Original Article
RNA-seq identifies determinants of oxaliplatin sensitivity in colorectal cancer cell lines

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Abstract: Oxaliplatin-based chemotherapy, such as FOLFOX, is the first-line therapy for advanced colorectal cancer (CRC) or metastatic CRC patients. However, the partial response of patients to these regimes and the severe peripheral neuropathy toxicity induced by oxaliplatin makes it urgent to figure out biomarkers for oxaliplatin sensitivity to select suitable patients who benefit from these treatments. In present work, 21 CRC cell lines with different sensitivities to oxaliplatin were applied to RNA-seq. The basal expression profiles of these cell lines were correlated to their response to oxaliplatin. Bioinformatics analysis suggested that expression of 58 genes was correlated, negatively or positively, to oxaliplatin response across the 21 CRC cell lines. These 58 genes were mainly enriched in small molecules biochemistry, Wnt/β-catenin signaling and EMT pathways. The latter two pathways were predicted to be activated in oxaliplatin-resistant CRC cell lines. Moreover, 15 genes were validated by qPCR that their expression levels were actually closely correlated to their response to oxaliplatin, in line with the biocomputation prediction. Taken together, our work might provide potential biomarkers for oxaliplatin sensitivity in CRC cell lines and therapeutic targets for combinational therapy with oxaliplatin.

Keywords: Colorectal cancer, oxaliplatin, RNA-seq

Introduction

Colorectal cancer (CRC) is one of the most common malignant diseases, with more than 1 million new cases and approximately 492000 patients’ death annually, and is the fourth cause of cancer-related deaths worldwide [1-3]. Approximately 5-25% of newly diagnosed CRC cases present with advanced disease [4], prognosis for these patients remains poor. Oxaliplatin, a third-generation platinum compound, is approved by FDA for adjuvant therapy of stage III CRC or initial therapy of advanced or metastatic CRC (aCRC or mCRC). Combination chemotherapy with oxaliplatin, fluorouracil (FU), and leucovorin (LV), known together as FOLFOX, or with oxaliplatin and capecitabine, known together as CapeOX, is first line therapy for aCRC or mCRC in National Comprehensive Cancer Network (NCCN) Guidelines Version 3.2012. Clinical trials showed that oxaliplatin-based combination chemotherapy exhibits 28-50% of objective response rate for aCRC or mCRC patients [5-7], that is, more than half of patients response poor to oxaliplatin-based therapy. Furthermore, Peripheral neuropathy (PN) is currently recognized amongst the major non-hematological dose-limiting toxicities of oxaliplatin [8-10]. Hence, it is of great importance to figure out oxaliplatin biomarkers to increase the response rate and to decrease the toxicity.

Oxaliplatin is proposed to exert its antitumor activity by cell cycle effects, the formation of DNA adducts and interstrand cross-links and the role of DNA repair proteins [11]. ERCC1 is a highly conserved protein and is an essential member of the nucleotide excision repair (NER) pathway, one of the major DNA repair systems in mammalian cells. The mRNA expression of ERCC1 has been demonstrated to have significant independent correlation with overall survival after FU/oxaliplatin therapy in patients with aCRC refractory to first-line chemotherapy [12]. However, there are contradictory reports
on the protein expression of ERCC1 to the response of CRC patients to oxaliplatin [13, 14]. Additionally, the mRNA expression of G-protein-coupled receptor galanin receptor 1 (GalR1) [15] and CXCR4 [16], and the protein expression of Bmal1 [17], XPF [18], topoisomerase-1 (Topo1) [19] and FAS [20], are associated with the response of CRC patients or cell lines to oxaliplatin. Despite several biomarkers were suggested to be involved in the response of cancer cells to oxaliplatin, none was validated in prospective clinical trials. Meanwhile, inconsistent reports shadow our understanding on these oxaliplatin biomarkers. Therefore, it is of great interest to uncover oxaliplatin markers in CRC cell lines.

