Ankyrin Repeat Domain 1, ANKRD1, a Novel Determinant of Cisplatin Sensitivity Expressed in Ovarian Cancer

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Abstract

Purpose: The standard of care for ovarian cancer includes platinum-based chemotherapy. It is not possible, however, to predict clinical platinum sensitivity or to design rational strategies to overcome resistance. We used a novel approach to identify altered gene expression associated with high sensitivity to cisplatin, to define novel targets to sensitize tumor cells to platin and ultimately improve the effectiveness of this widely used class of chemotherapeutics.

Experimental Design: Using differential display PCR, we identified genes differentially expressed in a mutagenized cell line with unusual sensitivity to cisplatin. The most highly differentially expressed gene was selected, and its role in determining cisplatin sensitivity was validated by gene transfection and small interfering RNA (siRNA) approaches, by association of expression levels with cisplatin sensitivity in cell lines, and by association of tumor expression levels with survival in a retrospective cohort of 71 patients with serous ovarian adenocarcinoma.

Results: The most highly differently expressed gene identified was ANKRD1, ankyrin repeat domain 1 (cardiac muscle). ANKRD1 mRNA levels were correlated with platinum sensitivity in cell lines, and most significantly, decreasing ANKRD1 using siRNA increased cisplatin sensitivity >2-fold. ANKRD1 was expressed in the majority of ovarian adenocarcinomas tested (62/71, 87%), and higher tumor levels of ANKRD1 were found in patients with worse outcome (overall survival, P = 0.013).

Conclusions: These findings suggest that ANKRD1, a gene not previously associated with ovarian cancer or with response to chemotherapy, is associated with treatment outcome, and decreasing ANKRD1 expression, or function, is a potential strategy to sensitize tumors to platinum-based drugs.
has given insight to specific molecular mechanisms that influence platinum sensitivity. For example, aberrations in recognition and repair of platinum-DNA adducts via the nucleotide excision repair pathway (3, 4), the ATM-CHK2 (5) and Fanconi anemia-BRCA1 pathways (6), in apoptosis (7, 8), and in platinum transmembrane transport (9–13) have been implicated in modulating the cellular pharmacokinetics and cytotoxicity of cisplatin. However, notwithstanding the number of drug resistance mechanisms that have been described, thus far none have a validated role in determining clinical response.

The aim of this study was to identify mechanisms underlying differential response to platinum-based therapy in ovarian cancer patients. To do this, we focused initially on molecular pathways that sensitize cancer cells to chemotherapy by examining differential gene expression in a model cell line with acquired, exquisite sensitivity to cisplatin following random mutagenesis (14). A striking finding was that expression of a gene homologous to ANKRD1 (ankyrin repeat domain 1 [cardiac muscle]) was specifically decreased in the sensitive cell line. To determine the potential clinical significance of this finding, ANKRD1 expression in a panel of ovarian cancer cell lines and ovarian cancer samples was compared with cisplatin response and survival following platinum-based treatment respectively.

Materials and Methods

Cell lines and culture conditions. The parental Chinese hamster ovary cell line, CHO-K1, and its cisplatin sensitive derivative MMS-2, were originally provided as a gift by Prof. I. Hickson and Dr. C. Robson (Imperial Cancer Research Fund, Oxford, United Kingdom; ref. 14). MMS-2 had been derived by exposure of the parental line to the mutagen ethylmethanesulfonate and colonies selected by replica plating. The CHO-MMS-2 line was subcloned to ensure a homogeneous platinum sensitive line, CHO-MMS-2-SC2, henceforth referred to as SC2.

