Loss of Expression of a New Member of the DNAJ Protein Family Confers Resistance to Chemotherapeutic Agents Used in the Treatment of Ovarian Cancer

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INTRODUCTION

The DNAJ proteins are a highly conserved family of proteins with the Escherichia coli heat shock protein, DNAJ (the human HSP403 orthologue), as its founding member (1). The defining feature of the HSP40 family is a highly conserved 70-amino acid residue, termed the DNAJ domain, that includes a signature tripeptide, HPD, that is critical for the function of the DNAJ domain (2). DNAJ proteins belonging to the HSP40 family contain four distinct domains including the DNAJ domain, whereas other proteins from this superfamily only possess the DNAJ domain (2). J-domains are present in diverse proteins and participate in complex biological processes. For example, HSP40 family J-domain proteins serve as cochaperones by recruiting HSP70 and accelerating ATP hydrolysis (3, 4). The DNAJ proteins participate in processes such as protein folding and translocation (5), cell cycle control by DNA tumor viruses (6–12), and regulation of protein kinases (13).

In this report, we describe the molecular cloning of a new member of the DNAJ domain protein family designated as MCJ. Collectively, our studies demonstrate that MCJ loss is common in human ovarian cancer, results from the deletion of one allele (LOH) and the silencing of the other by hypermethylation, and confers resistance to the three drugs most commonly used in the treatment of ovarian cancer. Here we show that stable transfectants expressing MCJ in OV167 are more sensitive to cisplatin, paclitaxel, and topotecan than parental and vector-transfected controls, implicating MCJ down-regulation in processes leading to decreased drug sensitivity.

MATERIALS AND METHODS

Cell Culture. Five of eight ovarian carcinoma cell lines (OV167, OV177, OV202, OV207, and OV266) were low-passage primary lines established at the Mayo Clinic (14), whereas OVCAR-5, SKOV-3, and the PC3 prostate cancer cell line were purchased from American Type Culture Collection (Manassas, VA). All cells were grown according to the provider’s recommendations.

Assessment of Methylation Control. The OV202 cell line was treated with varying concentrations of 5-aza-2′-dC, ranging from 1 to 5 μM the day after plating. After a 48-h exposure to 5-aza-2′-dC, the cells were harvested in Trizol (Life Technologies, Inc., Rockville, MD) for RNA extraction.

mRNA Differential Display. DD-PCR was performed on the short-term cultures of normal OCes and tumor cell lines as described by Liang and Pardee (15). Total RNA was extracted from the cell lines using Trizol and treated with RNase-free DNase I to eliminate genomic DNA contamination. Differential display of the expressed transcripts was performed using the RNA Image Kit (GenHunter Corp., Nashville, TN) according to the manufacturer’s instructions. Of the several bands identified that were differentially expressed, band 13 was absent in the tumor lane. This band was excised from the gel, reamplified with T11G and A6P primers, and sequenced using dye terminator technology by the Molecular Biology Shared Resource of the Mayo Foundation.

Strategy for Cloning the Gene. BLAST search of the isolated sequence identified several homologous ESTs in the database EST. The homologous ESTs were assembled into a contig with the use of Sequencer 3 (Gene Codes Corp., Ann Arbor, MI) software. The integrity of the full-length cDNA obtained by this electronic walking was confirmed by PCR analysis using PCR primers flanking each junction between EST clones. The entire cDNA contig was sequenced twice with overlapping primers.

