Glutathione peroxidase 3 is a candidate mechanism of anticancer drug resistance of ovarian clear cell adenocarcinoma

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Received July 26, 2008; Accepted September 24, 2008

DOI: 10.3892/or_00000144

Abstract. Ovarian clear cell adenocarcinoma has low sensitivity to platinum drugs. The molecular-biological mechanism of the low sensitivity has not been clarified. The objective of this study was to identify candidate genes associated with low sensitivity of clear cell adenocarcinoma to platinum drugs. Exhaustive gene profiling of 4 ovarian clear cell adenocarcinoma cell lines, KK, OVMANA, OVSAYO, and RMG-1 and 4 ovarian serous adenocarcinoma cell lines, KF, HRA, SHIN-3 and KOC-2S was performed by DNA microarray. Obtained candidate genes were suppressed by RNA interference and changes in the cisplatin sensitivity of clear cell adenocarcinoma cells were observed. Six genes including the *glutathione peroxidase 3* (*GPX3*) gene were identified to be highly expressed in clear cell adenocarcinoma by DNA microarray analysis. GPX3 suppression by RNA interference increased cisplatin sensitivity 3.3-4.2-fold in 3 of the 4 clear cell adenocarcinoma cell lines. GPX3 was identified to be a gene highly expressed in clear cell adenocarcinoma. Since GPX3 suppression increased the cisplatin sensitivity of clear cell adenocarcinoma cells, *GPX3* may be a candidate gene associated with the low cisplatin sensitivity of clear cell adenocarcinoma.

Introduction

The highest number of patients die of epithelial ovarian cancer in the gynecology field (1). Platinum-based combination chemotherapy and debulking surgery has recently improved the prognosis of progressive epithelial ovarian cancer, but clear cell adenocarcinoma is an exception and its prognosis remains poor. The important factor of poor prognosis of clear cell adenocarcinoma is its low sensitivity to known anticancer drugs, particularly platinum drugs. In our investigation of clinical cases, only 11% of patients responded to platinum-based chemotherapy for clear cell adenocarcinoma, which was markedly lower than the response rate in serous adenocarcinoma (73%) (2). Goff *et al* also reported that 70% of patients with stage III clear cell adenocarcinoma were resistant to platinum-based chemotherapy (3).

Regarding biological behavior of clear cell adenocarcinoma, Itamochi *et al* reported low proliferation activity (4,5). They showed that in an *in vitro* study, the doubling time of clear cell adenocarcinoma cells was 2 times or longer than that of serous adenocarcinoma cells (4), the ratios of clear cell adenocarcinoma cells in the G_2M, S and proliferation phases were low (4), and the ratio of Ki-67-positive cells was low in clinical cases of clear cell adenocarcinoma (5) and suggested that low proliferation activity of clear cell adenocarcinoma is a cause of the low sensitivity to platinum drugs. However, the molecular-biological mechanism of the low platinum sensitivity of clear cell adenocarcinoma has not been clarified.

The objective of this study was to identify candidate genes associated with cisplatin resistance of clear cell adenocarcinoma. Candidate genes associated with cisplatin resistance were exhaustively investigated in several cell lines of ovarian clear cell adenocarcinoma using DNA microarray. Obtained gene candidates were suppressed by RNA interference and changes in the cisplatin sensitivity of clear cell adenocarcinoma cells were observed.

Materials and methods

Cell cultures. Eight ovarian cancer cell lines were used in this study; four were of clear cell adenocarcinoma including KK (6), OVMANA (7), OVSAYO (7), and RMG-1 (8), and the other four were of serous adenocarcinoma including KF (9), HRA (10), SHIN-3 (11) and KOC-2S (12). All of the cells were maintained in Eagle's MEM supplemented with 10% fetal bovine serum (FBS) and were grown in Ham's F12 medium with 10% FBS.

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Key words: ovarian cancer, clear cell carcinoma, glutathione peroxidase 3, cisplatin, drug resistance

