Amplification of PVT1 Contributes to the Pathophysiology of Ovarian and Breast Cancer

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Abstract

Purpose: This study was designed to elucidate the role of amplification at 8q24 in the pathophysiology of ovarian and breast cancer because increased copy number at this locus is one of the most frequent genomic abnormalities in these cancers.

Experimental Design: To accomplish this, we assessed the association of amplification at 8q24 with outcome in ovarian cancers using fluorescence in situ hybridization to tissue microarrays and measured responses of ovarian and breast cancer cell lines to specific small interfering RNAs against the oncogene MYC and a putative noncoding RNA, PVT1, both of which map to 8q24.

Results: Amplification of 8q24 was associated with significantly reduced survival duration. In addition, small interfering RNA-mediated reduction in either PVT1 or MYC expression inhibited proliferation in breast and ovarian cancer cell lines in which they were both amplified and overexpressed but not in lines in which they were not amplified/overexpressed. Inhibition of PVT1 expression also induced a strong apoptotic response in cell lines in which it was overexpressed but not in lines in which it was not amplified/overexpressed. Inhibition of MYC, on the other hand, did not induce an apoptotic response in cell lines in which MYC was amplified and overexpressed.

Conclusions: These results suggest that MYC and PVT1 contribute independently to ovarian and breast pathogenesis when overexpressed because of genomic abnormalities. They also suggest that PVT1-mediated inhibition of apoptosis may explain why amplification of 8q24 is associated with reduced survival duration in patients treated with agents that act through apoptotic mechanisms.

Amplification of a region on chromosome 8q24 is one of the most frequent events in carcinomas, including serous ovarian and breast cancers, and has been associated with reduced survival duration in some studies (1, 2). The well-established oncogene MYC maps to this locus and likely contributes to the pathophysiology of cancers in which it is amplified. However, the PVT1 transcript also maps to this region and has been implicated in cancer pathophysiology as well (3). In mouse, for example, the prt-1 locus is a site of recurrent translocation in plasmacytomas (4, 5) and is a common site of tumorigenic retroviral insertion in lymphomas (6). In humans, the region homologous to prt-1 is a site of recurrent translocation between chromosomes 2 and 8 (7, 8) and its first exon is coamplified with MYC in colon carcinoma cell lines (9). PVT1 has been suggested as a MYC activator (10); however, little evidence exists to support that role. Moreover, evidence is now emerging that PVT1 may act as a noncoding RNA[12] that is strongly conserved between mouse and human.

We now present evidence that both PVT1 and MYC contribute to ovarian and breast cancer pathophysiology when

overexpressed by amplification at 8q24. First, we show that amplification at this locus is associated with reduced survival duration in ovarian cancer. We also show that down-regulation of either PVT1 or MYC expression using small interfering RNA (siRNA) technology inhibits proliferation in ovarian and breast cancer cell lines in which they are amplified and overexpressed but not in cell lines in which they are not overexpressed. In addition, we show that inhibition of PVT1 but not MYC induces an amplification/overexpression-specific apoptotic response. Our analyses of PVT1 transcripts are consistent with the interpretation that PVT1 exerts its pathophysiologic influence as a noncoding RNA.

**Materials and Methods**

**Cancer cell lines.** Ovarian cancer cell lines with and without amplification at 8q24 were selected from a collection of 30 cell lines that were either purchased from the American Type Culture Collection, European Collection of Cell Culture, Genetic Resource Centre for Biologic Material, and Interlab Cell Line Collection or generously provided by Drs. Gordon Mills and Robert Bast (M. D. Anderson Cancer Center, Houston, TX), Dr. Tom Hamilton (Fox Chase Cancer Center, Philadelphia, PA), Dr. Nelly Auersperg (University of British Columbia, Vancouver, British Columbia, Canada), and National Cancer Institute Drug Panel (listed in Supplementary Table S1). The known biological properties of the cell lines are summarized in Supplementary Table S1. Breast cancer cell lines with and without amplification at 8q24 were selected from a collection of 51 well-characterized lines described by Neve et al. (11).

**Nucleic acid extraction.** Genome DNA and total RNA were purified from cultured cells as described previously (11). Total RNA from a panel of normal human tissues was purchased from Clontech and used to measure relative expression levels of PVT1.

**Genome copy number and expression analysis.** Relative genome number was assessed in the 30 ovarian cancer cell lines using array comparative genomic hybridization with three bacterial artificial chromosome (BAC) arrays as described previously (12, 13). These included (a) Hum2.0 arrays composed of 2,465 BACs selected at approximately megabase intervals along the genome (12, 14), (b) arrays composed of 1,860 BACs selected to include genes known to be involved in cancer pathogenesis (15), and (c) arrays carrying 480 BACs selected to tile across 13 Mb at 3q24, 15 Mb at 8q24, and 30 Mb at 20q centered on regions of recurrent amplification associated with reduced survival duration in earlier studies (15). Global gene expression was assessed by hybridization to Affymetrix U133A arrays in the J. David Gladstone Institutes in the University of California at San Francisco as described.13 Hybridized arrays were visualized with Affymetrix Microarray Suite 5.0. The image files of all the arrays were then analyzed together with the robust multitarray average algorithms (16). Genome copy number and expression analyses of the breast cancer cell lines used in this study are described by Neve et al. (11).

