Expression status of candidate genes in mesothelioma tissues and cell lines

Running title: Mesothelioma and gene expression

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Abstract

In order to broaden the knowledge on the mechanisms of development of malignant pleural mesothelioma (MPM), a review of works on MPM-transcriptome was performed previously and 119 deregulated genes were identified. However, a poor consistency amongst studies was observed. Thus, the expression of these genes was further investigated in the present work using quantitative Real-Time PCR in 15 MPM and 20 non-MPM tissues. 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 the cell lines, 12 (*ASS1*, *CCNB1*, , *CDH11*, *COL1A1*, *CXADR*, *EIF4G1*, *GALNT7*, *ITGA4*, *KRT5*, *PTGIS*, *RAN*, *SOD1*,) in at least one cell line, whereas seven (*DSP*, *HEG1*, *MCM4*, *MSLN*, *NME2*, *NMU*, *TNPO2*) were close to the statistical significance. 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.

Key words: mesothelioma; gene expression; cancer genes; biomarkers; therapeutic targets.

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, for CEA (carcino-embrionic antigen), and SP1 (pregnancy specific antigen) [1], till nowadays for *PPL*, *UPK3B*, and *TFPI* [2], a number of alterations have been suggested as relevant for MPM. 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. Thus, in order to broaden the knowledge on the mechanisms of MPM development, a data mining combined with a literature review of transcriptomic studies (now on defined as RTS) was recently performed by our own research group [14]. Nine hundred thirty one genes were reported as deregulated in at least one publication, but, again, only 119 were found deregulated by at least two independent research groups. In summary, the poor consistency among studies prevented to formulate solid conclusions. For this reason, in the present work the expression status of the 119 putatively deregulated genes was investigated and compared between MPM and non-malignant mesothelial (NMM) tissues. Then, genes deregulated in a statistically significant way in tissues were further investigated in two MPM cell lines, assuming that genes differentially expressed both in tissues and cell lines may play a more relevant role for the disease. As a result, a series of novel genes putatively involved in MPM initiation, clonal evolution, or progression, is presented here in an attempt to contribute to the understanding of 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 Pathology, Medicine, Molecular, and Critical Area) and of the 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 and 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 at 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 RNA later (Qiagen, S.p.A, Milano, Italy) and placed at -80˚C right after the collection. They were not analysed until a clinical report with a precise diagnosis was issued from the pathologists, following the standard clinical routine that was based on microscopical 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.

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 have been kindly donated by the University Hospital San Martino, Genova, Italy. MeT-5A, Mero-14, and Mero-25 cells were verified for their identity, by analysing 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, Monza, Italy), 400 nM hydrocortisone (Sigma Aldrich Corp. St Louis, MO, USA), and 870 nM insulin (Life Technologies, Monza, Italy). MPM cells were cultured in DMEM medium (Lonza, Basel, Switzerland), 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. St Louis, MO, USA) according to standard protocols. In order to remove possible contaminating genomic DNA, the extracted RNA was treated with DNase buffer (Sigma Aldrich Corp. St Louis, MO, USA). Concentration of clean-up RNA was determined by a spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, CA). The integrity and purity of total RNA was 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* departing from 1µg of total RNA, on a final volume of 20µl (Bio-Rad Laboratories, Hercules, CA).

2.4 Selection of reference genes for quantitative Real-Time PCR (RT-qPCR)

In order to perform accurate RT-qPCR measurements, an exhaustive selection of scientific literature available on MPM was carried out and a list of possible reference genes was gathered. The following data mining analysis by Coremine Medical™ (http://www.coremine.com/medical/) revealed that most of these genes were involved in processes of development of several types of cancer, thereby raising doubts about their use as reference genes. Therefore, the mRNA expression levels of six genes, *HPRT1*, *B2M*, *RPLP0*, *TBP*, *GUSB*, and *PPIA* (genes known to be not involved in carcinogenesis), were measured in NMM and MPM tissues and cell lines and their stability was tested by *geNorm* [15]. Based on the average M and the pair-wise variation values, *RPLP0*, *TBP*, and *HPRT1* were determined as the most stable and employed as reference genes for all the experiments.