RNA preparation and DNA microarray analysis. Total RNA was extracted from ovarian cancer cells by the acid guanidinium method and was subjected to a synthesis of double-stranded cDNA with oligo-dT primer, which was then used to prepare biotin-labeled cRNA with the use of the GeneChip labeling system (Affymetrix, Santa Clara, CA, USA). The resultant cRNA was then hybridized to GeneChip HGU95Av2 microarray (Affymetrix) revealing the expression intensities of 12,625 probe sets in each sample. Detection of hybridization signals and the statistical analyses of the digitized data were performed with a GMS 418 array scanner (Affymetrix) and Gene Spring 3.2.2 software (Silicon Genetics, Redwood, CA, USA), respectively. The fluorescence intensity for each gene was normalized relative to the median fluorescence value for all human genes with a 'Present' or 'Marginal' call (Microarray Suite; Affymetrix) in each hybridization. In the hierarchical clustering analysis, similarity was measured by the Pearson's correlation with a separation ratio of 1.0. The details of the genes shown in the figures are available upon request.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. To verify the data obtained from microarrays, we carried out real-time RT-PCR analysis. Portions of unamplified cDNA were subjected to PCR with SYBR-Green PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA). The incorporation of the SYBR-Green dye into the PCR products was monitored in real-time with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems), thereby allowing determination of the threshold cycle (Ct) at which exponential amplification of PCR products begins. The Ct values for cDNAs corresponded to the GAPDH gene and target transcripts relative to that of GAPDH mRNA. The oligonucleotide primers for PCR were as follows: GAPDH cDNA, 5'-CGCGGGGCTCTCAGAACATCAT-3' and 5'-CCAGCCCCAGCGTCAAAGGTG-3'; glutathione peroxidase 3 (GPX 3) cDNA, 5'-AGCAGTATGCTGGCAA ATATGTCC-3' and 5'-CAGACCGAATGGTGCAAGCTC TTC-3'.

Selection of short hairpin RNA stable cell lines. The DNA oligonucleotides, encoding short hairpin RNA (shRNA) targeting the GPX3 (forward; CACCGGGAGAGTTTGCAC TATTAACGTGTGCTGTCCGTTAATGGTGCAAGCTCT TCCTTTTT, reverse; GCATAAAAAGGAAGAGCTTGCA CCATTAACGGACAGCACACGTTAATAGTGCAAACTC TCCC) were synthesized, annealed, and cloned into the *Bsp*MI site of the vector piGENE PURhU6 (13), which contains a human U6 promoter and a puromycin resistance gene. The shRNA expression plasmid (piGENE PURhU6/shGPX3) and the control plasmid (piGENE PURhU6) were transfected into four ovarian clear cell carcinoma cell lines by the standard calcium phosphate precipitation method (14). The cells were selected with the concentration of 1 μg/ml puromycin (Calbiochem, Darmstadt, Germany). Resistant clones were obtained after four weeks. The cells were subsequently maintained in the presence of 1 μg/ml puromycin.

Semiquantitative RT-PCR analysis. Using a semiquantitative RT-PCR method, we assessed transcription levels of GPX3 and GAPDH in the transfectants. Total RNAs were extracted from the transfectants by the acid guanidinium method and reverse-transcribed using Reverse Transcription System (Promega, Madison, WI). Each RT-PCR reaction consisted of 25 cycles of 30 sec at 94˚C, 30 sec at 55˚C and 1 min at 72˚C. Amplification of GAPDH revealed similar signal strengths in all samples, as a control for the integrity of each RNA template. PCR products were electrophoresed in 1.5% agarose gels. Primers used for amplification were described above.

