Oxidative phosphorylation                              | 48 of 131        | 7.97e-54|
| Parkinson's disease                                    | 47 of 142        | 3.28e-51|
| RNA degradation                                         | 23 of 77         | 3.48e-24|
| Non-alcoholic fatty liver disease (NAFLD)              | 43 of 149        | 8.69e-45|
| Retrograde endocannabinoid signaling                   | 42 of 148        | 1.54e-43|
| Spliceosome                                             | 34 of 130        | 3.52e-34|
| Alzheimer's disease                                     | 43 of 168        | 4.74e-43|
| Thermogenesis                                           | 50 of 228        | 3.39e-47|
| Huntington's disease                                    | 43 of 193        | 6.95e-47|
| Citrate cycle (TCA cycle)                              | 4 of 30          | 0.0028  |
| Metabolic pathways                                      | 52 of 1250       | 2.16e-17|
| Down-regulated genes                                    |                  |         |
| Spliceosome                                             | 60 of 130        | 4.04e-72|
| mRNA surveillance pathway                              | 21 of 89         | 4.61e-19|
| Circadian rhythm                                        | 7 of 30          | 1.92e-06|
| Longevity regulating pathway - multiple species         | 10 of 61         | 1.24e-07|
| Hypertrophic cardiomyopathy (HCM)                      | 10 of 81         | 9.66e-07|
| SNARE interactions in vesicular transport              | 4 of 33          | 0.0044  |
| RNA transport                                           | 19 of 159        | 1.75e-12|
| Longevity regulating pathway                            | 10 of 88         | 1.70e-06|
| RNA degradation                                         | 8 of 77          | 4.26e-05|
| Adipocytokine signaling pathway                         | 7 of 69          | 0.00018 |
| FoxO signaling pathway                                  | 12 of 130        | 9.57e-07|
| AMPK signaling pathway                                  | 10 of 120        | 1.89e-05|
| Ovarian steroidogenesis                                 | 4 of 49          | 0.0136  |
| Insulin resistance                                      | 8 of 107         | 0.00028 |
| Glucagon signaling pathway                              | 7 of 100         | 0.0012  |
| Oxytocin signaling pathway                              | 10 of 149        | 8.76e-05|
| Insulin signaling pathway                               | 9 of 134         | 0.00021 |
| Apelin signaling pathway                                | 9 of 133         | 0.00021 |
| Tight junction                                          | 11 of 167        | 4.24e-05|
| Up-regulated genes                                                                 | Count In Network: | FDR   |
|-----------------------------------------------------------------------------------|------------------|-------|
| Fluid shear stress and atherosclerosis                                            | 8 of 133         | 0.0011|
| Non-alcoholic fatty liver disease (NAFLD)                                         | 8 of 149         | 0.0021|
| Endocrine resistance                                                              | 5 of 95          | 0.0221|
| Signaling pathways regulating pluripotency of stem cells                         | 7 of 138         | 0.0059|
| MicroRNAs in cancer                                                               | 7 of 149         | 0.0080|
| mTOR signaling pathway                                                            | 7 of 148         | 0.0080|
| Proteoglycans in cancer                                                           | 9 of 195         | 0.0024|
| Rap1 signaling pathway                                                            | 9 of 203         | 0.0030|
| Leukocyte transendothelial migration                                              | 5 of 112         | 0.0401|
| Focal adhesion                                                                    | 8 of 197         | 0.0085|
| Thermogenesis                                                                     | 9 of 228         | 0.0059|
| Herpes simplex infection                                                          | 7 of 181         | 0.0206|
| PI3K-Akt signaling pathway                                                        | 10 of 348        | 0.0231|

Tab.2 Top DEGs that had interactions in the protein-protein interaction (PPI) network.