**Real-time quantitative PCR analysis.** Quantitative PCR (QPCR) was done essentially as described previously (17). Quantitative detection of specific nucleotide sequences was based on the fluorogenic 5′ nuclelease assay and relative expression was calculated as described (17). Assays were purchased as Assays-on-Demand from Applied Biosystems. The catalogue numbers of these assays are listed in Supplementary Table S2. The sequences of PCR primers and Taqman probe specific for PVT1 transcription unit were designed with ABI Primer Express 2.0 software based on the sequence of a published expressed sequence tag (EST) clone for human PVT1 (National Center for Biotechnology Information accession no. M34428). The primer sequences for PVT1 were CATCCGGCGCCACGCT (sense) and TCTATGATGCTGTAGTCCCA (antisense). The Taqman probe was 5′-FAM-CTAGCCATACCTGCTGAGGCTTCTCC-BHQ1-3′. Primer and probe concentrations of 500 and 200 nmol/L were used, respectively. "No reverse transcriptase" analyses were done on all samples to confirm that genomic DNA was not present. For normalization, cDNA equivalent to the amount of RNA used in target gene measurements was measured for ribosomal 18S, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin A.

**Transfection of siRNA.** siRNAs against PVT1 and MYC were either predesigned using the “BioTool” function available online14 and purchased from Integrated DNA Technologies or ordered from Dharmacon predesigned siGENOME Collection. Two siRNAs cognate to different parts of mRNA sequence of PVT1 (siPVT1a and siPVT1b) and two siRNAs cognate to different parts of MYC (siMYCa and siMYCb) were used in this study. The target sequence of siPVT1a was 5′-AGGCGACTCATGATGFACT-3′ and that of siPVT1b was 5′-GGCACTCTTCCAGGATCATA-3′. The target sequence of siMYCa was 5′-GAGGCGAGACACAAG-3′. siMYCb was predesigned by Dharmacon (siGENOME ON-TARGET duplex 17, MYC) and its target sequence was 5′-GGAC- TATCTGCTGTCGAC-3′. CY3-conjugated siRNA against Aequorea green fluorescent protein (siGFP) was designed and synthesized by Integrated DNA Technologies and used as a control for both transfection efficiency and nonsequence-specific siRNA effects. A predesigned control siRNA (siControl) from Dharmacon was also used as a second nonsequence-specific effect control.

**MYC Western blotting.** Total protein sample (10 μg) was resolved in a precast NuPage 4% to 12% Bis-Tris Gel (Invitrogen), electrophoresed at 200 V for 45 min, and transferred to a piece of Immobilon transfer membrane at 250 mA for 50 min. Each membrane was then blocked and incubated with a monoclonal anti-MYC antibody (clone 9E10, Santa Cruz Biotechnology) at room temperature for 1 h or at 4°C for overnight. Each blot was washed and incubated in buffer containing an anti-mouse IgG antibody (1:20,000) at room temperature for 1 h. Finally, each blot was soaked in enhanced chemiluminescent reagents (GE Global Research) for 1 min and exposed to an X-ray autoradiography film for 1 min to 1 h.

**Bromodeoxyuridine (BrdU) DNA analysis.** The effects of siRNAs on cell cycle were assessed by measuring bromodeoxyuridine (BrdU) incorporation during a 30-min pulse 2 days after siRNA transfection. Cells were pulsed with 10 μL of 1 mmol/L stock BrdUrd added to 1 mL culture medium in each well of a 24-well plate. Cells were subsequently trypsinized, fixed in 70% ethanol, and stored in 4°C for at least 1 h. The cells were pelleted, resuspended in 0.08% pepsin (in 0.1 N HCl), and incubated at 37°C for 20 min to free nuclei. HCl (2 N) was then applied to nuclei to denature dsDNA and neutralized with 2 volumes of 0.1 mol/L sodium borate. The nuclei were then incubated on ice with 1:5 dilution of a FITC-labeled anti-BrdUrd antibody (Becton Dickinson Immunocytometry Systems) for at least 30 min and stained with 50 μg/mL of propidium iodide at 37°C for at least 15 min. BrdUrd/DNA distributions were measured in a Becton Dickinson FACSCalibur flow cytometer (fluorescence-activated cell sorting). Alternately, cells were fixed with 70% ethanol after 30 min of BrdUrd labeling for at least 2 h in room temperature, stained with mouse anti-BrdUrd antibody (BD Biosciences) and Alexa Fluor 488 goat anti-mouse (Invitrogen) antibody, and counterstained with Hoechst 33342 (Sigma-Aldrich). Cells were scanned and recorded using a Cellomics High Content.

13 http://www.affymetrix.com
14 http://www.idtdna.com
Apoptosis. The apoptotic effect of siRNA silencing was assessed using high-content image analysis. At each time point, cells were either directly stained with 1 μmol/L YO-PRO-1 stain (Invitrogen) and 10 μg/ml Hoechst 33342 for 30 min at 37°C or fixed in 4% formaldehyde at room temperature and stained with Alexa Fluor 488-phalloidin (Invitrogen) for filamentous actin (F-actin) and 10 μg/ml Hoechst 33342 for nuclei. Apoptotic cells were detected and analyzed using Cellomics Multiparameter Apoptosis and Multiparameter Cytotoxicity Bioapplications for F-actin content and YO-PRO-1 DNA staining, respectively. Intensities of YO-PRO-1 and F-actin in cells treated with siRNA were analyzed and compared with those of cells treated with LipofectAMINE only or siGFP and siControl with appropriate Cellomics applications. Significance was determined using a Student's t test.

