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Experimental Exploration for Genes Related to Susceptibility and Resistance to Irinotecan

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Abstract. *Background/Aim: Anticancer drug resistance is an important issue in cancer treatment. Multiple genes are thought to be involved in resistance to anticancer drugs; however, this is still not fully understood. This study aimed to identify the genes involved in irinotecan resistance and their functional characteristics. Materials and Methods: Gene trap insertion mutant Chinese hamster ovary (CHO) cells were used in the experiments, and next-generation sequencing, gene-ontology enrichment, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were used to evaluate the biological functions of differentially expressed genes (DEGs). Results: In total, 2,134 DEGs were identified, including 1,216 up-regulated and 918 down-regulated genes. In KEGG pathways, microRNAs in cancer were significantly associated with up-regulated DEGs, while spliceosome and p53 signaling pathways were significantly associated with down-regulated DEGs. The pathway analysis identified several genes that might be involved in irinotecan resistance. Conclusion: Using CHO cells, the genes involved in irinotecan resistance and functional characteristics were predicted. These results provide new clues for predicting irinotecan resistance.*

Chemotherapy is a major component of cancer treatment strategies, along with surgery and radiotherapy (1). However, acquisition of resistance to anticancer drugs is an important issue in the treatment of malignant tumors, and the mechanism underlying resistance to non-specific cytotoxic anticancer drugs remains unclear.

For example, irinotecan is one of the cytotoxic anticancer agent. It is a key anticancer drug used to treat various types of cancers (2), and there are several reports on its mechanism of resistance acquisition (3); however, some aspects remain unclear. Therefore, elucidating all probable mechanisms of decreased sensitivity and resistance to irinotecan is an important issue in cancer treatment.

Chinese hamster ovary (CHO) cells are susceptible to genetic mutations caused by external gene transfection, and it is easy to analyze functional changes in these cells due to gene loss (4). For this reason, CHO cells have been commonly used to analyze the biological functions of drugs.

The aim of the study was to elucidate the genes associated with irinotecan resistance by using random genetic mutations CHO cell line. This study may reveal new mechanisms involved in irinotecan resistance, and it is hoped that this research will lead to the improvement and overcoming of cancer resistance to irinotecan.

Materials and Methods

Obtaining and maintaining cells and chemicals for use. Gene trap insertion mutant CHO cell lines were developed and kindly provided by Nobukuni *et al*. (4). Irinotecan hydrochloride hydrate, an antineoplastic agent, was obtained from Nippon Kayaku Co., (Tokyo, Japan).

Enrichment of anticancer irinotecan-insensitive cells and cloning. Gene trap insertion mutant CHO cells were spread on a 100-mm dish, incubated for 48 h, and subsequently reseeded in multiple dishes at 1×10^7 cells per 100-mm dish. A 60-fold diluted irinotecan solution was added to a 100-mm dish containing CHO cells and allowed to incubate for 4 h. Following a medium change, the cells were incubated for another 24 h. After 24 h, 60-70% of the surviving cells in the dish were passaged, and the same drug treatment procedure was repeated thrice to generate a pool of irinotecan-insensitive mutant cells. These mutant cells were defined as irinotecan-resistant CHO cell lines. The cloning of each cell line was performed by diluting and culturing the cell pool obtained by enrichment of wild-type and irinotecan-insensitive cells.

Total RNA isolation and sample adjustment. Total RNA was extracted from each clone using TRIzol Reagent (Invitrogen) and purified using the SV Total RNA Isolation System kit (Promega, Madison, WI, USA). Only rRNA was removed from the total RNA

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using rRNA sequence-specific oligos and mRNA was purified using the MGIEasy rRNA Depletion Kit (MGI Tech, Shenzhen, Guangdong, PR China). The library was created based on a protocol that maintains information about the direction of RNA transcription (MGIEasy RNA Directional Library Prep Set).

Next-generation sequencing (NGS) sequencing and data analysis. MGI DNBSEQ G400 FAST (MGI Tech, Shenzhen, PR China) was used to sequence the prepared libraries and obtain raw data in the FASTA format (Next-generation sequencing, read type: paired end, read length: 150b). Trimmomatic v 0.38 (5) was used to evaluate the quality of the sequencing reads and trimmed unnecessary sequences, such as the sequence of the sequencing adapter. Using hisat2 2.1.0, trimmed reads were mapped to the reference genome (CriGri-PICRH-1.0) sequence. RSEM 1.3.0, bowtie2 (6) was used to count the reads mapped to each gene in the reference genome. EdgeR (7) was used to normalize the read counts for each gene and calculate the count per million (CPM) value. This value was used for the comparative analysis of gene expression to calculate the *p*-values and differentially expressed genes (DEGs). We established the criteria for DEGs ($p<0.05$).