MS-PCR. The methylation state of MCJ was determined using the recently described technique of MS-PCR (17). DNA was modified with sodium bisulfite according to Herman et al. (17) with the following modifications. DNA (1–1.5 μg) was digested with EcoRI in a 50-μl reaction overnight. The digested DNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 0.1 volume of 5 M ammonium acetate and...
100% ethanol in the presence of 1 μl of 20 mg/ml glycogen (Boehringer Mannheim, Indianapolis, IN). The DNA pellet was washed twice with 70% ethanol, and the DNA was taken up in 90 μl of 10 mM Tris (pH 7.5) containing 1 mM EDTA (TE buffer). Ten μl of freshly prepared 3 M NaOH were added to each sample, and the DNA was denatured at 42°C for 30 min. After the addition of 10 μl of distilled water, 1020 μl of 3.0 m sodium bisulfite (pH 5.0), and 60 μl of 10 mM hydroquinone, the samples were incubated in the dark at 55°C overnight (16–20 h). Modified DNA was purified using the Wizard purification system (Promega Corp., Madison, WI) according to the manufacturer’s instructions, followed by denaturation with 0.3 M NaOH for 15 min at 37°C. The DNA was eluted in 50–100 μl of TE and stored at −20°C in the dark.

We sequenced portions of BAC 251N23 and obtained an additional 361 bp 5′ of the reported cDNA sequence (GenBank accession no. AF126473). Restriction site analysis of this additional sequence revealed the presence of a Smal site 75 bases upstream of the reported cDNA sequence. A pair of primers, MCJ-WT (5′-CGTGAACCGACCAGCGGCG-3′) at 108 bp upstream of the Smal site and MCJ-WTR (5′-CTTCCCTGACCCCTTCCG-3′) at 86 bp downstream of the Smal site, were used to detect unmodified DNA. Nucleotide sequences of primers specific for methylation-mediated, modified DNA were MCJ-MF (5′-CGTGAAGTTATCGTATTCGGT-3′) and MCJ-MR (5′-CTTCCCTGACCCCTTCCG-3′), which yielded a product of 195 bp. Primers used for the analysis of unmethylated sequences in the modified DNA were MCJ-UF (5′-GTTTTTTAAGTGTTGGGAT-3′) at 101 bp upstream of the Smal site and MCJ-UR (5′-TAAACTTACCTAAACTTTCC-3′) at 100 bp downstream of the Smal site, which yielded a product of 234 bp. The primers for amplifying unmethylated sequences were specifically chosen not to contain any CpG-rich sequences at the 3′ end of the primer. PCR was performed by the “hot-start” method (Taq gold; Perkin-Elmer) with an initial denaturation of 10 min, followed by 30 cycles of amplification at 56°C, annealing with primers amplifying methylated sequences and 50°C, and annealing for amplifying nonmethylated/modified DNA with UF/UR primers. Controls without DNA and positive controls with unmodified DNA were performed for each set of reactions.

5′ RACE. To obtain the missing 5′ end sequences, 5′ RACE was performed with poly(A) + RNA isolated from PC3 cells. Adapter ligation and PCR were performed according to the instructions provided in the Marathon Ready cDNA amplification kit (Clontech, Palo Alto, CA). Primers used for 5′ RACE were 5′-GCAAAGTACGACCGTGGACA-3′ and MCJ-5′-CTTCCCTGACCCCTTCCG-3′ and MCJ-3′-CCGTAGGACAACTAGTTAAGG-3′.

Northern Blot Analysis. Fifteen μg of total RNA were fractionated on 1.2% formaldehyde agarose gels and blotted in 1× SSC to nylon membranes (Amersham, Piscataway, NJ). The probes were labeled using the random primer labeling system (Life Technologies, Inc.) and purified using spin columns (100 TE) from Clontech. Filters were hybridized at 68°C with radioative probes in a microhybridization incubator (Model 2000; Robbins Scientific, Sunnyvale, CA) for 1–3 h in Express Hybridization solution (Clontech) and washed according to the manufacturer’s guidelines.