Viable cell count analysis. Cell number was measured at 8, 24, and/or 48 h after treatments using the CellTiter-Glo Luminescent assay (Promega) according to the manufacturer's instructions, and luminescence was recorded with a luminometer (BioTek FLx800, BioTek Instruments, Inc.).

5'-Rapid amplification of cDNA ends for determination of PVT1 transcript structure. The 5' NH2-terminal sequences of PVT1 transcripts were determined using 5'-rapid amplification of cDNA ends (5'-RACE) with total RNA from epithelial cells surgically scraped from normal ovary surface epithelium (OSE1157), an OSE cell line with extended life span in culture due to the transfection of SV40 large T antigen (IOSE29), and two immortalized ovarian cancer cell lines (CAOV4 and HEY). The reactions were carried out using a FirstChoice RLM-RACE kit (Ambion) following the manufacturer's instructions. Single-band PCR products from 5'-RACE were gel purified and cloned using a TOPO TA cloning kit (Invitrogen). Plasmids containing the desired 5'-RACE PCR product were isolated and purified from single bacterial colonies. They were then sequenced with M13 forward and reverse sequence primers. The sequences of individual PVT1 exons were queried on the Web site for potential "stem-loop" structures commonly observed in microRNAs (miRNA; refs. 19, 20).

Results

Genome copy number and transcriptional analyses. We applied fluorescence in situ hybridization (Fig. 1A) for analysis of 380 ovarian tumors arranged in tissue microarrays with probes to the MYC locus (8q24) and the chromosome 8 centromere. This analysis showed that a MYC locus copy number to centromere copy number ratio of ≥1.5 (amplification) was significantly higher in serous tumors (P < 0.0001) and was associated with reduced survival duration (P = 0.0170; Fig. 1B).

We assessed the mechanisms by which amplification at 8q24 contributes to ovarian and breast pathophysiology by analyzing the effects of reducing expression levels of transcripts encoded in the region of recurrent amplification in cell lines with and without amplification at this locus. We identified ovarian cell lines amplified at this locus by applying array comparative genomic hybridization to 30 ovarian cancer cell lines. Most regions of recurrent genome copy number abnormality, including amplification at 8q24 in the cell lines, were similar to those in primary serous ovarian tumors (Fig. 1C and D). The raw array comparative genomic hybridization data have been deposited to Tab2MAGE ArrayExpress (accession no. E-TABM-246).

Supplementary Table S3 describes similarities in recurrent genome copy number abnormalities between the cell lines and primary tumors. Figure 1E and F shows that the genome copy number profiles at 8q24 for several ovarian tumors and cell lines are similar and suggest a consensus region of amplification spanning ~1 Mb encoding MYC and PVT1.

We also analyzed mRNA expression of ~17,000 transcripts using Affymetrix Hu_U133A GeneChip microarrays in the 30 ovarian cancer cell lines [raw image .cel files and analyzed robust multiarray average data have been deposited to Tab2MAGE ArrayExpress (accession no. E-TABM-254)] and calculated Pearson’s correlations between transcription levels and genome copy number changes to identify transcripts that were significantly deregulated by the genome copy number aberrations in the collection of cell lines. Each transcript was paired with a BAC clone that was nearest to the gene in the genome. These analyses revealed 417 transcripts with Pearson’s correlations of >0.5 in both cell lines and in primary ovarian tumors, suggesting that the cell lines mirror much of the genome copy number–driven transcriptional deregulation found in primary tumors. The genes are listed in Supplementary Table S4. We used QPCR to analyze the expression levels of 57 transcripts (listed in Supplementary Table S2) encoded in regions of recurrent copy number abnormality previously implicated in the pathophysiology of ovarian cancer. We analyzed these transcript levels in 21 cell lines (bold highlighted in Supplementary Table S1) to determine the accuracy with which the microarray analyses estimated expression levels. Supplementary Figure S1 shows correlation coefficients between QPCR and microarray results calculated for each gene in the 21 ovarian cancer cell lines. The correlation coefficients between expression levels measured using QPCR and Affymetrix array analysis were mostly high (average correlation coefficient, 0.75), except for five genes, including PVT1, for which the correlation coefficients were very low to negative.

