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Original Article

RNA-seq reveals determinants for irinotecan sensitivity/resistance in colorectal cancer cell lines

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Abstract: Irinotecan is a topoisomerase I inhibitor approved worldwide as a first- and second-line chemotherapy for advanced or recurrent colorectal cancer (CRC). Although irinotecan showed significant survival advantage for patients, a relatively low response rate and severe adverse effects demonstrated the urgent need for biomarkers searching to select the suitable patients who can benefit from irinotecan-based therapy and avoid the adverse effects. In present work, the irinotecan response (IC50 doses) of 20 CRC cell lines were correlated with the basal expression profiles investigated by RNA-seq to figure out genes responsible for irinotecan sensitivity/resistance. Genes negatively or positively correlated to irinotecan sensitivity were given after biocomputation, and 7 (CDC20, CTNNAL1, FZD7, CITED2, ABR, ARHGEF7, and RNMT) of them were validated in two CRC cell lines by quantitative real-time PCR, several of these 7 genes has been proposed to promote cancer cells proliferation and hence may confer CRC cells resistance to irinotecan. Our work might provide potential biomarkers and therapeutic targets for irinotecan sensitivity in CRC cells.

Keywords: Colorectal cancer, irinotecan, RNA-seq, CITED2, CTNNAL1, FZD7

Introduction

Colorectal cancer (CRC) is one of the most common malignant diseases, with 945,000 new cases and approximately 492000 patients' death annually, and is the fourth cause of cancer-related deaths worldwide [1]. As the probability of developing CRC rises sharply with age, CRC is a great health challenge for countries whose population is ageing, such as the UK and China [2]. Approximately 5-25% of newly diagnosed CRC cases present with advanced disease [3], prognosis for these patients remains poor. Chemotherapy is used as adjuvant therapy for resectable CRC patients or palliative therapy for advanced/metastatic CRC patients, to prevent the recurrence or to improve the survival or quality of life. Irinotecan is a topoisomerase I inhibitor approved worldwide for the treatment of metastatic CRC (mCRC), as a first- and second-line chemotherapy for advanced or recurrent CRC. Irinotecan has demonstrated significant survival advantage for patients with tumors that have progressed on initial 5-fluorouracil (5-FU)-based chemotherapy compared with supportive care alone [4-6]. When used alone, irinotecan exhibits 20-30% of objective response rate (ORR) for advanced CRC patients [7]. Irinotecan-based combinational therapy, such as FOLFIRI (irinotecan together with leucovorin and 5-FU), can reach an ORR of approximately 50% for mCRC patients [8]. However, more than half of the patients response poor to irinotecan-based therapy, in addition, irinotecan has severe toxicities, such as delayed-onset diarrhea, neutropenia, nausea, and vomiting, these demonstrate the urgent need for finding irinotecan biomarkers to improve the therapy.

Previous studies suggested that the mRNA or protein expression levels of several DNA repair-related genes or ubiquitin-like genes or kinase genes were associated with irinotecan sensitivity, including APTX, BRCA1 and ERCC1 [9, 10], DNA topoisomerase I (Topo I) [7, 11], IFN-stimulated gene 15 (ISG15) [12], thymidine phosphorylase (TP) [13], protein kinase CK2 [14, 15]. However, few of these potential biomarkers were validated in prospective clinical

Table 1. IC50 doses of 21 CRC cell lines to irinotecan

Cell line	Irinotecan IC50 (μM)
SW620	1.53
LS180	3.11
COLO741	3.57
COLO205	7.23
LOVO	7.64
CX-1	8.69
GP2D	9.89
SW48	10.02
RKO	10.78
HCT116	14.84
HCT15	17.81
SW480	18.26
SW1116	27.22
DLD1	28.58
CACO-2	30.90
D2	36.60
SW837	46.42
GP5D	47.22
CO115	53.95
HT29	120
LS174T	150

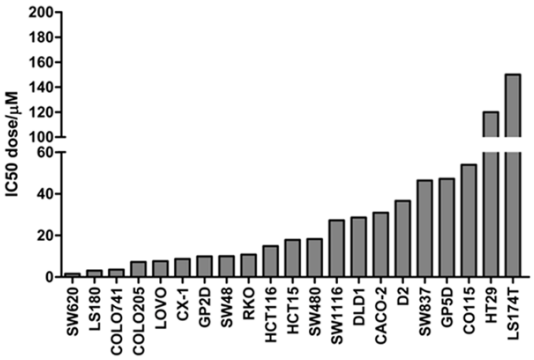

Figure 1. Determination of IC50 doses for 21 CRC cell lines. 21 CRC cell lines were treated with nine doses of irinotecan for 144 h, and then the cell viability was detected by MTS assay. The IC50 doses of these cell lines were calculated with the aid of GraphPad Prism 5.0 software via nonlinear regression.

trials. Hence it is of great interest to figure out irinotecan biomarkers in CRC cell lines in vitro.