Semiquantitative RT-PCR. Fifty-100 ng of reverse transcribed cDNA were used in a multiplex reaction with the forward MCJ-4 (5′-GGCGTACGCTGACCTGCA-3′) and reverse primer MCJ-5′ (5′-AGATAAGCTGTTGACGCAATC-3′) to yield a 595-bp product and GAPDH forward (5′-ACCCACTGGCAGGTGCTGAAACT-3′) and reverse (5′-TCCACCCATTGGTCTGA-3′) primers to yield a 450-bp product. The PCR reaction mixes contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 400 μM each of the forward and 50 μM of each primer, 0.05 μl of [32P]dCTP (10 μCi/μl), and 0.5 μl of Taq polymerase (Promega) in a 10-μl reaction volume. The conditions for amplification were 94°C for 2 min and then 30 cycles of 94°C for 30 s, 52°C–57°C for 30 s, and 72°C for 30 s in a Perkin-Elmer-Cetus 9600 Gene-Amp PCR system in a 96-well plate. The PCR products were denatured and run on 6% polyacrylamide sequencing gels containing 8 M urea. The gels were dried and autoradiographed for 16–24 h and scored for LOH. Multiple exposures were used before scoring for LOH. Allelic imbalance indicative of LOH was scored when there was >50% loss of intensity of one allele in the tumor sample with respect to the matched allele from normal tissue. The evaluation of the intensity of the signal between the different alleles was determined by visual examination by two independent viewers (V. S. and J. S.).

Estimation of MCJ Stable Transfectants. On the basis of the cDNA sequence of MCJ, two primers were synthesized to amplify a 536-bp fragment of MCJ from base 367-903 containing the entire ORF. A HindIII site was introduced into the forward primer 5′-CCTGAGCTTACCTGTTGACGCA-3′ and a BamHI site into the reverse primer 5′-CCGGGATCCCTTGTTGAC-3′ (restriction sites are underlined in the sequences). The PCR product was digested with both HindIII and BamHI, gel purified, subcloned into the cloning sites of the mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA), and transformed into Escherichia coli DH5α (Life Technologies, Inc.) cells. Mini-preparations of the ampicillin-resistant colonies were sequenced and verified. Exponentially growing cells of OV167 in 100-mm dishes were washed with serum-free medium and treated with a mixture of 5 μg of plasmid, 30 μl of LipofectAMINE, and 20 μl of Plus reagent. After a 3-h incubation, complete medium with serum was added. Beginning 24 h after the start of transfection, G418 was added to select the transfectants. Two stable clonal transfectants, MCJ 6 and MCJ 13, were subsequently generated. For controls, cells were similarly transfected with vector [pcDNA3.1(+) only] and selected.

Tissue Culture and Colony-forming Assays. Topotecan was kindly provided by the Pharmaceutical Resources Branch of the National Cancer Institute. Paclitaxel and cisplatin were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were obtained as described previously (16, 18, 19). Stock (1000-fold concentrated) solutions of paclitaxel and topotecan were prepared in DMSO and stored at −20°C prior to use. Cisplatin was prepared immediately before use as a 1000-fold concentrated solution in DMSO.

OV167 cell lines were cultured in MEM with Earle’s salts and nonessential amino acids containing 20% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine (medium A). Cells were passaged once weekly and maintained at 37°C in an atmosphere containing 95% air/5% CO2. To determine population doubling times, 1 × 105 cells were seeded in triplicate 100-mm tissue culture plates, incubated for intervals between 24 and 240 h, trypsinized, and counted on a hemacytometer. Colony-forming assays were performed as described previously (16).
In brief, subconfluent cells were released with trypsin, plated at a density of 4000 cells/plate in multiple 35-mm dishes containing 2 ml of medium A, and incubated for 14–16 h at 37°C to allow cells to attach. Graded concentrations of each drug or equivalent volumes of DMSO (0.1%) were then added to triplicate plates. After a 24-h treatment, plates were washed twice with serum-free MEM and incubated in drug-free medium A for an additional 14 days. The resulting colonies were stained with Coomassie Blue and counted manually. Diluent-treated control plates typically contained 75–200 colonies and served as a basis for estimates of colony-forming efficiency for the four lines.

