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Upregulated expression of BCL2, MCM7, and CCNE1 indicate cisplatin-resistance in the set of two human bladder cancer cell lines: T24 cisplatin sensitive and T24R2 cisplatin resistant bladder cancer cell lines

Sung Han Kim[1], Jin-Nyoung Ho[2,3], Hyunjin Jin[2,3], Sang Chul Lee[3], Sang Eun Lee[3], Sung-Kyu Hong[3], Jeong Woo Lee[4], Eun-Sik Lee[4], Seok-Soo Byun[3]

[1]Department of Urology, National Cancer Center, Goyang, [2]Biomedical Research Institute and [3]Department of Urology, Seoul National University Bundang Hospital, Seongnam, [4]Department of Urology, Seoul National University Hospital, Seoul, Korea

Purpose: The mechanism of resistance to cisplatin during treatment of bladder cancer (BC) has been a subject of intense investigation in clinical research. This study aims to identify candidate genes associated with resistance to cisplatin, in order to understand the resistance mechanism of BC cells to the drug, by combining the use of microarray profiling, quantitative reverse transcription-polymerase chain reaction (RT-PCR), and Western blot analyses.
Materials and Methods: The cisplatin sensitive human BC cell line (T24) and the cisplatin resistant BC cell line, T24R2, were used for microarray analysis to determine the differential expression of genes that are significant in cisplatin resistance. Candidate upregulated genes belonging to three well-known cancer-related KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (p53 tumor suppressor, apoptosis, and cell cycle) were selected from the microarray data. These candidate genes, differentially expressed in T24 and T24R2, were then confirmed by quantitative RT-PCR and western blot. A fold change ≥2 with a p-value <0.05 was considered significant.
Results: A total of 18 significantly upregulated genes were detected in the three selected cancer-related pathways in both microarray and RT-PCR analyses. These genes were *PRKAR2A, PRKAR2B, CYCS, BCL2, BIRC3, DFFB, CASP6, CDK6, CCNE1, STEAP3, MCM7, ORC2, ORC5, ANAPC1*, and *ANAPC7, CDC7, CDC27*, and *SKP1*. Western blot analyses also confirmed the upregulation of BCL2, MCM7, and CCNE1 at the protein level, indicating their crucial association with cisplatin resistance.
Conclusions: The *BCL2, MCM7*, and *CCNE1* genes might play distinctive roles in cisplatin resistance in BC.

Keywords: Cell line; Cisplatin; Drug resistance; Microarray analysis; Urinary bladder neoplasms

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Corresponding Author: Seok-Soo Byun
Department of Urology, Seoul National University Bundang Hospital, 82 Gumi-ro 173beon-gil, Bundang-gu, Seongnam 13620, Korea
TEL: +82-31-787-7349, FAX: +82-31-787-4057, E-mail: ssbyun@snubh.org

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INTRODUCTION

Cisplatin-based combination chemotherapy protocols such as methotrexate, vinblastine, doxorubicin and cisplatin, or gemcitabine and cisplatin have been a mainstay for advanced (or metastatic) bladder cancer (BC) treatment for a number of years and have prolonged the survival of these patients by up to 19 months [1-4]. However, about 30% of patients fail to respond to the initial chemotherapy and in most cases, the cancer recurs within one year, and patients usually die after 2–3 years of chemotherapy due to the emergence of cisplatin resistant (CR) cancer [1,2]. This widespread occurrence of CR has resulted in many studies that have investigated the molecular mechanisms underlying cisplatin resistance in BC with the aim of developing an effective therapeutic regimen for BC patients with CR.

In the last few decades, an increased understanding of genetic and molecular biological properties of both anticancer agents and anticancer resistance has led to the clinical development of mechanism-based therapeutics and targeted strategies for the clinical treatment of advanced BC [3,4]. Numerous experiments have also tried to understand and overcome CR in BC cells in the laboratory [5,6]. Recently, there has been a rapid progress in the understanding of CR using microarray analysis that permits a simultaneous and quick expression profiling of tens of thousands of genes, leading to the identification of genes that might be significant in CR [7,8]. Several previously published experiments from our laboratory have been performed to try to define the CR mechanism and CR-related genes and molecular pathways using microarray methods with BC cell lines including CR and reported multiple important findings of CR [5-11].

In this study, we performed microarray profiling, quantitative real time polymerase chain reaction (qRT-PCR), and western blot analyses on a CR and a cisplatin sensitive BC cell line, to investigate potential candidate genes that might be significant in CR. Knowing which genes are differentially expressed between the CR and sensitive cell lines will help us gain a better understanding of the resistance mechanism of cisplatin.

