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Putative chemosensitivity predictive genes in colorectal cancer cell lines for anticancer agents

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Abstract. In order to identify genes which could predict chemosensitivity in colorectal cancer, gene expression and chemosensitivity were examined in colorectal cancer cell lines. Gene expression profiling of 5 colorectal cancer and 3 normal cell lines was performed using a 22K spotted oligonucleotide microarray. The IC_{50}s of 17 anticancer drugs were determined using the MTT assay for chemosensitivity. The SOURCE database, KEGG Pathway database, and Molecular Diagnosis Score (MDS) were used for data analysis. Two representative colorectal cancer cell lines were identified which were resistant or sensitive to drugs commonly used for colon cancer treatment (5-FU, irinotecan and topotecan). Six hundred and eighty-three genes that were up- or down-regulated by >4-fold between the two cell lines were selected. Pathway analysis was performed with 147 of the 683 genes using the KEGG Pathway database. This analysis revealed 27 genes in the apoptosis, MAPK signaling, and focal adhesion pathways, which could explain the mechanism of chemosensitivity in colorectal cancer cell lines. In addition, the chemosensitivity of other colorectal cancer and normal cell lines was predictable with the selected 27 genes. These genes may act as putative predictive markers for chemosensitivity in chemo-naive colorectal cancer patients following functional analysis and clinical validation.

Introduction

Colorectal cancer is one of the most common cancers worldwide and the second leading cause of cancer-related death in the Western world. In addition, the incidence of colorectal cancer has rapidly increased within the past ten years in Asia (1). Many patients are diagnosed with advanced stage, and recurrences after surgery are still a major cause of death (1,2). Therefore, various chemotherapies have been introduced in order to improve surgical outcomes in colorectal patients by controlling local or distant recurrences.

There is still insufficient understanding of the targets of drug activity and the individual variability of patients to these chemotherapies. For example, the efficacy of 5-Fluorouracil (5-FU), the most frequently used agent for colorectal cancer, was only 20-39% despite various combinations of chemotherapy used until 2000 (3,4). Newer combinations of 5-FU and folinic acid with irinotecan and oxaliplatin have significantly improved the response rates to 40-50%. However, more than 50% of patients still undergo chemotherapy without any significant benefit.

These limitations are in part due to a lack of effective predictive markers for drug sensitivity or resistance, and a few predictive markers that have been identified have not been validated for clinical application (5-10). Although several genes have been reported to influence chemosensitivity, the sensitivity of cancer cells to particular anticancer drugs is known to be determined by many factors that influence overall sensitivity. Hence, an effort to identify a large and complex gene set related to the chemosensitivity of particular anticancer agents has been made.

Microarray technology facilitates the analysis of genome-wide expression profiles that can efficiently generate information in a large scale. This study utilized human 22K microarray spotting with 70-mer oligonucleotides which give a high quality result while avoiding errors related to clones and minimizing cross-hybridization compared to cDNA microarrays (11-17).

In this study, in order to identify candidate chemosensitivity predictive markers for colorectal cancer, drug-naive resistant colorectal cancer cell lines were compared with cell lines sensitive to several anticancer agents commonly used for colorectal cancer.

Materials and methods

Cell lines and culture. Five human colorectal cancer cell lines, COLO 205, DLD-1, HCT-15, HCT-116 and HT-29, and 3

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human colorectal normal cell lines, CCD-18-Co, CCD-841-CoN, and CCD-841-CoTr, were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were maintained in Minimum Essential Medium (MEM, Gibco, Grand Island, NY, USA) with 10% FBS (Omega Scientific, Australia) in 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco). Cells were incubated at 37˚C in a 5% CO_2 humidified atmosphere, and the medium was replaced every 3-4 days.