**Flow Cytometry.** Flow cytometry for cell cycle analysis was performed as reported previously (18). Briefly, cells were grown to 30–40% confluence in 100-mm dishware culture dishes, released by trypsinization, and sedimented at 200 x g for 5 min. All additional steps were performed at 4°C unless otherwise indicated. Samples were fixed in 5% ethanol, treated with RNase A, stained with propidium iodide, and analyzed by flow cytometry on a Becton Dickinson flow cytometer using the ModFit software (Verity Software House, Topsham, ME). Cellular accumulation of topotecan was assessed by FACS analysis as described previously (20). Briefly, cells grown to 50–60% confluence in 100-mm dishes were incubated for 1 h in the presence of 20 μM topotecan, trypsinized in the continued presence of topotecan, and examined by FACS using an excitation wavelength of 488 nm and an emission wavelength of 585 nm.

**Assessment of Cell Viability.** To directly assess cell viability, cells were grown to 30% confluence in 100-mm dishes, treated with 100 nM paclitaxel for 24 h, harvested at the indicated time points, and assessed for either their ability to exclude trypan blue or apoptotic morphology by staining with Hoescht 33258, as described previously (16, 18). Floating and adherent cells from each dish were combined prior to evaluation with trypan blue or Hoescht staining.

**RESULTS**

**Isolation and Characterization of a Novel cDNA Containing the DNAJ Domain.** We performed DD-PCR with primers HT11G and AP6 from the RNA Image kit (GenHunter Corp.) against low-passage cell lines established from primary ovarian tumors and short-term cultures of normal OCEs. Several fragments were isolated. One fragment (13) was expressed exclusively in the normal cell line and absent in three of seven tumor cell lines. This band was isolated from the gel by standard procedures, reamplified with the same set of primers, and sequenced. Comparative sequence analysis of this fragment using the BLAST alignment revealed that the 150-bp fragment showed 56% (63 of 112) identity and 73% (83 of 112) overall similarity. These two proteins are similar in two respects. In contrast to the majority of DNAJ-containing proteins, both MCJ and CEESD64F contain their DNAJ domains in the COOH-terminal half of the protein. In addition, both have a potential membrane-spanning domain (between residues 36–58 in MCJ and residues 5–23 in CEESD64F) extending beyond the DNAJ domain. The alignment of the putative protein encoded by the isolated sequences and CEESD64F (Fig. 2) showed 56% (63 of 112) identity and 73% (83 of 112) overall similarity. These two proteins are similar in two respects. In contrast to the majority of DNAJ-containing proteins, both MCJ and CEESD64F contain their DNAJ domains in the COOH-terminal half of the protein. In addition, both have a potential membrane-spanning domain (between residues 36–58 in MCJ and residues 5–23 in CEESD64F) at the NH2 terminus of each respective protein (Fig. 2).

**Expression of MCJ in Ovarian Tumor Cell Lines and Normal Tissues.** Primers MCJ1 (5'-TAACTAGTGGTCCCTA-3') and MCJ2 (5'-CAGTGGTGCCTAAGC-3') were synthesized based on the 720-bp sequence flanking the ORF. RT-PCR was performed on short-term cultures of normal OCEs and epithelial cell brushings from patients without cancer (Fig. 3A) and seven ovarian tumor cell lines including OV167, OV177, OV202, OV207, OV266, OVCAR5, and SKOV3. With the exception of OV167, OV202, and OV266, all other cell lines amplified the expected 540-bp fragment (Fig. 3B). Northern blot analysis confirmed the results obtained with RT-PCR (Fig. 3C). The probe used for Northern analysis was the full-length ORF probe generated by RT-PCR in the normal cell line. Expression analysis with the multiple-tissue Northern blot revealed that MCJ is expressed as ~1.2-kb message in all tissues examined (data not shown). Tests showed the highest level of expression, whereas expression in other normal tissues, including adrenal gland, total brain, fetal brain, kidney, lung, pancreas, prostate, and uterus, was much lower.