2.5 Primer design and RT-qPCR

For each gene, primer pairs were designed in adjacent exons to allow specific amplification of mRNA instead of genomic DNA, by using Primer3 (http://frodo.wi.mit.edu/), "Primer-BLAST" (www.ncbi.nlm.nih.gov/tools/primer-blast), "Beacon Designer", and "Netprimer" (PREMIER Biosoft; www.netprimer.com).

Primer specificity was verified by running the PCR products on agarose gel electrophoresis and by analyzing the melting curves, to exclude the presence of primer dimers. To assess the efficiency of each primer pair, five serial dilutions were prepared from a pool of control cDNAs and used as template to generate the standard curves. Each RT-qPCR was set up in a final volume of 25 µl using 2 µl of cDNA, 0.3 µM of forward and reverse primers and 5 µl of 5× Eva Green master mix with ROX as reference dye (Solis, Tartu, Estonia). The thermal cycling conditions were 15 min at 95°C followed by 15 s at 95 °C, 30 s at 60 °C (40 cycles) and 30 s at 65 °C.

When the primer pair did not meet the required criteria of specificity or showed a low efficiency or reproducibility (thresholds: r2>0.96, 3.11<slope<3.58), pre-designed TaqMan probes (Life Technologies, Monza, Italy) were employed. For the TaqMan assay, the reaction mixture consisted of 2 µl of cDNA template, 7 µl of deionized H2O, 1 µl of specific TaqMan Assay probe and primer mixture, and 10 µl of TaqMan® Gene Expression Master Mix (Life Technologies, Monza, Italy). The thermal cycling conditions were: 15 min at 95 °C followed by 15 s at 95 °C and 60 s at 60 °C (40 cycles). Primers and TaqMan ID assays are reported in Supplementary Table 1. Each sample was run in triplicate and the quality control of the derived expression values was performed according to MIQE guidelines [16]. When a sample did not meet any of the strict quality criteria for a given gene, the sample was not included in the statistical analysis for the considered gene. A gene was analyzed when the measurements of gene expression were available for a minimum of 75% of the samples, and all genes could be analyzed.

2.6 Statistical analyses

Gene expression levels from MPM samples were compared to NMM tissue samples by applying the one-tailed Wilcoxon test, in the Mann–Whitney version by R (www.r-project.org). The tested a priori hypothesis of up- or down-regulation for each gene was derived by Melaiu and colleagues paper [14]. For cell lines, two-tailed non parametric version of Dunnet-t test with Satterthwaite correction was adopted to compare gene expression levels of Mero-14 and Mero-25 with MeT-5A cell lines. The threshold of statistical significance for tissues and cell lines statistical analyses was fixed to 0.05 and multiple testing correction was performed by using Benjamini and Hochberg False Discovery Rate. The statistical analyses were performed by the R package.

WebGestalt (Gene Set Analysis Toolkit V2; http://bioinfo.van- derbilt.edu/webgestalt/) was employed to perform the overrepresentation analysis using the differentially expressed genes in tissues as input list. The whole human genome was selected as background.

The OS was calculated from the diagnosis of malignancy until the patient death (also due to other causes), or until the last follow-up visit for still alive patients. The data were available for 13 patients who were censored at last follow-up visit if still alive or lost. Survival analysis was carried out using Kaplan–Meier curves and the significance was verified by a log-rank test. All P-values were determined by two-sided tests and corrected with Bonferroni's adjustment in order to be considered statistically significant. Data analysis and summary graphs were produced by the software Statgraphics Centurion XV (StatPoint, Inc.).

3. Results

The expression levels of 119 candidate genes were measured in MPM and NMM tissues. The extent of differential expression was quantified by calculating the $\log_2$(fold change) (i.e. the $\log_2$(FC)), using NMM as control samples (see Table 2 for details). The table shows also information of the expected direction of deregulation (DOD), i.e. up- or down- regulation in MPM compared to normal pleura and represented as arrows, according to what summarized in RTS [14]. We found that 59 genes were differentially expressed (DE genes) between MPM and NMM in a statistically significant way, based on q-value: 51 were up-regulated and 8 down-regulated. All these DODs were comfortingly in agreement with the RTS. Forty-eight of the up-regulated genes showed a $|\log_2(FC)| \geq 1$. In addition to the 59 DE genes, 25 showed a differential expression between MPM and NMM close to the statistical significance (q-value<0.10) and, for 22 of them, the DOD was in agreement with that reported in RTS. Among these 22, 12 had also a $|\log_2(FC)| \geq 1$. Among the remaining 35 genes, 18 showed the DODs

expected from RTS although not reaching the statistical significance and 6 had also a $|\log_2(FC)| \geq 1$. Thus, overall, 99 genes out of the 119 candidates (83%) showed DODs in agreement with previous findings from transcriptomic studies and 66 showed a $|\log_2(FC)| \geq 1$.

