Frequent Coamplification and Cooperation between *C-MYC* and *PVT1* Oncogenes Promote Malignant Pleural Mesothelioma

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Introduction: Malignant pleural mesothelioma (MPM) is a deadly disease with poor prognosis and few treatment options. We characterized and elucidated the roles of *C-MYC* and *PVT1* involved in the pathogenesis of MPM.

Methods: We used small interfering RNA (siRNA)-mediated knockdown in MPM cell lines to determine the effect of *C-MYC* and *PVT1* abrogation on MPM cells undergoing apoptosis, proliferation, and cisplatin sensitivity. We also characterized the expression of *microRNAs* spanning the *PVT1* region in MPM cell lines. Copy number analysis was measured by quantitative polymerase chain reaction and fluorescence in situ hybridization.

Results: Copy number analysis revealed copy number gains (CNGs) in chromosomal region 8q24 in six of 12 MPM cell lines. MicroRNA analysis showed high *miR-1204* expression in MSTO-211H cell lines with four copies or more of *PVT1*. Knockdown by siRNA showed increased PARP-C levels in MSTO-211H transfected with *siPVT1* but not in cells transfected with si*C-MYC*. *C-MYC* and *PVT1* knockdown reduced cell proliferation and increased sensitivity to cisplatin. Analysis of the expression of apoptosis-related genes in the MSTO-211H cell line suggested that *C-MYC* maintains a balance between proapoptotic and antiapoptotic gene expression, whereas *PVT1* and, to a lesser extent, *miR-1204* up-regulate proapoptotic genes and down-regulate antiapoptotic genes. Fluorescence in situ hybridization analysis of MPM tumor specimens showed a high frequency of both CNGs (11 of 75) and trisomy (three copies; 11 of 75) for the *C-MYC* locus.

Conclusion: Our results suggest that *C-MYC and PVT1* CNG promotes a malignant phenotype of MPM, with *C-MYC* CNG stimulating cell proliferation and *PVT1* both stimulating proliferation and inhibiting apoptosis.

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Malignant pleural mesothelioma (MPM) is a highly aggressive cancer with a poor prognosis.[1] MPM's incidence in the United States has increased in recent years, and 2000 to 3000 people are diagnosed with the disease annually.[2,3] MPM predominantly affects men who have been exposed to asbestos in an occupational setting.[4–8] The risk of developing the disease increases with age (median age at diagnosis is 72 years; range, 45–85 years). MPM's three major histologic subtypes are epithelioid, biphasic, and sarcomatoid.[1] Epithelioid tumors are the most common and have the best prognosis of the three subtypes. However, all types are very difficult to treat and have a median overall survival (OS) duration ranging from 9 to 17 months,[9] with an overall 2-year survival rate of only 20%.[10] There is therefore a great need to identify new therapeutic targets and develop more effective therapies for patients with MPM.

A potential therapeutic target for MPM and one of the most common chromosomal amplification sites in cancer tissues is the 8q24 region that contains the genes *C-MYC* and *PVT1*.[11–13] *C-MYC* encodes a transcription factor that regulates the expression of multiple genes involved in cellular responses such as growth, proliferation, apoptosis, and differentiation.[14–16] Deregulated amplification and expression of the *MYC* locus occur in approximately 30% of human cancers, including colon, prostate, and breast carcinomas, and have been associated with poor prognosis.[11,17,18] *PVT1* is a candidate oncogene located adjacent to the *MYC* locus on chromosomal region 8q24.[18–20] *PVT1* has been shown to act as a noncoding RNA with many alternatively spliced isoforms.[12,21] The *PVT1* locus has recently been found to contain a cluster of at least six microRNAs (*miRNAs*) (such as *miR-1204*, *-1205*, *-1206*, *-1207-3p*, *-1207-5p*, and *-1208*) that span the *PVT1* region, adding further complexity to the locus.[12,21] Both *PVT1* copy number gains (CNGs) and *PVT1* overexpression have been implicated in the pathophysiology of many tumors, including breast and ovarian cancers and acute myeloid leukemia.[19,22] In addition, *PVT1* alteration has been shown to contribute to tumor survival and

chemoresistance.[22,23] However, the roles that *MYC*, *PVT1*, and *miRNAs* contained in the 8q24 chromosomal region play in MPM remain unclear. We therefore sought to elucidate these roles and the specific mechanisms of action of *C-MYC* and *PVT1* involved in the pathogenesis of MPM.

In the present study, we characterized the molecular abnormalities found in the 8q24 locus in MPM cell lines and in specimens from surgically resected MPMs and characterized the *miRNA* (*miR-1204, -1205, 1206, -1207-3p, 1207-5p*, and *-1208*) expression in MPM cell lines. We also determined the biological impact of small interfering RNA (siRNA)-mediated *C-MYC* and *PVT1* abrogation on MPM cellular processes such as apoptosis, cell proliferation, and response to cisplatin and then determined the effect of *C-MYC*, *PVT1*, and *miR-1204 knockdown* on the expression levels of apoptosis-related genes. Finally, we studied *C-MYC* and *PVT1* copy number and gene expression in MPM tumor specimens.