| Gene name                                                                 | Gene     | Betweenness Centrality | Degree |
|---------------------------------------------------------------------------|----------|------------------------|--------|
| NADH Ubiquinone Oxidoreductase Subunit A2                                 | NDUFA2   | 0.009082               | 48     |
| Succinate Dehydrogenase Complex Subunit D                                 | SDHD     | 0.013266               | 41     |
| LSM5 Homolog, U6 Small Nuclear RNA And MRNA Degradation Associated         | LSM5     | 7.02E-04               | 36     |
| DDB1 And CUL4 Associated Factor 4                                          | DCAF4    | 0                      | 25     |
| Cytochrome C Oxidase Assembly Factor Heme A:Farnesyltransferase            | COX10    | 4.08E-05               | 24     |
| RNA Binding Motif Protein 8A                                               | RBM8A    | 0.007638               | 75     |
| TIMP Metallopeptidase Inhibitor 1                                          | TIMP1    | 0.031803               | 20     |
| KH Domain Containing RNA Binding                                            | QKI      | 0.035524               | 17     |
| Trans-Golgi Network Protein 2                                               | TGOLN2   | 0.062555               | 16     |
| Prostaglandin-Endoperoxide Synthase 2                                      | PTGS2    | 0.018124               | 14     |

Tab.3 Involvement of the top networking DEGs in significant KEGG pathways. Gene enrichment analysis of the DEGs involved in resistance of CRC patients treated with FOLFIRI and their involvement in significant KEGG pathways.
| Gene      | SDHD | LSM5 | DCAF4 | COX10 | RBM8A | TIMP1 | QKI | TGOLN2 | PTGS2 |
|-----------|------|------|-------|-------|-------|-------|-----|--------|-------|
| Alzheimer disease | X    | X    |       |       |       |       |     |        |       |
| Amyotrophic lateral sclerosis | X    | X    |       |       |       |       |     |        |       |
| Arachidonic acid metabolism |     |      |       |       |       |       |     |        | X     |
| Biosynthesis of cofactors |     |      |       |       |       |       |     | X      |       |
| Biosynthesis of secondary metabolites | X    | X    |       |       |       |       |     |        |       |
| Butanoate metabolism |     |      |       |       |       |       |     |        | X     |
| Carbon metabolism |     |      |       |       |       |       |     |        | X     |
| Chemical carcinogenesis |     |      |       |       |       |       |     |        | X     |
| Citrate cycle (TCA cycle) |     |      |       |       |       |       |     | X      |       |
| C-type lectin receptor signaling pathway |     |      |       |       |       |       |     |        | X     |
| Diabetic cardiomyopathy | X    | X    |       |       |       |       |     |        |       |
| HIF-1 signaling pathway |     |      |       |       |       |       |     | X      |       |
| Human cytomegalovirus infection |     |      |       |       |       |       |     |        | X     |
| Human papillomavirus infection |     |      |       |       |       |       |     |        | X     |
| Huntington disease | X    | X    |       |       |       |       |     |        |       |
| IL-17 signaling pathway |     |      |       |       |       |       |     | X      |       |
| Kaposi sarcoma-associated herpesvirus infection |     |      |       |       |       |       |     |        | X     |
| Leishmaniasis |     |      |       |       |       |       |     |        | X     |
| Metabolic pathways | X    | X    |       |       |       |       |     |        | X     |
|                  | SDHD | LSM5 | DCAF4 | COX10 | RBM8A | TIMP1 | QKI | TGOLN2 | PTGS2 |
|------------------|------|------|-------|-------|-------|-------|-----|--------|-------|
| Microbial        |      |      |       |       |       |       |     |        |       |
| metabolism       |      |      |       |       |       |       |     |        |       |
| in diverse       |      |      |       |       |       |       |     |        |       |
| environments     |      |      |       |       |       |       |     |        |       |
| mRNA surveillance|      |      |       |       |       |       |     |        |       |
| pathway          |      |      |       |       |       |       |     |        |       |
| NF-kappa B       |      |      |       |       |       |       |     |        |       |
| signaling        |      |      |       |       |       |       |     |        |       |
| pathway          |      |      |       |       |       |       |     |        |       |
| Non-alcoholic    |      |      |       |       |       |       |     |        |       |
| fatty liver      |      |      |       |       |       |       |     |        |       |
| disease          |      |      |       |       |       |       |     |        |       |
| Ovarian steroidogenesis | | | | | | | | | |
| Oxidative        |      |      |       |       |       |       |     |        |       |
| phosphorylation  |      |      |       |       |       |       |     |        |       |
| Oxytocin         |      |      |       |       |       |       |     |        |       |
| signaling        |      |      |       |       |       |       |     |        |       |
| pathway          |      |      |       |       |       |       |     |        |       |
| Parkinson disease|      |      |       |       |       |       |     |        |       |
|                      |      |      |       |       |       |       |     |        |       |
| Pathways in       |      |      |       |       |       |       |     |        |       |
| cancer            |      |      |       |       |       |       |     |        |       |
| Pathways of       |      |      |       |       |       |       |     |        |       |
| neurodegeneration |      |      |       |       |       |       |     |        |       |
| - multiple        |      |      |       |       |       |       |     |        |       |
| diseases          |      |      |       |       |       |       |     |        |       |
| Porphyrin and     |      |      |       |       |       |       |     |        |       |
| chlorophyll       |      |      |       |       |       |       |     |        |       |
| metabolism        |      |      |       |       |       |       |     |        |       |
| Prion disease     |      |      |       |       |       |       |     |        |       |
| Regulation of     |      |      |       |       |       |       |     |        |       |
| lipolysis in      |      |      |       |       |       |       |     |        |       |
| adipocytes        |      |      |       |       |       |       |     |        |       |
| Retrograde        |      |      |       |       |       |       |     |        |       |
| endocannabinoid   |      |      |       |       |       |       |     |        |       |
| signaling         |      |      |       |       |       |       |     |        |       |
| RNA degradation   |      |      |       |       |       |       |     |        |       |
| RNA transport     |      |      |       |       |       |       |     |        |       |
| Serotonergic      |      |      |       |       |       |       |     |        |       |
| synapse           |      |      |       |       |       |       |     |        |       |
| Spliceosome       |      |      |       |       |       |       |     |        |       |
| Thermogenesis     |      |      |       |       |       |       |     |        |       |
| TNF signaling     |      |      |       |       |       |       |     |        |       |
| pathway           |      |      |       |       |       |       |     |        |       |
| VEGF signaling    |      |      |       |       |       |       |     |        |       |
| pathway           |      |      |       |       |       |       |     |        |       |
### Tab. 4 The prognostic relationship between high and low expression of specific genes involved in drug resistance to the overall survival of colon and rectum adenocarcinoma. 5-year survival factor obtained from The Human Protein Atlas database [35].