All the DE genes have been already studied in relation to various types of cancer in humans. Some of them, in particular *BIRC5, CCNB1*, *CDC2*, *DSP*, *FEN1*, *ITGA4, MKI67*, *PCNA, PDGFRB, SMARCA4, TIMP3,* and *TOP2A* count hundreds of citations in literature, whereas others such as *ANK2, CXADR, C10orf116, GALNT7, FBLN2, HCA112, HEG1, TNPO2,* and *XPOT,* have been reported only in few studies. Furthermore, *MSLN*, *CALB2*, *BIRC5*, *PDGFRB,* and *MKI67* have been already described thoroughly in MPM and the present results reinforce the notion that they should have an important role in this disease. Concerning the remaining genes, very little is known, as most of them have never been studied either in MPM tissues or in cell lines. DE genes were also evaluated in relation to their possible belonging to specific pathways and 19 different KEGG-pathways were found overrepresented with statistical significance. The 5 most relevant pathways are reported in Table 3 and involve cell-cycle regulation, cell–cell and cell–matrix interactions, DNA replication, and cell energy metabolism. Finally, DE genes were also evaluated as potential prognostic biomarkers. Patients' OS was inversely correlated with the expression of *BIRC5*, *NME2*, *DSP,* and *THBS2* in a statistically significant way at nominal level of 0.05 (following the analysis of Kaplan-Meyer curves), but none of the genes resulted associated with OS after the correction for multiple tests. A brief analysis is reported in table 4, more details and Kaplan-Meyre curves are provided in supplementary data (Fig. S2).

The 59 DE genes were further evaluated on Mero-14 and Mero-25 MPM cell lines (the non-MPM cell line Met-5A was adopted as reference). The expression levels of the candidate genes correlated in a statistically significant way when the two cell lines were compared to each other (r=0.84; slope=0.74;

std.error= 0.069; P-value<10^{-4}; Fig. 2). When tissues were compared with cell lines, 28 genes showed, in at least one cell line, the same DOD found in tissues, and, in particular:

• 9 genes (*CCNO*, *CFB*, *PDGFRB*, *SULF1*, *THBS2*, *TIMP3*, *ACSL1*, *XPOT*, *TACC1*) were deregulated with statistical significance in both the cell lines;

• 12 genes (*ASS1*, *COL1A1*, *CXADR*, *GALNT7*, *RAN*, *SOD1*, *EIF4G1*, *CCNB1*, *ITGA4*, *KRT5*, *CDH11*, *PTGIS*) reached the statistical significance in one cell line only;

• 7 genes (*DSP*, *HEG1*, *MCM4*, *MSLN*, *NME2*, *TNPO2*, *NMU*) showed a deregulation that did not reach the statistical significance in any of the cell lines.

The remaining genes did not show DODs in agreement to those found in tissues.

4. Discussion

The screening of gene expression confirmed most of the findings described by RTS. However, none of the DE genes allowed distinguishing clearly MPM from NMM (Supplementary Fig. 2). Available evidences suggest that MPM is heterogeneous [2,17], and it is not an easy task to develop a simple diagnostic assay based on a single marker. Thus, here it is reinforced the notion that a panel of biomarkers will be needed to perform a correct diagnosis.