RNA preparation. Total RNA was extracted from each cell line during the experimental growth phase using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The Yonsei reference RNA (Cancer Metastasis Research Center, Seoul, Korea) was prepared by pooling equivalent amounts of total RNA from the following 11 human cancer cell lines: YCC-B1 (breast cancer), HCT-116 (colon cancer), SK-HEP-1 (liver cancer), A549 (lung cancer), HL-60 (acute promyelocyte leukemia), MOLT-4 (acute lymphoblastic leukemia), HeLa (cervical cancer), Caki-2 (kidney cancer), T98G (glioblastoma), HT1080 (fibrosarcoma) and YCC-3 (gastric cancer) (18). The quantity and quality of the RNA was confirmed using an ND-1000 spectrophotometer (NanoDrop Technologies, USA) and gel electrophoresis.

Oligonucleotide microarray. Microarray analysis was performed using human spotted oligonucleotide microarrays (CMRC-GT, Seoul, Korea) containing 22,740 oligonucleotide probes of 70 bases with a reference design. The test RNA from each colorectal cancer cell line was labeled with Cy5 and individually co-hybridized with the Cy3-labeled reference RNA (CMRC, Seoul, Korea). One hundred micrograms of total RNA from each sample was used for the reverse transcription process with oligo-dT primer (Genotech, Daejun, Korea), SuperScript II (Invitrogen, USA), 5X first-strand buffer, 100 mM DTT, low-dT/dNTP mix, and Cy3- or Cy5-dUTP. The purified probes were combined and hybridized in 30% formamide, 5X SSC and 0.1% SDS at 42˚C for 16 h. Fluorescence was measured using a GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA) and the scanned images were processed using GenePix Pro 4.0 software (Axon Instruments).

For further analysis, raw Cy5/Cy3 values were $\log_2$-transformed. Systemic variations were corrected by normalization using intensity-dependent within-print tip normalization based on the Lowess function. After normalization, genes with more than one missing value in all experiments were filtered resulting in no missing proportion (NMP) of 100%. The values of repeated genes were adjusted by S-Plus 2000 software (Insightful, Seattle, WA, USA). Also, the genes which had signal intensities <100 were excluded from further analysis.

Chemosensitivity assay. Growth inhibition was measured in 5 human colorectal cancer cell lines with various concentrations of 17 anticancer drugs (5-fluorouracil, irinotecan, topotecan, doxorubicin, etoposide, mitomycin, docetaxel, paclitaxel, cisplatin, carboplatin, gemcitabine, vinblastine, vincristine, leucovorin, cyclophosphamide, methotrexate, and nimustine) using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, Saint Louis, MO, USA] assay. Control wells were treated with medium alone, without the anticancer drug. The absorbance at 540 nm was measured with a multi-well ELISA automatic spectrometer (Behring ELISA Processor II, Germany). Results were expressed as percent cell survival, calculated by the following formula: % survival = (mean absorbance of test wells - standard absorbance) / (mean absorbance of control wells - standard absorbance) x100. Percent cell survival at different drug concentrations was plotted to determine the growth inhibitory concentration. The drug concentration at which 50% of cancer cells survived (IC_{50}, μM) was calculated using Calcusyn software (Biosoft, Cambridge, UK). Since there was wide variation in the scale of data points for different drugs, the IC_{50} was transformed into a $\log_{10}$ scale.

Data analysis. To determine the representative resistant or sensitive cell lines to anticancer drugs commonly used for colorectal cancer, IC_{50}s of tested drugs in five colorectal cancer cell lines were compared. The genes differentially expressed between two cell lines were selected based on the fold change of ≥4-fold for up- or down-regulated genes. Hierarchical clustering analysis was performed with Cluster, and the resulting dendrogram was visualized using TreeView software (Eisen Lab, http://rana.lbl.gov/EisenSoftware.htm). Annotation of the selected genes was performed using the Stanford Online Universal Resource for Clones and Expressed sequence tags (SOURCE) (http://source.stanford.edu/cgi-bin/source/sourceSearch). The pathway searching was conducted with the KEGG Pathway database (http://www.genome.jp/kegg/pathway.html).

