EGFR And PDGFR Differentially Promote Growth In Malignant Epithelioid Mesothelioma Of Short- And Long-term Survivors

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Keywords: Mesothelioma, short-term- and long term survivors, tissue microarray, correlation analysis, signal pathways

Word count: 3249 words
ABSTRACT

Malignant pleural mesothelioma (MPM) is an asbestos-related tumour difficult to detect early and treat effectively. Asbestos causes genetic modifications and cell signalling events that favour the resistance of MPM to apoptosis and chemotherapy. Only a small number of patients, approximately 10%, survive more than 3 years. The aim of our study was to assess possible differences within signalling pathways between short-term survivors (survival <3 years; STS) and long-term survivors (survival >3 years; LTS) of MPM. In order to do this, 37 antibodies detecting proteins engaged in cell signalling pathways, enforcing proliferation, anti-apoptosis, angiogenesis, and other cellular activities were investigated by tissue microarray (TMA) technology. Epidermal growth factor receptor (EGFR) is expressed stronger in LTS whereas platelet derived growth factor receptor (PDGFR) signalling is more abundant in STS. The expression of TIE2/Tek, a receptor tyrosine kinases involved in angiogenesis, is differentially regulated via PDGFR and thus is more important in STS. Anti-apoptosis is upregulated in STS by signal transducer and activator of transcription 1 (STAT1)-survivin and related molecules, but not in LTS. Our study provides novel insights into the regulatory mechanisms of signalling pathways in MPM, which differentially promote tumour growth in LTS and STS. We demonstrate that small scale proteomics can be carried out by a powerful linkage of TMA, immunohistochemistry, and statistical methods to identify proteins which might be relevant targets for therapeutic intervention.
INTRODUCTION

Malignant pleural mesothelioma (MPM) is an asbestos-related tumour difficult to detect early and treat effectively.\[1\] Although MPM in general is a rare tumour, its incidence is increasing worldwide. It is estimated that in Europe, Australia, and Japan MPM will peak between 2015 and 2025, whereas in the United States MPM reached its peak incidence in 2004.\[2\]

Most cases are a direct consequence of asbestos exposure 30-40 years earlier. From the onset of symptoms, survival is from a few weeks to a few years \[3\], the median survival range being from 4-12 months in either treated or untreated patients.\[4\] Only a small number of patients, approximately 10%, survive more than 3 years, which seems to be restricted to the epithelioid subtype.\[5, 6\] Immunohistochemical analysis of Ki-67/MIB-1 \[7\], p27\(^{kip1}\) \[8\], p21\(^{cip1}\), and Cyclooxygenase-2 (COX-2) \[5, 9\] have been correlated with survival in MPM, but their significance has not been entirely clarified.

Asbestos causes genetic modifications and cell signalling events, most notably the upregulation of cell survival and growth pathways, as well as other proteins that favour the resistance of MPM to apoptosis and chemotherapy.\[10\] Various studies have confirmed the importance of signalling pathways in MPM including the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF) pathways, and their downstream signalling molecules such as the mitogen-activating protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K)/Akt kinase respectively.\[11, 12\]

The aim of our study was to compare the role of signalling pathways between short-term survivors (<3 years; STS) and long-term survivors (>3 years; LTS) of MPM, assessing possible differences correlated to the corresponding phenotype. In order to do this a large panel of antibodies detecting proteins engaged in these cell signalling pathways, enforcing proliferation, anti-apoptosis, angiogenesis, and other cellular activities in MPM were investigated by tissue microarray (TMA) technology. Due to the extreme rarity of LTS in MPM a comparison between STS and LTS in regard to signalling pathways has never been investigated.

