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Major carcinogenic pathways identified by gene expression analysis of peritoneal mesotheliomas following chemical treatment in F344 rats

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Abstract

This study was performed to characterize the gene expression profile and to identify the major carcinogenic pathways involved in rat peritoneal mesothelioma (RPM) formation following treatment of Fischer 344 rats with *o*-nitrotoluene (*o-NT*) or bromochloracetic acid (BCA). Oligo arrays, with over 20,000 target genes, were used to evaluate *o-NT*- and BCA-induced RPMs, when compared to a non-transformed mesothelial cell line (Fred-PE). Analysis using Ingenuity Pathway Analysis software revealed 169 cancer-related genes that were categorized into binding activity, growth and proliferation, cell cycle progression, apoptosis, and invasion and metastasis. The microarray data were validated by positive correlation with quantitative real-time RT-PCR on 16 selected genes including *igf1*, *tgfb3* and *nov*. Important carcinogenic pathways involved in RPM formation included insulin-like growth factor 1 (*IGF-1*), p38 MAPkinase, *Wnt/β-catenin* and integrin signaling pathways. This study demonstrated that mesotheliomas in rats exposed to *o-NT*- and BCA were similar to mesotheliomas in humans, at least at the cellular and molecular level. Published by Elsevier Inc.

Keywords: Rat; Mesotheliomas; Bromochloroacetic acid (BCA); *o*-Nitrotolulene; Carcinogenesis; Microarray

Introduction

Human malignant mesothelioma is an aggressive neoplasm of the serosal lining of the body cavities arising from mesothelial cells. There are approximately 2000 new cases of mesothelioma annually in the United States, and the total projected number of mesothelioma cases in the male population over the next 50 years (2003–2054) is approximately 71,000 (Price and Ware, 2004). The incidence of mesothelioma worldwide is currently increasing, and the estimated deaths over the next 30 years from mesothelioma in Western Europe alone could approach 250,000 (La Vecchia et al., 2000). The occurrence of mesothelioma is expected to peak between 2010 and 2020 as a consequence of the widespread mining and use of amphibole asbestos in the recent past and the long latent period (30–40 years) for tumor development. Mesothelioma is resistant to conventional therapeutic regimens, with the median survival in most cases of only 6–18 months after diagnosis.

Mesothelioma is almost exclusively associated with the exposure to fibrous dusts or asbestos with particular physical properties (Suzuki and Yuen, 2002). About 10 to 20% of all mesotheliomas have been documented in patients without previous exposure to asbestos (Carbone et al., 2003). Other factors such as chemical carcinogens, ionizing radiation,

Abbreviations: BCA, bromochloroacetic acid; DNA, deoxyribose nucleic acid; F344, Fischer 344; H and E, hematoxylin and eosin; IGF, insulin-like growth factor; IPA, ingenuity pathway analysis; NTP, National Toxicology Program; *o-NT*, *o*-nitrotoluene; RNA, ribose nucleic acid; RPM, rat peritoneal mesothelioma; RT-PCR, reverse transcription polymerase chain reaction; SV40, simian virus 40; TGF, transforming growth factor.

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chronic inflammation and SV40 viral exposure may also contribute to the development of mesothelioma (Bielefeldt-Ohmann et al., 1996). Accordingly, multiple active tumorigenic pathways are thought to be possible.

Mechanisms of human mesothelioma development have been studied using different in vitro and in vivo experimental systems. The rat has been used widely as an animal model of mesothelioma formation. Spontaneous mesothelioma in the rat occurs at an incidence of 2.7–3.6% and predominantly occurs in the peritoneal cavity but rarely in the thoracic cavity (Haseman et al., 1990). Males are frequently affected and the tunica vaginalis is believed to be the origin of the tumor (Crosby et al., 2000b). In studies performed by the National Toxicology Program several chemicals including *o*-nitrotoluene (*o-NT*) and bromochloroacetic acid (BCA) increased the incidence of mesotheliomas in rats (NTP, 2002, in preparation). *o-NT* is a high production chemical and caused tumor formation at multiple sites. A study of *o-NT*-induced mouse intestinal tumors indicate that the *Wnt/β-catenin* signaling pathway, K-ras/MAPkinase pathway and p53 pathway, and cyclin D1 play a role in large intestinal tumor development following chemical exposure (Sills et al., 2004). BCA is a prevalent haloacid by-product of drinking water disinfection and caused increased tumor formation in F344 rats.

Previous studies using microarray analysis have been performed to understand the molecular carcinogenesis of human and rat mesothelioma (Rihn et al., 2000; Sandhu et al., 2000; Singhal et al., 2003), to differentiate subtypes (Hoang et al., 2004a) and to differentiate it from lung tumors (Gordon et al., 2002). This study was designed to identify the major carcinogenic pathways involved in peritoneal mesothelioma formation following exposure to *o-NT* or BCA in Fischer 344 rats. Gene expression studies using rat mesotheliomas from *o-NT*- or BCA-treated Fischer 344 rats provided an opportunity to identify genes and signal transduction pathways which contributed to the carcinogenesis of rat mesotheliomas.