Molecular Diagnosis Score (MDS) analysis was utilized for scoring the expression of selected genes in each cell line (19). The MDS was defined as the sum of weighted expression of the selected genes in the two cell lines: $MDS_i = \sum S_k \log_2(r_{ik})$, where r_{ik} is the expression ratio of gene k of cell line i, and S_k is the sign for gene k which was determined as follows: first, the mean log ratio $\log_2(r_{ik})$ was calculated for gene k in resistant and sensitive cell lines. Next, the sign for each gene was determined: S_k=+1 if $MEAN_{sen} > MEAN_{res}$, and S_k=-1 if $MEAN_{sen} < MEAN_{res}$.

Results

Gene expression profiling of colorectal cancer and normal cell lines. To investigate the genetic characteristics of each cell line, gene expression profiling was performed on 5 colorectal cancer cell lines and 3 normal colorectal cell lines without drug treatment. A total of 18,099 genes that fulfill the NMP 100% for all eight cell lines were selected for further analysis. Unsupervised hierarchical clustering was performed using the $\log_2$ (red/green) ratio, and normal and cancer cell lines had significantly different gene expression patterns (Fig. 1A).

Chemosensitivity profiling of colorectal cancer cell lines. Seventeen anticancer agents were tested for growth inhibition in 5 colorectal cancer cell lines. Hierarchical clustering with $\log_{10}IC_{50}$ showed that the drugs were classified into several groups according to their mechanism of action, except

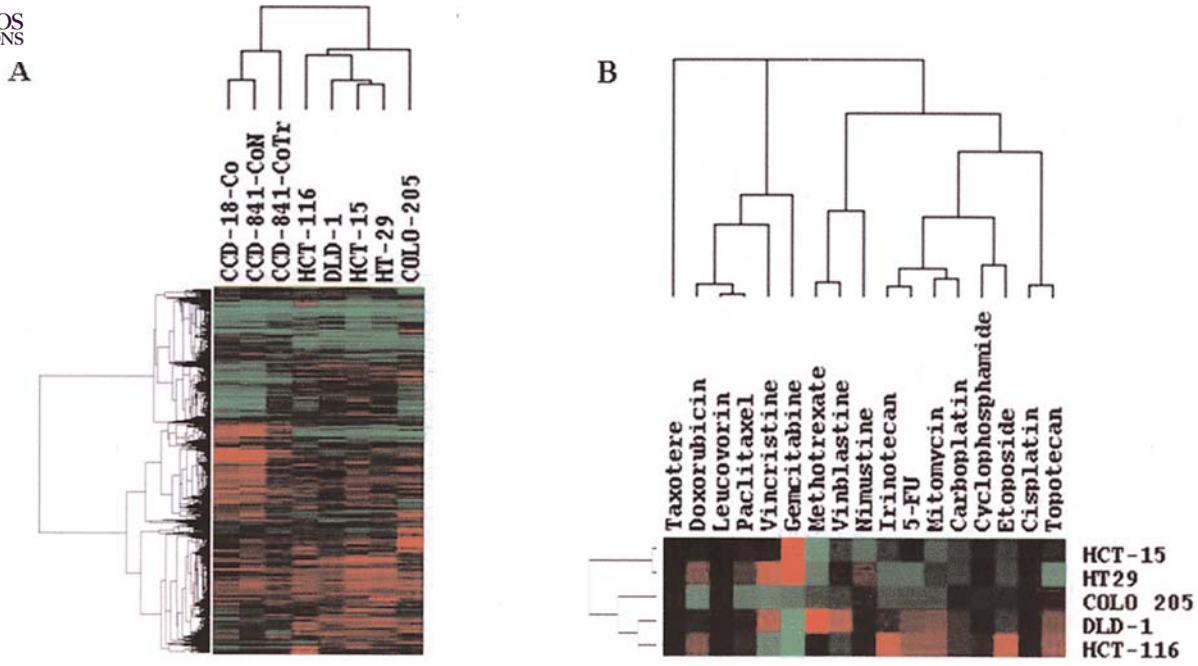

Figure 1. Gene expression and chemosensitivity patterns of colorectal cell lines. (A) Unsupervised hierarchical clustering of 3 colorectal normal and 5 cancer cell lines with 18098 filtered genes. (B) Unsupervised hierarchical clustering of 5 colon cancer cell lines and 17 anticancer drugs. The values represent $\log_{10}IC_{50}$.