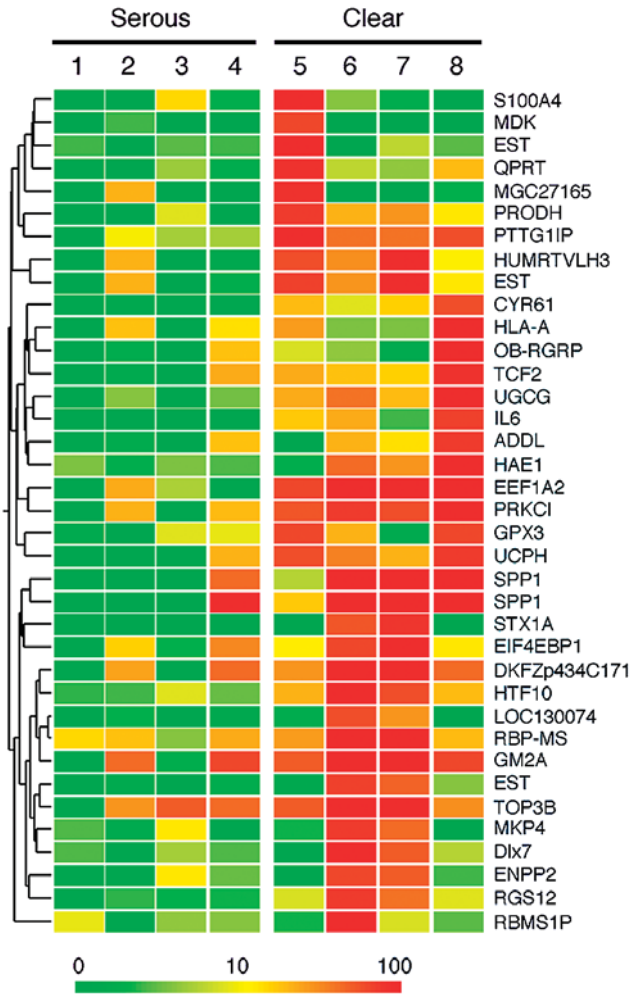

Figure 1. Identification of clear cell adenocarcinoma-specific genes. Hierarchical clustering of 34 probe sets were performed on the basis of their expression profiles in serous adenocarcinoma cell lines [HRA (1), KF (2), KOC-2S (3) and SHIN-3 (4)] and clear cell adenocarcinoma cell lines [KK (5), OVMANA (6), OVSAYO (7) and RMG1 (8)]. Each column represents a separate cell line and each row a single probe set on the microarray. Expression level of each probe set is shown color-coded as indicated by the scale at the bottom. Note that two distinct probe sets are assigned to the *PP1* gene on GeneChip HGU95Av2 array.

Colorimetric assay. The sensitivity of the transfectants to cisplatin (Bristol-Myers Squibb Co., Ltd., Tokyo, Japan) was investigated by colorimetric assay using Cell Proliferation kit II (XTT) (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany). The transfectants were exposed to cisplatin at concentrations of 1-128 μM for 24 h. The viable cell count measured by colorimetric assay was presented as a percent ratio to the count of the control untreated with cisplatin. A

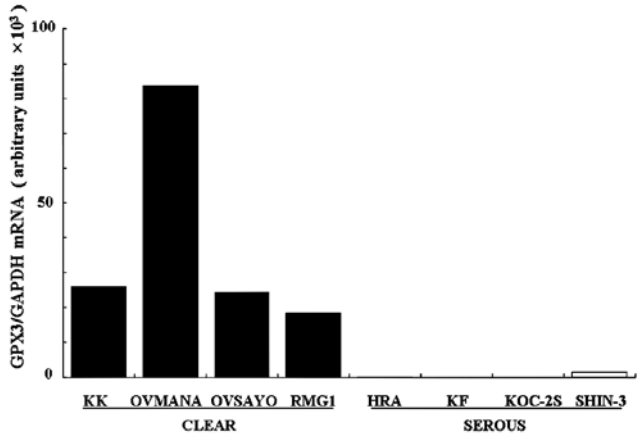

Figure 2. Quantitation of *GPX3* transcripts in ovarian cancer cell lines. Complementary DNA prepared from the ovarian cancer cells was subjected to real-time RT-PCR with primers specific for *GPX3* or *GAPDH* genes. The ratio of the abundance of the *GPX3* transcript to that of GAPDH mRNA was calculated as 2^n, where n is the Ct value for *GAPDH* cDNA minus the Ct value of the *GPX3* cDNA.

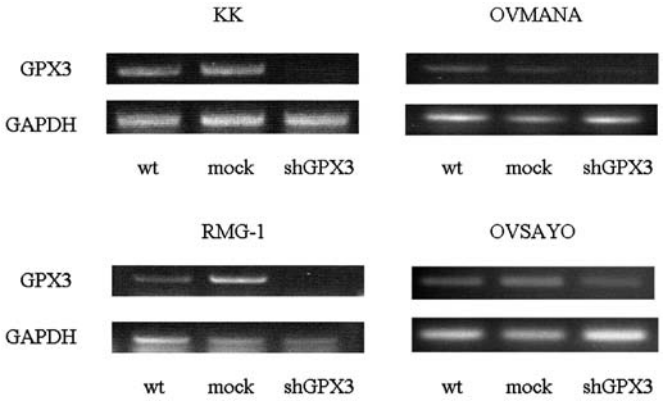

Figure 3. Expression of GPX3 in clear cell adenocarcinoma transfectants. GPX3 expression by the semiquantitative RT-PCR method was decreased in cells transfected with the shRNA expression plasmid in all 4 clear cell adenocarcinoma cell lines compared with the parent and control cell lines. The integrity of each RNA template was controlled through amplification of GAPDH.

dose-response curve was prepared and the 50% growth inhibitory concentration (IC_{50}) was obtained for cisplatin.