DAVID analysis and KEGG analysis. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/home.jsp) was used to visualize and intuitively analyze the characteristics of gene groups with expression variation. In this study, we analyzed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using DAVID. Statistical significance of the collected data from the DAVID analysis of GO and KEGG pathways was calculated using Fisher's exact test. Statistical significance was set at $p<0.05$.

Results

Number of differentially expressed genes obtained by sequencing. As described in the Materials and Methods section, four clones of wild-type cells and eight clones of irinotecan-insensitive cells were constructed. We examined the variation in expression at the mRNA level and identified 15,016 DEGs between wild-type and irinotecan-insensitive cells. Of the 15,016 genes, 2,512 DEGs showed statistically significant differences in expression between two types of cells. Of the 2,512 DEGs, there were 2,134 genes assigned to DAVID IDs. Of the 2,134 DEGs, there were 1,216 genes in irinotecan-insensitive cells with up-regulated reads relative to those in wild-type cells, and 918 genes were found in irinotecan-insensitive cells with down-regulated reads. The heat map of all 2,512 DEGs that showed significant differences is shown in Figure 1.

Functional annotation of gene ontology. Functional annotation using DAVID analysis was performed to clarify the types of functional changes that could be caused by gene alterations. Functional annotation of GO showed six terms classified by biological process (BP), of which significant differences were observed in intracellular signal transduction (GO: 0035556, $p=0.017$), response to heat (GO:0009408, $p=0.044$), and negative regulation of transcription, DNA-templated (GO:0045892, $p=0.048$) (Table I).

Functional annotation of KEGG pathway. To delve deeper into the functional annotation, KEGG pathway analysis was performed among the 2,134 DEGs. The main pathways commonly associated with the up-regulated DEGs were biosynthesis of antibiotics ($p=3.6\times10^{-9}$), carbon metabolism ($p=4.3\times10^{-7}$), biosynthesis of amino acids ($p=6.7\times10^{-6}$), microRNAs (miRNAs) in cancer ($p=5.6\times10^{-4}$), and glycerophospholipid metabolism ($p=6.6\times10^{-4}$). The main pathways commonly associated with down-regulated DEGs were spliceosome ($p=8.0\times10^{-5}$), p53 signaling pathway ($p=1.4\times10^{-3}$), RNA transport ($p=2.5\times10^{-3}$), protein processing in endoplasmic reticulum ($p=1.1\times10^{-2}$), and viral carcinogenesis ($p=1.1\times10^{-2}$). The gene trap vectors in CHO cells were randomly transfected with reporter vectors, and the disrupted genes were identified to analyze which gene functions had changed. Therefore, we focused on the down-regulated genes in the spliceosome and p53 signaling pathways.

DEGs and microRNAs potentially involved in irinotecan resistance. Loss of spliceosome function leads to increased expression of noncoding RNAs and microRNAs. Therefore, further DAVID analysis was performed to identify miRNAs associated with DEGs. In the pathway of miRNAs in cancer, the associations between DEGs and miRNAs are shown in Table II. In total, five miRNAs (Lte-7, microRNA-34/326, and microRNA-200/205) were selected for theoretical analysis of up-regulated DEGs (HMGA2, NOTCH, and ZEB2), which were possibly involved in multiple functions.

p53 signaling pathway - Cricetulus griseus (Chinese hamster). The p53 signaling pathway, one of the pathways that showed significant differences in the DAVID analysis, is shown in Figure 2. As shown in the figure, APAF-1, BBC3, BCL2L1, CHEK2, CCND2, CCNB2, and CCNG2 were up-regulated genes in the pathway. In contrast, GADD45, PIDD1, SERPINE1, CCNE2, CDK2, Noxa, ZMAT3, SESN3, and PPM1D were down-regulated. According to the total number of up- and down-regulated genes, the p53 signaling pathway was evaluated using the down-regulated pathway in the analysis.