| Gene     | Colon adenocarcinoma | Rectum adenocarcinoma |
|----------|-----------------------|------------------------|
|          | 5-year survival high  | 5-year survival low    |
| NDUFA2   | 72%                   | 58%                    |
| SDHD     | 71%                   | 52%                    |
| LSM5     | 43%                   | 69%                    |
| DCAF4    | 61%                   | 71%                    |
| COX10    | 71%                   | 57%                    |
| RBM8A    | 57%                   | 71%                    |
| TIMP1    | 53%                   | 69%                    |
| QKI      | 64%                   | 63%                    |
| TGOLN2   | 64%                   | 63%                    |
| PTGS2    | 69%                   | 59%                    |

To better characterize CRC cell lines and their respective SN-38 resistant variants, we have analyzed changes in mRNA expression of top-up/down-regulated best networking proteins that were identified in patients samples. The obtained data presented in Fig. 4A,B clearly shows that the data from CRC cell lines analysis partially overlapped with data obtained from CRC patients samples analysis. SN-38 - resistance-related changes in LoVo lines presents significant resemblance in changes of mRNA levels of NDUAF2, DCAF4, and PTGS2. Interestingly NDUAF2 and PTGS2 are two of the three most influential tested proteins involved in several important pathways (Table 3). HCT116 and their SN-38 resistant variant, in comparison to the patient samples, present similar (statistically significant) changes in mRNA expression of multifunctional PTGS2 and LSM5, that are involved in RNA degradation and spliceosome mechanism. The above-mentioned cell lines also present a similar tendency observed in CRC patients samples (yet not statistically significant change) in mRNA expression of RBM8A that is also involved in spliceosome and RNA transport. The same expression tendency was observed in the case of NDUFA2. On the other hand, the mRNA expression profile of SDHD presents inverse changes. In HT29 similar to patient samples mRNA expression of TIPM1 and TGOLN2 was downregulated in the SN38-resistant variant. The expression of NDUFA2 was also downregulated in the SN38-resistant variant however, its expression was upregulated in resistant patients. Surprisingly, COX10 which is the number one among up-regulated genes and one of the highest networking proteins, is not upregulated in any of SN-38 resistant variants.