MATERIALS AND METHODS

1. Cell lines and culture conditions

Three experimental paired sets of two BC cell lines used: a cisplatin-sensitive control cell line T24 (ATCC, Manassas, VA, USA) and a CR cell line T24R2 generated by the treatment of T24 cells with serial dilutions of cisplatin from 0.039 to 40.0 μg/mL [6,10]. T24R2 cells were resistant to treatment with cisplatin (Pfizer, Seoul, Korea) at concentration up to 2 μg/mL [11] and proven significantly more resistant to cisplatin treatment than other cell lines in our previous studies [6,9-12]. To measure cell chemosensitivity, exponentially growing cells were incubated with different concentrations of cisplatin (0.1–100 μg/mL) for four days, as previously described [6,9-11]. All assays were performed in triplicate. Preliminary experiments confirmed that cisplatin at these concentrations had no direct cytotoxic effect during first 24 hours of treatment. The half maximal inhibitory concentrations (IC_{50}) for T24R2 and T24 cell lines were determined treated with 20 μg/mL and 1.25 μg/mL of cisplatin, respectively (not shown in data) [6,10].

2. Microarray sample preparation and analysis of gene expression

Total RNA was extracted from T24 and T24R2 cell lines using the mirVanaTM isolation kit (mirVana microRNA Isolation Kit, Ambion [Life Technologies], Austin, TX, USA) in accordance with the manufacturer's instructions. RNA samples that had high RNA integrity numbers (RIN>9.0; RIN, developed by Agilent Technologies, Santa Clara, CA, USA) and had A260/A280 absorbance ratios ranging from 1.8 to 2.1 were used for cDNA synthesis. The amplification cycles of RNA to cDNA and cDNA to biotin-labeled aRNA were performed using the GeneChip IVT Express kit (Affymetrix, Santa Clara, CA, USA). The generation of biotin-labeled aRNA by *in vitro* transcription, hybridization to the array and washing and scanning were performed according to the manufacturer's instructions.

3. Microarray data normalization and analysis

GenPlex software ver. 3.0 (ISTECH Inc., Goyang, Korea) was used for analyzing the CEL file data. The MAS5 algorithm was used for obtaining the gene expression summary. GeneChip Human Genome HG-U133 Plus 2.0 array data was analyzed using the Microarray Suite, MicroDB, and the Data Mining Tool software (Affymetrix) containing 54,120 probe sets of 38,573 gene clusters in the UniGene database. After Global scaling normalization, the normalized data was then log transformed to base 2. The gene expression levels in T24R and T24R2 were normalized and analyzed by (Affymetrix). Gene clusters were analyzed using GeneCluster 1.0 (MIT, Cambridge, MA, USA). Patterns of altered gene expression between the cell lines were explored using the self-organizing map cluster analysis tool [13]. The genes showing altered expression were then categorized based on their biological function using