Discussion

In the present study, we screened mutants resistant to irinotecan to elucidate the related genes and molecular mechanisms underlying irinotecan sensitivity and/or resistance. The results showed 1,216 up-regulated DEGs and 918 down-regulated DEGs between wild-type cells and irinotecan-insensitive cells. KEGG pathway analysis using the DAVID tool showed that microRNAs, the p53 pathway,

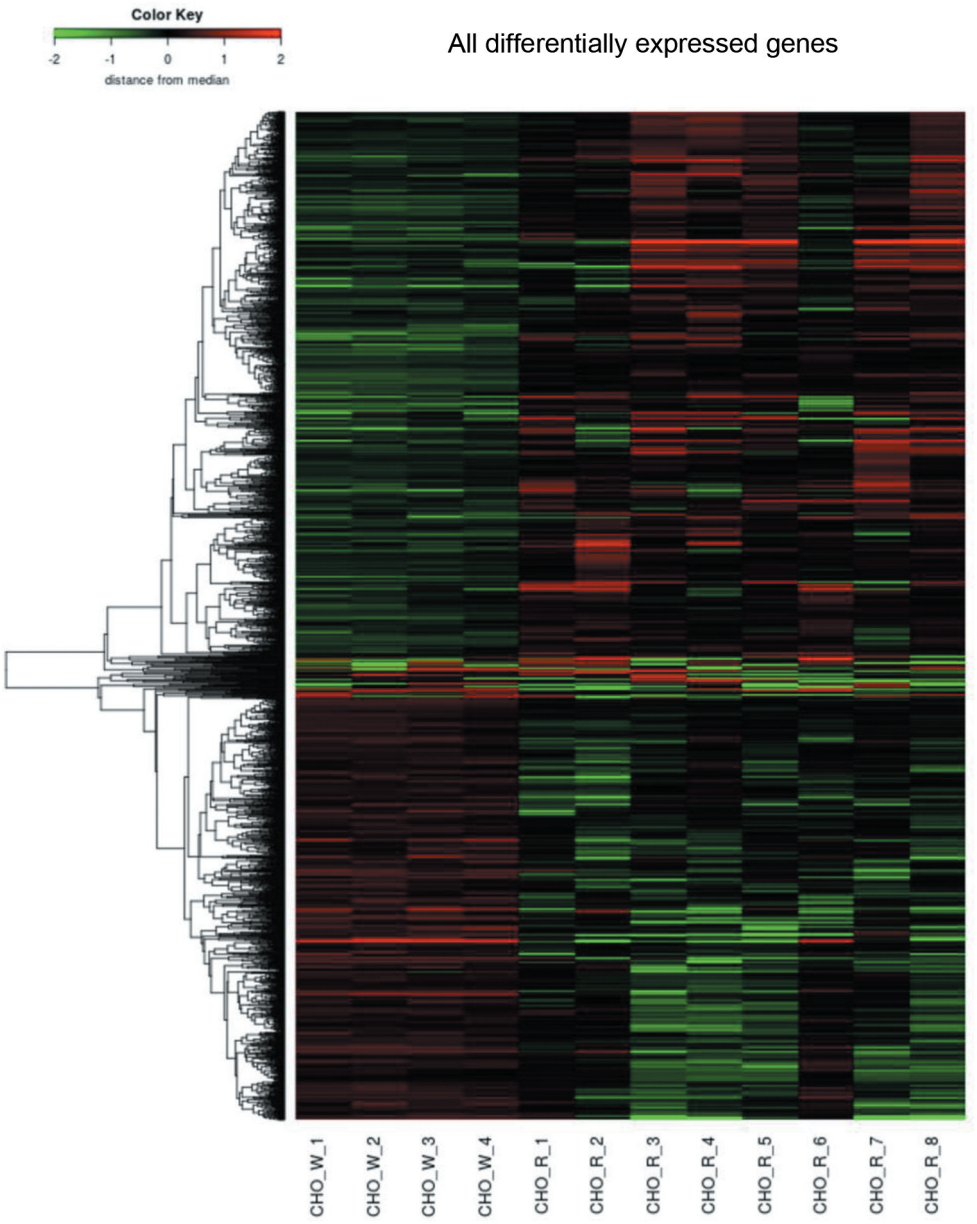

Figure 1. *Heat-map of all differentially expressed genes (DEGs). The four rows on the left represent wild-type cells and the eight rows on the right represent irinotecan-insensitive cells. The amount of gene expression between the two cell types was targeted.*

and several DEGs in the pathway may be involved in irinotecan resistance.