RNA-sequencing (RNA-seq) is a technology that uses the capabilities of next-generation sequencing to reveal a snapshot of RNA presence and quantity from a genome at a given moment in time. RNA-seq is widely used for biomarkers finding [21]. In present work, 21 CRC cell lines with different sensitivities to oxaliplatin were subjected to RNA-seq. The basal expression of CRC cell lines was correlated with the oxaliplatin response and genes responsible for oxaliplatin sensitivity were predicted and validated by quantitative real-time PCR.

Materials and methods

Cell culture

The human CRCC 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% CO2 at 37°C. Cells in the exponential growth phase were used for all the experiments.

Determination of IC50 dose by MTS assay

Cells (1x10^4/eight 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 oxaliplatin (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 (8x10^4) were grown in 2 ml of 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 [22]. The cDNA library was size-fractionated on a 2% TAE low melt agarose gel (Lanza catalog # 50080), a narrow slice (~2 mm) of the cDNA lane centered at the 300 bp marker was cut. The slice was extracted using the QiaEx 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 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 oxaliplatin 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 [23]. Several parameters were calculated: Pearson correlation $R$ between oxaliplatin IC50 doses and expression profile of some gene; distance ($d = 1 - R$) between oxaliplatin response and the expression profile of some gene; Relative distance: accumulated between oxaliplatin response and the accumulated expression profiles among a selected set of genes; False discovery rate (FDR) when $H$ (hypothesis) = gene expression significantly correlated with oxaliplatin response ($H = \text{corr}$); False discovery rate (FDR) when $H = \text{gene expression significantly counter-correlated with oxaliplatin response (H = counter-corr)}$.

**Quantitative real-time PCR (qPCR)**

Total RNA above isolated was synthesized to cDNA using PrimeScript 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
Determinants of oxaliplatin sensitivity in CRC cell lines

21 CRC cell lines were treated with 9 different doses of oxaliplatin for 144 h, and then the cell viability was determined by MTS assay. The IC50 doses of these cell lines to oxaliplatin were calculated with the aid of GraphPad Prism 5.0 software via nonlinear regression. The results showed that these cell lines harbor dramatic difference in sensitivity to oxaliplatin: SW620, LS174T and LOVO cell lines are sensitive to oxaliplatin, while HCT116, SW480 and D2 cell lines are resistant to oxaliplatin, the other cell lines are moderate sensitive to oxaliplatin (Figure 1). The IC50 dose of D2 cell line is 30-fold higher than that of SW620 cell line. Furthermore, the sensitivities of these cell lines to oxaliplatin were distributed in nearly a normal fashion. This normal distribution of oxaliplatin sensitivity is very suitable for biomarkers searching by correlation between oxaliplatin response and gene expression profile.

RNA-seq and biocomputation for oxaliplatin-responsible genes

And then the basal mRNA expression profiles of 21 CRC cell lines were investigated by RNA-seq. The gene expression was log2 transformed, the oxaliplatin sensitivities (IC50 doses) were log10 transformed, and these two sets of data were used for biocomputation of oxaliplatin-responsible genes (Figure 2). The process of data analysis was described in Material and Methods. Those genes with distance>1.67 and relative distance>0.10 were designed as expression-negatively correlated genes to oxaliplatin sensitivity, whereas those genes with distance<0.33 and relative distance<-0.10 were designed as expression-positively correlated genes to oxaliplatin sensitivity. There were 45 and 13, respectively, expression-negatively and expression-positively correlated genes. These 58 genes were list in Table 1.