Using mRNA expression profiles of selected genes, we performed clustering analysis, Fig. 5. HT29 and HTC116 with their respective SN-38 resistant variants differ enough to establish their phylogenetic group but are closely located in...
two respective arms. Interestingly, LoVo and LoVo SN38 cell lines present the highest diversity not only inside the resistant/parental group but also among all tested cell lines, located separately, on different arms of a phylogenetic tree.

To analyze the mutual changes between different DEGs, we decided to correlate mRNA expression profiles of previously described top 5 up and top 5 down-regulated, and the highest networking proteins and we performed a correlation matrix using Pearson correlation, Fig. 6. LoVo (pooled parental and their respective SN-38 resistant variant), similarly to patient samples, presented a positive correlation (yet not statistically significant in all cases) of the majority of top 5 upregulated genes of the best networking proteins: NDUFA2, SDHD, LSM5, and DCAF4 (COX10 positively correlates only with NDUFA2 and DCAF4). A negative correlation, similarly to patient samples, was observed among RBM8A, TGOLN2, and PTGS2. In the case of HT29 and HTC116 cell lines data were more ambiguous, as NDUFA2, SDHD, LSM5, DCAF4, COX10, RBM8A, TIMP1, QKI, TGOLN2, and PTGS2 presents mixed correlation to one another.

EMT was proven to play a role in the acquisition of chemoresistance on several anticancer drugs [32]. Observed in LoVo cell line shift towards mesenchymal phenotype upon acquisition of SN38 resistance with changes in EMT markers, vimentin and CDH2 encouraged us for analysis of EMT phenotype in FOLFIRI sensitive and resistant patients cohorts (GSE62080, n=21). First, using the mRNA level of 4 main EMT markers – E-cadherin (CDH1), N-cadherin (CDH2), vimentin (VIM), and fibronectin (FN) we divided patient samples into 3 groups: strongly epithelial (E), partially mesenchymal (E/M), and mesenchymal (M). However, all subgroups presented elevated expression levels of CHD1, suggesting a strongly epithelial, well-differentiated phenotype. This observation can be explained by the fact that GSE62080 data set is composed of cancer samples diagnosed at an early stage, with no metastatic occurrence. Thus, we decided to assign the patient samples to E or E/M group. (Fig. 7A). Interestingly, samples distribution proved, that FLOFIRI resistant E/M CRC samples present upregulation of FN and CDH2 rather than VIM (Fig. 7A). Nevertheless, in the resistant cohort (n=12) 7 samples were qualified into the E/M group and 5 into E while the sensitive cohort (n=9) E/M group was composed of 3 and E of 6 samples (Fig. 7B). These results showed the tendency of the resistant cohort to acquire a more mesenchymal phenotype. Furthermore, this heterogeneous composition of the E and E/M group, which consists of both resistant and sensitive patients samples in each phenotypical group, partially explained why no statistically significant upregulation of EMT markers between resistant and sensitive cohorts was observed in GSE62080 data set.

Since GSE62080 data sets include a low number of patients samples we have decided to apply the GSE18105 data set to analyze the mRNA expression level of previously selected top networking DEGs (up-regulated: NDUFA2, SDHD, LSM5, DCAF4, COX10 and down-regulated: RBM8A, TIMP1, QKI, TGOLN2, and PTGS2) in CRC patient samples. GSE18105 (https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE18105) database composed of mRNA profiles (n=111) obtained using an oligonucleotide microarray of normal tissue and CRC patients primary tumors with and without distant metastasis. Similar to previous analysis samples were divided into E, E/M, and M groups using mRNA levels of 4 main EMT markers – E-cadherin, N-cadherin, vimentin, and fibronectin. Only six out of ten tested proteins,i.e. NDUFA2, SDHD, LSM5, COX10, QKI, and TGOLN2, presented statistically significant changes in mRNA expression among E, E/M, and M patient groups (Fig. 8A). We observed that in M and E/M group similar to the resistant patients' group NDUFA2, SDHD, LSM5, COX10 and TGOLN2 was upregulated, additionally, the mRNA level of QKI was upregulated in E/M and M phenotype group unlike to the one observed in the resistant patients' group. Mesenchymal phenotype correlated upregulation of NDUFA2, SDHD, and COX10 that are involved (among the vast amount of different processes) in oxidative phosphorylation (OXPHOS) is extremely interesting. Several experiments proved that human CRC exhibit higher rates of oxidative phosphorylation than large intestine normal cells [33]. EMT
regulates ABC proteins expression [35]. Previously we proved that ABCC4 in CRC correlates with EMT and CRC cells with advanced EMT phenotype presents a higher level of ABCC4 contrary to ABCG2 level [22]. In the current study, we confirmed our previous observation that ABCC4 is upregulated in more mesenchymal phenotype presenting cells (M and E/M groups) whereas expression of ABCG2 is slightly (yet statistically significant) downregulated in advanced EMT (Fig. 8B). We also showed that ABCC1 correlates with mesenchymal phenotype, however to our biggest surprise, ABCC5 that was upregulated in SN-38 resistant variants of CRC cell line HT29 (and partially in LoVo) presents downregulated mRNA levels in both E/M and M patient groups. Finally, to prove that our E, E/M, and M groups are composed correctly, we have also tested the mRNA level of LGR5 [28], which was significantly upregulated in both the E/M and M group compared to E.