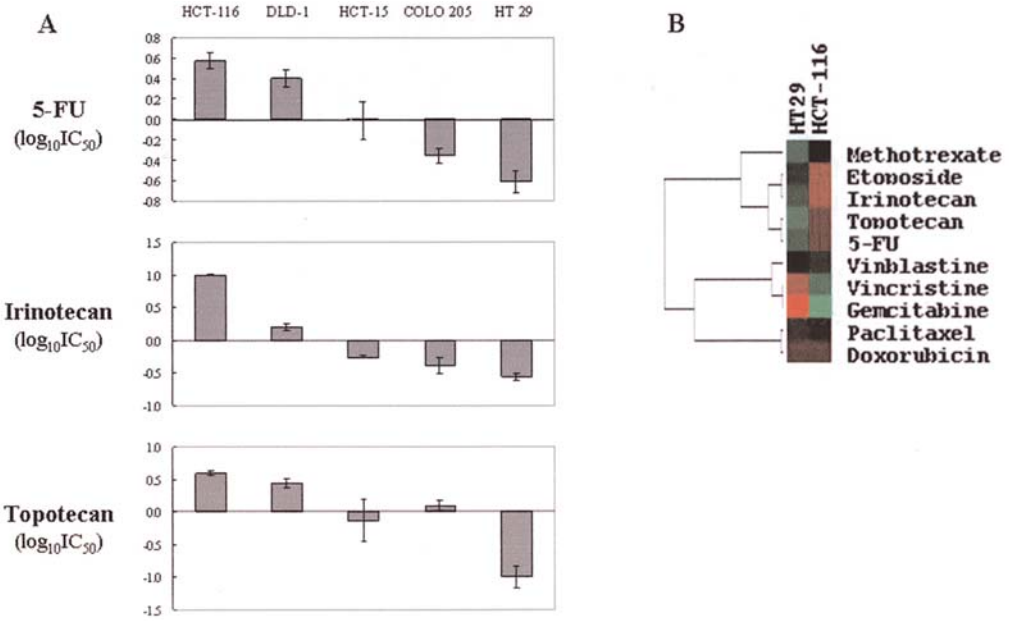

Figure 2. Comparisons of the chemosensitivity of colorectal cancer cell lines. (A) Histogram of $\log_{10}IC_{50}$s of 5 colorectal cancer cell lines for the 3 drugs commonly used for colon cancer. The most resistant and sensitive cell lines for the 3 drugs were HCT-116 and HT-29, respectively. (B) The hierarchical clustering of HT-29 and HCT-116 with 10 anticancer drugs. The 7 drugs carboplatin, cisplatin, cyclophosphamide, leucovorin, nimustine, and taxotere were excluded because their $\log_{10}[Max(IC_{50})/Min(IC_{50})]$ values were less than 1 $\log_{10}$ scale.

docetaxel, to which all tested cancer cell lines were sensitive. Topotecan, irinotecan, carboplatin, and cisplatin were clustered together, and paclitaxel and vincristine were clustered in a separate branche (Fig. 1B). To evaluate the variation in chemosensitivity among the cell lines, the $\log_{10}[Max(IC_{50})/Min(IC_{50})]$ for each drug was calculated. The median of the $\log_{10}[Max(IC_{50})/Min(IC_{50})]$ of the 17 drugs was 1.10, ranging from 0 to 5.25. The values for 7 drugs (carboplatin, cisplatin, cyclophosphamide, leucovorin, mitomycin, nimustine, and taxotere) were less than 1 $\log_{10}$ scale, a <10-fold difference, suggesting that the values from each cell line had no significant differences. Thus, those drugs were excluded from further analysis.