MATERIALS AND METHODS

Tumors Specimen and Cell Lines

From the tissue bank at The University of Texas M. D. Anderson Cancer Center, we obtained archived frozen and formalin-fixed, paraffin-embedded tissues for patients who had undergone surgical resection for MPM. We randomly selected 75 MPM samples of different histologic subtypes (37 epithelioid, 26 biphasic, and 12 sarcomatoid) for analysis. Detailed clinical and pathologic information on the patients is presented in Supplementary Table 1 (Supplementary Digital Content 1, http://links.lww.com/JTO/A598). The study protocol was approved by the M. D. Anderson Institutional Review Board. Of the 12 MPM cell lines used in this study, five (H2452, MET-5A, H2052, H28, and MSTO-211H) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Cellgro Mediatech, Manassas, VA) and seven (HCT-4012, Meso, HP3, HP5, HP7, HP9, and HP10) were acquired from Dr. Harvey Pass (New York University, New York, NY) and cultured in high-glucose Dulbecco's modified Eagle's medium (Cellgro Mediatech). All media formulations included 10% fetal bovine serum and antibiotics (Sigma-Aldrich, St. Louis, MO). All MPM cell lines had been tested for absence of mycoplasma using Universal Mycoplasma Detection Kit according to the manufacturer's instructions (ATCC, Manassas, VA) and cells were authenticated at University of Texas M.D. Anderson Cancer Center Core Facility.

Isolation of DNA and Copy Number Profiling

DNA was extracted from cell lines using DNAzol Reagent (Life Technologies, Grand Island, NY), and whole-genome single nucleotide polymorphism (SNP) array profiling was performed using Affymetrix SNP 6.0 chips (Agilent Technologies, Santa Clara, CA) in five MPM cell lines. CNGs were identified using the SNP-Fast Adaptive States Segmentation Technique 2 algorithm in Nexus 5.1 software (BioDiscovery, Hawthorne, CA) with the significance threshold for segmentation setting at $p < 5 \times 10^{-7}$. CNGs were defined with log2 ratio values of 0.2, and two or more than two CNGs were defined by log2 ratio values of 0.7.

Copy Number Analysis

We used fluorescence in situ hybridization (FISH) and real-time quantitative polymerase chain reaction (q-PCR) analyses to quantify 8q24 CNGs in MPM tumor specimens. We used directly labeled fluorescent chromosomal centromeric probes (CEP 8; SpectrumGreen; Vysis, Abbott Laboratories, Chicago, IL) for chromosome 8 and locus-specific probes for regions 8q24.12-q13 (*C-MYC* Spectrum Orange) (Vysis; Abbott Laboratories, Chicago, IL). FISH was performed according to the manufacturer's instructions. Copy number analysis was performed in 50 nuclei per tumor in at least four areas. Copy number alteration was defined as the presence of more than two gene copies per cell on average of the 50 cells. Trisomy was defined as the presence of three copy number alterations and CNG was defined as the presence of at least four copies. To enrich for malignant cell content for q-PCR analysis, tumor tissues were manually microdissected for subsequent DNA extraction from formalin-fixed, paraffin-embedded tissue sections. Tumor DNA was extracted using the Pico Pure DNA Extraction Kit (Arcturus; Life Technologies) according to the manufacturer's instructions. DNA samples with proportions of microdissected tumor cell greater than 70% were qualified for q-PCR analysis. *MYC* and *PVT1* gene copy numbers were examined by q-PCR using the comparative Ct method using the ABI 7300 real-time PCR system (Applied Biosystems, Grand Island, NY). The sequences of PCR primers used to CNG were for *C-MYC*: 5'-TCAAGAGGTGCCACGTCTCC-3' and 5'-TCTTGGCAGCAGGATAGTCCTT-3' (flanking exon 3), and for *PVT1*: 5'-ACAGTGATCTTCAGTGGTCTGG-3' and 5'-CGTGTGTCATTCCAGTGCAT-3' (flanking exon 2). Each PCR was carried out using Power SYBR Green PCR Master Mix (Applied Biosystems) at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. β-Actin was introduced as the endogenous reference gene, and TaqMan Control Human Genomic DNA (Applied Biosystems) was amplified as a standard control for calibration. *MYC* and *PVT1* gene copy number in normal human genomic DNA was set as 2, and copy number 4 or more was considered as CNG.[24] All sample and standard DNA reactions were set in triplicate to gauge reaction accuracy.

Isolation of mRNA and *miRNA* Analysis

Total RNA was extracted from frozen MPM tumor specimens and cell lines using TRI Reagent (Life Technologies). Spectrophotometric analysis using Nanodrop 1000 (Nanodrop; Thermo Fisher Scientific, Waltham, MA) was used to quantify RNA, and Agilent BioAnalyzer RNA nano-chips (Agilent Technologies) were used to gauge RNA quality. Affymetrix U133 Plus 2.0 gene expression arrays (Affymetrix, Santa Clara, CA) were used to determine global expression levels of genes, including *MYC* and *PVT1* in the total RNA extracted from tumor specimens. Using a High Capacity RNA-to-cDNA Kit and Taqman Gene Expression PCR Assays (Applied Biosystems), we performed quantitative reverse transcriptase (qRT) PCR analysis of the RNA extracted from cell lines to quantify *MYC* and *PVT1* levels. The sequences