In the present work genes involved in MPM were detected. They resulted positive to three stringent criteria: (i) deregulated in RTS, (ii) deregulated in MPM tissues, and (iii) deregulated in cell lines. It should be acknowledged that the cancer heterogeneity is so broad that two cell lines (such as Mero-14 and Mero-25) might not be fully representative for MPM. However, it is conceivable that cell lines keep the original de-regulated status of (at least a share of) genes playing a crucial role in the disease, in spite of the changes occurred as a consequence of the adaptation to the *in vitro* growth conditions. Thus, the present work had the merit to highlight *ACSL1*, *CCNO*, *CFB*, *PDGFRB*, *SULF1*, *THBS2*,

TIMP3, *XPOT*, *TACC1* as the strongest candidate cancer genes. However, also *ASS1*, *CCNB1*, *CDH11*, *COL1A1*, *CXADR*, *DSP*, *EIF4G1*, *GALNT7*, *HEG1*, *ITGA4*, *KRT5*, *MCM4*, *MSLN*, *NME2*, *NMU*, *PTGIS*, *RAN*, *SOD1*, *TNPO2* should be regarded as novel potential cancer genes worthy of further investigations. Among them, *CDH11* (found up-regulated also in peritoneal mesothelioma [18], *RAN* (proposed as co-target for MPM treatment) [19], and *THBS2* (whose antibody titer was proposed as tumor marker for the diagnosis and follow up of patients) [20], seem particularly interesting.

Concerning cellular pathways, alterations in those devoted to the control of cell cycle and DNA replication are common hallmarks of cancer. Also alterations of metabolic pathways have been already correlated with human cancer [21-23]. Intriguingly, genes belonging to cell-cell and cell-matrix interactions were found deregulated in the present work. *COL1A1* and *TIMP3* (encoding for components of the matrix), *ITGA4* (integrins), *THBS2*, and *MSLN* (surface glycoproteins) play fundamental roles in cell growth, survival, migration, epithelial-to-mesenchymal transition, and even metastasis [24-26]. It is suggested that a favorable tumor micro-environment is built also with the help of normal cells surrounding and or infiltrating the tumor. Future therapies for MPM could be aimed to interfere with these mechanisms.

A further aim of the study was to evaluate how gene deregulation performs as prognostic biomarker. The actual number of analyzed patients was not appropriate for a thorough investigation. An altered expression of four genes (*BIRC5*, *NME2*, *DSP*, and *THBS2*) showed a correlation with patients' OS only at nominal level of 0.05, that dropped after the adjustment for multiple tests. It could be hypothesized that higher expression of these genes were measured when biopsies have a low share of normal tissue. In other words, the expression of *BIRC5*, *NME2*, *DSP*, and *THBS2* could be a proxy for the staging of the tumor. However, this is an unlikely explanation because OS would have been associated with a larger number of DE genes. Although, the study of OS could have been hampered by

a limited statistical power, the results are consistent with previous works carried out on MPM and on large series of other, less rare, tumors. In fact, a poor OS was significantly correlated with high expression of survivin mRNA in pleural effusions of patients diagnosed for MPM and lung adenocarcinoma [27], and in tumor tissues of patients diagnosed for colorectal [28], and breast cancer [29]. A poor OS was observed also among patients with high expression in tumor tissues of *NME2* (pancreatic, melanoma, hepatocellular, breast, ovarian, and gastric carcinoma patients [30-33], *DSP* (oral cancer patients) [34], and *THBS2* (lung adenocarcinoma patients [35]. In summary, although MPM is a poorly studied cancer, some features are starting to emerge. Particularly, these findings provide some evidences on the role of genes involved in focal adhesion, metabolism and cell-matrix interaction. Further studies on the genes identified in the present work are needed to better ascertain their fruitful exploitation as therapeutic targets, or as diagnostic and prognostic biomarkers of MPM.

5. Conflict of interest

The authors declare no conflict of interest.

6. Acknowledgements

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7. References

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Figure Legends

Figure 1. Fifty-one genes deregulated in a statistically significant way in MPM tissues were assayed in Mero-14 and Mero-25 MPM cell lines. The Fig. shows the correlation of the results obtained in the two cell lines. Gene expression level (as the logarithm base 2 of the fold change) and its statistical significance are calculated using the non-MPM cell line Met-5A as reference. The correspondence between labels and genes is reported in legend. Three genes, *KRT5*, *CDH11*, and *PTGIS*, showed an up-regulation in Mero-25 cells with a Log_2(FC) of 1.685, 5.607, and 8.910 and P-values of 0.0198, $<10^{-5}$, and $<10^{-5}$, respectively but were undetectable in Mero-14 cells. The remaining genes (*C10orf116*, *HCA112*, *SPINT2*, *UPK1B*, *FBLN2*, *ANK2*, *AOC3* and *COL11A1*) were undetectable in both cell lines.