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Species	Direction	Gene	Sequence	Product (bp)
Human	hGAPDH	5'-forward-3'	TGC ACC ACC AAC TGC TTA G	177
		5'-reverse-3'	AGA GGC AGG GAT GAT GTT C	
Human	MCM7	5'-forward-3'	TCG GAT TGT GAA GAT GAA CA	132
		5'-reverse-3'	TAT ATT TCT GGG GCG ATT GA	
Human	ORC5	5'-forward-3'	TGA ACC CGT GGT TAA AGG AG	156
		5'-reverse-3'	CCC GGA TCT GTG TCA TCT TT	
Human	CDK6	5'-forward-3'	AGA GAC AGG AGT GGC CTT GA	185
		5'-reverse-3'	TGA AAG CAA GCA AAC AGG TG	
Human	CCNE1	5'-forward-3'	AGC GGT AAG AAG CAG AGC AG	189
		5'-reverse-3'	TTT GAT GCC ATC CAC AGA AA	
Human	ANAPC7	5'-forward-3'	GGC ACA GAT GTT GGA TCC TT	211
		5'-reverse-3'	AAT GGC CTT GGC TCC TAA AT	
Human	CDC7	5'-forward-3'	TCA GCA GTC CAC CAC AAA AG	169
		5'-reverse-3'	AGG GCT CTC ATG TGA AAT GG	
Human	CDC27	5'-forward-3'	TGC TGA CGT GTT TCT TGT CC	134
		5'-reverse-3'	TTG CAC TGC CTT TCA TTC TG	
Human	ANAPC1	5'-forward-3'	TTG GAA TTG CTC TTC CCA TC	107
		5'-reverse-3'	GGA AAG ATC CTG ACG TCC AA	
Human	ORC2	5'-forward-3'	TCA ATG CTC CTC TCA TGT GG	144
		5'-reverse-3'	TAA GTG GCA GGG ATC CAG AC	
Human	SKP1	5'-forward-3'	CAC TGG AGG CTG CTG ACA TA	126
		5'-reverse-3'	TAG GAG GGA AGC TGG AAA CA	
Human	CYCS	5'-forward-3'	CAA AAA CAA GGG CCA GAT GT	136
		5'-reverse-3'	GCT ACC ACA CTG GAC AGC AA	
Human	STEAP3	5'-forward-3'	AGA TCC TGG TGG ATG TGA GC	118
		5'-reverse-3'	ACA TTG AAG GCC TTG ACC AC	
Human	PRKAR2B	5'-forward-3'	GAG GGC ACT TGG CAA TTA AA	131
		5'-reverse-3'	CCA AGG CCA GCA CAT AAC TT	
Human	BCl2	5'-forward-3'	ATC GCC CTG TGG ATG ACT GAG	129
		5'-reverse-3'	CAG CCA GGA GAA ATC AAA CAG AGG	
Human	BIRC3	5'-forward-3'	ATG AAT GGA CAG CCC TGA AG	125
		5'-reverse-3'	GAA TGC TGC ACA GAG ACC AA	
Human	DFFB	5'-forward-3'	AAA CCA CCC ACA AGC TCA AC	148
		5'-reverse-3'	TTA AAA TGA TGC CCA CGT CA	
Human	CASP6	5'-forward-3'	GAG TTC GAG ACC AGC CTG AC	110
		5'-reverse-3'	TTC CCA GTA GCT GGG ATT GC	
Human	PRKA2A	5'-forward-3'	CAG AGG TTC AAC CCA CAC CT	117
		5'-reverse-3'	GTT GGA GAG ACA TGC CGT TT	

Fig. 1. List of primers used in quantitative real-time polymerase chain reaction studies of the 18 upregulated genes and two housekeeping controls.

Onto-Express and three biological functions, p53 tumor suppressor, apoptosis, and cell cycle were selected for further study. GenMAPP software was also used to represent gene expression data (http://www.genmapp.org).

4. RNA extraction and real-time PCR

To minimize the least direct effect of treatment, the T24R2 resistant cell line was used to extract RNA and protein after leaving at least two weeks interval from the removal of the treating drug from T24R2. Total RNA was extracted from T24R2 cells using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and the same way with those from T24 cells. cDNA was produced from 1 μg of total RNA using the oligo(dT) primer and Omniscript reverse transcriptase enzyme (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. qRT-PCR was done with the FastStart Universal SYBR Green Master (Roche Diagnostics, Indianapolis, IN, USA), a ready-to-use hot start reaction mix using a 7500 real-time PCR system (7500 real-time PCR system, Applied Biosystems, Foster City, CA, USA). *GAPDH* was used as the reference gene, and fold-change in gene expression was calculated making use of the comparative CT ($2^{-\Delta\Delta CT}$) method. Primer sequences are presented in Fig. 1.

5. Western blot analysis

Cells were harvested and lysed in radio immunoprecipitation assay buffer containing 50mM Tris-HCl (pH 8.0), 150mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1mM phenylmethylsulfonyl fluoride. Protein content in the cell lysates was measured using the Pierce bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein from the lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane. Membranes were

blocked with 5% milk for 1 hour at room temperature and incubated overnight at 4°C with the corresponding primary antibodies to BCL2, CCNE1, and MCM7, respectively (Cell Signaling Technology, Beverly, MA, USA). After incubation with secondary antibodies, protein bands were visualized using an enhanced chemiluminescence system (Pierce). Beta-actin was used to normalize for the amount of protein in each lane. Each experiment was performed in triplicate.

6. Statistical analysis

Data are shown as mean±standard deviation with at least 3 data sets obtained from biological replicates. The relationship between two or three variables and the numerical values obtained by real-time PCR and Western blot were analyzed using the Bonferroni adjusted Mann-Whitney U test and the Kruskal-Wallis test with $p<0.05$ considered significant. StatView ver. 4 (SAS Institute Inc., Cary, NC, USA) was used for statistical calculations. Differentially expressed genes (DEGs) were selected based on upregulation profiling with fold change >2.0 of log scale and p-value<0.05 after the microarray. All experiments were performed in triplicate.

