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Analysis of microRNA expression signatures in malignant pleural mesothelioma, pleural inflammation, and atypical mesothelial hyperplasia reveals common predictive tumorigenesis-related targets

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ABSTRACT

Pleural chronic inflammation (PP) and mesothelial hyperplasia (HP) may be critical to the development of malignant pleural mesothelioma (MPM). Nonetheless, studies searching for mechanistic links involving microRNA (miRNA) regulation among these interrelated processes have not been reported. Using PCR-Array, we identified the miRNAs expressed in pleural tissues diagnosed with MPM (n = 5), PP (n = 4) and HP (n = 5), as well as in non-cancerous/non-inflammatory tissue as the normal control (n = 5). We performed bioinformatics and network analysis of differentially expressed miRNAs to identify tumorigenesis-related miRNAs and their biological networks. The targets of four down-regulated miRNAs in MPM (mir-181a-5p, miR-101-3p, miR-145-5p and miR-212-3p), one in PP (mir-101-3p) and one in HP (mir-494) were significantly enriched in "pathways in cancer". Interactome networks revealed that >50% of down-regulated miRNAs in MPM targeted the signaling-activation molecule MAPK1, the transcription factor ETS1 and the mesenchymal transition-associated molecule FZDA, which have been associated with oncogenic function. Comparative analysis revealed that FZD4 was an overlapping gene target of down-regulated miRNAs that were associated with "pathways in cancer" in MPM, PP and HP. Moreover, MAPK1, ETS1 and Cox-2, a pro-inflammatory enzyme associated with over-expression in cancers, were among the 25 overlapping target genes in MPM and PP. This network analysis revealed a potential combinatory effect of deregulated miRNAs in MPM pathogenesis and indicated potential molecular links between pleural inflammation and hyperplasia with tumorigenesis mechanisms in pleura.

1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor, which develops following the neoplastic transformation of mesothelial cells in pleura (Kaufman and Pass, 2008). MPM is mostly associated with exposure to asbestos fibers, and it is characterized by a short overall survival following diagnosis, regardless of the individual or combination treatment applied (Bagheri et al., 2011; Ceresoli et al., 2007; Jakobsen and Sorensen, 2011; Kaufman and Pass, 2008; Ray and Kindler, 2009; Vogelzang et al., 2003). Because this disease is highly aggressive and refractory to current available therapies, it is important to characterize tumor-specific targets for therapy by understanding the mechanisms involved in the development of MPM.

Chronic inflammation due to asbestos exposure (Carbone and Yang, 2012; Matsuzaki et al., 2012; Sekido, 2013; Wang et al., 2004; Yang et al., 2006) and genetic factors is considered to play a key role in the pathogenesis of MPM (Carbone and Yang, 2012; Jean et al., 2012; Sekido, 2013), but the molecular mechanisms controlling the malignant transformation of mesothelial cells are still poorly defined. Recent studies have indicated that chronic inflammation could precede atypical hyperplasia and MPM after asbestos injection (Yang et al., 2010). However, inflammation was shown to precede the development of MPM without the use of asbestos in a xenograft model (Hillegass et al., 2010), indicating that inflammation alone could be critical for tumorigenesis. Indeed, chronic inflammation has often been linked to tumor initiation, progression and metastasis, regardless of etiology (Cha and DuBois, 2007; Schetter et al., 2010; Vakkila and Lotze, 2004).

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MicroRNAs (miRNAs) have emerged as crucial players in post-transcriptional gene regulation in several biological processes, including inflammation (Liu et al., 2014; O'Connell et al., 2007, 2012). The abnormal expression and/or function of miRNAs have been linked to multiple human diseases, including inflammatory disorders (Schetter et al., 2010; Q. Wang et al., 2014), cancers (Avila-Moreno et al., 2011; Chen and Stallings, 2007; Patnaik et al., 2010; Schetter et al., 2008), and both of these diseases (Altamemi et al., 2014; Xiang et al., 2014). MicroRNAs are small non-coding RNAs that regulate post-transcriptional gene expression by inducing mRNA degradation or inhibiting translation (Bartel, 2009; Carthew and Sontheimer, 2009). One relevant characteristic of miRNAs is their ability to target multiple genes and pathways simultaneously to lead to broad changes in specific pathways. Moreover, increasing evidence suggests that miRNAs regulate delicate biological processes and provide robustness via regulation of target networks (Ebert and Sharp, 2012). MicroRNAs can function as either tumor suppressors or tumor oncogenes, and the study of cancer-associated miRNAs has been relevant in elucidating the mechanisms of malignant transformation.

Although chronic inflammation may promote atypical hyperplasia and it may be a prior state to malignancy, no studies have investigated whether there are mechanistic links between miRNA regulation and malignant mesothelioma. In this study, we analyzed differentially expressed miRNAs in pleural tissues diagnosed with malignant pleural mesothelioma, chronic inflammation and hyperplasia using a bioinformatics approach to identify tumorigenesis-related miRNAs. We also examined the biological networks regulated by these miRNAs in pleura, as well as potential links between these interrelated-processes.

