Introduction

Although there have been innumerable clinical trials over the last few decades related to the prevention, detection, and treatment of cancer, acquired drug resistance remains an inevitable obstacle to successful chemotherapy in various types of cancer, causing treatment failure in over 90% of patients with metastatic cancer (Szakacs et al., 2006; Saraswathy and Gong, 2013). Acquired drug resistance may be the result of a multifactorial etiology that emerges due to a variety of reasons, including host environmental factors, as well as genetic or epigenetic alterations in cancer cells (Longley and Johnston, 2005; Chen and Sikic, 2012; Foo and Michor, 2014). According to the most well known reported studies, acquired drug resistance in cancer cells could be caused by three major mechanisms, as follows: (i) decreased uptake of water-soluble drugs which require membrane transporters to enter cancer cells, (ii) various changes in cancer cells that affect the capacity of cytotoxic drugs to kill them, including alterations in the cell cycle, increased repair of DNA damage, reduced apoptosis, and altered metabolism of drugs, and (iii) increased energy-dependent efflux of hydrophobic drugs.

Gemcitabine (2’-2’-difluorodeoxycytidine) is a deoxycytidine analog with a broad spectrum that was established as the first-line chemotherapeutic treatment for locally advanced and metastatic pancreatic cancer in the late 1990s; it has generally been used to treat a variety of solid tumors, including breast, ovarian, pancreatic and non-small cell lung carcinoma, especially in combination with the platinum-based drugs, cisplatin and carboplatin (Toschi et al., 2005; Mini et al., 2006; Toschi and Cappuzzo, 2009; Zhang et al., 2013; de Sousa Cavalcante and Monteiro, 2014). Gemcitabine is first transported into the cancer cell by nucleoside transporters, which include the concentrative and equilibrative transporters. When phosphorylated by deoxycytidine kinase (DCK) to generate its active forms, the diphosphate and triphosphate, gemcitabine interferes with DNA replication and inhibits cancer cell growth, by modulating dNTP pools via the inhibition of ribonucleotide reductase...
Materials and Methods

Selection of microarray datasets eligible for meta-analysis

We conducted a narrow search of microarray datasets for meta-analysis, according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines published in 2009. We collated data from microarray gene expression studies related to acquired-gemcitabine-resistant cancer cell lines from the PubMed, National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), and European Bioinformatics Institute ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) databases. For objective assessment, two independent reviewers extracted data from the original studies; any discrepancies that arose between these reviewers were resolved either by consensus or by consultation with a third reviewer. The keywords “gemcitabine,” “gemcitabine cancer,” “acquired gemcitabine resistance (resistant),” “acquired drug resistance (resistant),” and “gene and/or expression and/or profile” were used in the search for studies. We included a study in the analysis if it contained the following: (i) gene expression profiling of gemcitabine-resistant cancer cell lines or gemcitabine-resistant derivatives of cancer cell lines that had been generated by stepwise selection, and (ii) sufficient data and the correct platform to facilitate meta-analysis. Studies that reported non-human data or used intrinsically drug-resistant cells were excluded from the meta-analysis.

Meta-analysis of multiple microarray datasets

We carried out a cross-platform meta-analysis of gene expression profiles in the selected microarray datasets using the rank product method (RankProd package in the R software, http://www.r-project.org/) implemented in the web-based INMEX tool for meta-analysis of expression data (http://www.inmex.ca/INMEX/) (Xia et al., 2013; Song et al., 2014; Toro-Dominguez et al., 2014). Before meta-analysis of the three datasets, all genes or probe IDs from each dataset were annotated as Entrez database IDs, for data consistency, and the expression values for corresponding genes in the samples were log-transformed and quantile-normalized so that they had zero mean and unit variance. According to the RankProd algorithm non-parametric permutation test, which considers all possible pair-wise comparisons, the DEGs that appeared consistently in whole datasets were assigned to a higher rank, depending on the percentage of false-positives predicted in a given number of replicates, multiplied across the different microarray datasets.