Because we observed some discordances between transcript levels measured using QPCR and Affymetrix expression array analysis, we measured transcript levels of the transcripts for PVT1 and MYC using QPCR in 20 ovarian cancer cell lines. The Affymetrix U133A arrays used in this study carried probe sets 216240_at and 216249_at that were designed from EST clone M34428. The array signals for both probe sets were either undetectable or very low across all the lines, whereas the Taqman analyses designed from the same source EST sequence detected significant and variable expression levels in the same cell lines (Table 1). The Pearson’s correlation between PVT1 expression levels measured by microarray and by QPCR was only -0.02 and -0.01 for 216240_at and 216249_at, respectively, whereas the correlation between PVT1 transcript levels measured using QPCR and genome copy number at 8q24 was high (Table 1). We attribute these discordances to the poor performance of the probe sets for PVT1 on the microarrays. Table 1 compares array comparative genomic hybridization measurements of genome copy number at 8q24 and QPCR analyses of expression levels for PVT1 and MYC in 20 of the ovarian cancer cell lines. Genome copy number was assessed at the BAC array probe closest to PVT1 and MYC (clone

16 http://microrna.sanger.ac.uk/sequences/search.shtml

17 http://www.ebi.ac.uk/cgi-bin/microarray/tab2mage.cgi
Fig. 1. Recurrent copy number aberrations in ovarian tumors and cell lines. A, relative amplification of the chromosome 8q24 locus determined using fluorescence in situ hybridization with a spectrum orange–labeled probes for MYC (red) and a spectrum green–labeled probe for centromere of chromosome 8 (green) in a mucinous tumor case (probes from Vysis). The ratio of the number of copies of the MYC probe relative to the number of copies of centromere 8 was 6.8 in this case, indicating high level of amplification. B, Kaplan-Meier plot showing survival rates in 380 stage I to III ovarian tumors with and without amplification of chromosome 8q24 detected by fluorescence in situ hybridization. C and D, frequencies of significant increases or decreases in genome copy numbers are plotted as a function of genome distances of University of California at San Francisco July 2003 freeze (National Center for Biotechnology Information Build 34) for 30 cell lines (C) and primary tumors from study B of Kuo et al. (Genomic deregulation of transcription in serous ovarian cancers; prognostic markers and therapeutic targets, submitted for publication). D, positive values indicate frequencies of samples showing copy number increases and negative values indicate frequencies of samples showing copy number decreases. Gray bars, frequencies of log2 copy numbers >0.3 or < -0.3; black bars, frequencies of log2 copy numbers >0.9 or < -0.9. Solid vertical gray lines, chromosome boundaries; dotted vertical lines, centromere locations. Bottom, numbers of the even-numbered chromosomes. Data are arranged with chromosome 1pter to the left and chromosomes Xqter to the right. E, log2 copy number changes in seven ovarian cell lines that had amplification on chromosome 8q24. F, log2 copy numbers in ovarian tumors (Kuo et al. Genomic deregulation of transcription in serous ovarian cancers; prognostic markers and therapeutic targets, submitted for publication) showing copy number increases at 8q24. The MYC/PVT1 amplicon was defined by the minimal overlapping regions from tumors.
VYS08A2679). The starting site of this BAC clone overlaps with 5′ end of the MYC locus and 3′ end of the clone is ~50 kb downstream of the 5′ end of the PVT1 transcription unit. Both PVT1 and MYC transcript levels were strongly correlated with genome copy number in the 20 ovarian cancer cell lines tested. Interestingly, the correlation between copy number and expression level was higher for PVT1 than for MYC (0.89 and 0.64, respectively). This is due to the fact that some cell lines (e.g., OVCA432 and OVCA8) with amplification at 8q24 did not overexpress MYC, whereas transcription levels of PVT1 were high in all lines (e.g., CAOV4, HEY, OVCA432, and OVCA8), showing amplification at 8q24. In most cell lines, transcription levels of MYC and PVT1 were significantly higher where they were amplified than in cell lines in which they were not. However, PVT1 was highly expressed in cell line TOV21G, although it was not amplified, suggesting another mechanism of overexpression. We also compared transcription levels of PVT1 in 18 different normal tissues, 3 breast cancer lines, and 2 normal ovarian cell lines. Breast cell lines generally expressed PVT1 in trachea but not at levels found in the two ovarian cancer cell lines. Interestingly, the correlation between copy number and genome copy number changes at 8q24 in ovarian cancer cell lines was higher for PVT1 than for MYC (0.89 and 0.64, respectively). This is due to the fact that some cell lines with both overexpression and amplification of PVT1.

### Table 1. Expression levels of PVT1 and MYC and genome copy number changes at 8q24 in ovarian cancer cell lines

<table>
<thead>
<tr>
<th>Cell line name</th>
<th>PVT1 (normalized to the expression of Stratagene RNA reference pool)</th>
<th>Clone VYS08A2679</th>
<th>c-myc (normalized to the expression of Stratagene RNA reference pool)</th>
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<tr>
<td>A2780</td>
<td>0.91</td>
<td>-0.17</td>
<td>1.14</td>
</tr>
<tr>
<td>CAOV3</td>
<td>0.91</td>
<td>-0.04</td>
<td>0.09</td>
</tr>
<tr>
<td>CAOV4*</td>
<td>21.36</td>
<td>3.15</td>
<td>2.49</td>
</tr>
<tr>
<td>DOV13</td>
<td>2.05</td>
<td>-0.07</td>
<td>0.60</td>
</tr>
<tr>
<td>ES-2</td>
<td>1.29</td>
<td>0.32</td>
<td>0.86</td>
</tr>
<tr>
<td>HEY*</td>
<td>5.09</td>
<td>0.63</td>
<td>2.88</td>
</tr>
<tr>
<td>OCC1</td>
<td>0.67</td>
<td>-0.44</td>
<td>0.30</td>
</tr>
<tr>
<td>OV90</td>
<td>0.25</td>
<td>-0.02</td>
<td>0.62</td>
</tr>
<tr>
<td>OVCA420</td>
<td>1.89</td>
<td>0.54</td>
<td>1.92</td>
</tr>
<tr>
<td>OVCA429</td>
<td>3.28</td>
<td>0.09</td>
<td>0.72</td>
</tr>
<tr>
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<td>0.91</td>
<td>1.25</td>
</tr>
<tr>
<td>OVCA433</td>
<td>3.14</td>
<td>0.04</td>
<td>0.62</td>
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<tr>
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<td>1.47</td>
<td>0.52</td>
<td>0.45</td>
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<td>0.73</td>
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<td>1.00</td>
</tr>
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<td>0.80</td>
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<td>0.76</td>
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<td>SW626</td>
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<tr>
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<tr>
<td>TOV21G</td>
<td>5.74</td>
<td>0.00</td>
<td>0.82</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>$r^2 = 0.89$</td>
<td>$r^2 = 0.64$</td>
<td></td>
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*Cell lines with both overexpression and amplification of PVT1.