2. Methods

2.1. Samples and patients

The samples consisted of formalin-fixed paraffin-embedded (FFPE) pleural biopsies from subjects diagnosed with epithelioid malignant pleural mesothelioma (MPM group, n = 5), pachypleuritis/chronic inflammation (PP group, n = 4) and atypical mesothelial hyperplasia (HP group, n = 5). All samples were collected from 2009–2010 from the Archive of the Pathological Anatomy Unit of the Instituto Nacional de Enfermedades Respiratorias "Ismael Cosio Villegas" (INER), Mexico. The patients included in this study did not receive any adjuvant chemotherapy or radiation therapy prior to sample collection.

All MPM samples were diagnosed by immunohistochemistry using at least four of the following markers: calretinin positive, cytokeratin 5/6 positive, thyroid transcription factor-1 (TTF-1) negative, carcinoembryonic antigen (CEA) negative, and BerEP4 negative. An independent pathologist validated the original diagnosis. Only samples with a matching clinical diagnosis of MPM were included in this study. MPM samples were macro-dissected to include only tissues containing >80% neoplastic cells. The PP samples were characterized by a thickening of the pleural membranes, organized fibroconnective tissue and the presence of chronic inflammatory infiltrating cells. The HP samples were characterized by the presence of enlarged and polymorphous surface mesothelial cells with large nuclei and prominent nucleoli. The HP samples were subclassified as mesothelial hyperplasia of unspecified malignancy and reactional inflammatory hyperplasia (presence of an underlying inflammatory reaction).

Adjacent noncancerous and noninflammatory tissues from independent subjects were used as the normal control samples (Ctrl group, n = 5). The pathologist verified the absence of cancer cells and inflammatory features in the normal control tissues used in this study. The clinical data were obtained retrospectively and included information on gender, age, clinical symptoms, radiologic exams, asbestos exposure, tobacco smoking and presence of pleural effusion.

2.2. RNA extraction

Total RNA was extracted from the FFPE samples (four slices of 20 μm, approximately 4 × 3 mm^3) using the RecoverAll total nucleic acid isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA quantification was assessed with a Qubit 2.0 fluorometer using the RNA Assay kit. Typically, the yield was 30–180 ng/μL.

2.3. MicroRNA profiling

Quantitative global profiling of tissue miRNAs was performed using the TaqMan Array Human MicroRNA Panel v2.0 (Applied Biosystems, CA, USA), which includes Cards A and B in a 384-well format. Card A contains 384 TaqMan MicroRNA Assays enabling the simultaneous quantitation of 377 mature human mature miRNAs plus 4 endogenous controls. Card B contains assays for 290 mature human mature miRNAs plus 7 endogenous controls. Quantitative reverse transcription polymerase chain reactions (RT-qPCR) were performed according to the manufacturer's instructions. Briefly, 75 ng of total RNA was reverse

Table 1
Clinical and pathological characteristics of MPM, PP, HP, and adjacent noncancerous/noninflammation tissue (Ctrl) specimens.

ID	Age (years)	Gender (F/M)	Smoker (yes/no)	Asbestos exposure	Pleural effusion	Diagnosis (histopathology)	Category of sample
MPM-1	50	F	Yes	Yes	Yes	MPM subtype epithelioid	Mesothelioma (stage III)
MPM-2	48	F	No	No	Yes	MPM subtype epithelioid	Mesothelioma (stage III)
MPM-3	65	M	Yes	No	Yes	MPM subtype epithelioid	Mesothelioma (stage III-B)
MPM-4	49	F	No	No	No	MPM subtype epithelioid	Mesothelioma (stage no reported)
MPM-5	54	M	Yes	Yes	Yes	MPM subtype epithelioid	Mesothelioma (stage III-A)
PP-1	47	M	Yes	No	Yes	Chronic pachypleuritis	Pachypleuritis
PP-2	58	M	Yes	Yes	Yes	Pachypleuritis with focal reaction to foreign object	Pachypleuritis
PP-3	71	F	No	No	Yes	Acute & chronic fibrinous pachypleuritis	Pachypleuritis
PP-4	53	F	Yes	Yes	Yes	Adjacent[a] chronic pachypleuritis	Pachypleuritis
HP-1	21	M	No	No	Yes	Mesothelial hyperplasia	Mesothelial hyperplasia
HP-2	28	M	Yes	No	Yes	Atypical mesothelial hyperplasia with chronic pachypleuritis	Reactional inflammatory hyperplasia
HP-3	86	F	No	No	No	Adjacent** mesothelial hyperplasia	Mesothelial hyperplasia
HP-4	21	M	No	No	No	Atypical mesothelial hyperplasia with pleuritis	Reactional inflammatory hyperplasia
HP-2 (b)	28	M	Yes	No	Yes	Atypical mesothelial hyperplasia with chronic pachypleuritis	Reactional inflammatory hyperplasia
CTRL-1	71	F	No	No	Yes	Normal tissue	Normal control
CTRL-2	51	F	No	No	No	Normal tissue	Normal control
CTRL-3	21	F	No	No	Yes	Normal tissue	Normal control
CTRL-3 (b)	21	F	No	No	Yes	Normal tissue	Normal control
CTRL-4	53	M	No reported	No reported	Yes	Normal tissue	Normal control

[a] Adjacent PP to MPM tissue sample.

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