GO hierarchy and KEGG pathway enrichment analysis

In order to discern biological attributes of the identified DEGs in the acquired-gemcitabine-resistant cancer cell lines, functional enrichment via Gene Ontology (GO) hierarchy and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics resources (http://david.abcc.ncifcrf.gov/), with a significance threshold of p<0.05.

Gene regulatory network analysis

In order to determine the gene regulatory network of the identified DEGs, we carried out enrichment analysis for potential transcription factors and microRNAs, based on a comparison of upstream DNA sequences with an assembly of previously discovered gene sets retrieved from the Molecular Signatures database (MSigDB, http://www.broadinstitute.org/gsea/msigdb/index.jsp), with a significance threshold of p<0.05 (Shi et al., 2014). The hypergeometric algorithm and Benjamini-Hochberg adjustment were used for statistical processing and multiple-test revision of the network analysis, respectively.

Gene co-expression network analysis

In order to predict the biological activity of the identified DEGs at the gene level, we constructed a...
gene co-expression network of the top 50 up- and down-regulated DEGs using the GeneMANIA web server (http://www.genemania.org/) (Warde-Farley et al., 2010; Molina-Navarro et al., 2014). The relationships between genes in the network were determined by GO term (biological process)-based weighting, and filtered by including only gene co-expression relationships with a significance threshold for weight value of p>0.05. Within the network, distinct modules were identified based on the fast-greedy HEN (G) algorithm, using the Community Clusters GLay plug-in (http://cytoscape.wodaklab.org/wiki/CommunityStructureLayout) in the Cytoscape software (http://www.cytoscape.org/) (Gupta et al., 2012; Firoz et al., 2014). Overrepresented biological functions within each module were examined using the functional enrichment analyses in the DAVID and g:Profiler (http://biit.cs.ut.ee/gpro) programs.

Protein–protein interaction network analysis

In order to predict the biological activity of the identified DEGs at the protein level, the top 50 (20) up- and down-regulated DEGs were imported into a protein–protein interaction (PPI) network downloaded from the Biological General Repository for Interaction Datasets (BioGRID, http://thebiogrid.org/, (Pan, 2012). The PPI network was screened on a genome-wide scale using the Cytoscape software. Within the PPI network, we identified the hub proteins of distinct protein clusters using the Cytoscape plug-in, ClusterONE (http://apps.cytoscape.org/apps/clusterone) (Nepusz et al., 2012). Overrepresented biological functions within each protein cluster surrounding a hub protein were examined by the functional enrichment analyses in the DAVID and g:Profiler online programs.

Results

Selection of microarray datasets related to AGR for meta-analysis

From microarray datasets in the NCBI GEO database, we extracted 22 GEO samples within three GEO series (GSEs) related to AGR that met our criteria (see Materials and Methods; Figure 1A). All three GSEs were solely derived from cancer cell lines that had acquired drug resistance by stepwise treatment with gemcitabine, such as lung adenocarcinoma, pancreatic cancer, and epidermoid carcinoma (Table 1). With regard to the microarray platforms used, GSE 35141 was obtained using Illumina BeadChip (Illumina, San Diego, CA) and the other two datasets (GSE 6914 and GSE 3344) were obtained using Affymetrix GeneChip (Affymetrix, Santa Clara, CA).

Table 1. Characteristics of Individual Studies Selected from GEO of NCBI for Meta-analysis

| Dataset | Sample PC | AGR | Drug | Cancer cell line | Platform |
|---------|-----------|-----|------|------------------|----------|
| GSE 6914 | 4         | 4   | Gemcitabine | lung adenocarcinoma (Calu3) | Affymetrix Human Genome U133A |
| GSE 35141 | 6         | 6   | Gemcitabine | pancreatic cancer (PK) | Agilent-014850 Whole Human Genome Microarray 4x44K |
| GSE 3344 | 1         | 1   | Gemcitabine | epidermoid carcinoma (KB) | Affymetrix Human Genome U95 Version 2 |

*GEO gene expression omnibus, PC: parental control, AGR: acquired gemcitabine resistance