4. Discussion

Approximately 50-60% of patients who are diagnosed with CRC will eventually develop metastatic disease. Most often, metastases develop after first-line therapy treatments for local disease thus the drug resistance is still the major problem in CRC treatment [36, 37]. Gene profiling, among other methods, has been used to identify genes involved in the progression and the prognosis of CRC or to establish current CRC classification based on four molecular subtypes (CMS1-CMS4) [38, 39]. Previously, we identified NMU as a predictive marker of CRC invasiveness [40]. In the current study to bridge the gap between in vitro and in vivo study, we combined and compared the expression profiles of cell lines and patient samples from the publicly available database to select the new candidate genes for irinotecan resistance. Many attempts to identify chemotherapy predictive biomarkers of treatment response and resistance in CRC were made. However, most of these biomarkers, except for the KRAS and BRAF genes, are not accurately predicting treatment response. As such, additional studies are urgently needed to identify and validate novel biomarkers to improve the therapy for CRC patients [39].

CRC cell lines have previously been shown to recapitulate the mutational and transcriptional heterogeneity of primary tumors [41, 42]. To understand the irinotecan resistance mechanism various CRC cell lines resistant to SN-38 were selected. By studying the parental drug-sensitive cells to the drug-resistant cells by molecular biology and cellular methods, particularly acquired MDR-associated molecules have been studied [43]. In our study, we analyzed the expression profile of three cell lines, HT29, HCT116, LoVo, and their SN38 resistant variants. We applied those cell lines as they present various genetic, epigenetic, and molecular subtypes, and to some extent, they may reflect the molecular heterogeneity of CRC [41]. Since it has been demonstrated that assessment of multiple biomarkers provides an accurate prediction of drug response than a single biomarker in our study we analyzed the main enzymes involved in irinotecan transport and processing and additionally main EMT players as the mesenchymal phenotype is associated with irinotecan resistance [22, 39]. First, we concluded that SN-38 treatment changed the expression profile of all tested cells lines irrespective of their molecular background and origin. The expression of prominent SN38/irinotecan transporters, i.e. ABCB1, ABCC1, and ABCC2 has significantly upregulated in the majority of SN-38 resistant variants and the expression profile of ABC transporters was specific for each cell line. This observation is in line with many other reports in vitro studies concerning ABC transporters' function in cancer [44]. We also observed various changes in the expression profile of the main EMT markers in each of the tested cell lines. EMT is the best-known case of tumor cell plasticity which appears to influence sensitivity to various chemotherapeutic drugs and EMT is an important regulator of ABC transporters. It was demonstrated that the promoters of ABC transporters carry several binding sites for EMT-inducing transcription factors [45]. According to our previous data, the phenotype conversion induced by the Snail transcription factor decreased ABCG2 and increased the ABCC4 expression level in HT29 cells [40]. In the current study, we observed that LoVo SN-38 resistant variant which exhibited mesenchymal phenotype showed the upregulation of ABCG2 expression. The possible
explanation for the inconsistency may be the fact that LoVo and HT29 present different genetic profiles and belong to various CRC molecular subtypes, LoVo to CMS1, HT29 - CMS3, or that LoVo SN-38 resistant variants acquired mesenchymal feature through SN-38 treatment. Our analysis of the main enzyme responsible for the irinotecan activation (CES1/2) and deactivation indicated the different expression profiles among the tested lines. The changes in their expression are relevant in the case of irinotecan administration, however, in the case of the analysis of CRC cell lines their expression profiles are less interesting since the majority of their activity related to irinotecan in the body is located in the liver [13]. In our tested CRC cell lines, they may process inactive irinotecan metabolite - NPC into active SN-38. The clustering analysis confirmed that all SN-38 resistant variants presented significantly different profiles compared to the respective parental cell lines, and among three tested CRC lines, LoVo showed the most evident changes in the mRNA profile of tested genes under SN38 treatment. To verify our above observation we analyzed the expression profile of the patients with advanced colon cancer sensitive or resistant to first-line treatment of FOLFIRI. We observed that in the patients’ samples no statistically significant changes were observed in mRNA expression levels of neither ABC transporters nor irinotecan processing enzymes between the sensitive and resistant patients’ groups. We confirmed the upregulation of Lrg5 that is a marker of advanced CRC, in the resistant patients’ group concomitantly the expression profiles of EMT players from this group demonstrated the epithelial phenotype. The epithelial phenotype may be related to the 5FU resistance [46] as patients were treated with 5FU within FOLFIRI scheme.