The human cell lines used were as follows: ovarian cancer cell lines, Caov-3 and SK-OV-3 (American Type Culture Collection), A2780, COLO 316, C80-135, JAM, NIH-OVCAR3, PEO1, and PEO14; HOSE 17.1, an immortalized normal ovarian epithelial cell line, used with permission from Prof. S. Mok (15); breast cancer cell lines T-47D, and MDA-MB-231, obtained from EG & G Mason Research Institute, Worcester, MA; HCC1937 and BT-483 from the American Type Culture Collection; and BRE-80, a normal mammary epithelial cell line, obtained from Dr. L. Huschtscha, Children’s Medical Research Institute, Sydney, Australia (16). Cell lines were maintained under standard conditions and routinely tested for Mycoplasma.

Clonogenic assay for cisplatin sensitivity. Cells in exponential growth phase were seeded at 103 cells per 100 mm culture dish, in duplicate. After attaching, the cells were exposed to serum-free media containing cisplatin (DBL) for 2 h then cultured in complete media for 6 d, the volume of 100 A

Cell proliferation assay. Cells were seeded into 96-well plates in a volume of 100 μL at 500 to 2,000 cells per well. The following day 50 μL of complete medium containing cisplatin was added to duplicate wells to give a final drug concentration ranging from 625 nmol/L to 20 μmol/L. MTS assays [based on color conversion by viable cells MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] were carried out as described by the manufacturer (Promega) on day 3 following drug addition. IC50 (concentration required to reduce cell survival to 50%) values were calculated for each cell line from survival curves, using data generated from between two and seven independent experiments.

Differential display PCR. Differential display–PCR was used to identify genes differentially expressed between CHO-K1 and SC2 cells. The method was essentially as described by Liang et al. (17), using a commercially available kit and primer sets (Genomyx). Negative controls lacking either reverse transcriptase or RNA template were included, as was a HeLa RNA positive control supplied by the manufacturer (Genomyx). Differential display–PCR products were resolved on a 6% denaturing polyacrylamide gel. We did independent, duplicate harvests of RNA from the CHO clones and did the reverse transcription–PCR reaction in duplicate on each of these harvests. Bands were only selected if the difference between clones was consistent across at least 3 out of the 4 replica lanes. Selected bands were cut from the dried polyacrylamide gel, amplified by PCR, isolated by cloning into a bacterial vector (pGEMT-Easy Cloning Kit; Promega), and sequenced using an ABI automated sequencer (PE Applied Biosystems). All sequence searches were done using data and tools available at the Australian National Genomic Information Service and the National Center for Biotechnology Information Web site.8

Cloning full-length CHO ANKRD1. The full-length open reading frame of CHO Ankd1 was obtained using the SMART 5’-3’-RACE cDNA Amplification kit (BD Biosciences Clontech). Rapid amplification of cDNA ends was carried out according to the manufacturer’s protocol. The two initial primers, forward 5’-AACATGATGGTGCTGA-GACTAGACAGAAGCC-3’ and reverse 5’-GCACCATCATGGTCGAC-CAGTGAGTCT-3’, were designed from the sequence isolated from the differential display–PCR. An additional internal primer was required to obtain the full-length sequence, 5’TGGCGCTGAGAACAAACTGCG-ACTGTG-3’ at position 383 bp (primers synthesized by Sigma Genosys). The full-length sequence was then compared with all known ANKRD1 genes using Clustal W accurate for multisequence alignment (18) and Boxshade (Hofman K and Baron MD) and Biomanager by the Australian National Genomic Information Service.7

Isolation of RNA and Northern blot analysis. Total RNA was isolated by lysis in guanidinium isothiocyanate solution followed by GCl centrifugation using standard methods or by using Tri-reagent according to the manufacturer’s instructions (Sigma Aldrich), and Northern blot analysis was done by standard techniques. An oligonucleotide probe to ANKRD1 was designed from the common sequence of the published human, rat, and mouse ANKRD1 cDNA (5’-GTCAGGCGTTTCAGCCCAAA-3’), and a oligonucleotide designed to the 18S rRNA sequence (5’-ACGGTGACTGATGTCTGTCCTCGAAC-3’) was used to control for equal loading and transfer on Northern blots. Probes were end-labeled with [32P]dATP (5,000 Ci/mmol) using T4 polynucleotide enzyme (Promega), and unincorporated label was removed using G25 Sephadex spin column. Following hybridization, the filters were washed then exposed to either radiographic film (BioMax; Eastman Kodak) or a phosphoimager screen (Molecular Dynamics). Lane intensity was compared using the Image Quant program (Molecular Dynamics). Filters were then stripped and reprobed for 18S.