RESULTS

Microarray analysis found a total of 1,163 upregulated and 1,465 downregulated genes in T24R2 and T24 cells, with 1,164 and 147 DEGs with more than twofold and fourfold changes, respectively. Statistical analysis differentiated 432 gene ontology associations (classified into 294 biological processes, 67 cellular components, and 71 molecular functions) among 905 up- and 1,302 downregulated gene ontology associations. Furthermore, 231 KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were found among 327 up- and 543 downregulated pathways differentially regulated in T24 and T24R2 cell lines (Fig. 2). After analysis of the data according to the Gene Set Enrichment Analysis computational method (http://www.broadinstitute.org/gsea), we identified 448 significant candidate gene sets in the differentially expressed genetic suppressor element genes and 69 KEGG pathways.

The n-fold change and statistical p-values were used after regression normalization between T24 and T24R2 data sets, and genes from three major cancer-related pathways (p53, apoptosis, and cell cycle) were selected for further evaluation. We identified 4 up- and 2 downregulated genes from the p53 pathway, 7 up- and 1 downregulated gene from the apoptosis pathway, and 10 up- and 1 downregulated gene from the cell cycle pathway (Table 1). Several genes with upregulated expression from three pathways were selected for the further analysis to confirm their role in CR mechanism.

For the qRT-PCR, 18 genes were found with more than a 2-fold difference in expression levels and their qRT-PCR results were consistent with the microarray data. These results suggested that these 18 genes were indeed differentially expressed in the two cell lines and were most likely related to CR in BC cells (Fig. 3).

BCL2 (B-cell CLL/lymphoma 2), CCNE1 (cyclin E1), CDK6 (cyclin-dependent kinase 6), ORC5 (origin recognition complex, subunit 5), CYCS (Cytochrome c, somatic), MCM7 (minichromosome maintenance complex component 7), and PRKAR2B showed a fold change greater than 3.0 in both microarray and qRT-PCR, suggesting a high correlation with CR. Western blot analysis of BCL2, CCNE1, and MCM7 showed significantly greater differences in expressions levels between T24 and T24R2 (Fig. 4).

DISCUSSION

Microarrays have been used to measure alterations in gene expression profiles induced by several chemotherapeutic agents in many urological cancers [7,8,14]. Cisplatin, a platinum-based chemotherapeutic agent, is currently the backbone for several chemotherapy combinations used in treating metastatic and locally recurring BC as well as other cancers. Many studies have tried to define the mechanism of CR, and the molecular biology of the genes involved for modeling future targeted therapies [6,8]. However, the changes in gene expression profiles, and the mechanism of CR in BC cells after cisplatin-based combination chemotherapy in patients with progressive BC are not entirely understood.

In this study, alterations in the gene expression profiles between the BC cell lines T24 and T24R2 were determined using a high-throughput microarray experiment. Cellular and molecular responses to cisplatin treatment are likely to be mediated by a variety of regulatory pathways. The molecular response to cisplatin in BC cells involved inhibition or induction of genes with specific functions in signal transduction, cell proliferation, cell cycle control, transcriptional and translational regulation, protein degradation, and cellular metabolism, as well as apoptosis and tumor suppression. These results demonstrate that CR was acquired and regulated by genes important in fundamental cellular processes and might thus be responsible for metastasis and recurrence of progressive BC.