To select the new candidate genes for irinotecan resistance we have analyzed the top 250 differently expressed genes between resistant and sensitive patient groups using protein-protein interaction (PPI) network and then we selected five up, such as NDUFA2, SDHD, LSM5, DCAF4, COX10, and down-regulated genes, RBM8A, TIMP1, QKI, TGOLN2, and PTGS2 respectively, with highest networking score. Those genes were not reported as irinotecan resistance-associated thus we check their utility as the prognostic markers. We showed using the KEGG database the involvement of selected DEGs in significant cellular pathways. We selected the biological processes that might be changed in response to chemotherapy treatment. The oxidative phosphorylation was the most upregulated pathway while the processes related to RNA processing (RNA spliceosome, surveillance, transport, and degradation) were the most downregulated. These findings are consistent with the previous observation that cells to survive under chemotherapy treatment reduce transcription rate and increase energy demand. Several experiments proved that human CRC cells exhibit even higher rates of oxidative phosphorylation than large intestine normal cells [35]. The elevated production of ATP may be connected to the increase in drug efflux operated by ABC transporters and/or to the increased migration of advanced CRC cells. The irinotecan-resistant Non-Small Cell Lung Cancer (NSCLC) cells are characterized by increased oxidative phosphorylation and treatment with gossypol (pan-ALDH inhibitor) and phenformin (OXPHOS inhibitor) reverses irinotecan resistance in tested xenograft model of human NSCLC [33]. CRC patient 5-year survival analysis showed that the prognostic value of selected DEGs depends on cancer location, i.e. colon vs. rectum, and additionally, they may be favorable or unfavorable factors. These results are difficult to discuss, however, they are in line with current knowledge that in the case of CRC, actual tumor location is one of the most important prognostic factors [39].

To integrate the obtained data and facilitate the selection of cell lines as appropriate research models, we reversed the standard data flow axis from “cell lines to patients” into “patients derived data to cell lines”. Although in vivo irinotecan resistance mechanisms are more complicated than in vitro, investigating such mechanisms in vitro can provide strategies to overcome acquired drug resistance in CRC [47]. Expression of the top networking up and down-regulated genes (NDUFA2, SDHD, LSM5, DCAF4, COX10, RBM8A, TIMP1, QKI, TGOLN2, and PTGS2) was differently expressed in the majority of irinotecan resistant variants of CRC cell lines, and often presented the reversed tendency than observed in vivo. The obtained data demonstrated that in vivo irinotecan resistance mechanisms are more
complicated than in vitro and suggest that not one, but many different mechanisms, signaling pathways, and other factors such as other drugs or tumor microenvironment, may act simultaneously or complementarily in the development of irinotecan resistance in CRC. We can assume that our results may need to be verified by a specific irinotecan treatment patient cohort. In the case of CRC irinotecan is commonly applied to the patients in FOLFIRI scheme. Our current results may also imply why plenty of promising in vitro studies fail the clinical implementation and why the mechanisms leading to irinotecan resistance are still poorly characterized. However, cell lines still represent a mainstay to functionalize molecular data as they allow experimental manipulation, global and detailed mechanistic studies, and high-throughput applications.