Identification of up- or downregulated DEGs in the meta-analysis

From cross-platform microarray meta-analysis based on the RankProd algorithm, we identified a total of 158 DEGs, including 76 up- and 82 down-regulated genes with a significance threshold of p<0.05. The top 20 up- and down-regulated genes among the total DEGs are listed in order of significance (by p value) in Table 2. The up-regulated DEGs with the lowest value of p<1.0x10^-10 were CALB1, ADAM28, TRIM22, MSMB, TLE4, INHBB, ADH1C, IL1R2, and TRIM21. The down-regulated DEGs with the lowest observed value of p=0.00250 were ARHGAP29 and PTX3. The up- and down-regulated DEGs with the largest mean log2 fold change were CALB1 (calbindin 1, 28 kDa) and ARHGAP29 (Rho GTPase activating protein 29), respectively. In order to interpret the biological significance of the identified DEGs in different cancer cell lines with AGR, we attempted the systemic approach of using various in silico analyses that might identify gene regulation, gene co-expression, and PPI networks, accompanied by functional enrichment analysis (Figure 1B).

Functional and pathway enrichment analysis of all the identified DEGs

A total of 158 DEGs identified by the meta-analysis were classified according to GO hierarchy functional category (biological process, molecular function,
and cellular component) and KEGG pathway, with a significance threshold of p<0.05 (Table 3). The most overrepresented GO terms under biological process were enriched in the following descending order: “Defense response” (GO 0006952), “Response to extracellular stimulus” (GO 0009991), and “Response to drug” (GO 0042493). The most enriched GO terms under molecular function and cellular component were “Tumor necrosis factor receptor superfamily binding” (GO 0032813) and “Extracellular region (GO 0005576). The most enriched KEGG pathway terms were (descending order): “Cytokine-cytokine receptor interaction” (Hsa 04060), “Metabolism of xenobiotics by cytochrome P450” (Hsa 00980), and “Regulation of actin cytoskeleton” (Hsa 04810).

Table 2. The top 20 Most Strongly Up- or Down-regulated Genes in the DEGs Identified by Meta-analysis