LEGEND FIGURE 1:

Mero 14-cells: ***for P-value≤10^{-5}, **for P-values≤10^{-3}, *for P-values≤0.05;
Mero-25 cells: †††for P-value≤10^{-5}, ††for P-values≤10^{-3}, †for P-values≤0.05.
*SULF1****†††; *PDGFRB****†††; *THBS2****†††; *AKR1C1****†††; *GALNT7****; *CFB****††; *CXADR*†††; *COL1A1*†††; *RAN*†; *SOD1*†; *HEG1*; *TIMP3**†††; *NME2*; *TNPO2*; *ITGA4****; 1=*ASS1*†††; 2=*MCM4*; 3=*CCNO**†††; 4=*XPOT****††; 5=*MSLN*; 6=*PCNA*; 7=*DSP*; 8=*EIF4G1*†††; 9=*NMU*; 10=*PPARA*; 11=*CCNB1*†; 12=*TACC1**†; 13=*PKM2*; 14=*GAPDH*†††; 15=*ALDOA*; 16=*KRT18**; 17=*HELLS****; 18=*CENPF****†; 19=*SMC4****; 20=*KIF23****; 21=*ACSL1****†; 22=*NUSAP1****; 23=*CHEK1****; 24=*EFEMP1**†††; 25=*FEN1****; 26=*MKI67****†††; 27=*TOP2A****†††; 28=*CCNB2****†; 29=*SMARCA4****†††; 30=*BIRC5****†††; 31=*CALB2****; 32=*CDC2****†††; 33=*FANCI****†††

Supplementary Figures

Figure S1. Kaplan-Meyer OS curves distinguishing MPM patients with intra-tumor gene expression levels over the median (dotted lines) from patients with gene expression levels below the median (solid lines). Only genes with expression levels showing a statistically significant difference at nominal level of 0.05 between the groups of patients are presented. None of the genes was associated in a statistically significant way with OS, following Bonferroni's adjustment. Patients with the expression levels of the considered genes over the median showed poorer prognosis as compared to patients with gene expression levels below the median, with OSs of 20.8 months vs. 40 (P=0.016), 16.6 vs. 45 (P=0.016), 15.6 vs. 43.6 (P=0.03), and 18.2 vs. 40.8 (P=0.05), for *BIRC5*, *NME2*, *DSP*, and *THBS2*, respectively.

Figure S2.

Box-and-Whisker-Plots of four deregulated genes showing the strongest statistical significance when comparing MPM and NMM tissues.

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
			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

Table 2. Statistical analyses testing differences in gene expression levels (expressed as logarithm base 2 of the fold change) between MPM and NMM. Arrows show the direction of deregulation (DOD, ↑ up- or ↓ down-regulation) in MPM as compared to NMM, as expected from RTS. Results in disagreement are labeled with the symbol **↕;** q-values= Benjamini and Hochberg False Discovery Rate. In bold are highlighted the 59 differentially expressed genes.