IPA showed that Wnt/β-catenin signaling and EMT pathways were activated

And then these 58 genes were subjected to the Ingenuity Pathway Analysis (IPA). The IPA results showed that these genes were mainly enriched in small molecules biochemistry (cholesterol and cysteine biosynthesis), Wnt/β-catenin signaling and epithelial-mesenchymal transition (EMT) pathways (Figure 3A). The Wnt/β-catenin signaling and EMT pathways were predicted to be activated, based mainly on the highly expression of FZD5, NOTCH1, and HNF1A in the oxaliplatin-resistant cell lines (Figure 3B and Supplementary Figure 1).

qPCR validation

And then 16 genes were selected to validate their predictive effects on oxaliplatin sensitivity in COLO741 and LS174T cell lines by qPCR assay. Among the 16 genes, 11 genes were
Determinants of oxaliplatin sensitivity in CRC cell lines

predicted to be expression-negatively correlated to oxaliplatin sensitivity, while the other 5 genes (AP1M1, C190RF25, CYR61, FZD2 and KCND1) were predicted to be expression-positively correlated to oxaliplatin sensitivity. LS174T is sensitive to oxaliplatin, while COLO741 is relative resistant to oxaliplatin. The fold changes of expression in LS174T relative to COLO741 were log2 transformed and plotted (Figure 4). The qPCR results showed that expression trends of 15 out of these 16 genes were consistent between RNA-seq data and qPCR data, except that of C170RF25. These results suggested that the biocomputation prediction on oxaliplatin-responsible genes was reliable. FZD5, NOTCH1, and HNF1A were validated to be highly expressed in oxaliplatin-resistant cell lines.

Discussion

Oxaliplatin-based chemotherapy, such as FOLFOX, is the first-line therapy for aCRC or mCRC patients. However, the partial response of patients to these regimes and the severe peripheral neuropathy toxicity induced by oxaliplatin makes it necessary to select suitable patients who benefit from these treatments. Therefore, it is great importance to figure out biomarkers for oxaliplatin sensitivity to improve the therapy.
In present work, 21 CRC cell lines with different sensitivities to oxaliplatin were applied to RNA-seq. The basal expression profiles of these cell lines were correlated to their response to oxaliplatin. Bioinformatic analysis suggested that 58 genes were correlated, negatively or positively, to oxaliplatin response across the 21 CRC cell lines. These 58 genes were proposed by IPA to be mainly enriched in small molecules biochemistry, Wnt/β-catenin signaling and EMT pathways. The latter two pathways were predicted to be activated in oxaliplatin-resistant CRC cell lines. Moreover, 15 genes were validated by qPCR that their expression levels were actually closely correlated to their response to oxaliplatin, in line with the biocomputation prediction.

Within the 58 oxaliplatin-responsible genes, several genes other than FZD5, NOTCH1 and HNF1A deserve further investigation. CYP51A1 encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. Several cytochrome P450 genes, such as CYP24A1 [24], CYP2W1 [25] and CYP1A1 [26], were positively correlated to resistance to chemotherapy drugs in various cancers. MYB encodes a transcriptional factor. MYB protein plays an essential role in the regulation of hematopoiesis and may play a role in tumorigenesis. MYB expression has been proposed to be associated with the castration resistance of prostate cancer cells [27]. Furthermore, MYB is suggested to regulate the expression of EMT-related genes [28]. PARP15 is a macrodomain-containing transcriptional repressor with poly (ADP-ribose) polymerase activity [29]. PARP family member has been suggested to modulate resistance of cancer cells to chemotherapy drug [30], therefore, the combination treatments with PARP inhibitors and some chemotherapy drugs are widely used regimens in some types of cancer. In our data, the mRNA expression of CYP51A1, MYB and PARP15 was negatively correlated with response of CRC cell lines to oxaliplatin, consistent with above reports.

Taken together, our work might provide potential biomarkers for oxaliplatin sensitivity in CRC cell lines and therapeutic targets for combinational therapy with oxaliplatin.

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Disclosure of conflict of interest

None.

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References

Determinants of oxaliplatin sensitivity in CRC cell lines


Determinants of oxaliplatin sensitivity in CRC cells


Supplementary Table 1. Primers for qPCR validation

<table>
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<tr>
<th>GENE</th>
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<tr>
<td>FZD5</td>
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Supplementary Figure 1. EMT was predicted to be activated in oxaliplatin-resistant cell lines.