Cell transfection. Rat Ankrd1 cDNA contained in the pcDNA3.1 expression vector, under the control of a CMV promoter (a kind gift from the laboratory of Prof. L. Kedes, University of Southern California), was transfected into SC2 cells using Lipofectamine 2000 (Invitrogen Life Technologies). Individual transfected clones were selected and maintained in 400 μg/mL G-418 (Gibco).

siRNA-mediated inhibition of ANKRD1 expression. Three small interfering RNAs (siRNAs) to ANKRD1 were designed using the Silencer siRNA Target Finder software (Ambion) which designs hairpin siRNA encoding DNA oligonucleotide insert sequences for the pSilencer

7 http://www.angis.org.au
The gene-specific inserts were siRNA41 5’-AGTCCAGTTGTGAGGAAA-3’ (sense), 5’-TTCCTACCACTCTGACT3’ (antisense); siRNA58 5’-TTCCTGATATGCTGGAT-3’ (sense), 5’-ATCCAGCATATCAGCAGGA-3’ (antisense); and siRNA775 5’-TGGACGAGGAAGAATTT-3’ (sense), 5’-GAATATCTGTTTGCTCA-3’ (antisense). The sense and antisense templates were synthesized commercially (Sigma Genosys), annealed, and the double-stranded inserts were cloned into the psiBlue vector according to the manufacturer’s instructions. Each siRNA and the negative control plasmid, provided by the manufacturer that expresses a siRNA with low homology to any known sequences in the human, mouse, and rat genomes, were then transfected into CHO-K1 cells using Lipofectamine 2000. Transfected cells were selected and maintained in media containing 50 μg/mL hygromycin B (Invitrogen).

Real-time quantitative PCR. Total RNA was reverse-transcribed into cDNA using Superscript III First Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. Samples were analyzed on the ABI PRISM 7700 real-time PCR system (PE Applied Biosystems) using Platinum Quantitative PCR Supermix-UDG (Invitrogen) with 250 nmol/L of each primer set. Amplification conditions were 50°C for 2 min, 95°C for 10 min, then 50 cycles of 95°C for 15 s and 60°C for 1 min. Primers for ANKRD1 were designed using the LUX primer design software (Invitrogen), and labeled with FAM: 5’-GAACTTCGGCA-CATCCACAGCTC-FAM-3’ (sense) and 5’-AGAACAGCGAGGCA-GAGC-3’ (antisense). ANKRD1 mRNA concentration was determined by comparison of C, values with a standard curve. Values were corrected for equal cDNA loading using VIC-labeled primer and probe sets for 18S and rodent Gapdh (glyceraldehyde-3-phosphate dehydrogenase) or human P0 (acidic phosphoprotein P0; also known as 36B4) for rodent and human samples, respectively (Applied Biosystems).

Patients. The study population consisted of 71 patients with epithelial ovarian cancer or primary peritoneal cancer treated at Westmead Hospital, Sydney from 1987 to 2002. All patients underwent laparotomy for diagnosis, staging, and tumor debulking, and received chemotherapy according to standard protocols specifying cisplatin or carboplatin, usually in combination with cyclophosphamide or paclitaxel. Tumor tissue examined was excised at the time of primary surgery, before the administration of chemotherapy, and was stored for research with patient consent. This project was approved by the institutional Human Research Ethics Committee. The clinical characteristics of the cohort are described in Table 1.