Gene ID	Log_2(FC)	q-value	Gene ID	Log_2(FC)	q-value
↑***KIF23***	1.879	**$1.70x10^{-4}$**	↑***NMU***	4.080	**$1.72x10^{-4}$**
↑***CDH11***	1.737	**$3.30x10^{-4}$**	↑***MKI67***	1.583	**$1.39x10^{-3}$**
↑***COL1A1***	4.680	**$2.40x10^{-3}$**	↑***PTGIS***	1.739	**$2.40x10^{-3}$**
↑***CCNB2***	3.411	**$2.40x10^{-3}$**	↑***CCNB1***	1.867	**$2.91x10^{-3}$**
↑***TOP2A***	3.073	**$3.24x10^{-3}$**	↑***CHEK1***	1.488	**$3.24x10^{-3}$**
↓***ACSL1***	-1.682	**$3.25x10^{-3}$**	↑***HEG1***	1.935	**$4.56x10^{-3}$**
↑***PKM2***	2.774	**$4.77x10^{-3}$**	↑***FEN1***	1.784	**$5.39x10^{-3}$**
↑***CENPF***	1.974	**$6.58x10^{-3}$**	↑***FANCI***	2.533	**$6.58x10^{-3}$**
↓***FBLN2***	-1.871	**$6.58x10^{-3}$**	↑***SPINT2***	4.642	**$8.99x10^{-3}$**
↑***EIF4G1***	1.601	**$8.99x10^{-3}$**	↑***NUSAP1***	1.460	**$9.83x10^{-3}$**
↓***ANK2***	-0.331	**0.0103**	↑***CALB2***	5.670	**0.0105**
↓***AOC3***	-1.316	**0.0105**	↑***GAPDH***	0.946	**0.0105**
↑***SULF1***	1.472	**0.0151**	↑***ASS1***	2.282	**0.0151**
↑***MCM4***	0.872	**0.0163**	↑***TIMP3***	1.039	**0.0163**
↑***CCNO***	3.172	**0.0163**	↑***KRT5***	1.487	**0.0163**
↑***DSP***	4.685	**0.0163**	↑***SMC4***	0.914	**0.0163**
↓***C10orf116***	-2.132	**0.0188**	↑***SMARCA4***	0.606	**0.0190**
↑***TNPO2***	3.803	**0.0190**	↑***GALNT7***	2.276	**0.0190**
↑***MSLN***	1.841	**0.0221**	↑***EFEMP1***	0.931	**0.0234**
↓***TACC1***	-0.650	**0.0234**	↓***PPARA***	-1.367	**0.0235**
↑***COL11A1***	1.503	**0.0247**	↑***XPOT***	0.334	**0.0270**
↑***HCA112***	2.887	**0.0276**	↑***BIRC5***	2.052	**0.0276**
↑***THBS2***	4.985	**0.0290**	↑***CDC2***	0.910	**0.0314**
↑***ITGA4***	1.043	**0.0331**	↓***AKR1C1***	-0.219	**0.0354**
↑***RAN***	3.719	**0.0354**	↑***PDGFRB***	2.791	**0.0354**
↑***NME2***	1.939	**0.0354**	↑***CFB***	1.054	**0.0368**
↑***SOD1***	2.418	**0.0373**	↑***CXADR***	3.512	**0.0387**
↑***UPK1B***	5.202	**0.0430**	↑***PCNA***	2.518	**0.0430**
↑***HELLS***	1.237	**0.0454**	↑***KRT18***	0.859	**0.0479**
↑***ALDOA***	1.748	**0.0479**			

↑*THBS1*	2.988	0.0552	↑*PLK2*	0.810	0.0552
↕*BUB1B*	-0.103	0.0552	↓*EGR2*	-0.782	0.0552
↑*SSBP1*	2.605	0.0560	↕*COL6A1*	0.850	0.0570
↑*EEF2*	1.867	0.0601	↑*RAD21*	4.809	0.0605
↑*CRIP1*	1.455	0.0627	↓*EPAS1*	-0.739	0.0695
↑*FGF9*	2.935	0.0712	↑*VCAN*	1.481	0.0716
↓*NR4A2*	-0.333	0.0722	↑*VEGFA*	2.138	0.0738
↑*S100A10*	0.510	0.0761	↑*MCM2*	0.820	0.0782
↑*PGM1*	0.897	0.0788	↑*CCT2*	0.710	0.0790
↑*LGALS3BP*	1.891	0.0791	↕*CDK4*	-1.036	0.0794
↑*PGK1*	2.466	0.0847	↑*CDK7*	0.428	0.0927
↑*CCNH*	3.054	0.0953	↓*EGR3*	-1.940	0.0953
↓*FBLN5*	-0.412	0.0969			
↕*FBLN1*	0.341	0.110	↑*CDK2AP1*	1.479	0.111
↑*RCN2*	3.537	0.120	↑*EID1*	0.526	0.120
↕*AURKA*	-0.233	0.123	↓*PECAM1*	-0.335	0.126
↓*SFRP1*	-0.482	0.126	↑*FAS*	0.314	0.136
↑*PTGS2*	0.134	0.162	↑*SYNE1*	6.506	0.164
↕*IFITM1*	-1.966	0.164	↑*NR3C1*	1.083	0.172
↑*RHOB*	0.277	0.172	↕*PDGFRA*	0.003	0.178
↑*PSMD11*	0.371	0.178	↑*DAP*	0.027	0.187
↑*FGF2*	1.611	0.257	↓*CAV1*	-0.823	0.305
↕*CCND2*	1.412	0.330	↑*SFRP2*	1.337	0.363
↕*DAB2*	-0.158	0.379	↑*FHL1*	0.030	0.415
↕*IARS*	-0.631	0.422	↕*ANXA4*	-0.362	0.422
↕*S100A11*	-0.408	0.422	↕*CDKN1A*	1.398	0.469
↕*METAP1*	-0.361	0.470	↕*UBXN4*	-0.256	0.476
↕*KIF5B*	-0.460	0.482	↕*TGFBR2*	-0.247	0.482
↕*VWF*	0.391	0.486	↕*IGFBP4*	1.760	0.506
↑*ITGA6*	0.016	0.511	↕*ASPA*	-1.544	0.511
↑*BLMH*	0.092	0.511			