| Enterz ID | Gene symbol | Log2 FC | p value | Gene name |
|-----------|-------------|---------|---------|-----------|
| 793       | CALB1       | -2.20630|         | calbindin 1, 28kDa |
| 10863     | ADAM28      | -1.99019|         | metalloepitope domain 28 |
| 10346     | TRIM22      | -1.78943|         | tripartite motif containing 22 |
| 4477      | MSMB        | -1.76001|         | microeminoprotein, beta- |
| 7091      | TLE4        | -1.73700| <1.0E-5 | transducin-like enhancer of split 4 |
| 3625      | INHB       | -1.40772|         | inhibin, beta B |
| 126       | ADH1C       | -1.33476|         | alcohol dehydrogenase 1C (class I), gamma polypeptide |
| 7850      | ILIR2      | -1.31214|         | interleukin 1 receptor, type II |
| 6737      | TRIM21      | -1.11247|         | tripartite motif containing 21 |
| 25849     | PARM1       | -1.66358| 0.00250 | prostate androgen-regulated mucin-like protein 1 |
| 1004      | CDH6        | -1.63775| 0.00272 | cadherin 6, type 2, K-cadherin (fetal kidney) |
| 51176     | LEF1        | -1.49318| 0.00357 | lymphoid enhancer-binding factor 1 |
| 2697      | GJA1        | -1.45898| 0.00307 | gap junction protein, alpha 1, 43kDa |
| 4856      | NOV         | -1.70069| 0.00357 | nephroblastoma overexpressed |
| 124       | ADH1A       | -1.34103| 0.00437 | alcohol dehydrogenase 1A (class I), alpha polypeptide |
| 1002      | CDH4        | -1.48827| 0.00466 | cadherin 4, type 1, R-cadherin (retinal) |
| 10231     | RCAN2       | -1.21286| 0.00588 | regulator of calcineurin 2 |
| 6304      | SATB1       | -0.38100| 0.00611 | superoxide dismutase 3, extracellular |
| 6240      | RRM1        | -0.93649| 0.00631 | ribonucleotide reductase M1 |
| 6649      | SOD3        | -0.88427| 0.00636 | superoxide dismutase 3, extracellular |
| 9411      | ARHGAP29    | 1.94459| 0.00250 | Rho GTPase activating protein 29 |
| 5806      | PTX3        | 1.54702| 0.00250 | pentraxin 3, long |
| 50810     | HDGFPR3     | 1.60953| 0.00285 | hepatoma-derived growth factor, related protein 3r |
| 72        | ACTG2       | 1.28665| 0.00300 | actin, gamma 2, smooth muscle, enteric |
| 8187      | ZNF239      | 1.37927| 0.00333 | zinc finger protein 239 |
| 6696      | SPPI        | 1.34688| 0.00333 | secreted phosphoprotein 1 |
| 26150     | RIBC2       | 1.32767| 0.00333 | RIB43A domain with coiled-coils 2 |
| 6414      | SEPP1       | 2.21272| 0.00400 | selenoprotein P, plasma, 1 |
| 7292      | TNSF4       | 1.50729| 0.00461 | tumor necrosis factor (ligand) superfamily, member 4 |
| 7431      | VIM         | 1.61811| 0.00470 | vimentin |
| 1382      | CRABP2      | 1.24545| 0.00473 | cellular retinoic acid binding protein 2 |
| 1635      | DCK         | 2.13356| 0.00500 | deoxycytidine kinase |
| 2313      | FLI1        | 1.39167| 0.00500 | Fli-1 proto-oncogene, ETS transcription factor |
| 4633      | MYL2        | 1.28665| 0.00500 | myosin, light chain 2, regulatory, cardiac, slow |
| 5272      | SERPINB9    | 1.02120| 0.00500 | serpin peptidase inhibitor, clade B (ovalbumin), member 9 |
| 147       | ADRA1B      | 0.82309| 0.00500 | adrenergic receptor alpha 1B |
| 2947      | GSTM3       | 0.80997| 0.00521 | glutathione S-transferase mu 3 (brain) |
| 10669     | CGREF1      | 1.78892| 0.00533 | cell growth regulator with EF-hand domain 1 |
| 6515      | SLC2A3      | 1.68493| 0.00545 | solute carrier family 2 (facilitated glucose transporter), member 3 |
| 894       | CCND2       | 1.13272| 0.00545 | cyclin D2 |

Gene regulation network analysis of the top 50 up- and down-regulated DEGs

In order to identify the network regulating gene expression of the top 50 up- and down-regulated DEGs, which might directly influence AGR, we analyzed potential regulatory elements that target the DEGs depending on their upstream DNA sequence (Table 4). The target sites of the following transcription factors were significantly enriched in the DEGs: JUN, LEF1, NFAT, MAZ, MLLT4, and TCF1. The target sites of the following microRNAs were also significantly enriched in the DEGs: miR-200B/200C/429, miR-19A/19B, miR-520G/520H, miR-524, miR-23A/23B, miR-153, miR-409, miR-145, miR-9, and miR-129.

Gene co-expression network analysis of the top 50 up- and down-regulated DEGs

We constructed a co-expression network for the top 50 up- and down-regulated DEGs, with gene-correlation interactions consisting of 143 nodes and 764 edges, by mapping the DEGs onto a massive database of functional-interaction datasets in the GeneMANIA web...
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The network was further subdivided into five functional modules that were closely connected by >20 nodes, using the fast-greedy HEN (G) algorithm of the Cytoscape GLay plug-in, followed by functional enrichment analysis according to GO hierarchy and KEGG pathway. For example, “Module 1,” of maximum size in the network, was significantly enriched using biological terms of the GO hierarchy such as “Programmed cell death” (GO 0012501) and “Response to organic substance” (GO 0010033).

The smallest module, “Module 4,” was significantly enriched using biological terms such as “Cell proliferation” (GO 0008283) and “Calcium signaling pathway” (Hsa 04020), with regard to GO hierarchy and KEGG pathway, respectively.