Clinical definitions. Surgical staging was assessed in accordance with the classification of the Fédération Internationale des Gynécologues et obstétriciens (FIGO). Optimal debulking was defined as ≤1 cm (diameter) residual disease, and suboptimal debulking was >1 cm (diameter) residual disease. Progression-free survival was defined as the time interval between the date of diagnosis and the first confirmed sign of disease recurrence based on the Gynecologic Cancer Intergroup criteria (19). Six patients who progressed on primary treatment were not included in progression-free survival analyses. Overall survival was defined as the interval between the date of diagnosis and the date of death.

Tissues and RNA isolation. Tissues were snap-frozen and stored in liquid nitrogen. Representative sections from each case were reviewed by a pathologist, blinded to the outcome data, for confirmation of histologic subtype and grading according to the Universal grading system (20). Only cases of invasive serous adenocarcinoma were included. For each case, RNA was extracted from crossections or pieces of snap-frozen tumor specimens, and an adjacent H&E-stained section was taken to verify tissue quality and content. Total RNA was isolated from frozen sections using the Absolutely RNA MicroPrep kit (Stratagene) following homogenization with a hand-operated pestle grinder in the lysis buffer provided; or from pulverized frozen tissue pieces by lysis in guanidinium isothiocyanate solution followed by CsCl centrifugation using standard methods. RNA quality was assessed using an Agilent 2100 Bioanalyzer and RNA Integrity Number software (Agilent Technologies). RNA Integrity Number values were routinely >8 (median, 9.1), indicating high-quality, intact RNA. ANKRD1 mRNA levels were measured by real-time quantitative PCR, and analysis was done on coded samples with the operator blinded to all clinical data.

Statistical analyses. The data were analyzed using the SPSS software package for Windows version 14 (SPSS, Inc.). The Spearman rank correlation coefficient was used to quantify the degree of association between ANKRD1 gene expression level, in the cell lines with detectable ANKRD1, and cell sensitivity to cisplatin. One-way ANOVA with Bonferroni correction for pairwise multiple comparisons was used to compare chlomerocytic survival following cisplatin treatment in transfected cells, and one-way ANOVA with Dunnett correction for multiple comparisons was used to analyze siRNA-mediated reduction of ANKRD1 expression. Kruskal-Wallis and Jonckheere-Terpstra tests were used to determine any association between ANKRD1 expression (as a continuous variable) and histologic grade or FIGO stage. Kaplan-Meier survival curves were used to illustrate the survival distribution in subgroups of interest. Cox proportional hazards models were fitted to the progression-free and overall survival data. The estimated hazards ratios, together with their 95% confidence intervals, were used to quantify the degree of association between survival and the variable of interest. Backward stepwise variable selection was used to identify the independent predictors of survival. Two-tailed tests with a 5% significance level were used throughout.

Results

ANKRD1 is differentially expressed in CHO lines with differing sensitivity to cisplatin. A stringent differential display PCR strategy was used to compare gene expression between CHO-K1 and SC2, a cisplatin-sensitive mutant subclone with >7-fold lower IC50 for cisplatin (Fig. 1A).

Four sequences were consistently differentially expressed in the sensitive or parental cell line. Those corresponding to ribosomal protein L39 and Bcr/Abl-regulated protein (EST

| Table 1. Clinical and histopathologic characteristics of serous adenocarcinoma cohort |
|---------------------------------|-----------------|
| Patient characteristics (n = 71) | No. patients (%) |
| Age, y                          | 59 (22-83)      |
| Median                         | 4 (5.6)         |
| Range                          | 2 (2.8)         |
| Stage                          | 1 (2.8)         |
| I                             | 61 (85.9)       |
| II                            | 6 (8.5)         |
| III                           | 3 (38.0)        |
| Histologic grade               |                 |
| None or microscopic            | 10 (14.1)       |
| ≤1                            | 24 (33.8)       |
| >1                            | 37 (52.1)       |
| Status                         |                 |
| Dead                          | 58 (81.7)       |
| Alive                         | 13 (18.3)       |
| Follow-up, mo                  |                 |
| Median (75% CI)               | 32.1 (16.1-57.2) |