Results

DNA microarray analysis. Expression intensities of >12,000 human probe sets were examined in a total of 8 samples. To identify genes whose expression was specific to the clear cell adenocarcinoma subtype, we first calculated the mean expression levels of each gene in both clear cell adenocarcinoma and serous adenocarcinoma group. With the use of GeneSpring software, we then searched for genes whose expression profiles were similar, with a minimal correlation of 0.99, to that of a hypothetical 'clear cell adenocarcinoma-specific gene' with a mean expression level of 0.0 arbitrary unit (U) in the serous adenocarcinoma and of 200.0 U in the clear cell adenocarcinoma. From the resulting genes, we then selected those whose expression level was ≥60.0 U in at least one of the clear cell adenocarcinoma group. A total of 34 probe sets (corresponding to 33 human genes) were finally identified to be specific to the clear cell adenocarcinoma cell lines, including those for secreted phosphoprotein 1 (SPP1), eukaryotic translation elongation factor 1α2 (EEF1A2), hereditary angioedema (HAE) 1, pituitary tumor-transforming gene 1 (PTTG1IP), UDP-glucose ceramide glucosyltransferase (UGCG) and GPX3.

Expression profiles of these clear cell adenocarcinoma-specific genes were shown as a dendrogram, or 'gene tree,' in which genes with similar expression profiles among the samples were clustered near each other (Fig. 1).

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. To confirm the group-specific expression of these genes, we measured their mRNA level by the quantitative real-time RT-PCR method. As shown in Fig. 2, the relative expression level of *GPX3* to *GAPDH* was, for instance, highly induced in the clear cell adenocarcinoma cell lines, but negligible in the serous adenocarcinoma cell lines.

Expression of GPX3 in clear cell adenocarcinoma transfectants. As shown in Fig. 3, GPX3 expression by the semiquantitative RT-PCR method decreased in cells transfected with the shRNA expression plasmid in all 4 clear cell carcinoma cell lines compared with the parent and control lines. The integrity of each RNA template was controlled through GAPDH amplification.

Cisplatin sensitivity. The dose-response curves of the 4 types of clear cell adenocarcinoma transfectants to cisplatin are shown in Fig. 4 and the IC_{50} values (μM) in Table I. The ratio of the IC_{50} value in cells transfected with the shRNA expression plasmid to that in the control cells was defined as the sensitivity index. In KK, OVMANA, and RMG-1, the sensitivity index was 3.3, 4.0 and 4.2, respectively, showing an increase in cisplatin sensitivity by the suppression of GPX3 expression. In OVSAYO, the sensitivity index was 1.1, showing no definite change.

Table I. IC_{50} value (μM) and sensitivity index for cisplatin in the 4 types of clear cell adenocarcinoma transfectants.

	IC_{50} (μM)	Sensitive index
KK/mock	25.0±0.5	-
KK/shGPX3	7.5±0.9	3.3
OVMANA/mock	54.9±2.0	-
OVMANA/shGPX3	13.9±0.5	4.0
RMG-1/mock	54.7±4.4	-
RMG-1/shGPX3	12.9±0.3	4.2
OVSAYO/mock	26.6±0.4	-
OVSAYO/shGPX3	24.5±2.5	1.1