In present work, 21 CRC cell lines with different sensitivities to irinotecan were subjected to RNA-seq. The basal expression of CRC cell lines was correlated with the irinotecan response and genes responsible for irinotecan sensitivity were predicted and validated by quantitative real-time PCR.

Materials and methods

Cell culture

The human CRC cell lines, SW620, LS180, COLO741, COLO205, LOVO, CX-1, GP2D, SW48, RKO, HCT116, HCT15, SW480, SW1116, DLD1, CACO-2, D2, SW837, GP5D, CO115, HT29, and LS174T were purchased from ATCC or China Center for Type Culture Collection. These cell lines were maintained in RPMI 1640 or DMEM medium (Gibco) supplemented with 10% FBS (Hyclone), penicillin (100 IU/ml) and Streptomycin (100 μg/ml) (Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells in the exponential growth phase were used for all the experiments.

Determination of IC50 dose by MTS assay

Cells (1×10^3/each well) were grown in 100 μl of RPMI 1640 or DMEM medium containing serum per well in a 96-well plate. After 24 h, the cells were treated with irinotecan (0, 0.0100, 0.0316, 0.100, 0.316, 1.00, 3.16, 10.0, 31.6, 100 μmol/L, respectively) for 144 h. Every treatment was triplicate in the same experiment. Then 20 μl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 1 to 4 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer's protocol. The cell viability was calculated relative to the untreated cells, respectively. The IC50 calculation was performed with GraphPad Prism 5.0 software via nonlinear regression.

RNA-seq

Cells (8×10^4) were grown in 2 ml of RPMI 1640 or DMEM medium containing serum per well in a 6-well plate with duplication. All the samples were homogenized with 1 ml Trizol (Invitrogen, Life Technologies) and total RNAs were extracted according to the manufacturer's instruction.

Preparation of cDNA followed the procedure described in Trapnell et al. [16]. The cDNA library was size-fractionated on a 2% TAE low melt agarose gel (Lonza catalog # 50080), a narrow slice (~2 mm) of the cDNA lane centered at the 300 bp marker was cut. The slice was