Abbreviation: CI, confidence interval.
were both increased in the sensitive clone by 2- and 1.7-fold, respectively. The sequence corresponding to ribosomal protein L19 was decreased in the sensitive clone by 2-fold (Table 2). The most highly differentially expressed sequence corresponded to Ankrd1 (ankyrin repeat domain 1), which was decreased 9.3-fold in the sensitive clones compared with parental CHO-K1 cells (Fig. 1B).

Ankrd1 is also known as cardiac ankyrin repeat protein, Carp (21), cardiac Adriamycin-responsive protein (22), and is homologous to the human gene, C-193 (23), and muscle ankyrin repeat protein (MARP; ref. 24). Full length Ankrd1 was cloned from CHO-K1, and sequencing revealed a 957 bp open reading frame with high homology between species and an amino acid identity of 91%, 92%, and 93% to human, mouse, and rat, respectively (Fig. 1C; GenBank accession EU882035). As with all other ANKRD1 proteins identified to date, the predicted hamster protein sequence contains a nuclear localization signal (at position 94 to 98 aa), five tandem ankyrin-like repeats

![Image](image-url)

**Table 2.** Genes differentially expressed in CHO lines with differing sensitivity to cisplatin

<table>
<thead>
<tr>
<th>Gene</th>
<th>ID*</th>
<th>Direction of change in sensitive cells</th>
<th>Fold change †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ankyrin repeat domain 1 (ANKRD1)</td>
<td>NM_014391.2</td>
<td>Decreased</td>
<td>9.3</td>
</tr>
<tr>
<td>Ribosomal protein L19</td>
<td>NM_000981</td>
<td>Decreased</td>
<td>2</td>
</tr>
<tr>
<td>Ribosomal protein L39</td>
<td>NM_001000.2</td>
<td>Increased</td>
<td>2</td>
</tr>
<tr>
<td>Bcr/Abl regulated protein</td>
<td>EST 251111</td>
<td>Increased</td>
<td>1.7</td>
</tr>
</tbody>
</table>

† Determined by Northern blot analysis or real-time PCR.
(between positions 127 to 283 aa), and multiple consensus casein kinase II and protein kinase C phosphorylation sites.

**Altered Ankr1 expression modulates cisplatin sensitivity.** To test whether Ankr1 was directly involved in modulation of cisplatin response, expression was increased in the cisplatin-sensitive SCG2 cells by gene transfection. Ankr1 transfection resulted in significantly increased clonogenic survival, following cisplatin exposure, compared with vector-control transfected cells, indicating decreased cisplatin sensitivity (P = 0.008, one-way ANOVA with Bonferroni correction for pairwise multiple comparisons; Fig. 2A).

To confirm that Ankr1 expression was important to platinum response, expression was decreased in CHO-K1 using a siRNA-based approach. Three vectors containing siRNA oligonucleotides, designed to regions encoding the N- and COOH terminus or a central portion of Ankr1, were each stably expressed in CHO-K1 cells. All three gene-specific siRNAs significantly reduced Ankr1 transcript levels (Fig. 2B) to <20% of CHO-K1 control (P < 0.001, one-way ANOVA with Dunnett correction for multiple comparisons). Reduced expression of Ankr1, by siRNA, was associated with increased sensitivity to cisplatin (Fig. 2C), the cisplatin IC50 being at least 2-fold lower in CHO-K1 cells with reduced Ankr1 expression compared with control. The extent of the effect on cisplatin sensitivity was similar among all three Ankr1 siRNA vectors. Ankr1 expression and cisplatin sensitivity were unaffected by transfection with the negative siRNA control.