http://www.hgmp.mrc.ac.uk/NIX

Supplementary Figure S2) were obtained from OSE1157 5′-RACE PCR products from CAOV4 and HEY cells. The 5′-RACE of IOSE29 and CAOV4 cells produced multiple bands. The single bands from OSE1157 and HEY cells were cut, gel purified, cloned into TOPO TA cloning vectors, and sequenced with M13 primers flanking the inserted PCR products. Two different sequences (#1 and #2 in Supplementary Fig. S2) were obtained from OSE1157 5′-RACE PCR products from HEY cells. The 5′-RACE sequence was identical to the 5′-RACE sequence #2 from OSE1157. We then searched human EST databases using National Center for Biotechnology Information nucleotide-nucleotide BLAST (blastn) based on our 5′-RACE sequences and the sequence of a cDNA clone (National Center for Biotechnology Information accession no. BC033263) that was previously considered to be a full-length clone for PVT1. The 125 bp at the 5′ ends of both 5′-RACE sequences overlapped with the 5′ end of the BC033263 sequence. ESTs that had >95% sequence homology with the query sequences were assembled to predict full-length transcripts. The web tool, NIX (Nucleotide Identify X software), was used to identify exons from these EST alignments. Nine exons were predicted for each of the two 5′-RACE full-length transcripts (Fig. 2) assembled from sequences of 5′-RACE #1 and #2 and BC033263. The two transcripts shared exons 2 to 9 but had different first exons. We termed the first exon that corresponds to 5′-RACE sequence #1 as exon 1a and the exon that corresponds to 5′-RACE sequence #2 as exon 1b. Exon 1a is upstream of exon 1b in the genome (Fig. 2). To determine whether the predicted full-length transcripts exist in the
transcriptome, primers against exon 1a or 1b and exon 9 were used to amplify PVT1 cDNAs from the HEY cells and normal testis. PCR products were then cloned and sequenced. As shown in Fig. 2, multiple alternatively spliced variants were identified from these PCR products. Predicted exons 4 and 8 were missing in all of the 22 PCR products that we have cloned. We also assessed the sequences of individual PVT1 exons detected by PCR for possible stem-loop structures that could signify the presence of transcripts that will be bound and cleaved by Drosha to liberate 70-nucleotide miRNA precursors (21). Sequences homologous to known stem-loop structures in different species were found in predicted exons 5, 6, 7, and 9; however, the significance indices of these predictions were low in all cases.

Biological responses to inhibition of PVT1 and MYC expression. Because our main goal in this study was to determine how PVT1 and MYC contribute to ovarian cancer pathophysiology when overexpressed by amplification or other genomic mechanisms, we compared biological responses to inhibition of PVT1 and MYC in ovarian and breast cancer cell lines with and without amplification and overexpression of these two genes.

We assessed the biological effects of inhibiting mRNA levels of PVT1 using siRNAs in the ovarian cancer cell lines CAOV4, HEY, OVCA432, and OVCAR8 where 8q24 is amplified and PVT1 is overexpressed and in cell lines A2780, CAOV3, OV90, and SKOV3 where PVT1 is not amplified or overexpressed. We compared these responses to responses to siRNA inhibition of MYC expression in a subset of these lines. After 48 h, >50% knockdown in PVT1 mRNA level was achieved in all ovarian cancer lines treated with 120 nmol/L siPVT1a and at least 80% knockdown was achieved in HEY cells treated with either 120 nmol/L siPVT1a or siPVT1b. Representative semiquantitative reverse transcription-PCR agarose gel electrophoresis and Taqman QPCR analyses of PVT1 are shown in Fig. 3A and B, respectively. Figure 3A also shows similar PVT1 knockdown levels in three breast cancer cell lines (SUM159PT, HBL100, and SKBR3). Notably, siRNA knockdown of PVT1 expression was accompanied by a slight decrease in MYC protein expression in CAOV4 but not in any of the other cell lines (Fig. 3C). Figure 3C shows that 200 nmol/L siMYCa also reduced the level of MYC protein expression to >50% at 48 h. To minimize off-target effects of high concentration of siRNA, we also assessed responses to a different siRNA against MYC (siMYCb) that reduced the MYC mRNA level in HEY cells to <12% of that in siControl-transfected cells (Fig. 3D) at 120 nmol/L. Eight other siRNAs targeting different parts of PVT1 transcript (see Fig. 2) were also tested for knockdown in HEY cells but none of these reduced PVT1 mRNA levels significantly.