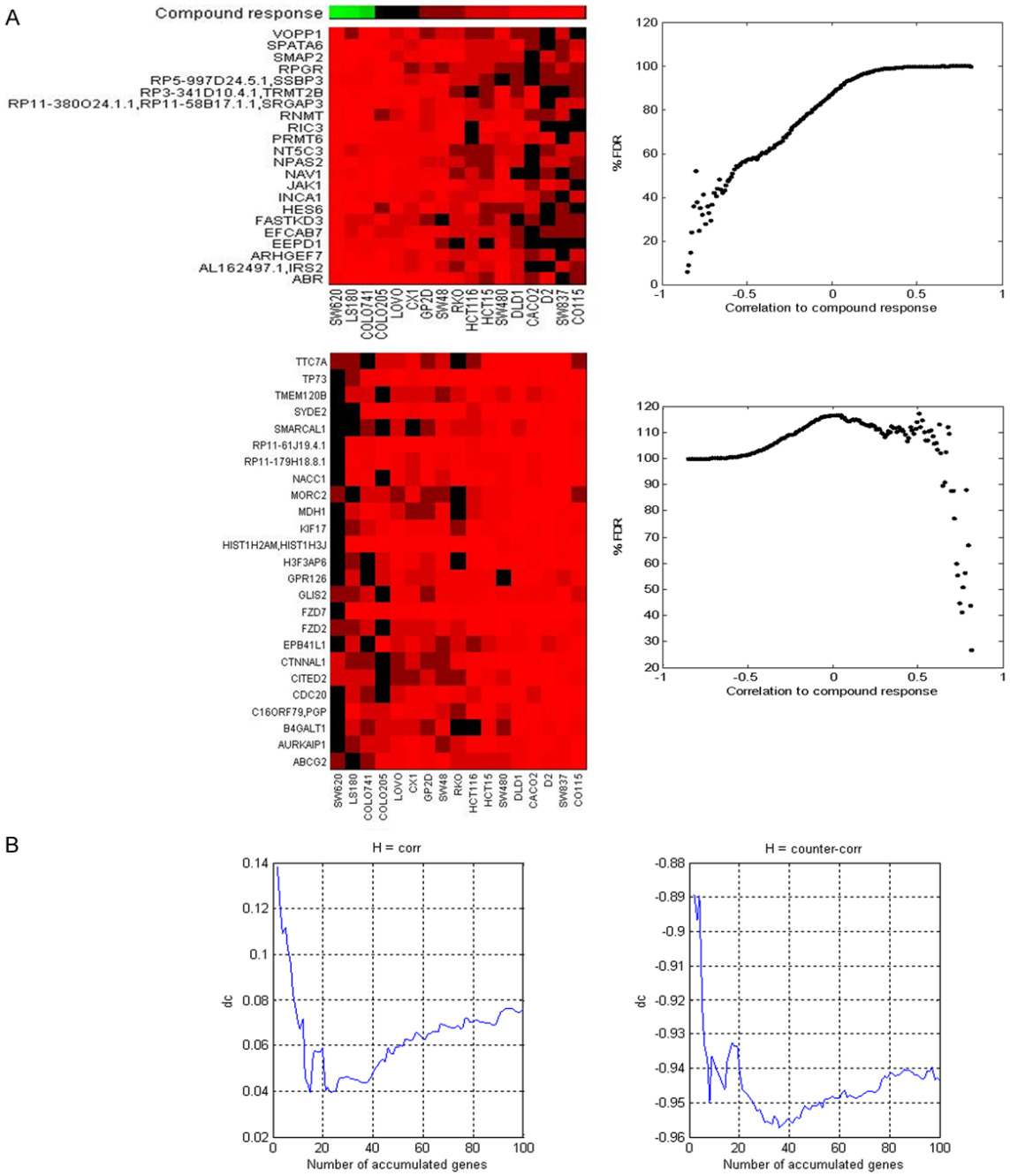

Figure 2. Identification of genes correlated with compound response. A: In upper panel, we showed that genes with expression counter-correlated with compound response (FDR < 20%); in lower panel we showed that genes with expression correlated with compound response (FDR < 30%). B: The correlation/counter-correlation was improved when gene expression were accumulated, where we are able to estimate the size of gene panel in the predictor.

extracted using the Qia Ex II kit (Qiagen catalog # 20021), and the extract was filtered over a Microcon YM-100 microconcentrator (Millipore catalog # 42409) to remove DNA fragments shorter than 100 bps. One-sixth of the filtered sample volume was used as template for 15 cycles of amplification using the paired-end primers and amplification reagents supplied with the Illumina ChIP-Seq genomic DNA prep kit. Each library was loaded into its own single Illumina flow cell lane, producing an average of 14.5 million pairs of 51-mer reads per lane (8.4

Table 2. Top 20 genes negatively correlated to irinotecan sensitivity

Gene symbol	d	d_c
CDC20	0.185	0.185
RP11-61J19.4.1	0.218	0.138
TP73	0.232	0.119
H3F3AP6	0.236	0.109
KIF17	0.239	0.112
AURKAIP1	0.246	0.102
SMARCAL1	0.270	0.096
CTNNAL1	0.292	0.083
MDH1	0.299	0.078
SYDE2	0.302	0.070
HIST1H2AM, HIST1H3J	0.322	0.067
FZD2	0.324	0.072
EPB41L1	0.326	0.046
B4GALT1	0.327	0.043
GPR126	0.328	0.040
FZD7	0.333	0.056
TTC7A	0.336	0.058
RP11-179H18.8.1	0.336	0.057
MORC2	0.340	0.057
CITED2	0.344	0.059

million purity filtered read pairs), or nearly 1.5 Gb of total sequence for each sample. Transcripts were assembled from the mapped fragments sorted by reference position.

Biocomputation for irinotecan sensitivity-related genes

We applied an elastic net regression algorithm combined with a bootstrapping procedure to derive predictive models that explained the drug sensitivity profiles based on the basal expression profiles investigated by RNA-seq, as described before [17]. Several parameters were calculated: Pearson correlation R between irinotecan IC50 doses and expression profile of some gene; Correlation distance (d = 1-R) between irinotecan response and the expression profile of some gene; Correlation distance (dc) accumulated between irinotecan response and the accumulated expression profiles among a selected set of genes; False discovery rate (FDR) when H (hypothesis) = gene expression significantly correlated with irinotecan response (H = corr); False discovery rate (FDR) when H = gene expression significantly counter-correlated with irinotecan response (H = counter-corr).

Quantitative real-time PCR (qPCR)

Total RNA above isolated was synthesized to cDNA using Prime Script RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR with mixture of oligo-dT and Random Primer (9 mer). The primers used for qPCR validation were list in Supplementary Table 1. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control Actb. Gene expression was calculated relative to expression of Actb endogenous control and adjusted relative to expression in COLO205 cells.