Table 3. The Top 15 enrichment of GO Hierarchy and KEGG Pathway for the Total DEGs

| GO ID           | GO Term                                      | Genes | p value  |
|-----------------|----------------------------------------------|-------|----------|
| GO_CC:0005576   | Extracellular region                          | 45    | 9.92E-07 |
| GO_MF:0032813   | Tumor necrosis factor receptor superfamily binding | 5    | 3.91E-04 |
| GO_BP:0006952   | Defense response                              | 15    | 3.17E-03 |
| GO_BP:0009991   | Response to extracellular stimulus            | 8    | 6.28E-03 |
| GO_BP:0042493   | Response to drug                              | 7    | 2.05E-02 |
| GO_BP:0008284   | Positive regulation of cell proliferation     | 10   | 2.19E-02 |
| GO_BP:0043067   | Regulation of programmed cell death           | 15   | 3.02E-02 |
| GO_MF:0008083   | Growth factor activity                         | 6    | 3.61E-02 |
| GO_BP:0051046   | Regulation of secretion                        | 6    | 5.04E-02 |
| GO_BP:0007267   | Cell-cell signaling                           | 11   | 7.44E-02 |
| GO_CC:0031988   | Membrane-bounded vesicle                      | 11   | 8.21E-02 |
| GO_BP:0006355   | Regulation of transcription, DNA-dependent    | 20   | 8.99E-02 |
| GO_CC:0031090   | Organelle membrane                            | 13   | 1.03E-01 |
| GO_BP:0043933   | Macromolecular complex subunit organization   | 8    | 1.74E-01 |
| GO_MF:0030528   | Transcription regulator activity               | 16   | 1.95E-01 |

Table 4. The potential regulatory elements of the top 50 up- and down-regulated DEGs

| Transcription factor | Target sequence | Genes | p value  |
|----------------------|-----------------|-------|----------|
| JUN                  | TGANTCA         | 20    | 1.93E-09 |
| LEF1                 | CTTTTGT         | 26    | 3.56E-09 |
| NFAT                 | TGAAAA          | 25    | 7.38E-09 |
| MAZ                  | GGGAGGRR        | 27    | 1.54E-01 |
| MLLT4                | TTGTTT          | 25    | 3.46E-01 |
| TCF1                 | WRGTTAATNA      | 9     | 2.51E-07 |
| TGAACNNN             |                 | 4     | 5.30E-01 |

| microRNA            | Target sequences | Genes | p value  |
|---------------------|------------------|-------|----------|
| MIR-200B/200C/429   | CAGTATT          | 14    | 1.02E-09 |
| MIR-19A/19B         | TTTGCAC          | 11    | 1.84E-06 |
| MIR-520G/520H       | CACTTTG          | 7     | 1.88E-05 |
| MIR-524             | CTTTGTG          | 9     | 2.00E-05 |
| MIR-23A/23B         | AATGTTG          | 6     | 1.05E-04 |
| MIR-153             | CATGTTG          | 8     | 1.05E-04 |
| MIR-409-3P          | AACATTTC         | 5     | 1.34E-04 |
| MIR-145             | AACTGGG          | 6     | 1.63E-04 |
| MIR-9               | TAGCTTT          | 6     | 1.70E-04 |
| MIR-129             | GCAAAAA          | 5     | 4.33E-04 |

Figure 2. Functional Modules Clusters in Gene Co-Expression Network of the Identified DEGs. From gene co-expression network of the top 50 up- and down-regulated DEGs, five functional module clusters were identified. The node and edge of each module cluster stand for genes with the DEGs and interaction of the genes, respectively. The color of node signifies as follows: Light blue-up-regulated DEGs, Light red-down-regulated DEGs, and Light brown-additional genes in GeneMANIA.
hub proteins (SYT1, GJA1, LEF1, SATB1, RRM1, and BATF) represented up-regulated DEGs, the other six (FHL1, CCND2, SORBS2, FGF2, DCK, and SERPINB9) represented down-regulated DEGs. By constructing a PPI network for the top 20 up- and down-regulated DEGs, we also observed the whole schematic diagram that the two kinds of DEGs interact directly or indirectly in the network (Figure 4).