Materials and methods

Frozen tumor tissues. Treatment of Fischer 344 (F344) rats with either *o-NT* or BCA significantly increased incidences of peritoneal mesotheliomas (NTP, 2002, In preparation). Four mesotheliomas from *o-NT*-treated rats and 4 mesotheliomas from BCA-treated rats were selected for Oligo microarray analysis (Table 1). At necropsy, the tumors were collected, minced and flash frozen in liquid nitrogen and stored at −80 °C until subsequent RNA isolation.

Table 1
Frozen rat peritoneal mesotheliomas that were used for microarray analysis from Fischer 344 rats following exposure to *o*-nitrotoluene (*o-NT*) or bromochloroacetic acid (BCA) for 2 years

Sample #	Chemical	Dose	Histotype
o-NT #1	*o-NT*	1250 ppm	Epithelioid
o-NT #2	*o-NT*	1250 ppm	Epithelioid
o-NT #3	*o-NT*	2000 ppm	Epithelioid
o-NT #4	*o-NT*	2000 ppm	Epithelioid
BCA #1	BCA	250 mg/l	Epithelioid
BCA #2	BCA	250 mg/l	Epithelioid
BCA #3	BCA	500 mg/l	Epithelioid
BCA #4	BCA	1000 mg/l	Epithelioid

Other sections were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 5 μm sections for H and E staining for routine histological examinations.

Non-transformed mesothelial cell lines. Non-transformed mesothelial cell lines, Fred-PE and Fred-PL, were provided by Dr. DeAngelo (Environmental Protection Agency, RTP, NC). Fred-PE and Fred-PL were prepared from the peritoneal and pleural cavities of normal F344 male rats, respectively (Crosby et al., 2000a). The cell lines were grown and subcultured as described previously (Crosby et al., 2000a). Mesothelial cell origin was confirmed by immunohistochemistry using antibodies against pan-cytokeratin (Dako, Carpinteria, CA) and vimentin (Dako, Carpinteria, CA). Both Fred-PE and Fred-PL were positive for both pan-cytokeratin and vimentin by immunohistochemistry (data not shown). For gene expression analysis by microarray technology as well as real-time RT-PCR method, we used Fred-PE of passage 8 as a source of reference RNA. Because normal mesothelial cells are small and form only a single cell layer in the peritoneum, making it difficult to obtain sufficient amounts of normal mesothelial cells directly from the rats for this study, the non-transformed mesothelial cell line was used. To evaluate the effect of multiple passages and the anatomical origin of the cell lines on the expression profile, the Fred-PE of passage 21 and the Fred-PL of passage 8 were included in real-time RT-PCR analysis, respectively.

RNA isolation, linear amplification label protocol and feature extraction. Total RNAs from tumor tissue and Fred-PE of passage 8 were isolated with a RNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. The quality of the RNA was evaluated by 260/280 nm absorbance ratio, formaldehyde agarose gel electrophoresis and an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNAs from eight frozen RPMs comprised of four tumor samples each from the *o-NT* and the BCA treatment groups were included for the comparisons. Two hybridizations, with fluor reversals, were performed for each RNA pair (Fred-PE vs. tumor). Four tumors from each treatment group were analyzed resulting in 8 arrays each for BCA and *o-NT*.

Gene expression analysis was conducted using Agilent Rat Oligo array with over 20,000 genes (Agilent Technologies, Product Number G4130A). Total RNA was amplified using the Agilent Low RNA Input Fluorescent Linear Amplification Kit protocol. Starting with 500 ng of total RNA, Cy3 or Cy5 labeled cRNA was produced according to manufacturer's protocol. For each two-color comparison, 750 ng of each Cy3 and Cy5 labeled cRNAs was mixed and hybridized using the Agilent In Situ Hybridization Kit protocol. Hybridizations were performed for 17 h in a rotating hybridization oven using the Agilent 60-mer Oligo microarray processing protocol. Slides were washed as indicated in this protocol and then scanned with an Agilent Scanner. Data were obtained using the Agilent Feature Extraction software (v7.5), using defaults for all parameters. The default normalization performed in feature extraction is a linear normalization across all the data followed by a non-linear normalization (LOWESS).

Rosetta resolver (v5.0) and determination of signature genes. Images and GEML files, including error and *p* values, were exported from the Agilent Feature Extraction software and deposited into Rosetta Resolver (version 5.0) (Rosetta Biosoftware, Kirkland, WA). The Resolver system performs a squeeze operation that creates ratio profiles by combining replicates while applying error weighting. A *p* value is generated and propagated throughout the system. The resolver system allows users to set thresholds, below which genes of a *p* value are considered to be significantly expressed. The resolver system then combines ratio profiles to create ratio experiments using an error-weighted average as described in Stoughton and Dai (2002). Intensity plots were generated for each ratio experiment and genes were considered "signature genes" if the *p* value was less than 0.001, and the fold difference was more than 1.5-fold throughout all biological replicates. Genes with a ratio (tumor/Fred PE) of ± 1.5 and a *p* value of 0.001 or less in all 4 biological replicates within each treatment group were analyzed further.

Identification of signaling pathway. The Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Mountain View, CA) was utilized to identify networks of interacting genes and other functional groups. A fold-change cutoff