From several mechanisms involved in the regulation

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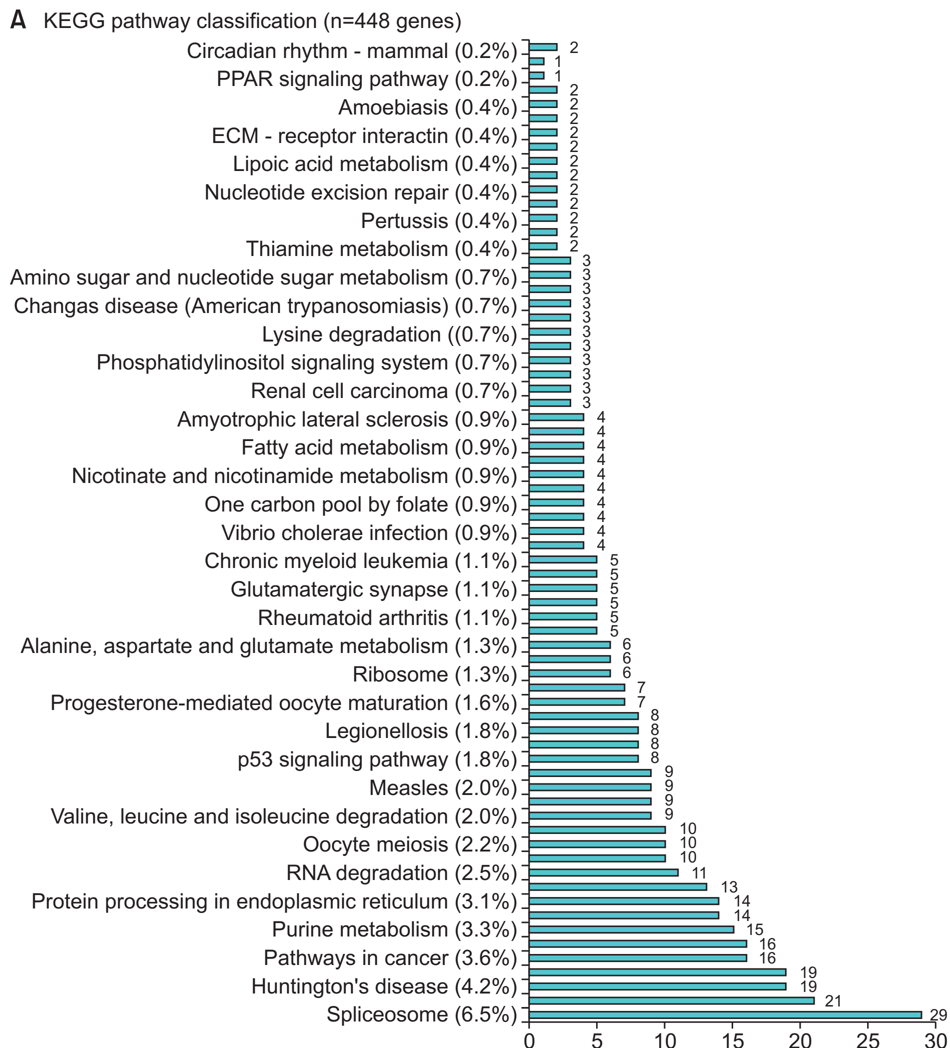

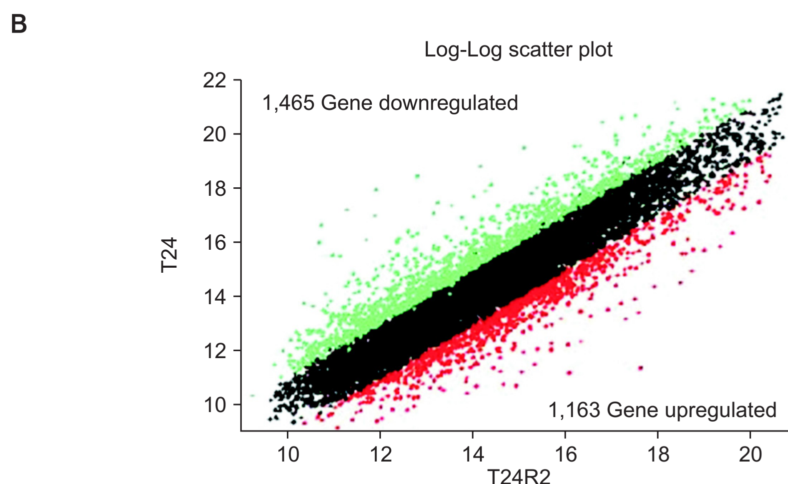

Design	Cutoff	Regulation	Significant gene	Gene ontology	Pathway
T24-ctr vs. T24R2-ctr	2.0	Down	1,465	1,302	543
		Up	1,163	905	327

Fig. 2. Differential gene expression in T24 and T24R2 cell lines. (A) KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway classification of differentially regulated genes. (B) Logarithmic scatter plot of microarray results. ECM, extracellular membrane.

Table 1. List of 18 significant upregulated genes with symbol, name, pathway, function, and fold change (FC) between T24 and T24R2