Various mechanisms have been implicated in modulation of cellular response to cisplatin, including altered transmembrane transport and capacity to repair DNA adducts. We used siRNA to reduce the expression of Ankr1 in parental CHO-K1 cells and measured whole cell platinum accumulation, accumulation of platinum on DNA, and removal of platinum from DNA, but found no evidence for a role of Ankr1 in these proposed mechanisms of platin resistance (Supplementary Figs. S1-S4).

**ANKRD1 is expressed in ovarian and breast cancer cell lines and ANKR1 levels correlate with platinum sensitivity.** ANKR1 mRNA levels were measured in a series of 16 human ovarian and breast cell lines by real-time quantitative PCR and found to be expressed in 5 of 9 ovarian cancer cell lines, as well as in the normal ovarian cell line HOSE 17.1 and in 1 of 5 breast cancer cell lines (Fig. 3A). The cell lines varied by >20-fold in the level of ANKR1 mRNA, with the highest level of expression found in the COLO 316 > Caov-3 > OVCAR3 > MDA-MB-231 > Caov-3 ≈ HOSE 17.1 > PEO14 > OVCAR3.

Cisplatin sensitivity was measured in the seven cell lines that expressed ANKR1 at appreciable levels (Fig. 3B), and a highly significant rank correlation was observed between ANKR1 levels and platinum response (rank correlation coefficient, 0.883; P = 0.008). The cell lines with highest expression of ANKR1, COLO 316 and Caov-3, were the least sensitive to cisplatin, and the most sensitive cell line, OVCAR3, expressed a low level of ANKR1 (Fig. 3A and B). These data support the conclusion that ANKR1 has a central role in determining cellular response to cisplatin in these lines.

Cell lines that did not express ANKR1 had variable cisplatin sensitivity (not shown), indicating that increased expression of ANKR1 is only one potential mechanism for modulating cisplatin sensitivity within the cell lines examined.

**ANKRD1 in human ovarian adenocarcinomas.** To determine whether ANKR1 was expressed in human ovarian adenocarcinomas and whether the level of expression was associated with clinical outcome, ANKR1 mRNA was measured by real-time quantitative PCR in tumor specimens from a retrospective cohort of 71 serous ovarian cancer patients (Fig. 4A). The majority of cases were of moderate or high grade and from patients with advanced stage disease, reflecting the most common epithelial ovarian cases treated with platinum-based chemotherapy (Table 1). ANKR1 mRNA was detectable in the majority of cases (62 of 71, 87%), and relative expression was moderate to high in 31% (22 of 71; Fig. 4A). There was no association between the level of ANKR1 expression and either FIGO stage (P = 0.74, Kruskal-Wallis; P = 0.38, Jonckheere-Terpstra test) or histologic grade (P = 0.73, Kruskal-Wallis; P = 0.83, Jonckheere-Terpstra test), but it should be noted that there were few patients with early stage or low grade tumors in this cohort.

The level of ANKR1 mRNA, considered as a continuous variable, was significantly associated with overall survival (P = 0.013, Cox regression analysis; n = 71). Patients with a moderate to high tumor level of ANKR1 had a worse outcome compared with patients whose tumors had low ANKR1 expression (Fig. 4C). When age, residual disease, FIGO stage, and ANKR1 levels were entered as possible explanatory variables into a Cox regression model (using Backward Stepwise Variable selection), ANKR1 expression was found to be an independent predictor of overall survival (hazards ratio, 1.9; 95% confidence interval, 1.15-3.15; P = 0.013). ANKR1 expression was also found to be an independent predictor of progression-free survival (hazards ratio, 1.7; 95% confidence interval, 1.01-2.80; P = 0.046, Cox regression model with Backward Stepwise Variable selection; n = 65), where patients with higher ANKR1 expression relapsed earlier than patients with low tumor ANKR1 levels (Fig. 4B).