In our study, we showed that by integration of the expression profile data each CRC cell line is a resource to select relevant models for studies of irinotecan resistance mechanisms.

**Conclusion**

Irinotecan represents the backbone of CRC chemotherapy. Currently, after more than two decades of clinical usage, the mechanism of irinotecan resistance remains still the subject of investigation. Although in vivo irinotecan resistance mechanisms are more complicated than in vitro, investigating of latter can provide strategies to overcome acquired drug resistance in CRC. However, CRC cell lines are a limited model often presenting different mechanisms of utilizing drug resistance, thus usage of at least two different cells lines is essential. Importantly, identification of irinotecan processing enzymes as potential game-changing factors in acquired irinotecan resistance seems to be pointless, as the process of irinotecan activation in vivo is located mainly in the liver but not in the tumor – contrary to in vitro cell line-based experiments. The significance of ABC proteins seems to be less obvious, as patients' samples presented no significant changes between resistant and sensitive cohorts. FOLFIRI resistant patients' cohort present a tendency for activation of EMT, that enches both drug resistance and migration, and this observation require further analysis. Nevertheless, our analysis provided several potential irinotecan resistance markers, previously not described as such.

**Abbreviations**

5-FU – 5 fluorouracil

ABC - ATP-binding cassette

ABCB1 - ATP binding cassette subfamily B member 1

ABCC1 - ATP binding cassette subfamily C member 1

ABCC2 - ATP binding cassette subfamily C member 2

ABCC4 - ATP binding cassette subfamily C member 4

ABCC5 - ATP binding cassette subfamily C member 5

ABCG2 - ATP binding cassette subfamily G member 2

ALDH - aldehyde dehydrogenase

APC - aminopentane carboxylic acid (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin)
CES1 - carboxylesterase 1  
CES2 - carboxylesterase 2  
CMS - consensus molecular subgroups  
COX10 - Cytochrome C Oxidase Assembly Homolog 10  
CPT-11 - Camptothecin-11  
CYP3A4 - cytochrome P450 family 3 subfamily A member 4  
CYP3A5 - cytochrome P450 family 3 subfamily A member 5  
DAVID - Database for Annotation, Visualization and Integrated Discovery  
DCAF4 - DDB1 and CUL4 associated factor 4  
DEGs - differently expressed genes  
FDR - false discovery rate  
FN - fibronectin  
FOLFIRI - chemotherapy combination scheme: folinic acid (leucovorin), fluorouracil and irinotecan  
FOLFOX - chemotherapy combination scheme: folinic acid (leucovorin), fluorouracil and oxaliplatin  
GEO - Gene Expression Omnibus  
hBChE - butyrylcholinesterase  
HIF-1 - hypoxia-inducible factor 1  
IL-17 - interleukin 17  
KEGG - Kyoto Encyclopedia of Genes and Genomes  
LCM - laser capture microdissection  
LGR5 - leucine rich repeat containing G protein-coupled receptor 5  
LSM5 - U6 small nuclear RNA and mRNA degradation associated protein 5  
MDR - multidrug resistance  
NPC - (7-ethyl-10[4-amino-1-piperidino]carbonyloxy camptothecin)  
NSCLC - non-small cell lung cancer  
OATP - organic-anion-transporting polypeptides  
OXPHOS - oxidative phosphorylation
RNA - ribonucleic acid

SN-38 - 7-ethyl-10-hydroxycamptothecin

TWIST-1 - Twist family BHLH transcription factor 1

UGT1A1 - UDP glucuronosyltransferase family 1 member A1

UGT1A10 - UDP glucuronosyltransferase family 1 member A10

UGT1A7 - UDP glucuronosyltransferase family 1 member A7

UGT1A9 - UDP glucuronosyltransferase family 1 member A9

VEGF – vascular endothelial growth factor

VIM – vimentin

Declarations

Authors’ contributions

J.K. designed the analysis, extracted and analyzed the expression data, wrote the manuscript, J.B. conceived of the study, wrote, revised and edited manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The expression level of irinotecan resistance-related proteins in CRC cell lines. Data were obtained from GOE database: GSE42387 (using GPL16297 Agilent-014850 Whole Human Genome Microarray 4x44K G4112F platform). Changes in mRNA expression of (A) ABC proteins, (B) irinotecan metabolism-related enzymes and (C) EMT markers were measured in every cell line variant in triplicate. Normality test (Shapiro-Wilk’s) followed by Mann-Whitney U test (for not normally distributed data) or T-test (for normally distributed data). * p>0.05, ** p> 0.005, ***p>0.001
Figure 2

Hierarchical clustering analysis of CRC cell lines using mRNA expression profile of irinotecan resistance-related proteins. A bidirectional hierarchical clustering heat map was visualized using the data visualization platform Orange. The expression values between SN38-resistant variants and corresponding parental cell lines are presented a grayscale, black represents low and white - high expression values (6.1-17.23).