Knockdown of PVT1 or MYC inhibits proliferation. We determined the effects of PVT1 and/or MYC knockdown on
cell proliferation by measuring changes in fractions of cells in the G1, S, and G2-M phases of the cell cycle estimated from BrdUrd/DNA distributions measured for cells pulse labeled with BrdUrd at 8, 24, and/or 48 h after siRNA transfection and by counting viable cells using the CellTiter-Glo Luminescent assay that measures ATP levels in metabolically active cells. Table 2 shows that siPVT1a strongly inhibited BrdUrd incorporation in four PVT1-amplified/overexpressed cell lines but not in any of the nonamplified lines at 48 h. In HEY and OVCAR8, the reduction in the fraction of cells in S phase was accompanied by a significant accumulation of cells in G1 phase of the cell cycle. Treatment with 120 nmol/L siPVT1a and 200 nmol/L siMYCa produced similar levels of inhibition of PVT1 incorporation in CAOV4 and HEY cells in which both PVT1 and MYC are amplified and overexpressed (Table 2). Neither G1 cell cycle arrest nor reduction in S phase was seen in any of the four PVT1/MYC-nonamplified/overexpressed cell lines. siPVT1b had even stronger antiproliferation effects in CAOV4 and HEY cells than siPVT1a (Table 2). We also evaluated the effect of siRNA knockdown with siPVT1a on cell growth using CellTiter-Glo Luminescent assays. Table 2 shows that the number of viable cells in siPVT1a-transfected HEY cells started to decrease relative to that of cells treated with LipofectAMINE alone or siControl at 8 h. By 24 h, the viable cell count was only 40% of that of control cultures. In contrast, siPVT1a had no effect on cell viability in two ovarian cell lines in which PVT1 was not amplified or overexpressed.

To determine the generality of the phenotype resulting from PVT1 knockdown, we also compared the effect of siPVT1a transfection in two breast cancer cell lines (SUM159PT and HBL100) in which PVT1 is both amplified and overexpressed with that in a breast line (SKBR3) where PVT1 is only amplified but not overexpressed. As shown in Table 2, transfection of siPVT1a decreased the proportion of BrdUrd-incorporating cells in SUM159PT and HBL100 but not in SKBR3.

**Knockdown of PVT1 but not MYC increases apoptosis.** We assessed the effects of inhibiting PVT1 expression on programmed cell death in cells with and without PVT1 amplification/overexpression by measuring membrane permeability (22), cell morphology, and F-actin reorganization (23, 24) using high-content image analyses. YO-PRO-1 dye uptake increases when cells lose membrane integrity during cell death, whereas F-actin reorganization results in increased Alexa Fluor 488-phalloidin binding that has been associated with earlier stages of apoptosis (24). Beginning at 8 h after transfection, siPVT1a significantly increased YO-PRO-1 dye uptake and F-actin staining relative to LipofectAMINE controls in HEY and CAOV4 cell lines in which PVT1 is amplified and overexpressed (Fig. 4A and B). Increased apoptosis in siPVT1a-transfected cells was further confirmed with Annexin V staining in CAOV4 cells (data not shown). In contrast, transfection of siPVT1a in three of the four nonamplified/overexpressed lines produced no significant changes, except in SKOV3 cells where F-actin staining increased significantly after

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**Fig. 3.** Expression of PVT1 and MYC in control and siRNA-treated cells. **A,** agarose gel electrophoresis images of semiquantitative reverse transcription-PCR specific for PVT1 transcript in CAOV4, HEY, SUM159PT, HBL100, and SKBR3 cells treated with conditions as indicated. GUS expression was tested as the sample loading control. **B,** % PVT1 mRNA level knockdown measured by Taqman QPCR in HEY cells transfected with three concentrations (as indicated in the graph) of siPVT1a and siPVT1b compared with cells transfected with siControl. **C,** Western blots with anti-MYC antibody in CAOV4 and HEY cells transfected with siPVT1a and siMYCa. In these experiments, the null control was cells that were incubated with Opti-MEM only during transfection. The LipofectAMINE (Lipo) control was cells that were mock transfected with LipofectAMINE 2000 at a maximum concentration used in each experiment (5–7.5 mg/mL). **D,** % MYC mRNA level knockdown measured by Taqman QPCR in HEY cells transfected with different concentrations (as indicated in the graph) of siMYCb compared with cells treated with siControl.
siPVT1a transfection ($P = 0.001$). Apoptosis induced by siRNA knockdown of PVT1 expression was more pronounced in CAOV4 and HEY cells transfected with siPVT1b compared with cells transfected with siPVT1a. No effect on apoptosis was seen in the nonamplified cell line OV90 transfected with either siPVT1a or siPVT1b (Fig. 4C). Transfection with 120 nmol/L siMYCb did not significantly affect apoptosis in any of the cell lines tested (Fig. 4B and C). Increased apoptosis was also seen in the breast cancer cell lines in which PVT1 was amplified and overexpressed following transfection of siPVT1a but not in SKBR3 where PVT1 was not overexpressed (data not shown).

