Mutation Research 771 (2015) 6–12

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Expression status of candidate genes in mesothelioma tissues and cell lines

Ombretta Melaiu[a], Erika Melissari[b], Luciano Mutti[c], Elisa Bracci[a], Chiara De Santi[a], Caterina Iofrida[b], Manuela Di Russo[b], Alfonso Cristaudo[d], Alessandra Bonotti[d], Monica Cipollini[a], Sonia I. Garritano[e], Rudy Foddis[d], Marco Lucchi[f], Silvia Pellegrini[b], Federica Gemignani[a,*], Stefano Landi[a]

[a] Department of Biology, University of Pisa, Pisa 56126, Italy
[b] Department of Surgical, Medical and Molecular Pathology and of Critical Care, University of Pisa, Pisa 56126, Italy
[c] Chair in Cancer Research, Environment & Life Sciences, College of Science & Technology, Room: 301a, Cockcroft Building, University of Salford, Salford, M5 4WT, UK
[d] Section of Occupational Medicine, School of Medicine and Surgery, University of Pisa, Pisa 56126, Italy
[e] Laboratory of Computational Oncology, Centre for Integrative Biology, CIBIO, University of Trento, Trento 38121, Italy
[f] Cardiac and Thoracic Department, Division of Thoracic Surgery, University of Pisa, Pisa 56126, Italy

ARTICLE INFO

Article history:
Received 24 June 2014
Received in revised form 14 October 2014
Accepted 4 November 2014
Available online 13 November 2014

Keywords:
Mesothelioma
Gene expression
Cancer genes
Biomarkers
Therapeutic targets

ABSTRACT

In order to broaden knowledge on the pathogenesis of malignant pleural mesothelioma (MPM), we reviewed studies on the MPM-transcriptome and identified 119 deregulated genes. However, there was poor consistency among the studies. Thus, the expression of these genes was further investigated in the present work using reverse transcriptase-quantitative PCR (RT-qPCR) in 15 MPM and 20 non-MPM tissue samples. Fifty-nine genes showed a statistically significant deregulation and were further evaluated in two epithelioid MPM cell lines (compared to MET-5A, a non-MPM cell line). Nine genes (*ACSL1*, *CCNO*, *CFB*, *PDGFRB*, *SULF1*, *TACC1*, *THBS2*, *TIMP3*, *XPOT*) were deregulated with statistical significance in both cell lines, 12 (*ASS1*, *CCNB1*, *CDH11*, *COL1A1*, *CXADR*, *EIF4G1*, *GALNT7*, *ITGA4*, *KRT5*, *PTGIS*, *RAN*, *SOD1*) in at least one cell line, whereas 7 (*DSP*, *HEG1*, *MCM4*, *MSLN*, *NME2*, *NMU*, *TNPO2*) were close but did not reach the statistical significance in any of the cell line. Patients whose MPM tissues expressed elevated mRNA levels of *BIRC5*, *DSP*, *NME2*, and *THBS2* showed a statistically significant shorter overall survival. Although MPM is a poorly studied cancer, some features are starting to emerge. Novel cancer genes are suggested here, in particular those involved in cell–cell and cell–matrix interactions.

1. Introduction

Malignant pleural mesothelioma (MPM) is a cancer of the pleural cavity with a long latency (>20 years) between the triggering event (i.e., exposure to asbestos) and diagnosis. Still nowadays, patients with MPM have poor prognosis, with overall survival (OS) typically ranging between 6 and 13 months. Understanding the mechanisms involved in the carcinogenesis of MPM is essential to detect clinically useful biomarkers and therapeutic targets. Since 1987, alterations in a number of genes have been suggested as relevant for MPM, including CEA (carcino-embryonic antigen) and SP1 (pregnancy specific antigen) [1], and *PPL*, *UPK3B*, and *TFPI* [2]. However, to date, the aberrant expression of *EGFR* [3], calretinin (*CALB2*) [4], mesothelin (*MSLN*) [5], *MKI67* [6], *MTOR* [7], CD146 (*MCAM*) [8], *MUC1* [9], *PDGFRB* [10], survivin (*BIRC5*) [11], and the presence of mutations within *NF2*, *CDKN2A*, *CDKN2B* [12], and *BAP1* [13] are the sole findings reported in a convincingly high number of independent studies.

In order to broaden knowledge of the pathogenic mechanisms of MPM development, our research group performed a data-mining study combined with a literature review of transcriptomic studies (defined as RTS) [14]. We found that 931 genes were reported as deregulated in at least one publication, but only 119 of them were found differentially expressed by at least two independent research groups. This poor consistency among studies prevented us from formulating solid conclusions. For this reason, in the present work we further investigated the expression status of the 119 putatively deregulated genes and compared the results between MPM and non-malignant mesothelial (NMM) tissues. Those genes, whose

* Corresponding author at: Department of Biology, University of Pisa, Via Derna, 1, 56126 Pisa, Italy. Tel.: +39 050 2211528; fax: +39 050 2211527.
E-mail address: federica.gemignani@unipi.it (F. Gemignani).

http://dx.doi.org/10.1016/j.mrfmmm.2014.11.002
0027-5107/

differential expression in tissues reached the statistical significance, were further investigated in two MPM cell lines, assuming that genes differentially expressed both in tissues and cell lines might play a more relevant role for the disease. Our results indicated a series of novel genes putatively involved in MPM initiation, clonal evolution, or progression, and that may contribute to an enhanced understanding of the molecular mechanisms of mesothelial carcinogenesis.

