Stathmin is Overexpressed in Malignant Mesothelioma

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Abstract. Background: Malignant pleural mesothelioma is a highly aggressive cancer, with low overall survival. The pathogenesis of mesothelioma is poorly understood. The aim of this study was to identify potential genes overexpressed in mesothelioma. Materials and Methods: A cDNA microarray was used to identify potential genes that are activated in mesothelioma cell lines. Overexpression of stathmin, a cytosolic protein that regulates microtubule dynamics, was found. RT-PCR, Western blot, and immunohistochemistry were used to confirm overexpression in both cell lines and tumor samples. Results: Using RT-PCR and Western blot, stathmin overexpression was confirmed in seven mesothelioma cell lines. Increased stathmin protein expression was also found in seven out of eight mesothelioma tumor samples. Finally, stathmin expression in a mesothelioma tumor was confirmed by immunohistochemistry. Conclusion: For the first time, stathmin was shown to be overexpressed in malignant mesothelioma. The overexpression of stathmin in mesothelioma may offer a potential therapeutic target and further studies are warranted.

Malignant pleural mesothelioma is an aggressive cancer which arises from the pleural lining of the lung (1). Approximately 3,000 patients are diagnosed with mesothelioma annually in the United States and the incidence is expected to increase (2-4). Most patients present at a relatively late stage and have a poor prognosis. Curative resection is usually not possible and medical therapies have had limited benefit. The median survival time is 12 months (5, 6).

Targeted therapies may improve the survival for patients with mesothelioma, but the pathogenesis of mesothelioma is poorly understood. Asbestos exposure is linked to the majority of cases, but its exact role in oncogenesis is still unclear (7, 8). Unlike many other epithelial cancers, the activation of ras genes and inactivation of Rb and p53 genes do not seem to be necessary for the development of mesothelioma (9, 10). Alterations of several molecular pathways, including epidermal growth factor receptor, cell cycle regulatory genes, and developmental pathways have been linked to mesothelioma (11, 12). There is also evidence that Simian virus 40 (SV40) may contribute to the development of mesothelioma, but the exact genetic alterations leading to mesothelioma remain unknown (11, 13).

The aim of this study was to identify potential genes involved in the pathogenesis of mesothelioma.

Materials and Methods

Cell lines. Mesothelioma cancer cell lines were obtained from the following sources: LRK1A and REN through a generous gift from Dr. Steven Albelda (University of Pennsylvania, Philadelphia, PA, USA), NCI-H2052, H28, MSTO-211H, and H513 from American Type Culture Collections (ATCC, Manassas, VA, USA), MS-1 and NCI-H290 from NIH (Frederick, MD, USA) and LP9 were from the Cell Culture Core Facility at Harvard University (Boston, MA, USA). We should note that LP9 are mesothelial cells that are not activated by SV40. All cell lines except LP9 were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml). LP9 was cultured in M199 containing 15% medium plus 10 ng/ml EGF and 0.4 µg/ml HC. All cells were cultured at 37°C in a humid incubator with 5% CO2.

Frozen cell pellets (–80°C) for use in the reference pool (WM-115, NTERA-2, Colo 205, MCF7, Hs 578T, RPMI 8226, Hep G2, SW-872, OVCAR-3, HL-60, & MOLT-4) were provided by the UCSF cancer center microarray core facility in medium containing 10% DMSO.

Human tissues. Fresh mesothelioma tissues and adjacent normal pleural tissues from patients undergoing primary resection of their tumors were collected at the time of surgery and immediately snap-frozen in liquid nitrogen (IRB H8714-22942-01). These tissue samples were kept at –80°C in a liquid nitrogen freezer prior to use. Final pathologic diagnosis was confirmed by a pathologist from the University of California, San Francisco, USA. Patient identifiers were coded to protect confidentiality.

cDNA microarray. The cDNA microarray chips used in this experiment were from a print labeled Poly-L HPLower9K.7 and were prepared by the UCSF Array Core Facility. These chips
contain approximately 8,600 ESTs. H28, MSTD-211H, H513, H290, H2052, MS1, LRK1A, and LP9 cell lines were labeled with Cy5 and run against a control reference pool labeled with Cy3. In subsequent analysis, abnormal cell lines were compared to the LP9 cell line, which was used as a control pleural cell line. Expression ratios were generated by dividing ratios from averaged abnormal cell line runs (n=3) by the ratios from the averaged LP9 runs (n=4).