We also treated the eight ovarian cell lines with paclitaxel as a positive control for apoptosis induction. Paclitaxel (100 nmol/L) induced massive apoptosis in six of the eight cell lines as expected, with the exception of two PVT1-amplified/overexpressed cell lines (HEY and OVCA432; Fig. 4A).

### Discussion

Several published findings implicate PVT1 in aspects of cancer pathophysiology. Examples include observations that rearrangement of the region at 8q24 encoding MYC and PVT1 is frequently involved in human leukemias and lymphomas (4, 5), the regions is frequently amplified in solid tumors (2), and a site of recurrent tumorigenic viral integration in mice (25). MYC is well established as an oncogene in this region. We now provide functional evidence for the importance of increased expression of PVT1 in cancer through analysis of cell lines with and without amplification at 8q24. These cell lines were selected from a collection of 30 ovarian cell lines described in this article and 51 breast cancer cell lines described elsewhere (11). Our analyses of both collections show that the recurrent genome aberrations and the resulting deregulation of gene expression are highly concordant between primary tumors and the cell lines. Thus, the aspects of amplification-dependent cancer pathophysiology discovered in the cell lines are likely to be obtained in primary tumors as well.

The strongest evidence for the importance of PVT1 in cancer pathophysiology is our observation that siRNA silencing of PVT1 expression decreases cell proliferation and increases apoptosis in breast and ovarian cancer cell lines in which it was amplified and overexpressed but not in cell lines where it is not amplified/overexpressed. The amplification/overexpression-specific response phenotypes argue that the observed effects are due to down-regulation of PVT1 rather than to off-target effects of siRNA. The PVT1 specificity of the response is further

### Table 2. Effects of PVT1 and MYC siRNAs on cell proliferation in ovarian and/or breast cancer cell lines

<table>
<thead>
<tr>
<th>MYC/PVT1 amplified</th>
<th>Lipo 120 nmol/L siGFP</th>
<th>120 nmol/L siPVT1a</th>
<th>200 nmol/L siMYCb</th>
<th>Cellomics BrdUrd incorporation assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAOV4 G1</td>
<td>56</td>
<td>48</td>
<td>60</td>
<td>G1 58</td>
</tr>
<tr>
<td>S</td>
<td>17</td>
<td>19</td>
<td>2</td>
<td>S 24</td>
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<tr>
<td>HEY G1</td>
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<td>66</td>
<td>61</td>
<td>G1 51</td>
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<tr>
<td>S</td>
<td>41</td>
<td>1</td>
<td>4</td>
<td>S 32</td>
</tr>
<tr>
<td>OVCA432 G1</td>
<td>36</td>
<td>43</td>
<td>53</td>
<td>G1 42</td>
</tr>
<tr>
<td>S</td>
<td>39</td>
<td>33</td>
<td>9</td>
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<tr>
<td>OVCA432 G1</td>
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<td>26</td>
<td>57</td>
<td>G1 32</td>
</tr>
<tr>
<td>S</td>
<td>42</td>
<td>45</td>
<td>3</td>
<td>S 39</td>
</tr>
<tr>
<td>MYC/PVT1 nonamplified</td>
<td>Lipo 120 nmol/L siGFP</td>
<td>120 nmol/L siPVT1a</td>
<td>200 nmol/L siMYCb</td>
<td>Cellomics BrdUrd incorporation assay</td>
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<td>-------------------</td>
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</tr>
<tr>
<td>CAOV3 G1</td>
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<td>60</td>
<td>58</td>
<td>G1 58</td>
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<tr>
<td>S</td>
<td>24</td>
<td>21</td>
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<td>S 24</td>
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<tr>
<td>OV90 G1</td>
<td>51</td>
<td>47</td>
<td>43</td>
<td>G1 51</td>
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<tr>
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<tr>
<td>SKOV3 G1</td>
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<tr>
<td>S</td>
<td>17</td>
<td>17</td>
<td></td>
<td>S 17</td>
</tr>
</tbody>
</table>

**NOTE:** BrdUrd/propiodum iodide cell cycle distributions were measured by fluorescence-activated cell sorting analyses or by Cellomics High Content Imaging System. Viable cell counts were assessed using the CellTiter-Glo Luminescent cell viability assay.

Abbreviations: FACS, fluorescence-activated cell sorting analysis; CTG, CellTiter-Glo Luminescent; Lipo, LipofectAMINE.
supported by our observation that the same amplification/overexpression-specific response phenotype was seen using two different siRNAs against PVT1.

PVT1 has been suggested to function as a MYC activator. However, our demonstration that PVT1 inhibition does not alter MYC levels in most of the cell lines where it influences both apoptosis and proliferation argues against this. Moreover, inhibition of PVT1 but not MYC induces apoptosis in cell lines where they are both amplified and overexpressed. If PVT1 were acting through MYC, the apoptotic response should have been observed after inhibition of MYC. Therefore, we conclude that PVT1 acts independently of MYC in generation of the apoptotic phenotype.