**Fig. 1.** cDNA nucleotide sequence and the putative protein sequence of MCJ. Highlighted, transmembrane domain at the NH2 terminus and DNAJ domain at the COOH terminus of the protein. Boxed, signature tripeptide (HFD). Underlined, polyadenylation signal. Small arrowheads, positions of the introns.
Cloning the Full-Length cDNA by 5′ RACE. This cDNA generated by EST-based walking was 720 bp in length. This cDNA contained a polyadenylation signal, AATAAA. However, the size of the transcript estimated by Northern analysis was 1.2 kb. To generate this cDNA, RACE was performed with the 5′ end of this cDNA, 5′ RACE was performed with the Marathon Ready cDNA kit using RNA isolated from PC3 cells under conditions recommended by the manufacturer. We obtained an additional 354 bp of sequences with 5′ RACE. Reanalysis of the 5′ RACE sequences revealed that the ORF generated from the original 740-bp sequence was not changed (GenBank accession no. AF126743). In addition, the putative initiation codon occurs within a strong Kozak sequence was not changed (GenBank accession no. AF126743). In addition, the putative initiation codon occurs within a strong Kozak sequence.

Expression Analysis of MCJ in Primary Ovarian Tumors. To determine whether MCJ was expressed in primary ovarian serous adenocarcinomas, we analyzed the expression of MCJ in 18 stage III and 3 stage IV ovarian adenocarcinomas by semiquantitative RT-PCR in the ovarian cell lines. Lane 1, OV167; Lane 2, OV177; Lane 3, OV202; Lane 4, OV207; Lane 5, OV266; Lane 6, OVCAR5; Lane 7, SKOV3; Lane 8, water control. The lane to the left of Lane 1 is a marker. C, autoradiograph showing the Northern hybridization results in the same cell lines (with MCJ ORF as probe) as in A. Lane 1, OV167; Lane 2, OV177; Lane 3, OV202; Lane 4, OV207; Lane 5, OV266; Lane 6, OVCAR5; Lane 7, SKOV3. D, tubulin hybridization of the corresponding samples.

Transcriptional Induction in the OV202 Cell Line by 5-aza-2′-dC Treatment. Because there was an absence of expression of MCJ mRNA in the OV202 cell line by both RT-PCR and Northern analysis, we were interested in whether methylation of this gene resulted in absence of its expression in this cell line. Therefore, we treated the OV202 cell line with the methyltransferase inhibitor 5′-deoxy-5-azacytidine to determine its effect on the transcription of the MCJ gene. After 2-day exposure to concentrations of 5-aza-2′-dC ranging from 1 to 5 μM, RNA was extracted from control and subjected to RT-PCR to assess MCJ mRNA expression. There was a dose-dependent increase in the expression of this message after treatment with 5-aza-2′-dC (Fig. 6), which is an inhibitor of DNA methyltransferases (24, 25). Because the reexpression of this message seems to be linked to the methylation status of this gene or to some other regulatory gene controlling the expression of this gene, we named this gene MCJ.

MS-PCR of MCJ in Cell Lines and Primary Tumors. On the basis of the results obtained with 5-aza-2′-dC, we tested cell lines lacking MCJ expression for CpG island methylation using MS-PCR (17). To distinguish unmodified from modified DNA, primers that encompassed regions containing multiple cytosines were chosen. In addition, restriction site analysis revealed the presence of rare restriction sites, such as SacII and EgrI, in these regions. Thus, CpG pairs