Results

21 CRC cell lines response differentially to irinotecan

21 CRC cell lines were treated with 9 different doses of irinotecan for 144 h, and then the cell viability was examined by MTS assay. The IC50 doses of these cell lines to irinotecan were calculated with the aid of GraphPad Prism 5.0 software via nonlinear regression (**Table 1** and **Figure 1**). The results showed that the sensitivities of these cell lines to irinotecan were distributed in nearly a normal fashion: SW620, LS180, COLO741, COLO205 and LOVO cell lines were sensitive to irinotecan, SW837, GP5D, CO115, HT29, and LS174T cell lines were resistant to irinotecan, while the other 11 CRC cell lines were moderate sensitive to irinotecan. This normal distribution of irinotecan sensitivity is very suitable for biomarkers searching by correlation between irinotecan response and gene expression profile.

RNA-seq and biocomputation for irinotecan-sensitivity-related genes

And then the basal expression profiles of 20 CRC cell lines (except SW1116 cell line) were investigated by RNA-seq. The gene expression was log2 transformed, the irinotecan sensitivities (IC50 doses) were log10 transformed, and these two sets of data were used for biocomputation of irinotecansensitivity-related genes (**Figure 2**). The process of data analysis was described in Material and Methods. The top 20 genes negatively and positively correlated to irinotecan sensitivity were list in **Table 2** and **Table 3**, respectively. The expression of CDC20, TP73, CTNNAL1, FZD7, and CITED2 was negatively correlated with irinotecan sensitivity, while the expression of ABR, ARHGEF7, and

Table 3. Top 20 genes positively correlated to irinotecan sensitivity

Gene symbol	1-d	$1\text{-}d_c$
EFCAB7	-0.861	-0.861
INCA1	-0.795	-0.889
ABR	-0.792	-0.897
EEPD1	-0.791	-0.890
HES6	-0.774	-0.923
RNMT	-0.769	-0.934
SPATA6	-0.749	-0.938
ARHGEF7	-0.747	-0.950
AL162497.1, IRS2	-0.747	-0.936
NPAS2	-0.747	-0.939
RPGR	-0.745	-0.941
RP5-997D24.5.1, SSBP3	-0.730	-0.943
JAK1	-0.729	-0.944
SMAP2	-0.727	-0.946
NAV1	-0.725	-0.938
NT5C3	-0.724	-0.935
RP3-341D10.4.1, TRMT2B	-0.722	-0.933
RP11-380O24.1.1, RP11-58B17.1.1, SRGAP3	-0.722	-0.934
VOPP1	-0.720	-0.934
RIC3	-0.719	-0.940

RNMT was positively correlated with irinotecan sensitivity.

qPCR validation

And then 7 genes (CDC20, CTNNAL1, FZD7, CITED2, ABR, ARHGEF7, and RNMT) were selected to perform qPCR in COLO205 and LS174T, two cell lines sensitive and resistant to irinotecan, respectively. The results showed that all the 7 genes' expression detected by qPCR assay was in line with the RNA-seq data: CDC20, CTNNAL1, FZD7 and CITED2 genes were highly expressed in LS174T, while ABR, ARHGEF7, and RNMT genes were highly expressed in COLO205 (**Figure 3**). Although ABR, RNMT and CDC20 genes were not too much differentially expressed between these two cell lines, the expression difference was significant ($p < 0.05$, data not shown). The most significantly expression-altered genes were ARHGEF7 and CITED2, the expression of these two genes was 0.13-fold and 3.40-fold in LS174T cells compared to that in COLO205 cells.

Discussion

Irinotecan is a topoisomerase I inhibitorapproved worldwide as a first- and second-line chemotherapy for advanced or recurrent CRC. Although irinotecan showed significant survival advantage for patients, a relatively low response rate and severe adverse effects demonstrated the urgent need for biomarkers searching to select the suitable patients who can benefit from irinotecan-based therapy and avoid the adverse effects.

In present work, the irinotecan response (IC50 doses) of 20 CRC cell lines were correlated with the basal expression profiles investigated by RNA-seq to figure out genes responsible for irinotecan sensitivity/resistance. Genes negatively or positively correlated to irinotecan sensitivity were given after biocomputation, and 7 of them were validated in two CRC cell lines by qPCR.

Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (CITED2), a novel MYC-interacting transcriptional modulator, functions as a molecular switch of transforming growth factor-α (TGF-α)-induced proliferation and transforming growth factor-β (TGF-β)-mediated quiescence. Ectopic CITED2 expression enhanced tumor growth in nude mice; furthermore, CITED2 knockdown caused tumor shrinkage and increased overall host mouse survival rates. Expression of CITED2/MYC/E2F3/p21 (CIP1) signaling molecules was associated with poor prognosis of lung cancer patients [18]. Moreover, CITED2 expression has been associated with the resistance to cisplatin in tumor cells [19, 20] and *in vivo* metastasis of breast cancer cells and *in vitro* invasion of colon cancer cells [21, 22]. These reports suggested that CITED2 is implicated in tumor initiation and progression, chemotherapy resistance, metastasis and prognosis of various cancers. In our work, CITED2 was highly expressed in irinotecan-resistant CRC cells, suggesting that CITED2 may serve as a potential biomarker for irinotecan resistance. The underlying mechanism by which high expression of CITED2 confer cancer cells resistance warrants further investigation in more cancer cell lines and tissues. Previous studies have demonstrated that expression of several crucial molecules is regulated by CITED2, such as NF-κB [23], estrogen receptor [24], OCT4 [25], HIF1α [26] and VEGF [27]. In our opinion, TGF-α signaling and NF-κB signaling pathways may be

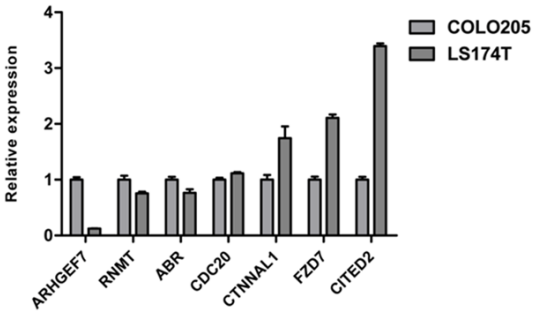

Figure 3. qPCR validation. 7 genes were validated in two CRC cell lines (COLO205 and LS174T) by qPCR. The gene expression was calculated relative to that in COLO205 cells.

suitable targets for next research to uncover the mechanism of CITED2 function.

Catenin, alpha-like 1 (CTNNAL1), also called as α-Catulin, is proposed to downregulate E-cadherin and hence promote melanoma progression and invasion [28], drive metastasis by activating ILK and driving an αvβ3 integrin signaling axis in non-small cell lung cancer and head and neck squamous cell carcinoma [29, 30]. Furthermore, as a Rho signaling component, CTNNAL1 can regulate NF-κB through binding to IKK-beta, and confers resistance to apoptosis [31]. CTNNAL1 promotes tumor growth by preventing cellular senescence, and its knockdown induces senescence in cancer cells [32]. In our results, CTNNAL1 was highly expressed in irinotecan-resistant CRC cell lines, which is in line with previous reports in theory. The mechanism by which CTNNAL1 confer resistance to irinotecan may be lie in EMT signaling and NF-κB signaling pathways, which needs further study.

FZD7, receptor for Wnt signaling proteins, has a critical role in cell proliferation in triple negative breast cancer [33], its downregulation decreases survival, invasion and metastatic capabilities of colon cancer cells [34]. FZD7 has been suggested to be a potential therapeutic target in colorectal cancer [35]. Therefore, it is reasonable that the high expression of FZD7 confer CRC cells resistance to chemotherapy drugs.

Taken together, high expression of CITED2, CTNNAL1 and FZD7 may promote CRC cells proliferation and hence confer CRC cells resistance to irinotecan, which warrants further investigation in more cancer cell lines and tissues. Our work might provide potential biomarkers and therapeutic target for irinotecan sensitivity in CRC cells.

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Disclosure of conflict of interest

We have no conflict of interest to declare.

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Supplementary Table 1. Primers for qPCR validation

Gene	Forward	Reverse
Actb	CACCATGTACCCTGGCATT	GTACTTGCGCTCAGGAGGAG
ABR	CAAAGTGAAAGGTCCCAAGG	ATGGGCTTGTAGAGCAGAGC
ARHGEF7	GCCTGGATAAATACCCTACGC	GGATGGCTTCCGTCAGGAT
CDC20	GTGGATTGGAGTTCTGGGAATG	AGAGCTTGCACTCCACAGGTACA
CITED2	TCGTTTTTGTAGCCTTGACATTC	AACAACGAAAAAGACCAAGTTAGC
CTNNAL1	AAAGCCAGACAAGCCTGACTCT	AGCAAACCCAGCTTAAGTCCAA
FZD7	GCGATGTGAATCGTCAAAGGT	CTCGGAGCCGGGAGAAAC
RNMT	CTTGTGCATTTGGGTTATCTCAAG	CTGGATTTGGCCTCTATTTTGC