Fig. 4. Effects of PVT1 and MYC siRNAs on apoptosis in ovarian and/or breast cancer cell lines measured by the Cellomics High Content Imaging System. A, cell permeability (YO-PRO-1 dye intake) and microfilament reorganization (F-actin staining) in PVT1-amplified/overexpressed and PVT1-nonamplified ovarian cell lines treated with siPVT1a and paclitaxel for 48 h. For the amplified lines, the white columns represent data from CAOV4, the columns with black stripes are for HEY, the dotted columns are for OVCAR4, and the solid black columns represent results from OVCAR8. For nonamplified cell lines, the gray columns represent data from A2780, the columns that have white hatched stripes against a black background represent CAOV3, the columns with checkered pattern are for OV90, and the columns with cross-hatched lines represent SKOV3.

B, apoptosis induced by transfection of 120 nmol/L siPVT1a at 8, 24, and 48 h in PVT1-amplified/overexpressed and PVT1-nonamplified ovarian cell lines. All the siRNAs were transfected at 120 nmol/L concentration. The heights of the columns in bar graphs represent fold changes in apoptotic cell proportions from LipofectAMINE-treated control cells. * experiments in which the difference in total fluorescent intensity was significant between siPVT1a-transfected cells and LipofectAMINE-treated control cells (P < 0.05).

C, comparison of apoptotic effects (measured by YO-PRO-1 dye intake) induced by 120 nmol/L siPVT1a and siPVT1b transfection at 48 h in three ovarian cell lines.
The strong induction of apoptosis resulting from siRNA inhibition of PVT1 suggests that PVT1 amplification contributes to the oncogenic phenotype, at least in part, by suppressing apoptosis. This suggests the interesting possibility that amplification at 8q24 might have two simultaneous oncogenic functions: overexpression of MYC, which stimulates proliferation, and overexpression of PVT1, which not only stimulates proliferation but also inhibits the apoptotic response normally associated with overexpression of MYC. The apoptosis suppression function of PVT1 may also explain why its overexpression is associated with reduced survival duration in patients treated with platinum plus taxane–based therapies. Platinum compounds produce apoptotic responses through production of DNA cross-links (26), whereas taxanes trigger apoptotic responses by stabilizing otherwise dynamic microtubules that are important for centrosome and mitotic spindle function (27). Overexpression of PVT1 may contribute to resistance to these agents by suppressing the apoptotic mechanisms through which they work. This possibility is partially supported by our finding that two ovarian cancer cell lines with high-level PVT1 expression do not exhibit significant apoptotic responses to treatment with paclitaxel at concentrations that induced apoptosis in the other cell lines. However, PVT1 is not the only determinant of response because two other PVT1–amplified/overexpressed cell lines exhibit a significant apoptotic response to paclitaxel.

Elucidation of the mechanism(s) by which PVT1 overexpression contributes to suppression of apoptosis and proliferation is complicated by the fact that PVT1 is transcribed into multiple splice forms that vary in form and abundance between cell lines (data not shown; ref. 9). However, our observation that siRNAs complementary to sequences in exons 2 and 3 both produced transcripts that were specific to cell lines with amplification and overexpression of PVT1 suggested that transcripts containing these two exons are functionally important.

Mechanistic interpretation is further complicated by the observation that PVT1 seems to be a noncoding RNA because the longest open reading frame predicted from our assessment of PVT1 sequences is 150 amino acids encoded in the first two exons. The noncoding RNAs most strongly implicated in cancer thus far are miRNAs (28). These 20– to 22-nucleotide RNAs are the result of enzymatic processing of larger transcripts and may operate in cancer by blocking translation of target genes to which they are complementary. Deregulated expression of several miRNAs has been associated with poor disease outcome in chronic lymphocytic leukemia, colorectal neoplasia, lung cancer, and Burkitt’s lymphoma (28). miRNAs are also frequently located at fragile sites and genomic regions that involved cancers (29). Thus, we investigated the possibility of PVT1 as a miRNA. Our computational analyses show that the predicted sequences of PVT1 transcripts do not seem to have the stem–loop structures normally associated with miRNAs (20, 30, 31). A recent study by Dr. Huppi has identified seven putative miRNAs within the ~400-kb PVT1 genomic locus. The precursor sequence of one of these overlaps with exon 1b in our current study, but it also extends beyond the consensus splice site of the exon. The precursor sequences of the other six miRNAs have no association with any of the annotated PVT1 exons and might be results of extensive alternative splicing found in this locus (Fig. 2). This may explain our failure to identify potential miRNA precursor sequences in our predicted PVT1 transcripts, which contain mostly known exons. Thus, the mechanism by which PVT1 exerts its pathologic function remains unclear.

In conclusion, we have used our well-characterized cell line collection to show that amplification at 8q24 increases expression of both MYC and PVT1 and that both of these deregulated transcripts seem to contribute to ovarian and breast cancer pathophysiology. We have shown that PVT1 is most likely a noncoding RNA that acts independently of MYC and, when amplified and overexpressed, acts to increase proliferation and inhibit apoptosis.

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References

10. Fotedar R, Cory S, Graham M, Webb E, Collisson EA, Huppi, and Natasha J. Kaplen for helpful discussions. We thank Dr. Huppi for a pre-publication copy of his manuscript on PVT1 miRNAs.


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