Selection of specific drugs and cell lines. Among the 10 drugs which showed differences in growth inhibition between the

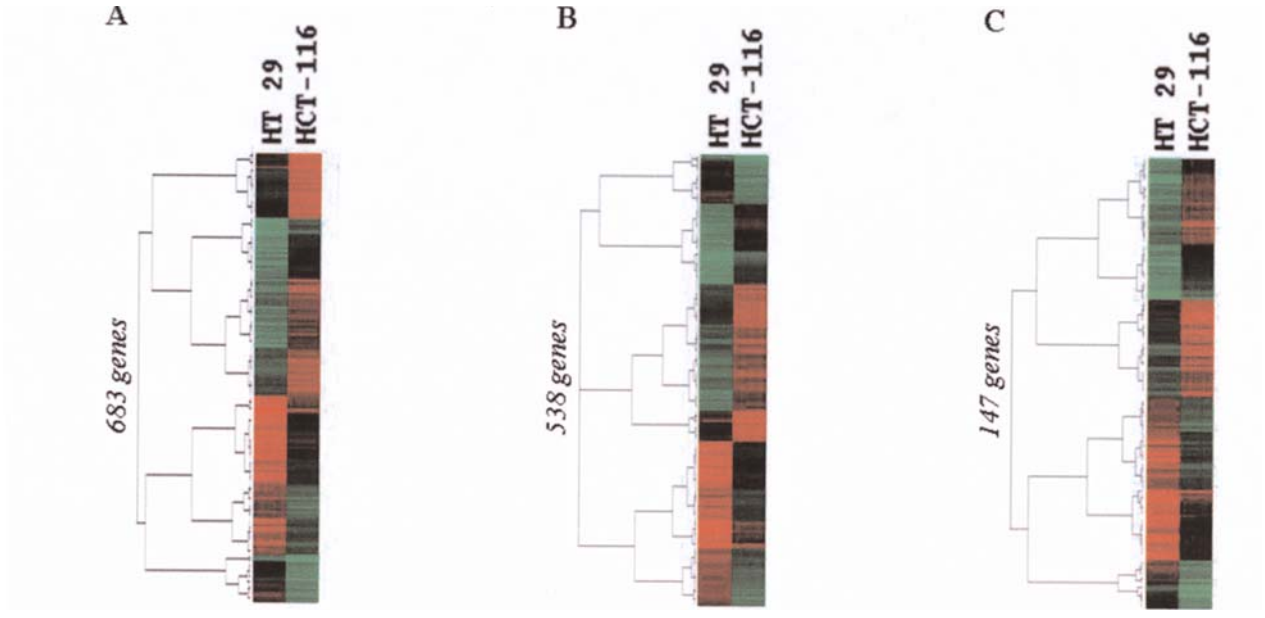

Figure 3. Expression patterns of selected genes related to chemosensitivity in HT-29 and HCT-116 cell lines. (A) Cluster of selected 683 genes. (B) Annotated 538 genes in the SOURCE database. (C) The 147 genes in the KEGG Pathway database.