**Discussion**

We have described a novel role for ANKR1 as a regulator of cisplatin sensitivity that is potentially important in the clinical treatment response in ovarian cancer. This conclusion is supported by the demonstration that decreasing ANKR1 expression sensitized ovarian cells to cisplatin and expression of ANKR1 was negatively correlated with cisplatin sensitivity in a panel of human cancer cell lines. The potential clinical significance of these in vitro findings was then examined by comparison of ANKR1 expression in ovarian cancer samples with survival in a cohort of patients treated with platinum-based chemotherapy. Consistent with the hypothesis that ANKR1 may play a role in the determination of clinical cisplatin response, tumor levels of ANKR1 were significantly associated with progression-free (P = 0.046) and overall survival (P = 0.013), independent of other prognostic features, with high tumor levels found in patients with a poorer outcome.

Many studies have attempted to identify genes affecting differential cisplatin response in ovarian cancer using resistant cell lines derived by exposing a sensitive cell line to increasing doses of cytotoxic drugs. However, the clinical relevance of resistance mechanisms generated in this way has been questioned, because clinical treatment regimens repeatedly expose tumors to a fixed-drug concentration and this could select for resistance via different mechanisms (25). In contrast,
our approach has been to use a cell line model where random mutagenesis resulted in clones with exquisite sensitivity to cisplatin. In this model, neither the parent cell line (which is intrinsically relatively platinum-resistant) nor the sensitive clones have ever been exposed to chemotherapeutic drugs. Rather than seeking to identify genes associated with resistance, our approach has been to identify the gene determinants of drug sensitivity as the ultimate clinical goal is to increase the sensitivity of tumors to chemotherapeutic agents.

This strategy led to the identification of ANKRD1 as a gene of interest. ANKRD1 is expressed in skeletal muscle (21), lung (21, 24), placenta (24), and in endothelial cells (23), and has been implicated in neovascularization during wound healing (26). The predicted ANKRD1 protein sequence contains five tandem ankyrin-like repeats suggesting a protein-protein interaction capability and multiple consensus protein phosphorylation. Its sequence contains a predicted nuclear localization signal.
signal consistent with a proposed function in gene- and tissue-specific transcriptional repression (22).

There are few reports of ANKRD1 expression in neoplasia. We found ANKRD1 to be expressed in 5 of 9 ovarian cancer cell lines, in 1 of 5 human breast cancer cell lines, and in the majority of serous ovarian adenocarcinomas tested. ANKRD1 has been reported to be expressed in rhabdomyosarcomas (27) and in hepatoma cell lines (28), but to our knowledge this is first report of ANKRD1 expression in human ovarian and breast cancer cells. Moreover, there are few published studies of ANKRD1 expression in response to chemotherapeutic agents, and the data that are available are contradictory. ANKRD1 was shown to be down-regulated by doxorubicin in cardiac myocytes in culture (22), a response mediated through the generation of hydrogen peroxide and activation of protein kinase C (29). In vivo studies in pigs, however, have shown that long-term exposure to high levels of doxorubicin resulted in increased ANKRD1 expression (30). Parthenolide, an NF-κB inhibitor that has been suggested for combinational chemotherapy to overcome drug resistance (31), also increased expression of ANKRD1 in hepatoma cell lines (28), but the underlying mechanisms, potential interactions between

Fig. 4. Association of ANKRD1 expression in serous ovarian adenocarcinomas with patient outcome. A, RNA was extracted from frozen ovarian tumor specimens, and ANKRD1 mRNA was measured by real-time quantitative PCR. Kaplan-Meier curves for (B) progression-free survival and (C) overall survival are used to illustrate the comparison of cases with low or moderate/high ANKRD1 expression, measured by real-time quantitative PCR. Cox regression analysis was done considering the level of ANKRD1 mRNA as a continuous variable (progression-free survival, \( P = 0.046 \); overall survival, \( P = 0.013 \)). Patients had not relapsed or died were censored, represented by vertical tick at the last point of contact and are weighted in the analysis. The numbers of patients who remained at risk of relapse or death at each time point are indicated in tables beneath each survival curve.
ANKRD1 and NF-κB, and the implications for response to therapy are far from clear.