LOH Analysis MCJ in Primary Ovarian Tumors. Sequencing a portion of the 420G23 BAC revealed a dinucleotide repeat consisting of 14 CA repeats ~80 bases downstream of the 3′ end of MCJ. We made primers (MCJ′NF and MCJ18) flanking this repeat and found that this sequence was polymorphic in humans. We then performed LOH analysis with this new microsatellite in the primary ovarian tumor samples that showed lower levels or absence of expression of MCJ. In the tumors tested, this marker was 75% informative. None of the benign tumors but 48% of the high-stage tumors showed loss of this marker (Fig. 5).
near the 3' end of the primers could provide maximum discrimination between methylated versus unmethylated sequences. Primers (wild-type, methyl-specific, and primers that would amplify unmethylated sequences) were synthesized flanking these restriction sites at nucleotide position 331 and at nucleotide position 484 of the MCI cDNA sequence to amplify a 154-bp product. PCR amplification of bisulfite-modified DNA with these methyl-specific primers yielded a product both in matched normal (WBC) and tumor DNA. Sequencing of these products revealed no differences in the methylation status of either the SacII, EagI, or other CpG sites within this sequence between the normal and tumor samples. This indicated clearly to us that the methylation site specific for lower levels of expression was probably present 5' to this sequence.

To check for other potential CpG sites, we sequenced the BAC 251N23 and obtained an additional 361 bp of sequences. Restriction site analysis of this additional sequence revealed the presence of a SmaI site 75 bases upstream of the reported cDNA sequence. Primers were designed to amplify the methylated and unmethylated sequences at this position, as described in “Materials and Methods.” Using this set of primers, we amplified methylation-specific products both in normal and tumor DNA (data not shown). However, sequencing these products with the reverse primer revealed that the SmaI site showed the presence of both methylated unconverted Cs as well as Ts (Gs and As, respectively, in the sequence Fig. 7, A and D) in all of the normal blood DNA samples. In tumor samples expressing MCI (tumors 183 and 270), only the unmethylated fully converted Ts (A in the opposite strand) are seen. Panels B and C in Fig. 7 show the sequence of the MS-PCR product amplified with methyl-specific primers in the blood and tumor DNA, respectively, of patient 183. In tumor samples with complete loss of MCI expression (tumors 202, 220, 332, 485, 97, and 107), only the nonconverted methylated Cs (G as seen in Fig. 7E) were visible at the SmaI site. The sequence of the MS-PCR product amplified with methyl-specific primers in the blood and tumor DNA, respectively, of patient 485 is shown in Fig. 7, D and E. In addition, in tumor samples with complete loss of MCI expression, we saw the loss of the other allele by LOH (Fig. 7E, inset, for tumor 485). Table 2 lists the results of the RT-PCR expression analysis, along with the MSP-PCR results and LOH status, in 18 high-stage tumors with and without the loss of expression of MCI. In tumors 202, 220, 332, 485, and 107 (which have all lost MCI expression), there is a loss of one allele (LOH analysis) and loss of expression of the other allele, attributable to methylation in the same tumor. In tumors with lower levels of expression (tumors 121, 124, 323, and 282) or normal MCI expression (tumors 183, 417, and 531), we did not see LOH of the MCI allele (Fig. 7C, inset). This marker, however, was uninformative in some of the samples. In tumors 183 and 270, the presence of a clear RT-PCR product also corresponded with the presence of only unmethylated alleles at this site (Fig. 7C). In samples with lower levels of expression and no LOH, the presence of both methylated and unmethylated alleles was seen at this site (Table 2).

Genomic Organization and FISH Mapping of MCI. We isolated two different BACs by screening the Research Genetics BAC pools. Sequencing of these two BACs with cDNA-specific primers revealed that the coding region of MCI is interrupted by introns. We assembled the exonic sequences to the sequence of the BAC 335G18 that was available on the HTGS database. MCI spans ~83 kb of genomic DNA and is interrupted by five introns. The primers spanning the intron/exon sequences are listed in Table 3. The 3' end BAC 421G23 was used to map MCI to chromosome 13q14.1 by FISH analysis (data not shown). This mapping confirmed the EST-based mapping of one of the ESTs (AA812596) used to build the cDNA contig.

Mutational Analysis of MCI in Primary Tumors. Primers (Table 3) were synthesized from intronic sequences flanking individual exons. Individual exons were amplified from matching blood and tumor DNA from several patients and sequenced directly to check for mutations within the coding sequences. Whereas several sequence polymorphisms were seen, no tumor-specific mutations were detected in any of the exons.