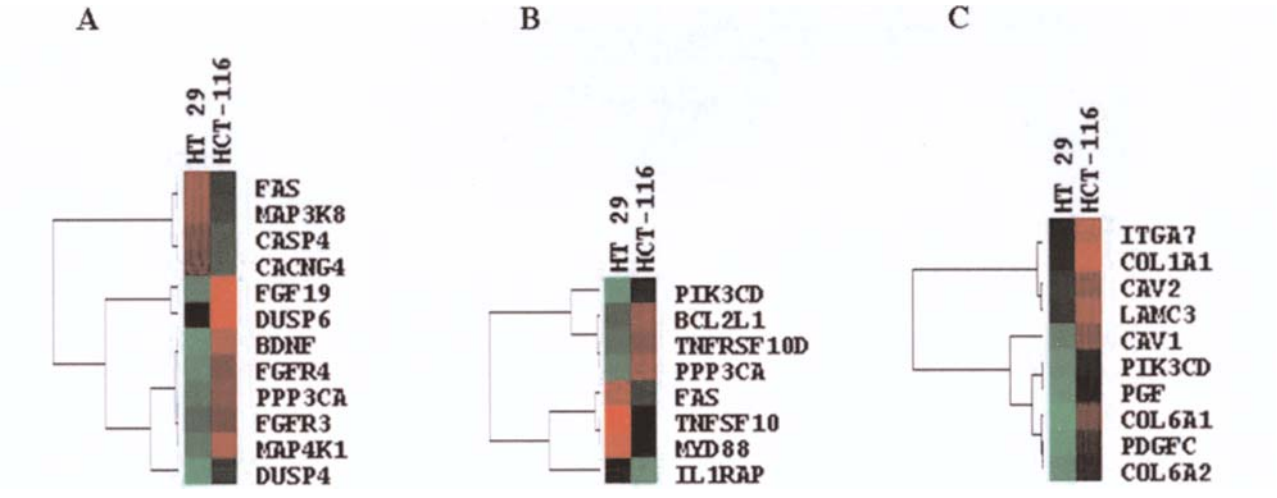

Figure 4. Predictive gene sets based on the different pathways. (A) The 12 genes in the MAPK signaling pathway. (B) The 8 genes in the apoptosis pathway. (C) The 10 genes in the focal adhesion pathway.

cell lines, the focus of the study was on drugs generally used for colon cancer: 5-FU, irinotecan, and topotecan. Analysis of the $\log_{10}IC_{50}$ of 5 cancer cell lines against 3 drugs showed that the HCT-116 cell line was the most resistant cell line and HT-29 was the most sensitive cell line (Fig. 2A). In addition, the chemosensitivity patterns of two cell lines were reversed in other drugs, such as tubulin inhibitors that are not used for colorectal cancer (vinblastine, vincristine, and paclitaxel, Fig. 2B). Thus, HCT-116 and HT-29 were identified as the representative cell lines for resistance and sensitivity to 3 drugs (5-FU, irinotecan, and topotecan) respectively.

Genes differentially expressed between resistant and sensitive colorectal cancer cell lines. To identify the differentially expressed genes between a resistant cell line (HCT-116) and a sensitive cell line (HT-29), 683 genes were identified that were ≥4-fold up- or down-regulated between the two cell lines. Among the selected 683 genes, 538 genes were annotated in the SOURCE database, and the clusters of the 683 and 538 genes from the 2 cell lines are shown in Fig. 3A and B, respectively. Of 538 genes, 54.6% belong to the biological process of cellular metabolism and 51.6% belong to the process of primary metabolism (Babelomics, http://babelomics.bioinfo.cipf.es/).

In order to identify the candidate biological pathways which could explain the difference in chemosensitivity, specific pathways which included the selected genes were found using the KEGG Pathway database. Of the 538 annotated genes, 147 were in the KEGG database, and they were included in 117 different pathways (Fig. 3C). Among the pathways, 12 genes were identified in the MAPK signaling pathway and 8 in the apoptosis pathway (Fig. 4A and B). Ten genes found in