Symbol	Name	Pathway	Function	FC in microarray	FC in RT-PCR
ANAPC1	Anaphase promoting complex subunit 1	Cell Cycle	Oocyte meiosis	2.4	1.6
ANAPC7	Anaphase promoting complex subunit 7	Cell Cycle	Oocyte meiosis	2.7	1.9
BCL2	B-cell CLL/lymphoma 2	Apoptosis	Ligand-mediated signaling	2.7	6.6
BIRC3	Baculoviral IAP repeat-containing 3	Apoptosis	caspase 6, apoptosis-related cysteine peptidase, mRNA	2.6	1.2
CASP6	Caspase 6 (apoptosis-related cysteine peptidase)	Apoptosis	Induction & cellular component disassembly of apoptosis	2.3	2.7
CCNE1	Cyclin E1	Cell Cycle, p53 signal	Regulation of cyclin-dependent protein kinase activity in mitotic cell cycle	3.3	2.6
CDC7	Cell division cycle 7 homolog (S. cerevisiae)	Cell Cycle	Cell cycle	2.6	2.0
CDC27	Cell division cycle 27 homolog (S. cerevisiae)	Cell Cycle	Oocyte meiosis	2.5	2.4
CDK6	Cyclin-dependent kinase 6	Cell Cycle, p53 signal	Mitotic cell cycle; Cell division protein kinase 6 gene	3.4	2.7
CYCS	Cytochrome c, somatic	Apoptosis, p53 signal	Nuclear gene encoding mitochondrial protein, mRNA	4.3	2.8
DFFB	DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase)	Apoptosis	DNA fragmentation involved in apoptotic nuclear change	2.3	2.9
MCM7	Minichromosome maintenance complex component 7	Cell Cycle	DNA replication	6.3	5.1
ORC2	Origin recognition complex, subunit 2	Cell Cycle	Cell cycle	2.2	2.1
ORC5	Origin recognition complex, subunit 5	Cell Cycle	Cell cycle checkpoint	3.5	3.9
PRKAR2A	Protein kinase, cAMP-dependent, regulatory, type II, beta	Apoptosis	Insulin signaling pathway	2.2	2.0
PRKAR2B	Protein kinase, cAMP-dependent, regulatory, type II, beta	Apoptosis	Insulin signaling pathway	5.9	5.3
SKP1	S-phase kinase-associated protein 1	Cell Cycle	Oocyte meiosis	2.1	2.5
STEAP3	STEAP family member 3	p53 signal	Six transmembrane prostate protein 3 mRNA	2.9	2.2

RT-PCR, reverse transcription-polymerase chain reaction.