Figure 3

Protein-protein interaction (PPI) network of differentially expressed genes (DEGs). PPI network was created from 250 differently expressed genes obtained from the GSE62080 database using STRING version 11.0 online software, The PPI pairs were imported into Cytoscape software as described in the Materials and Methods section. The lines represent the interaction relationship between nodes.
mRNA expression level in CRC cell lines of 5 top networking up (A) and down (B) regulated DEGs from FOLFIRI resistant patients cohort. Data were obtained using GOE database: GSE42387 (GPL16297 Agilent-014850 Whole Human Genome Microarray 4x44K G4112F platform). Normality test (Shapiro-Wilk’s) followed by Mann-Whitney U test (for not normally distributed data) or T-test (for normally distributed data). * p>0.05, ** p> 0.005, ***p>0.001

Figure 5
Hierarchical clustering analysis of CRC cell lines using mRNA expression of top networking DEGs from FOLFIRI resistant patients cohort. A bidirectional hierarchical clustering heat map was visualized using data visualization platform Orange. The expression values between corresponding irinotecan-resistant and parental CRC cell lines are presented a gray scale, black represents low and white high expression values (6.29-15.02). To analyze the mutual changes between different DEGs, we decided to correlate mRNA expression profiles of previously described top 5 up and top 5 down-regulated, and the highest networking proteins and we performed a correlation matrix using Pearson
correlation, Fig.6. LoVo (pooled parental and their respective SN-38 resistant variant), similarly to patient samples, presented a positive correlation (yet not statistically significant in all cases) of the majority of top 5 upregulated genes of the best networking proteins: NDUFA2, SDHD, LSM5, and DCAF4 (COX10 positively correlates only with NDUFA2 and DCAF4). A negative correlation, similarly to patient samples, was observed among RBM8A, TGOLN2, and PTGS2. In the case of HT29 and HTC116 cell lines data were more ambiguous, as NDUFA2, SDHD, LSM5, DCAF4, COX10, RBM8A, TIMP1, QKI, TGOLN2, and PTGS2 presents mixed correlation to one another.

**Figure 6**

Pearson correlation matrix of FOLFIRI resistance related top networking DEGs. Parental and corresponding resistant variants of CRC cell line mRNA expression data (GSE42387) A) HT-29, B) HTC116 and C) LoVo, were pooled to create Pearson correlation matrix using JASP 0.14.1.0 software; * p>0.05, ** p> 0.005, ***p>0.001
Figure 7

EMT phenotype of FOLFIRI resistant and sensitive patients. Patient samples n=21 from GSE62080 were divided into 2 groups: strongly epithelial (E) and partially mesenchymal (E/M) based on mRNA level of 4 main EMT markers – E-Cadherin (CDH1) N-Cadherin (CDH2), Vimentin (VIM) and Fibronectin (FN) using Orange software (A). Sample distribution among E and E/M group (B).
Figure 8

mRNA expression level of ABC proteins (A) and irinotecan-resistance related DEGs (B) from FOLFIRI resistant patients cohort in different EMT states. Patient samples n=111 from GSE18105 were divided into 3 groups: strongly epithelial (E), partially mesenchymal (E/M), and mesenchymal (M) based on mRNA level of 4 main EMT markers – E-Cadherin (CDH1) N-Cadherin (CDH2), vimentin, and fibronectin using Orange software. Next, mRNA expression of irinotecan resistant-related DEGs (A) and ABC proteins (B) was visualized for each patient in its respective group using JASP 0.14.1.0 software. Normality test (Shapiro-Wilk’s) followed by Mann-Whitney U test (for not normally distributed data) or T-test (for normally distributed data). * p>0.05, ** p> 0.005, ***p>0.001

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