**Discussion**

Gemcitabine is a molecular-targeted cancer drug for the standard chemotherapeutic treatment of patients with various solid tumors, but its clinical impact is limited by the high degree of inherent or acquired drug resistance; no definitive genetic factors have been reported to be solely responsible for the AGR of cancers. Investigating alterations in gene expression, which characterize the response of cancer cells to gemcitabine treatment during the process of AGR, would help us to understand the underlying mechanisms of drug resistance and improve the efficacy of therapeutic strategies for this deadly disease. In this context, we performed a cross-platform meta-analysis of three independent microarray datasets and attempted an integrative analysis of three systemic molecular networks (gene regulation network, gene co-expression network, and PPI network) for the identified DEGs. In the case of the top 20 up- and down-regulated DEGs, most of the genes have been reported to be involved in carcinogenesis of many tumor and cancer types, suggesting that they may be potential key factors in the AGR process. Functional enrichment analysis of all the identified DEGs revealed that they were mainly classified as related to biological functions such as the cell cycle, homeostasis, immune response, apoptosis, replication, and signal transduction that are associated with the general process of carcinogenesis. In particular, the following GO and KEGG enrichment terms had direct relevance to the mechanisms by which cancers acquire their gemcitabine-resistant property: the GO terms, “Response to drug,” “Regulation of programmed cell death,” “Regulation of secretion,” and “Regulation of transcription, DNA-dependent,” and the KEGG terms, “Metabolism of xenobiotics by cytochrome P450,” “Drug metabolism,” “Pyrimidine metabolism,” “MAPK and Wnt signaling pathway,” “Endocytosis,” and “Pathways in cancer” (Nakano et al., 2007; Toschi and Cappuzzo, 2009; Tufman and Huber, 2010; Hung et al., 2012; de Sousa Cavalcante and Monteiro, 2014). In evaluating the biological significance of the identified DEGs in the complex process of AGR, comprehensive information on the topological positions of the DEGs within a network at the transcriptome level is no less valuable than the fold-change and p values of individual DEGs. Systemic analysis of the gene regulation network of the identified DEGs and their potential regulatory elements (targets of transcription factors and microRNAs) may facilitate a macroscopic view of AGR, by looking into regulatory mechanisms governing the expression and the function of (many different) genes and cellular processes, respectively, in cancers that acquire gemcitabine resistance. In practice, most of regulatory
elements enriched by the top 50 up- and down-regulated DEGs were reported to be involved as oncogenes or tumor suppressors in a variety of human cancers including colon, gastric, prostate, lung, pancreatic, and breast cancer. For example, among transcription factors, it was reported that activation of the JUN-JNK complex was required for development of AGR in lung cancer H1299 cells, and increased expression of NFAT was correlated with tumor cell survival against apoptosis in drug-resistant pancreatic cancer (Teraishi et al., 2005; Griesmann et al., 2013). LEF1 (lymphoid enhancer-binding factor 1) was identified as a mediator of the Wnt/β-catenin signaling pathway during metastasis of lung adenocarcinoma (Bleckmann et al.). In the case of microRNAs, it was reported that the miR-200 family could serve as regulators of EMT in metastasis of ovarian, breast, and pancreatic cancer, and the attenuated expression of miR-200b/200c was found in gemcitabine-resistant pancreatic cancer cells (Ali et al., 2010). The microRNA, miR-145, is known to be a novel regulator of MUC13 that is highly involved in the progression of pancreatic cancer, and miR-19a was discovered as a prognostic factor for poor outcome in patients with non-small cell lung cancer (Lin et al., 2013; Khan et al., 2014). In another systemic approach for identifying expression patterns of DEGs that may be related to AGR in cancers, we evaluated the functional enrichment of DEGs into distinct modules, where they are co-located and form functional interactions with each other, within the network that was constructed by mapping the DEGs onto the massive gene co-expression database of the GeneMANIA online resource. Five modules, composed of the top 50 up- and down-regulated DEGs, were identified; genes within these modules and other genes already known to be in the network were largely involved in processes that are representative of AGR, including indefinite cell proliferation, for example, via abnormal apoptosis (modules 1, 3, and 4), intercellular membrane transport of small molecules (modules 2 and 4), deregulated transcription (modules 3 and 4), and arrested replication escape by reactivation of DNA synthesis (module 3). In parallel with the two above-mentioned network analyses, clustering and enrichment of functional hub clusters were analyzed at the protein level within the PPI network in order to identify hub proteins with a high degree of interaction. In many studies, hub nodes have been found to be necessary factors for the specific function that is executed by their corresponding network in an organic system, and play important functions in maintaining that network within the system. The twelve functional hub DEGs identified in the PPI network were enriched using GO terms for biological processes with a close relationship to the AGR process, such as abnormal apoptosis (hub clusters 3, 4, 5, 9, and 12), membrane transport of small molecules (hub cluster 1), deregulated transcription (hub clusters 2, 6, 8, and 11), and arrested replication escape by reactivation of DNA synthesis (hub clusters 7, 8, and 10). By comparing four lists of DEGs, for the gene regulation network, gene co-expression network, PPI network, and the top 50 up- and down-regulated DEGs, seven DEGs were shortlisted as AGR candidate genes from the total of 158 DEGs identified by meta-analysis; these included four up-regulated DEGs (SYT1, CCND2, GJA1, and LEF1) and three down-regulated DEGs (FHL1, CCND2, and SORBS2) that appeared in all four lists. In particular, GJA1, LEF1, and CCND2 were affiliated to the lists of the top 20 up- and down-regulated DEGs more likely to be crucial for the etiology of AGR.