Functional Analysis of MCI in OV167. A parental MCI-nonexpressing primary ovarian carcinoma cell line (OV167), vector transfected control, and two stable MCI clones (6 and 13) were tested for the expression of MCI by semiquantitative RT-PCR. Only the two MCI transfectants expressed the MCI transcript (Fig. 8A). Examination of the four OV167 lines demonstrated no consistent differences between MCI-high (clones 6 and 13) and MCI-nonexpressing (OV167 and empty vector transfectant) lines with respect to doubling time (i.e., proliferation rate). In particular, doubling times for the parental OV167 and the vector control were 5.0 and 3.0 days, respectively, whereas doubling times for the two MCI transfectants (clones...
trypan blue staining). Hoescht staining showed that the MCJ-expressing lines were more sensitive to paclitaxel-induced cytotoxicity (data not shown). Hoescht staining showed that the MCJ-expressing lines were similarly more sensitive to paclitaxel-induced apoptosis (Fig. 8D).

Although the MCJ-high and MCJ-nonexpressing lines did not vary significantly with respect to doubling time, we had some concern that the observed resistance of the MCJ-deficient lines to the cell cycle-dependent agents paclitaxel and topotecan might be attributable to differences in cell cycle distributions. To evaluate this possibility, we examined cell cycle distribution in all four lines. As shown in Fig. 8E, the cell cycle distributions of the four lines were similar and could not, therefore, explain the observed differences in drug sensitivity.

In an effort to determine whether differential drug accumulation
might be responsible for the observed differences in drug sensitivities of MCJ-high and MCJ-low lines, we also examined topotecan accumulation in the four lines by FACS analysis (20). These studies showed no significant difference between the lines (data not shown), eliminating the possibility that differential drug accumulation was responsible for the observed differences in drug sensitivities.

DISCUSSION

In the United States, ovarian cancer is the fourth most common cause of cancer-related deaths among women. Approximately 23,000 women are diagnosed with and ~14,000 women die from ovarian cancer annually in the United States (26). Although women with low-stage ovarian cancer have a good prognosis, most women are diagnosed with late-stage disease and eventually succumb to their cancer (27). Much progress, therefore, remains to be made in the early diagnosis and treatment of ovarian cancer. A major concern in treating ovarian cancer patients is the frequent development of resistance to chemotherapy. Whereas most patients initially respond to the commonly used chemotherapeutic drugs, resistance to these drugs usually develops, and the patients eventually succumb to the disease. Many mechanisms have been postulated to explain this resistance (28–33), but these remain to be tested in clinical materials. Accordingly, there is considerable interest in identifying genes that could differentiate between chemo-sensitive and chemoresistant ovarian tumors.

Similar to cancers of other tissues, multiple genetic alterations are common in ovarian carcinomas. Alterations in tumor suppressor genes such as p53 (34), pRB (35), and NOEY2 (36) have been implicated in ovarian carcinogenesis. Chromosomal regions of loss have frequently identified new tumor suppressor genes involved in either the initiation, progression, or metastasis of cancer-related genes. In the present study, we report the discovery of a novel gene (MCJ) that we identified using DD-PCR between ovarian tumor cell lines and short-term cultures of normal OSEs. Expression of this gene was either absent or reduced in a majority of primary ovarian tumors and ovarian carcinoma cell lines. In specimens lacking MCJ expression, one allele was lost and the other silenced by methylation. Interestingly, a comparison of the MCJ-expressing and MCJ-nonexpressing low-passage primary ovarian carcinoma cell lines implicates MCJ loss in conferring resistance to the three drugs most commonly used in the treatment of ovarian cancer. These findings have potentially important implications for ovarian cancer development and treatment.