Table 3. Five of the top 19 deregulated pathways detected by WebGestalt, based on the genes resulted deregulated in a statistically significant way.

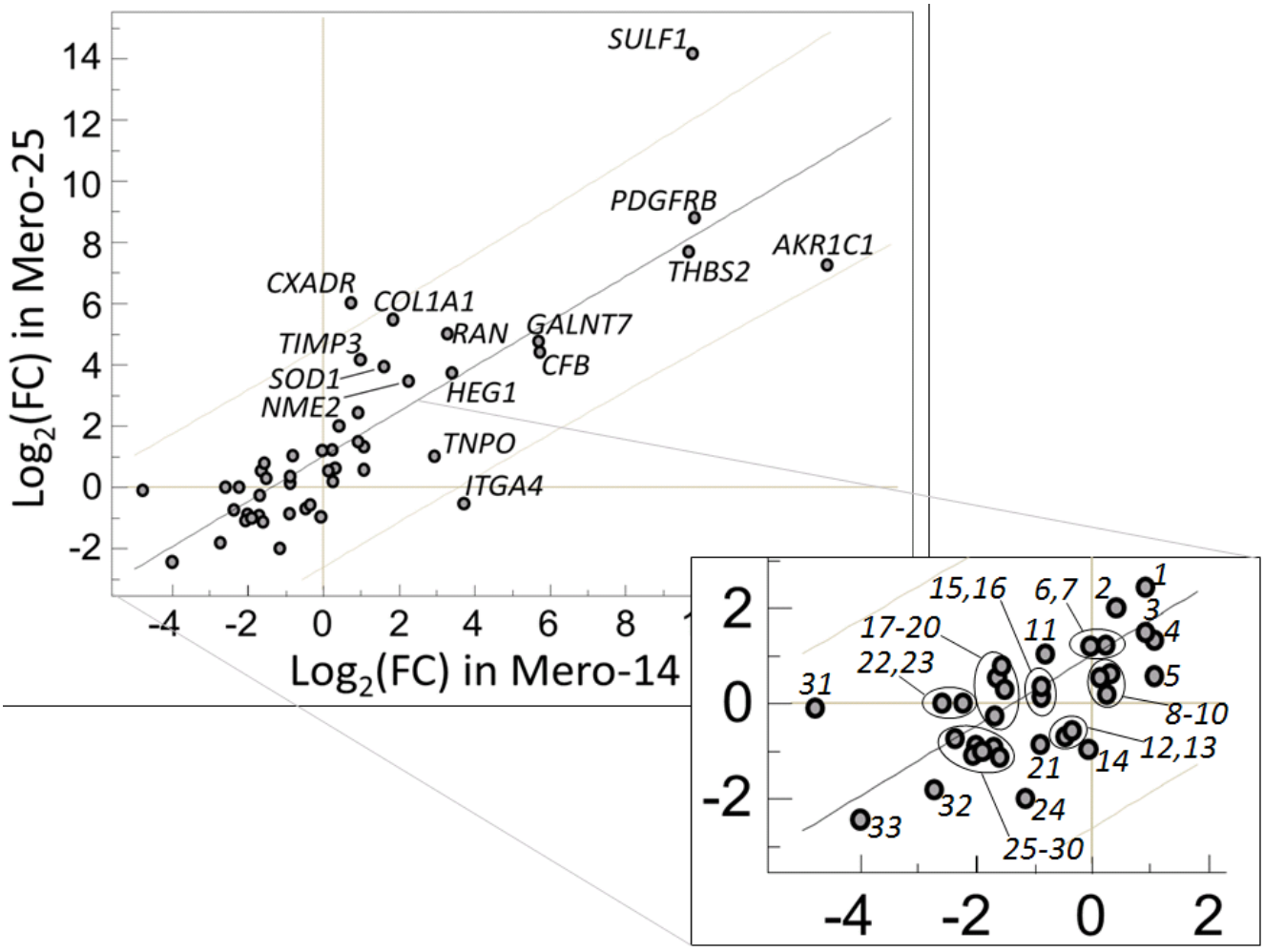
KEGG Pathway	Genes mapped in the pathway	Adjusted P-value
Cell cycle	*MCM4, CHEK1, PCNA, CCNB1, CCNB2*	1.23×10^{-5}
Focal adhesion	*COL11A1, THBS2, COL1A1, ITGA4, PDGFRB*	4.29×10^{-5}
Extracellular Matrix-receptor interaction	*COL11A1, THBS2, COL1A1, ITGA4*	4.29×10^{-5}
DNA replication	*MCM4, PCNA, FEN1*	7.20×10^{-5}
Metabolic pathways	*ASS1, AOC3, GALNT7, GAPDH, ACSL1, PTGIS, ALDOA, NME2*	8.77×10^{-4}