2. Materials and methods

2.1. Tissue collection

Consecutive series of NMM and MPM specimens were collected in collaboration with the units of Thoracic Surgery (Department of Surgical, Medical and Molecular Pathology and of Critical Area) and of Occupational Medicine (Department of Translational Research and New Technologies in Medicine and Surgery) at the University Hospital of Pisa, Italy. Control tissues (20) were normal pleura from patients who underwent surgery for early-stage lung cancer (6 lung adenocarcinomas and 14 lung squamous cell carcinomas). Pleural specimens were collected far from the tumor site, eye-inspected by surgeons and analyzed by pathologists in order to collect a small portion of pleura not containing evidence of lung cancer spread. MPM biopsies were collected during thoracoscopy before any treatment. About 73% of MPM patients had an ascertained positive history of exposure to asbestos, and approximately half of them had also a positive cigarette smoking history. The complete list of volunteers is reported in Table 1.

All samples were stored in the RNA later reagent (QIAGEN, S.p.A, Milano, Italy) and placed at −80 °C right after the collection. They were not analyzed until a clinical report with a precise diagnosis was issued from the pathologists, following the standard clinical routine that was based on microscopic inspection and immunohistochemical analyses of slides with antibodies to detect a panel of biomarkers (CK5/6, calretinin, vimentin, CK-Pan, EMA, TTF1, BerEP4, CEA). In order to focus on a specific histotype, 15 epithelioid MPM were selected and retrieved for RNA extraction and further analyses. According to the Helsinki declaration, volunteers gave informed consent for the research. The local ethical committee approved the study.

Table 1
Main characteristics of volunteers enrolled into the study; LA = lung adenocarcinoma; LSC = lung squamous cell carcinoma.

Code	Sex	Age	MPM
M01	F	40	Epithelioid
M02	M	72	Biphasic
M03	M	69	Epithelioid
M04	M	56	Biphasic
M05	M	86	Epithelioid
M06	F	77	Epithelioid
M07	M	69	Epithelioid
M08	M	69	Epithelioid
M09	M	87	Sarcomatoid
M10	F	69	Sarcomatoid
M11	M	87	Epithelioid
M12	M	64	Epithelioid
M13	F	60	Epithelioid
M14	M	73	Epithelioid
M15	M	68	Epithelioid
M16	M	61	Epithelioid
M17	M	48	Biphasic
M18	M	64	Biphasic
M19	M	64	Epithelioid
M20	M	61	Epithelioid
M21	M	65	Sarcomatoid
M22	M	65	Epithelioid
Code	**Sex**	**Age**	**NMM**
N01	M	74	LA
N02	F	72	LSCC
N03	M	85	LA
N04	M	76	LA
N05	M	77	LA
N06	M	65	LSCC
N07	F	65	LSCC
N08	M	76	LSCC
N09	M	80	LSCC
N10	M	63	LSCC
N11	F	69	LSCC
N12	F	76	LSCC
N13	F	69	LSCC
N14	M	68	LSCC
N15	M	68	LSCC
N16	F	82	LSCC
N17	M	76	LSCC
N18	F	76	LSCC
N19	F	59	LA
N20	M	77	LA

2.2. Cell cultures

A mesothelial non-MPM cell line (MeT-5A) and two epithelioid MPM cell lines (Mero-14 and Mero-25) were used. MeT-5A cells were purchased from the ATCC (American Type Culture Collection) whereas Mero-14 and Mero-25 MPM cells were kindly donated by the University Hospital San Martino, Genova, Italy. MeT-5A, Mero-14, and Mero-25 cells were verified for their identity by analyzing the genetic markers reported in the certification. The MeT-5A cell line was grown in Medium199 with HEPES (Life Technologies, Monza, Italy) supplemented with 10% fetal bovine serum (Sigma–Aldrich Corp., St. Louis, MO, USA), and 1% pen–strep (Lonza, Basel, Switzerland), 3.3-nM epidermal growth factor (EGF, Life Technologies), 400-nM hydrocortisone (Sigma–Aldrich Corp.), and 870-nM insulin (Life Technologies). MPM cells were cultured in DMEM medium (Lonza) supplemented with 10% fetal bovine serum and 1% pen–strep. Cells were incubated at 37 °C in a 5% CO_2-humidified atmosphere.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from individual samples by using Tri-Reagent (Sigma–Aldrich Corp.) according to standard protocols. In order to remove any contaminating genomic DNA, the extracted RNA was treated with DNase (Sigma–Aldrich Corp.). Concentration of clean-up RNA was determined by a spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, CA). The integrity and purity of total RNA were further verified by electrophoresis on ethidium bromide agarose gel, inspecting the 18 and 28S ribosomal RNA bands. The reverse transcription (RT) was performed using the *iSCRIPT cDNA Synthesis Kit* starting from 1 μg of total RNA, in a final volume of 20 μl (Bio-Rad Laboratories).

2.4. Selection of reference genes for RT-qPCR

In order to perform accurate RT-qPCR measurements, an exhaustive review of the scientific literature available on MPM was carried out, and a list of possible reference genes was gathered. Data-mining analysis by Coremine Medical™ (http://www.coremine.com/medical/) revealed that most of these genes were involved in carcinogenesis, thereby raising doubts about their use as reference genes. However, six genes not related to cancer were identified (i.e. *HPRT1*, *B2M*, *RPLP0*, *TBP*, *GUSB*, and *PPIA*) and their expression level was measured in NMM and MPM tissues and cell lines. The stability of these genes was tested by *geNorm* [15] and, according to the average *M* and the pair-wise variation