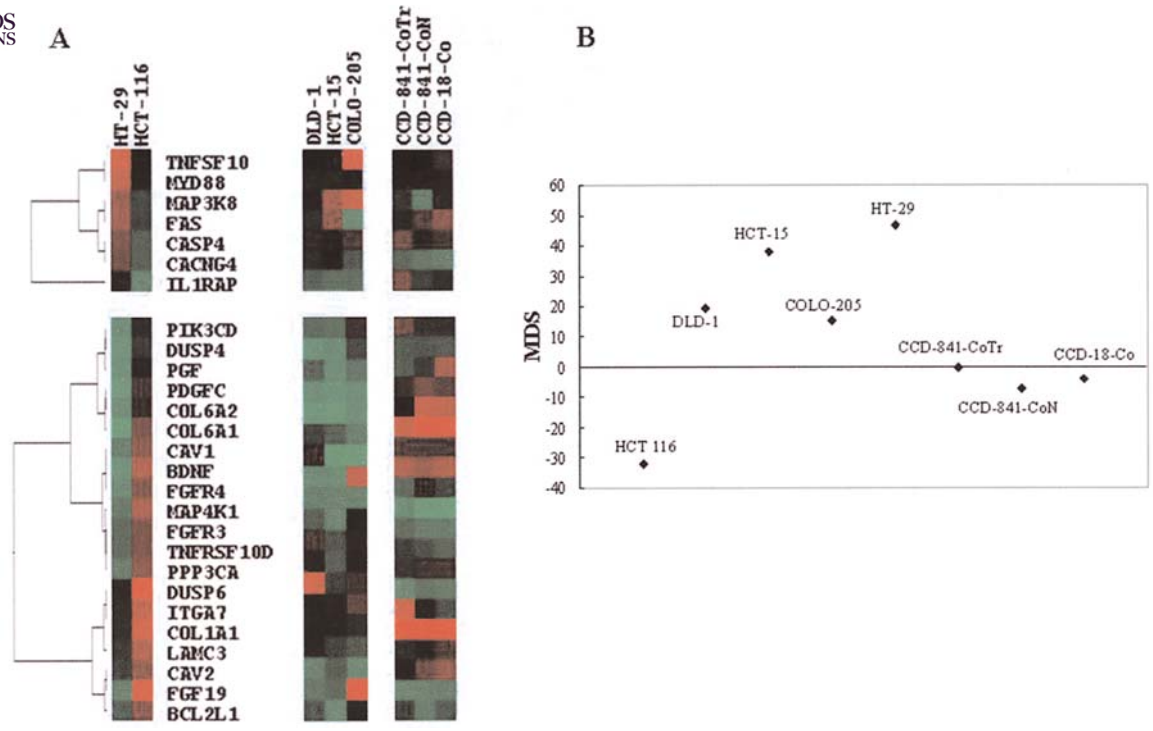

Figure 5. Molecular Diagnosis Score (MDS) analysis of colorectal cancer and normal cell lines using 27 genes selected via pathway searching. (A) Cluster of 27 genes in HT-29 and HCT-116 cell lines (left) and their expression patterns in other colorectal cancer cell lines, COLO-205, HCT-15, and DLD-1, and normal cell lines, CCD-18-Co, CCD-841-CoN, and CCD-841-CoTr (right). (B) MDS values calculated in 5 cancer and 3 normal cell lines.

the focal adhesion pathway were overexpressed in a resistant cell line, HCT-116, compared to a sensitive one (Fig. 4C). In combining the apoptosis and MAPK signaling pathways, we suggest that genes involved in the apoptotic and proliferative pathways could control the innate chemosensitivity in colorectal cancer cell lines. In the apoptotic pathway, FAS, TNFSF10, MYD88, and IL1RAP were overexpressed in sensitive HT-29 cells, and the antiapoptotic genes PIK3CD and BCL2L1 were overexpressed in resistant HCT-116 cells. These results suggest that the chemosensitivity might be related to the extrinsic apoptotic pathway. Also in the MAPK signaling pathway, proliferative genes were overexpressed in resistant HCT-116 cells, but antiproliferative genes such as FAS and CASP4 were overexpressed in HT-29 cells.

MDS analysis of cancer and normal cell lines. Using the 27 genes selected via the pathway analysis, the MDS was calculated for 5 cancer cell lines. The MDSs of the representative HCT-116 and HT-29 cell lines were -32.1 and +46.9, respectively (Fig. 5A, left). MDSs of the other cancer cell lines, COLO-205, HCT-15, and DLD-1, were between those of HT-29 and HCT-116, suggesting that the selected 27 genes could predict the chemosensitivity of colorectal cancer cell lines. In addition, the pattern of MDSs of 5 cancer cell lines was similar to the chemosensitivity profiling (Fig. 2A).

The MDS of 3 normal colorectal cell lines with the same 27 genes was calculated in order to examine the normal colorectal cell lines' chemosensitivity. The MDSs of CCD-18-Co, CCD-841-CoN, and CCD-841-CoTr were -3.9, -7.0, and 0, respectively, supporting our prediction that these normal cell lines are relatively resistant to the commonly used chemotherapeutic agents (Fig. 5B, right).