Total RNA was extracted from the cell lines using an RNeasy Mini or Midi kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. Cell pellets for the reference pool were isolated using TRizol® Reagent (Invitrogen, Carlsbad, CA, USA). 10 μg of total RNA was used for first-strand cDNA synthesis for each dye-coupling.

Initially, 10 μg of total RNA was mixed with 5 μg oligo-(dT)18-20 and heated to 70ºC for 10 min and then incubated on ice for 10 min. To synthesize cDNA, a reverse transcription reaction was carried out containing 10 μg of total RNA, 5 μg oligo (dT)18-20, 0.6 μL of 5x aa-dUTP/dNTPs (30 mM 5-(3-aminoallyl)-2'-deoxyuridine-5'- triphosphate aa-dUTP (Sigma), 50 mM each of dATP, dCTP, dGTP, and 20 mM dTTP), 6 μL of 5X buffer, and 1.9 μL of 200 U/μl SuperScript Reverse Transcriptase (Invitrogen). The reaction was incubated for 2 h at 37ºC.

Residual RNA was hydrolyzed with 0.5 mM EDTA and 1N NaOH at 65ºC for 15 min. The reaction was then neutralized by the addition of 1M Tris-HCL. We used a Micron 30 concentrator to remove residual Tris and concentrate the samples. The resulting cDNA pellets were resuspended in 0.05 M NaHCO for 15 min at room temperature and combined with NHS-ester Cy3 or Cy5 (Amersham, Piscataway, NJ, USA) monofunctional dye in DMSO. Reactions were allowed to couple for 1 hour at room temperature after which the reaction was quenched with the addition of 4M hydroxylamine. Test samples labeled with Cy5 were combined with purified using a QIAquick PCR purification system (Qiagen). The labeled products were used with their mean ratio of expression (across all mesothelioma cell lines).

Table I. cDNA microarray analysis was performed on RNA extracted from 7 mesothelioma cell lines (LRK1A, H2052, 211H, H290, MS1, H513, and H28) and compared to RNA from a normal pleural cell line (LP9). The 10 genes with the greatest ratios of expression (compared to LP9) are listed with their mean ratio of expression (across all mesothelioma cell lines).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Average ratio of expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHBG</td>
<td>12.8</td>
</tr>
<tr>
<td>ITIH2</td>
<td>11.7</td>
</tr>
<tr>
<td>SCY4</td>
<td>8.3</td>
</tr>
<tr>
<td>KIA0125</td>
<td>6.0</td>
</tr>
<tr>
<td>OP18 (Stathmin)</td>
<td>5.4</td>
</tr>
<tr>
<td>KIAA0255</td>
<td>5.0</td>
</tr>
<tr>
<td>CD48</td>
<td>4.8</td>
</tr>
<tr>
<td>SUOX</td>
<td>4.1</td>
</tr>
<tr>
<td>KIAA0973</td>
<td>4.1</td>
</tr>
<tr>
<td>H2BFL</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Results

The cDNA expression array showed multiple genes were overexpressed in mesothelioma cell lines compared to the LP9 cell line (Table I). Stathmin was one of the most strongly overexpressed genes. It was overexpressed in all 7 mesothelioma cell lines. The average level of expression was 5.4-fold higher than in the LP9 cell line (Figure 1).

Having detected stathmin overexpression by cDNA array, we confirmed the results by RT-PCR. All mesothelioma cell lines expressed stathmin more strongly than the LP9 cell lines (Figure 2).

Having shown that stathmin mRNA levels were increased in mesothelioma cell lines, we then showed that stathmin protein levels were increased in the cell lines. Western blot dilution. After incubation with the primary antibody the tissue sections were treated with 3% hydrogen peroxide and Normal Goat Serum (Vector Labs, Burlingame, CA, USA). After the incubation with the primary antibody, tissue sections were incubated with the secondary biotinylated Goat anti-Rabbit (Vector Labs) followed by avidin-biotin immuno-peroxidase. The sections were visualized using diaminobenzidine chromogen (Sigma Aldrich, St. Louis, MO, USA) and counterstained with hematoxylin (Thermo Shandon, Pittsburgh).