After MCJ was identified by DD-PCR, analysis of the ORF revealed that MCJ is a new member of the DNAJ family of proteins with sequence identity between MCJ and other DNAJ domain-containing proteins ranging from 30 to 50%. The major difference between MCJ and most other DNAJ-like proteins is the location of the DNAJ domain. Expression analysis of MCJ on a multiple-tissue Northern blot showed that this gene was highly expressed in testis. In this respect, it is similar to the Drosophila melanogaster DNAJ protein, DNAJ60 (37). Iliopoulos et al. (37) have shown that DNAJ60 encodes a putative protein of 217 amino acids with a molecular mass of 27.7 kDa and a pI of 10.5 that may play an important function during spermatogenesis and/or in the male genital tract. Whereas we have no evidence at present about a testis-specific function of MCJ, it is interesting to note that both MCJ and DNAJ60 are extremely basic proteins with similar pIs of 10.35. Another member of the DNAJ family of proteins with testis-specific expression is MSJ-1 (38). However, sequence analysis of MCJ revealed that it had no significant homology to MSJ-1.

We have shown that the absence of expression of MCJ is related directly to the methylation status of this gene. In the OV202 cell line, induction of MCJ is observed after 5-aza-2'-dC treatment. This is the first report linking methylation to the absence of expression of a DNAJ-like protein. The cell lines with loss of expression of MCJ were all cell lines derived from primary tumors harvested at the time of surgery, and therefore, the methylation pattern seen in these cell lines is a de novo effect and not the result of following exposure to chemotherapeutic agents in vivo. LOH analysis on 13q14.1 identifies this as a new region of LOH in ovarian cancer in the region of MCJ. We have shown LOH of the marker identified only 80 bases downstream of the 3’ end of the MCJ gene and that there is loss of an MCJ allele in some of the tumors not expressing MCJ. Whereas we have not found any tumor-specific mutations in the MCJ coding region, we have seen loss of expression of this gene both by LOH and hypermethylation in the same tumor. Although there are no reports of a DNAJ domain protein acting as a tumor suppressor, our data clearly indicate the mechanism for loss of function of this gene is probably attributable to loss of DNA sequences by deletion (LOH) and to hypermethylation. In this regard, this gene fits the criteria as a class II tumor suppressor.

To examine the effects of differences in MCJ expression, we evaluated the cytotoxic effects of cisplatin, paclitaxel, and topotecan in cells lacking or expressing MCJ. Cisplatin/carboplatin and Taxol are the most effective drugs in the treatment of ovarian cancer, and the combination of carboplatin/paclitaxel has been widely accepted as standard treatment for advanced ovarian cancer (28). Several lines of evidence support the idea that there is a direct correlation between the induction of apoptosis and drug sensitivity. For example, inactivation of the p53 gene could confer resistance to cisplatin and DNA-damaging agents as measured by both the induction of apoptosis and resulting antiproliferative effects (33, 34). It is clear from several studies that there may be multiple mechanisms involved in determining drug resistance (30, 31). Our preliminary studies suggest that loss of MCJ in ovarian carcinoma may be of potential functional significance. In particular, MCJ loss appears to be associated with de novo resistance to the antineoplastic agents paclitaxel, topotecan, and cisplatin in the OV167 cell line. On the other hand, it is important to note that the magnitude of resistance (2–3.5-fold) conferred by MCJ loss is less than resistance conferred by some other means. For example, overexpression of P-glycoprotein can confer a much greater level of resistance to paclitaxel (16, 39). On the other hand, the 2–3.5-fold resistance to these agents might be significant in the clinical setting, particularly when combined with other resistance-inducing changes. We speculate, therefore, that MCJ loss may have potential prognostic significance in ovarian cancer. The impact of MCJ loss within the context of the full spectrum of genetic alterations in ovarian cancer, however, remains to be more fully elucidated. It would be very informative to look at sequential tumor specimens derived from patients undergoing multiple surgeries to try to correlate the in vitro and in vivo chemoresistance characteristics.

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