Discussion

This study focused on genes which could affect the innate chemosensitivity of colorectal cancer to various anticancer drugs, rather than on the molecular consequences of chemotherapy. Thus, we used untreated colorectal cancer cell lines instead of cell lines with acquired drug-resistance (20), or cell lines immediately following drug treatment (21). Although we used a small number of cell lines, we were able to identify representative cell lines resistant and sensitive to particular anticancer drugs used for colorectal cancer and identify the genes which could predict chemosensitivity. This approach could help to identify candidate markers for predicting chemosensitivity.

At first, we examined the expression of known target genes for 5-FU, irinotecan, and topotecan, which could affect chemosensitivity. The expression ratios (HCT-116/HT-29) of Thymidylate synthetase (TYMS), Thymidine phosphorylase (TP), Dihydropyrimidine dehydrogenase (DPYD), and Uridine phosphorylase 1 (UPP1) were <2-fold. Topoisomerase I (TOP1), a target of irinotecan and topotecan, showed no significant difference between the 2 cell lines; however, mitochondrial Topoisomerase I (TOP1MT) was 2.2-fold overexpressed in HCT-116 cells compared to HT-29 cells. These known target genes were not selected in our analysis. These results reflect the difficulty in predicting chemosensitivity with a small number of genes, so it is important to identify many genes which could be related to chemosensitivity using genome-wide analysis.

Then we identified a large number of genes, 683, differentially expressed between representative resistant and sensitive cell lines. These genes could suggest many possible

mechanisms related to chemosensitivity. However our goal was to identify the genes related to each other and to chemosensitivity by pathway analysis. Using the pathway analysis, a well-known mechanism of chemosensitivity, apoptosis, was evaluated. Calcineurin A-α, included in the Ca^{2+}-induced cell death pathway, was overexpressed in the resistant HCT-116 cell line. This result inferred that the apoptotic pathway which participated in chemosensitivity was the extrinsic apoptotic pathway, not the Ca^{2+}-induced cell death pathway. Because of the stringent selection process, many genes were excluded from the group used for pathway analysis. Hence, we examined other genes simultaneously involved in the apoptotic and MAPK signaling pathways. In the apoptotic pathway, Caspases 1, 3 and 10 were overexpressed in HT-29 cells compared to HCT-116 cells (fold changes: 2.5, 2.4 and 7.0, respectively). Caspase 10 had a very significant fold change but its signal intensity was lower than 100, thus the gene was not selected. On the other hand, ERK and KRAS, in the MAPK signaling pathway, were overexpressed in HCT-116 cells compared to HT-29 cells (fold changes: 2.6 and 2.3, respectively). The expression of these genes strengthened the hypothesis that apoptotic and MAPK signaling pathways can affect chemosensitivity together. Remarkably, all of the genes in the focal adhesion pathway were overexpressed in HCT-116 cells compared to HT-29 cells. There was no evidence that these genes directly affected chemosensitivity, but many studies have found that the focal adhesion kinase was related to apoptosis and the proliferation of cancer cells (22-25). The integrin antagonist has also been shown to decrease the survival of colon carcinoma cells (26). These studies support our result of overexpression of focal adhesion genes in the resistant cell line.

To evaluate the predictive capacity of 27 selected genes and classify blind cell lines as to their chemosensitivity, we performed MDS analysis. We regarded positive MDS values as that of HT-29, and negative values as that of HCT-116, representative sensitive and resistant cell lines, respectively. All other colorectal cancer cell lines had positive MDS values, but normal colorectal cell lines had 0 and negative values. The results supported that these 27 genes were useful in predicting chemosensitivity. The fact that all normal cell lines did not exceed 0 was also reasonable because normal cell lines are thought to be resistant to anticancer cytotoxic drugs due to their low proliferative rate.

Interestingly, chemosensitivity patterns of HCT-116 and HT-29 were exactly reversed upon treatment with tubulin inhibitors, vinblastine, vincristine, and paclitaxel. There could be another pathway linked with a microtubule-associated pathway, which may be dominant to the apoptosis pathway in chemosensitivity.

With further studies, including functional analysis and clinical validation, these selected genes may act as predictive markers for chemosensitivity in chemo-naive colorectal cancer patients.

Acknowledgements

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