In cell lines that expressed ANKRD1 we found a relationship between the level of expression and survival following cisplatin treatment, and decreasing ANKRD1 expression with siRNA, increased cisplatin sensitivity, providing supporting evidence that ANKRD1 has a role in determining cellular response to cisplatin. Existing data on the role of ANKRD1 in cancer are limited, and the mechanisms underlying the association between expression of ANKRD1 and cisplatin response are currently unclear. There is evidence, however, implicating ANKRD1 in a number of molecular mechanisms that are potentially relevant to tumor response and progression. For example, although downstream targets of ANKRD1 have not been completely defined, the related protein ANKD2 has been shown to bind PML and p53, and to enhance the p53-mediated expression of the cell cycle inhibitor p21WAF1/CIP1 (32). Such an association between the MARP family of proteins and p53 expression in this study. In contrast, however, ANKD1 was up-regulated in hepatoma cells in response to fenretinide, an anticancer retinoid, and in this context ANKRD1 has been suggested to be proapoptotic (28). In our cell line model, the sensitive mutant cell line that had lost expression of Ankrd1 had at least three defects related to cisplatin sensitivity: increased drug influx, decreased glutathione levels, and defective DNA repair (34). Ankrd1 may be involved in one of these pathways, but we found no evidence that it is involved in platin transport or DNA repair. Alternatively, the mechanism of Ankrd1 action may be unrelated to these resistance mechanisms.

The differences in patient outcome based on ANKRD1 expression began to become evident on Kaplan-Meier plots at ~12 months after diagnosis (i.e., 3-4 months after the end of chemotherapy), and although the numbers were small, the majority of patients in our cohort with higher ANKRD1 expression relapsed and died within the next 12 months, i.e., within 24 months of diagnosis. Further validation, in specific patient cohorts, is required to determine whether the basis of this difference in patient outcome is related to increased sensitivity to chemotherapy in tumors with low ANKRD1 expression and/or whether high expression is specifically related to poor chemotherapy response, either intrinsic or acquired.

Gene expression microarray studies have suggested that ANKRD1 is a target of TGF-β/Wnt signaling. ANKRD1 was overexpressed in mammary tumors that developed in MMTV-Wnt1 transgenic mice (35), and the Wnt pathway has been implicated in ovarian tumorigenesis (36, 37). ANKRD1 has also been shown to be a component of a Titin-based signaling complex (38). Titin, the largest polypeptide known, has a central structural role in muscle, but in nonmuscle cells Titin is involved in chromosome condensation and chromosome segregation during mitosis (39). Intriguingly, Titin has recently been found to have a high probability of carrying cancer-associated “driver” mutations, but this has yet to be proven in a biological setting (40).

We showed an inverse correlation between expression levels of ANKRD1 and platinum sensitivity in breast and ovarian carcinoma cell lines, and changing ANKRD1 expression levels altered cisplatin cytotoxicity. Using tumor biopsies from ovarian carcinoma patients, we showed that expression levels of ANKRD1 in tumors are associated with the clinically observed response to platinum chemotherapy, and also that ovarian carcinoma expression levels are associated with survival. These results suggest that ANKRD1, or a molecular pathway involving ANKRD1, has a direct prosurvival effect following exposure to cisplatin and a novel role in determining chemosensitivity and clinical outcome in ovarian cancer. ANKRD1 therefore represents a novel target for pharmacologic inhibition to sensitize tumors to platin and increase the clinical effectiveness of these important chemotherapeutic agents.

Disclosure of Potential Conflicts of Interest
A. deFazio, A.D. Guminiski, and P.R. Harnett hold a patent but have received no financial benefit.

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References


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