Western blot. Whole cells were homogenized and lysed with M-Per mammalian protein extraction reagent for all cell lines and with T-Per protein extraction reagent for all tissue samples (Pierce, Rockford, IL, USA). Lysates were centrifuged for 14,000 xg for 5 min, and the supernatant was collected. The supernatant was separated on 4-15% gradient SDS-polyacrylamide gels and transferred to Immobilon-P (Millipore, Bedford, MA, USA) membranes for Western blotting. Membranes were incubated with stathmin antibody (Calbiochem, San Diego, CA, USA). Antigen-antibody complexes were detected by enhanced chemiluminescence blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Beta-actin served as a loading control (Sigma Chemical Co., St. Louis, MO, USA).
analysis revealed that stathmin was expressed in all malignant mesothelioma cell lines, but not in LP9 cells (Figure 3). Next, we showed that stathmin protein levels were increased in 7 of 8 mesothelioma tumor samples. Matched, normal pleural tissue was unavailable for 4 of the tumor samples, but stathmin protein was detected in all these tumor samples. In contrast, none of the 4 matched, normal pleural tissues expressed detectable levels of stathmin (Figure 4).

Finally, immunohistochemistry showed that mesothelioma tissues stained for stathmin as well (Figure 5).

Discussion

We have shown that stathmin is overexpressed in mesothelioma. cDNA expression arrays showed that stathmin mRNA levels are increased in all 7 mesothelioma cell lines we tested. We also found stathmin protein levels to be increased in all the cell lines and in 7 of 8 tumor tissues. This was confirmed with immunohistochemistry. Although we had a small sample number, these findings suggest that stathmin overexpression is common in mesothelioma and may play a role in its pathogenesis.

Stathmin, also known as oncoprotein 18, metablastin, phosphoprotein 19 and LAP18, is a highly conserved cytosolic phosphoprotein that helps regulate cell growth and migration through regulation of microtubule stability (14). Stathmin promotes microtubule depolymerization and sequesters tubulin (15, 16). In addition to regulating cellular progression through mitosis, stathmin plays a role in mediating cell migration and perhaps metastasis (15).

Stathmin has been shown to be overexpressed in multiple cancers, including leukemia, breast cancer, lung cancer, hepatocellular cancer, ovarian cancer and prostate cancer (17-23). To our knowledge, stathmin expression has not been previously reported in mesothelioma and a PubMed search of the keywords "stathmin and mesothelioma" revealed no hits.

Stathmin expression may be a marker for proliferation. Curmi et al. showed that overexpression of stathmin correlates with highly proliferative breast cancer tumors (18). Jeha et al. showed that stathmin increases the proliferation rate of leukemia cells (24). In hepatocellular cancer, stathmin overexpression was found to be associated with larger tumor size, tumor grade and higher stage. It also correlated with a lower 5-year survival rate, independent of tumor stage (21).
Stathmin overexpression may also confer chemotherapy resistance. Two of the most commonly used classes of chemotherapy drugs, vinka alkaloids and taxanes, interfere with microtubule dynamics. Because stathmin also acts on microtubules, there has been interest in its effect on resistance to these drugs. Alli et al. found that stathmin overexpression in breast epithelial cell lines decreased sensitivity to paclitaxel and to vinblastine, and Rosell et al. found that stathmin expression

Figure 2. RT-PCR was performed on RNA isolated from 7 mesothelioma cell lines and a normal pleural cell line (LP 9). Stathmin was overexpressed in all 7 mesothelioma cell lines. RT-PCR was performed using GAPDH primers as control.

Figure 3. Western blot analysis was performed on protein isolated from 8 mesothelioma cell lines and a normal pleural cell line (LP 9). Proteins were separated by SDS-PAGE and transferred to a membrane. They were then incubated with anti-stathmin antibody. Stathmin was overexpressed in all 8 mesothelial cell lines, but not in the normal pleura. Anti-beta-actin antibody was used as loading control.
correlated with response vinorelbine chemotherapy in non-small cell lung cancer (25, 26). However, Nishio et al. found that stathmin overexpression increases sensitivity to vindesine (a vinka alkaloid) in lung cancer cell lines (27). Further studies correlating stathmin expression with chemotherapy sensitivity in mesothelioma may help guide therapy.

Our cDNA microarray identified multiple genes that were overexpressed in mesothelioma cell lines. Due to the recent studies implicating its role in proliferation and chemotherapy resistance, we chose to focus on stathmin for the purposes of this study. However, we are in the process of validating the expression levels of other genes found to be either overexpressed or underexpressed in mesothelioma tissues. Due to the almost universally poor prognosis among mesothelioma patients, correlation between stathmin expression and clinical outcome could not be performed.
Our experiments showed increased levels of stathmin in malignant mesothelioma, but did not address what role stathmin plays in the actual pathogenesis of mesothelioma. Future experiments could address this question by transfecting LP9 cells with stathmin or by treating mesothelioma cells with stathmin siRNA. Future studies on stathmin as a potential therapeutic target in mesothelioma are warranted.

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References

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