In GO analysis, intracellular signal transduction, response to heat, negative regulation of transcription, and DNA-templated were extracted as groups with significant differences. Among the genes related to intracellular signal transduction that showed the most significant differences in GO analysis, Akap13, Arhgap29, Arhgef2, Ect2, and Neurl2 all belonged to the Rho GTPase signaling pathway. This pathway is involved in the formation of actin-rich membrane protrusions and the invasion of cancer cells (8). Alterations in the Rho pathway include the epithelial mesenchymal transition (EMT) itself, which has been reported as a cause of multiple anticancer drug resistance (9). Functional analysis of GO in this study suggests that EMT is involved in the development of irinotecan resistance; in particular, the Rho pathway may be important in the acquisition of resistance. Huang *et al*. performed RhoA functional analysis of the acquisition of irinotecan resistance in a colorectal cancer cell line (10), which supports the present results.

Table I. *Functional annotation chart of gene ontology.*

Gene ontology term - Biological process		*p*-Value[a]	Gene symbol
Intracellular signal transduction	[GO:0035556]	0.017	Akap13, Depdc5, Arhgap29, Arhgef2, Shc1, Def8, Ect2, Myo9a, Neurl2, Net1, Psen2, Spsb1, Socs1, Socs4
Response to heat	[GO:0009408]	0.044	Dnaja1, Dnaja3, Hsp90aa1, Hspa2
Negative regulation of transcription, DNA-templated	[GO:0045892]	0.048	Cbfa2t2, Limd1, Hspa8, Hmga1, Id3, Nelfb
Response to cold	[GO:0009409]	0.058	Hsp90aa1, Hspa2, Hspd1
Regulation of transcription from RNA polymerase II promoter	[GO:0006357]	0.075	Fos, Ldb1, Atf3, Atf4, Atf5, Batf, Irf5
DNA-templated transcription, initiation	[GO:0006352]	0.091	Taf12, Tbpl1

[a]To compare categorical data among all genes, the Fisher's exact test was performed.

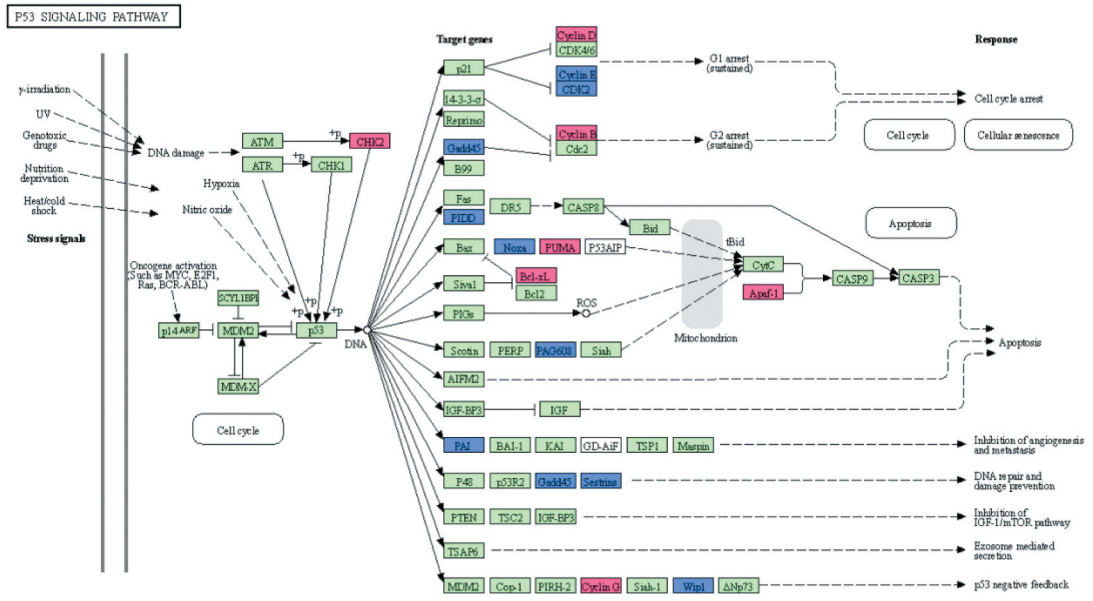

Figure 2. *Pathway map of p53 signaling pathway. Colored genes (green, red, and blue) represent those found in CHO cells. Genes shown in red: significantly up-regulated read counts in irinotecan-insensitive cells compared to wild-type cells. Genes shown in blue: significantly down-regulated read counts in irinotecan-insensitive cells compared with wild-type cells. CHO, Chinese hamster ovary; UV, ultraviolet radiation; IGF-1, insulin-like growth factor 1; mTOR, mammalian target of rapamycin.*

Functional annotation of KEGG pathway analysis showed that pathways associated with up-regulated DEGs were mainly biosynthesis of antibiotics and miRNAs in cancer, while the pathways associated with down-regulated DEGs were mainly the spliceosome and p53 signaling pathways. In the miRNA pathway in cancer, HMGA2, NOTCH, and ZEB2 were identified as up-regulated DEGs commonly found in multiple functions. Among these genes, HMGA2 has been reported to protect cancer cells from the topoisomerase type 1 poisoning effect of irinotecan (11). NOTCH has been reported to modulates irinotecan sensitivity *via* multidrug resistance-associated proteins (12). ZEB2 is also involved in EMT (9), which is implicated in resistance to several anticancer drugs.