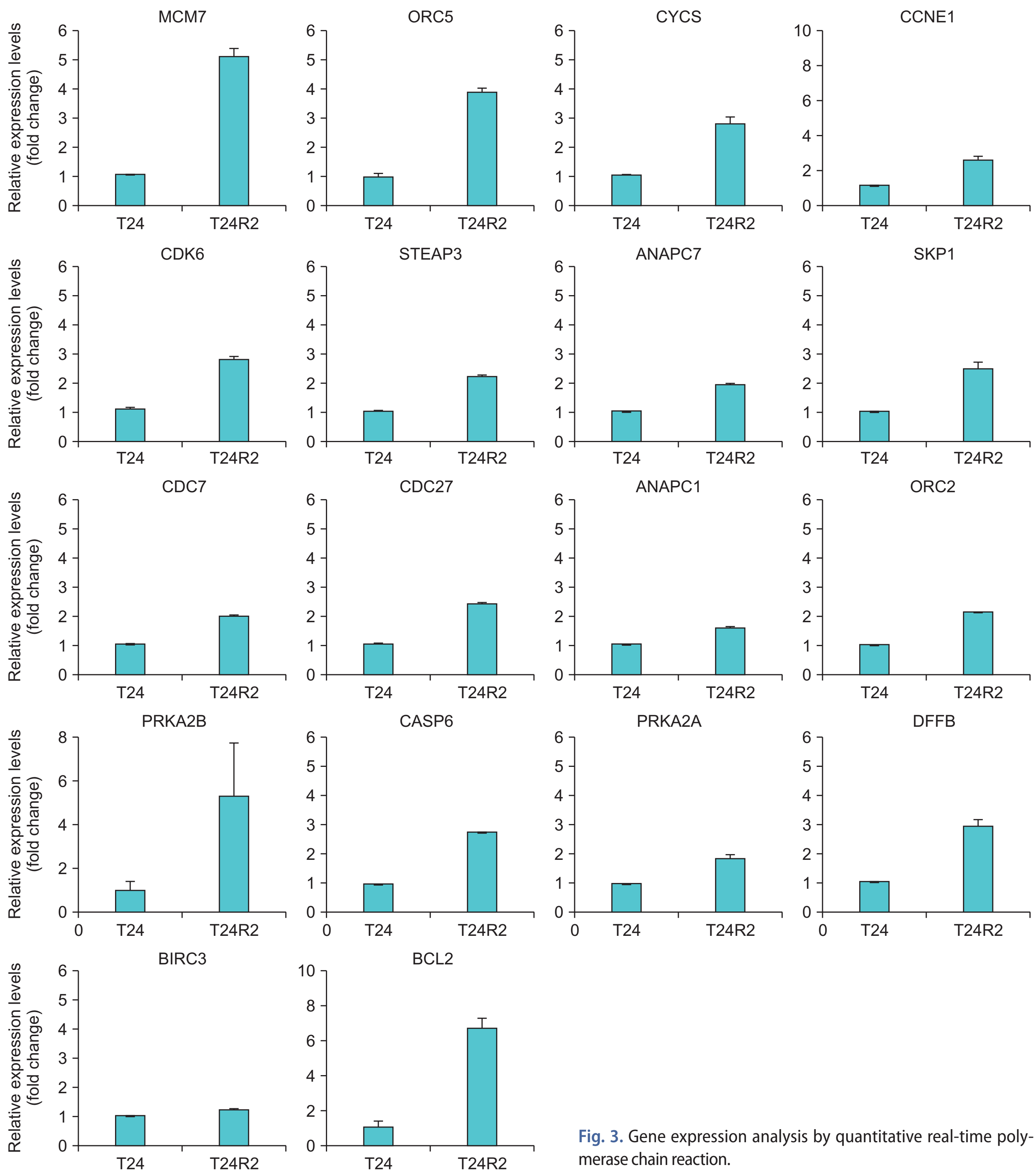

Fig. 3. Gene expression analysis by quantitative real-time polymerase chain reaction.

of CR in BC cells, we chose for further analysis three major biological pathways known to play a role in cancer and resistance development, namely, apoptosis, cell cycle, and p53 tumor suppressor pathway [4,15]. Apoptosis can be induced via an intrinsic (mitochondrial) and an extrinsic (cytoplasmic) pathway [16]. These two pathways converge on the activation of downstream caspases that cleave regulatory and structural molecules [17]. The cell cycle pathway was selected because the major target of cisplatin is DNA. The p53 tumor suppressor plays an important role as a transcription factor in the cell cycle arrest, DNA repair, and cell death. The loss of p53 results in the decreased incidence of apoptosis, inactivation of cell cycle checkpoints, and genomic instability [18]. The BC cell lines used in this

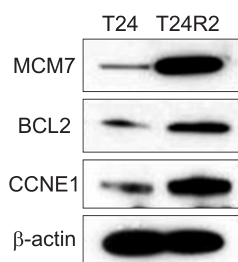

Fig. 4. Results of the Western blot analyses of BCL2, CCNE1, and MCM7.

study have already been confirmed as CR BC cell lines with the change of p53 expressions in our previously published studies [6,9-11]. Therefore, these three pathways were chosen from the KEGG Pathway database (http://www.genome.jp/kegg/pathway.html), a collection of pathway maps summarizing molecular interactions for various areas, such as genomes, biological pathways, diseases, drugs, and chemical substances. KEGG Pathway database is utilized for bioinformatics research, including data analysis in genomics, metagenomics, metabolomics, and other "-omics" studies.

The mechanism of CR is based on the premise that cisplatin targets DNA in BC. First, cisplatin reduces the accumulation of resistant cells due to the involvement of an influx mechanism by passive diffusion. Second, CR occurs due to an increase in trapped intracellular cisplatin in association with glutathione in resistant cells. Third, increased repair of damaged DNA or a tolerance to DNA damage occurs in CR cells. Finally, CR cells display a complex pleiotropic phenotype such as cross-resistance to antimetabolites and reduction in of uptake of sugars and amino acids [15]. Other proven explanations involve an increased expression of DNA topoisomerase I [19], increased abundance of thioredoxin [20], alterations in the expression of BCL2, Bcl-XL, Bax family proteins in association with a reduced apoptotic response [8,21], and down-regulation of Bcl-XL in cells sensitized to cisplatin [22].