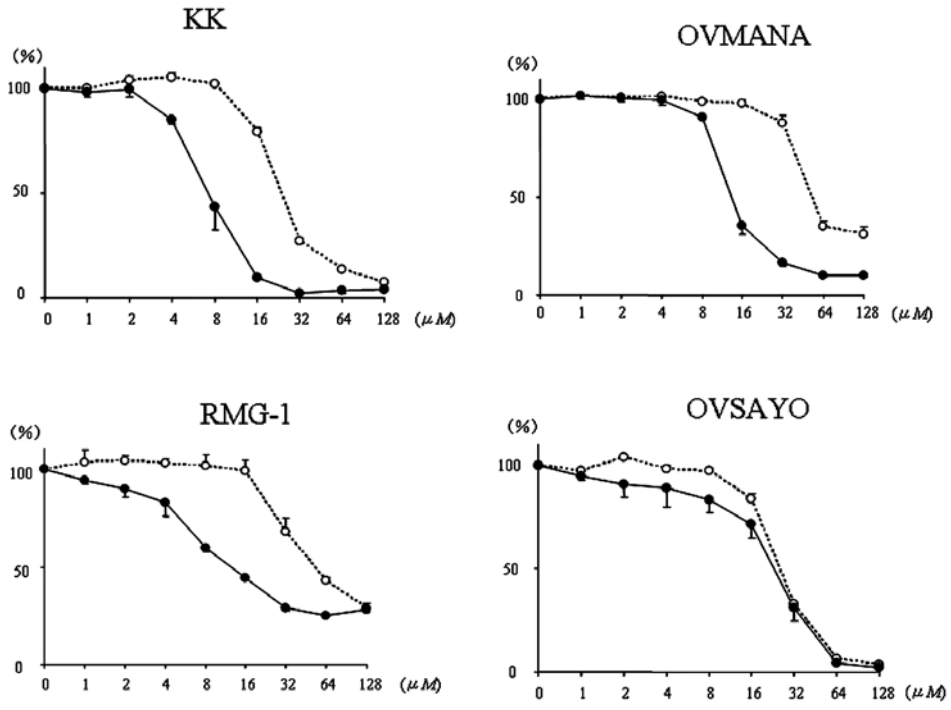

Figure 4. Dose-response curves of the 4 types of clear cell adenocarcinoma transfectants to cisplatin. Open circle, control cells; solid circle, cells transfected with the shRNA expression plasmid. The average of three independent experiments is shown and error bars indicate SD.

Discussion

Among the 33 genes that were up-regulated in the clear cell adenocarcinoma cell lines, SPP1 has been reported to be involved in bone metabolism, EEF1A2 in the repair of DNA damage in lymphoid cells, HAE1 in hereditary angioedema (as the responsible gene), PTTG1IP in the development of pituitary tumor and UGCG in the biosynthesis of glycosphingolipids.

GPX3 is an oxygen radical-metabolizing enzyme. Since GPX3 functions in the detoxification mechanisms of various substances (15,16), it was interesting to find *GPX3* in the specific genes to clear cell adenocarcinoma which is a chemoresistant malignancy. Studying clinical specimens of ovarian cancer, Hough *et al* (17) reported that *GPX3* was up-regulated in clear cell adenocarcinoma, similar to the results of our study. Thus, analyses of both cell lines and fresh clinical specimens have indicated that the up-regulation of the *GPX3* gene may be one of the molecular characteristics of clear cell adenocarcinoma.

GPX3 suppression by RNA interference definitely increased cisplatin sensitivity in 3 of the 4 clear cell adenocarcinoma cell lines. This experiment showed that GPX3 is involved in cisplatin sensitivity, suggesting that low cisplatin sensitivity is due to high GPX3 expression in clear cell adenocarcinoma, which in many cases highly expresses GPX3 (17).

For the anticancer drug resistance mechanism of cancer cells, promotion of excretion of anticancer drugs from cells, promotion of DNA repair and inhibition of apoptosis are generally considered. Previous study reports of anticancer drug resistance of clear cell adenocarcinoma, particularly cisplatin resistance, are discussed below.

Multidrug resistance-associated protein (MRP) and P-glycoprotein (P-gp) excrete anticancer drugs from cells in a ATP-dependent manner and decrease intracellular accumulation of anticancer drugs and are closely associated with multidrug resistance of various cancers. However, according to Itamochi *et al*, MPR or P-pg expression was not related to cisplatin sensitivity in clear cell adenocarcinoma either *in vitro* or clinical cases (4).

There is almost no previous data on DNA repair system in clear cell adenocarcinoma. Only Reed *et al* reported that ERCC1 and XPB associated with DNA repair were highly expressed in clear cell adenocarcinoma (18). However, an increase in DNA repair in clear cell adenocarcinoma has not been directly demonstrated.

Tsuchiya *et al* found a new candidate gene associated with anticancer drug resistance of clear cell adenocarcinoma (19). They nominated hepatocyte nuclear factor-1ß (HNF-1ß) for the candidate gene based on DNA microarray analysis of 4 clear cell adenocarcinoma cell lines. They suggested that *HNF-1ß* is a gene involved in the low sensitivity, based on the findings that HNF-1ß was highly expressed in many patients with clear cell adenocarcinoma and inhibition of HNF-1ß expression induced apoptosis of clear cell adenocarcinoma cells *in vitro*. Although association of *HNF-1ß* gene with low cisplatin sensitivity was not directly demonstrated, this is an interesting study.

In summary, we identified *GPX3* as a gene highly expressed in clear cell adenocarcinoma by DNA microarray and real-time RT-PCR. GPX3 suppression by RNA interference increased the cisplatin sensitivity of clear cell adenocarcinoma cells. *GPX3* was suggested to be a candidate gene associated with low cisplatin sensitivity of clear cell adenocarcinoma.

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