On the other hand, miRNAs predicted to regulate these genes were Let-7, microRNA-34/326, and microRNA-200/205. There have some experimental reports of attempts to improve chemosensitivity by targeting Let-7 (13). MicroRNA-34 targets NOTCH in ovarian cancer and has been noted to function as a tumor suppressor gene (14). Others have reported that microRNA-200 is involved in oxaliplatin resistance in colorectal cancer (15). In addition to these genes, the present analysis identified several other up-regulated genes that have not been reported for the

Table II. *DEGs and miRNAs potentially involved in irinotecan resistance*.

Differentially expressed genes		Assumed affected microRNAs
Up-regulated	Down-regulated	
HMGA2		Let-7
NOTCH		miRNA34, miRNA-326
ZEB2		miRNA-200, miRNA-205
HDAC4, FoxP1		miRNA-1
VEGF-A		miRNA-126
DDIT4		miRNA-221
CAT-1, HO-1		miRNA-122
SOCS1		miRNA-155
MRP1		miRNA-7, miRNA-345
RASSF1		miRNA-10
RPS6KA5		miRNA-103
PDGFRA		miRNA-29
SOS2		miRNA-195, miRNA-143
SHC1		miRNA-7
EZH2		miRNA-101
	ITGA5	miRNA-31
	CDC25	Let-7
	CONE2	miRNA-15/16
	CYP24	miRNA-125
	PLAU	miRNA-193
	SIRT1	miRNA-34

DEGs, Differentially expressed genes; miRNA, microRNA.

acquisition of chemotherapy resistance, and these genes may also contribute to overcoming irinotecan resistance through microRNA-mediated gene regulation.

As p53 is known to be the master regulator of cells, it is involved in many cellular processes, and is closely related to cancer development and anticancer drug resistance (16). In this analysis, down-regulation of nine genes (CCNE2, CDK2, GADD45, PIDD1, Noxa, ZMAT3, SERPINE1, SESN3, and PPM1D) in the p53 signaling pathway was observed. Among these genes, GADD45 and CCNE, which belong to the cell cycle pathway, have been reported to be involved in anticancer drug resistance (17, 18). Similarly, it has been reported that Noxa, which belongs to the apoptotic pathway, is involved in reducing the efficacy of irinotecan therapy through its inactivation (19). Among the many functions of p53, the cell cycle and apoptosis are known to be some of the key functions in cancer development and anticancer drug resistance (20). Therefore, these down-regulated genes, which are inferred to be involved in irinotecan resistance in this study may be useful as biomarkers of irinotecan resistance.

The present study had several limitations. First, most of the findings in this study were obtained from cell culture systems using CHO cells. Confirmation experiments after candidate gene extraction should be performed using normal human-derived cancer cell lines. However, as for the results, there may not be a significant difference, as functions related to anticancer drug resistance have been shown in human cancer cells. Second, in recent years, cancer cells have attracted attention for their ability to evade the immune system (21). Therefore, it is possible that the results of this study cannot be reproduced using cancer cells, for example, the genes suggested to be involved in irinotecan resistance in each pathway do not match. In the future, it will be necessary to confirm the reproducibility of this study using human cells and even human cancer cells.

In conclusion, the present study identified DEGs that might be associated with irinotecan resistance. The identification of genes related to irinotecan resistance and susceptibility will provide new clues to overcome irinotecan resistance and lead to the development of new methods for predicting drug susceptibility.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Study concept and design, analysis, and interpretation of data, drafting and revision of the article: Ogata S, Sudo T, Nobukuni Y, and Akagi Y. Cell management and experimentation, revising the manuscript: Ogata S, Sudo T, Nobukuni Y, and Ogata N. Provided important suggestions on the study and revised the manuscript: Fujita F, Yoshida T, Koushi K, Noguchi T, and Fukuda J. All Authors read and approved the final manuscript.

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