This study showed that 18 genes were differentially expressed between T24 and T24R2 suggesting that CR had a specific effect on BC cells. BCL2, CCNE1, CDK6, CYCS, ORC5, MCM7, and PRKAR2B levels showed an increase of more than 3 folds. Western blot analysis also showed an upregulation of BCL2, CCNE1, and MCM7, which was in agreement with microarray and RT-PCR results. The BCL2 gene is already known to be upregulated in CR of BC [10,21]. One of our previous studies demonstrated that induction of a BCL2 related gene by nuclear factor-kappa B and BCL2 up-regulation via the inhibition of Bax translocation to the nucleus contributed to CR in human BC cells. Furthermore, we found that cytochrome c played an important role in the suppression of the antiapoptotic and activation of the proapoptotic proteins belonging to the BCL2 family. As a result, permeability of mitochondrial membrane changed, and cytochrome c was released into the cytosol [9-12]. Upon binding to cytochrome c, apoptotic protease activating factor (Apaf-1) initiated formation of the apoptosome and the activation of caspase-9, which subsequently activates other caspases and induces apoptosis. In this study, we found that *CYCS* gene encoding cytochrome c was upregulated together with BCL2/Xl, BIRC3, DFFB, and especially CASP6 among other caspase families relating to the apoptosis pathway. Previously, it was shown that down-regulation of BCL2 by RNA interference restores sensitivity to cisplatin-induced apoptosis in T24R2 cells using the retrovirus vector-mediated RNAi system after inhibiting BCL2 expression, suggesting that BCL2 upregulation was involved in the resistance to cisplatin-induced Bax translocation and apoptosis in T24R2 cells [9,10].

CCNE1 (cyclin E1) deregulation leads to chromosomal instability and has been associated with the development of many cancers, including BC [22,23]. Together with CDK6 and ORC5, CCNE1 plays an important role in both p53 and cell cycle pathways, thus affecting cell proliferation and cancer progression [24,25]. In this study, activation of other signaling pathways along with CCNE1 during particular stages of the cell cycle showed that cyclin E1 functioned as a regulatory subunit of CDK2, which activity is required for G1/S cell cycle transition. Cyclin E1/CDK2 complex is associated with NPAT protein, a nuclear protein mapped to the ATM locus, and involved in its phosphorylation [26]. This process contributes to the cell cycle-regulated expression of histone genes and plays a critical role in promoting cell cycle progression in the absence of Rb protein [23,27].

MCM7 is also known to play a crucial role in cell proliferation [28]. In the cell cycle, CDC7, MCM2–7 complex, ORC1–6 complex, and other *CDK* genes are involved in the initiation of DNA replication. Assembly of these proteins into the pre-replication complex (pre-RC) is pivotal for the initiation of DNA replication, while converting the pre-RC to the non-active post-RC allows to control the process of genome duplication at the particular origin [29]. In our study, CDC7, ORC2 and ORC5, and other CDKs were found upregulated in parallel with MCM7. CDC7 phosphorylated MCM proteins in the pre-RC to activate the putative replicative helicase essential for DNA replication initiation and elongation to trigger the DNA biosynthesis. Besides BCL2, CCNE1 and MCM7, CDK6, CYCS, ORC5, MCM7, and PRKAR2B were also found to be upregulated with fold change above 3, although this increased expression was not

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confirmed by western blot analysis. According to previous reports, CDK6, CYCS, ORC5, MCM7, and PRKAR2B might have important roles in CR. CDK6 is a cyclin-dependent kinase, associated with D-type cyclins, with an important role in cell cycle regulation, especially in cell proliferation and the progression of p53 tumor suppressor pathway [24]. CYCS is also responsible for many cancers related to both apoptosis and p53 tumor suppressor pathways [28]. ORC5 is extensively involved in cellular proliferation in many cancers [30]. PRKAR2B is a cAMP-dependent, regulatory, type II alpha protein kinase regulating protein transport from endosomes to the Golgi apparatus and the endoplasmic reticulum in apoptotic pathways.

Certain limitations existed that this study did not deal simultaneously with other up- and downregulated genes due to limited resources and the functional aspects of the three identified genes, BCL2, CCNE1 and MCM7, in the mechanism of CR should be also demonstrated in animal and human models. To confirm direct involvement of CCNE1 and *MCM7* genes in CR, further study excluding their genetic expression using small interfering RNA or small RNA in T24R2 would be experimented like *BCL2* gene. Additionally, this study had redundant problems for the high dimensional gene expression data and the unknown combination effect of those genes in CR mechanism, in spite of findings of potential candidate genes for CR and explaining their adequate roles in CR mechanism. However, these limitations might be addressed in future in-depth studies to find out the roles of these DEGs in CR BC, and new additional sets of CR cell lines with sophisticated bioinformatics methodologies would be needed to try to find out the concrete roles of our gene profiles involved in CR mechanism to consider the relationships among genes and to remove redundancy as well.

CONCLUSIONS

A panel of 18 genes was upregulated in the CR T24R2 cell line with respect to the sensitive T24 line. Of these, the expression levels of BCL2, CCNE1, and MCM7 were confirmed by microarray profiling, quantitative RT-PCR, and Western blot analyses and might have important roles in CR in patients with BC.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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