Table 4. Data showing the expression of specific genes in relation to the censored status. None of the genes analysed with Kaplan-Meyer (see supplementary data, Fig. S1) reached the statistical significance after Bonferroni's correction. L= gene expression below the median, H= gene expression over the median, N/A= sample excluded following quality control.

	BIRC5			DSP			NME2			THBS2		
Status (Censored)	**L**	**H**	**N/A**	**L**	**H**	**N/A**	**L**	**H**	**N/A**	**L**	**H**	**N/A**
Alive	5	1	0	3	1	2	3	1	2	2	1	3
Deceased	0	4	3	2	4	1	3	4	0	3	4	0
% of deceased patients	0	80		40	80		50	80		60	80	

Fig. 1. Fifty-one genes deregulated in a statistically significant way in MPM tissues were assayed in Mero-14 and Mero-25 MPM cell lines. The figure shows the correlation of the results obtained in the two cell lines. Gene expression level (as the logarithm base 2 of the fold change) and its statistical significance are calculated using the non-MPM cell line Met-5A as reference. The correspondence between labels and genes is reported in legend. Three genes, KRT5, CDH11, and PTGIS, showed an up-regulation in Mero-25 cells with a Log2(FC) of 1.685, 5.607, and 8.910 and P values of 0.0198, <10−5, and <10−5, respectively but were undetectable in Mero-14 cells. The remaining genes (C10orf116, HCA112, SPINT2, UPK1B, FBLN2, ANK2, AOC3 and COL11A1) were undetectable in both cell lines. Mero 14-cells: ***for P value ≤10−5, **for P values ≤10−3, *for P values ≤0.05; Mero-25 cells: †††for P value ≤10−5, ††for P values ≤10−3, †for P values ≤0.05. SULF1***†††; PDGFRB***†††; THBS2***†††; AKR1C1***†††; GALNT7***; CFB***††; CXADR†††; COL1A1†††; RAN†; SOD1†; HEG1; TIMP3*†††; NME2; TNPO2; ITGA4***; 1 = ASS1†††; 2 = MCM4; 3 = CCNO*†††; 4 = XPOT***††; 5 = MSLN; 6 = PCNA; 7 = DSP; 8 = EIF4G1†††; 9 = NMU; 10 = PPARA; 11 = CCNB1†; 12 = TACC1*†; 13 = PKM2; 14 = GAPDH†††; 15 = ALDOA; 16 = KRT18*; 17 = HELLS***; 18 = CENPF***†; 19 = SMC4***; 20 = KIF23***; 21 = ACSL1***†; 22 = NUSAP1***; 23 = CHEK1***; 24 = EFEMP1*†††; 25 = FEN1***; 26 = MKI67***†††; 27 = TOP2A***†††; 28 = CCNB2***†; 29 = SMARCA4***†††; 30 = BIRC5***†††; 31 = CALB2***; 32 = CDC2***†††; 33 =

FANCI***†††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SOD1
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NME2
TNPO
ITGA4
Log2(FC) in Mero-25
Log2(FC) in Mero-14
15,16
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