LEF1 belongs to a family that shares conserved amino acid sequence homology with high-mobility group protein 1 (Nguyen et al., 2009; Bleckmann et al., 2013). Many previous studies showed that a LEF1/TCF4 complex is closely associated with poor survival in patients with different cancers (colon, gastric, breast, lung, and pancreatic), via regulation of cell proliferation, migration, invasion, and metastasis, as a transcription factor that mediates the Wnt/β-catenin signaling pathway in the nucleus. Another upregulated DEG, GJA1 (encoding gap junction protein, alpha 1, 43 kDa), is a member of the connexin gene family that encode components of gap junctions, which act as intercellular channels for the diffusion of low molecular weight materials from cell to cell (McLachlan et al., 2006; Li et al., 2007). GJA1, better known as connexin 43 (Cx43), was reported to function as a tumor suppressor that inhibits tumor growth, via regulation of EMT and angiogenesis, in breast and prostate cancer. Gene expression analysis of cisplatin-sensitive and -resistant ovarian cancer cells showed that GJA1 expression was highly elevated in cisplatin-resistant ovarian cancer cells, by whole-genome oligonucleotide microarray analysis, and by immunoblotting and immunofluorescence analyses using a Cx43-specific antibody. CCND2 (cyclin D2), identified by the down-regulated DEG, is known to function as a regulatory subunit of cyclin-dependent kinases that are involved in the G1/S transition in the mitotic cell cycle (Koyama-Nasu et al., 2013). Alteration of CCND2 gene expression promoted phosphorylation and subsequent inactivation of the retinoblastoma tumor suppressor protein, RB1, which causes dysregulation of the G1/S transition as a common event in the tumorigenesis of many cancers. There was no choice but to exclude DCK (deoxycytidine kinase) and RRM1, already known as molecular targets in cytotoxic mechanisms of gemcitabine, from the final gene lists; although they were contained in module 3 of the gene co-expression network and hub clusters 8 and 13 of the PPI network (highly related to AGR), we could not analyze their potential regulatory elements in the gene regulation network, owing to computational limitation using databases containing only experimentally discovered relationships.

In conclusion, by performing a cross-platform meta-analysis of three microarray datasets for different cancer cell lines with AGR, we have identified a total of 158 candidate DEGs that have a high probability of being involved in the molecular mechanism of AGR. We have also provided a comprehensive overview of the gene expression pattern of the AGR-related DEGs by attempting integrated in silico analysis of three molecular networks. This topological approach of integrative network analysis could help to provide new insights into the complex nature of AGR and may be useful for studying prospective chemotherapeutic strategies.
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Acknowledgements

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