METHODS

Histological examination and clinical data

70 MPMs were derived from surgically resected material, either open biopsies or pleurectomies. The cases were diagnosed between 1987 and 2003 and classification was made according to the World Health Organization (WHO 2004) by each of the contributing authors (P.M., F.D., H.Z., C.E.C., B.M., P.T.C., R. A., A.R.G., F.G.S., H.H.P.) At least 3 positive and two negative markers have been applied, to confirm the diagnosis of a mesothelioma according published recommendations.\[13\] 48/70 cases of epithelioid MPM (collected in USA, UK, Turkey, Italy, France, and Austria) with available clinical information and sufficient formalin-fixed, paraffin embedded material were selected for TMA construction. Asbestos exposure was confirmed in one third of the patients in both groups, in the others no exposure data were available. Informed consent from patients was provided with the data sheet submitted with the tissue. In addition the study was approved by the local Ethical Commission.
Taking 36 months as a cut-off level for survival, 26 patients were identified as long-term survivors (survival > 36 months; LTS) and 22 patients as short-term survivors (survival < 36 months; STS). The survival time was determined from the time of diagnosis until the last follow-up or death. Fifteen out of 20 females and 11 out of 28 males belonged to LTS. Clinical data were collected for all patients (tab 1).

Table 1. Clinical data for the 48 epithelioid MPM patients involved in the study.

<table>
<thead>
<tr>
<th>MPM patients</th>
<th>male</th>
<th>female</th>
<th>age range (mean)</th>
<th>survival range (mean)</th>
<th>MIB1/Ki-67 cases &lt; 30%</th>
<th>MIB1/Ki-67 cases &gt; 30%</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTS (26)</td>
<td>11</td>
<td>15</td>
<td>41 - 78 (63.7)</td>
<td>36 - 116 (50.7)</td>
<td>24/26 (92.3%)</td>
<td></td>
</tr>
<tr>
<td>STS (22)</td>
<td>17</td>
<td>5</td>
<td>30 - 71 (57.4)</td>
<td>&lt;1 - 31 (9.1)</td>
<td>13/22 (40.9%)</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>28</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tissue microarray construction

For TMA construction, an H&E stained section was cut from each paraffin-block and re-examined by an experienced pathologist (HH.P.). Five representative tumour regions were morphologically identified and marked on the H&E stained sections. Tissue cylinders with a diameter of 0.6 mm were punched from the marked areas of each block and brought into a recipient paraffin-block, using a manual instrument (Beecher instruments, Sun Prairie, WI; USA). Five µm-thick sections were cut from each TMA and prepared for immunohistochemistry as previously described.[14] Every 15th section was stained with H&E and controlled for the presence of the epithelioid subtype. To overcome the problem of tumour heterogeneity and increase the number of accessible slides, each donor tissue block was punched 6-10 times for the construction of two recipient blocks, each containing 243-tissue cores. In these 243-tissue cores, also 13 adjacent parenchyma cores were included.

Immunohistochemistry

Immunohistochemical (IHC) staining with 37 antibodies was performed on TMA sections, according to the recommendations of the manufacturer. In addition, antibodies were pretested on different tissues prior to their use on TMA. Antibodies used in the study for simplicity of reading are given in the abbreviated form (in alphabetically order): AMPKα2, β-Catenin, CREB binding protein, c-Fos, c-Jun, c-Met, c-Myc, Cyclin D1, EGFR1, ERK2, Gab1, Grb2, GSK3, IGF1R, IGF1Rα, JAK1, MAP4K-1, MECP2, p-m-Tor (Ser2448), NFKB p65, pAKT (Ser473), PCNA, Src, p27Kip1, PDGFR-α, PDGFR-β, PI3K p110α, p-ELK, p-p70S6K(Ser411), p-Paxillin, STAT1, STAT3, STAT5, Survivin, TGF-β, TIE2, and VEGF. A list of the antibodies with additional information about source, dilution, antigen retrieval, and detection is given in the Supplementary tab 1.
Scoring
IHC analysis was carried out by one pathologist (HH.P.) without knowledge of patient survival data or core distribution within the TMA. The protein expression was recorded semi-quantitatively. For each core the staining intensity (0: no staining, 1+: weak, 2+: moderate, 3+: strong) together with the percentage (0-100 %, in 10 % increments) of cells expressing the protein was recorded. Staining scores were calculated by multiplying the percentage of positive cells by the staining intensity. The obtained product scores, ranging from 0-300, were used for statistical analysis.

Statistics
The null hypothesis that there is no difference in immunohistochemical parameters between LTS and STS was assessed with Goeman's global test the R-package globaltest 3.0.2 (url http://www.bioconductor.org/repository/release1.5/package/html/globaltest.html) with a simulated permutation test criterion. Individual immunohistochemical parameters were tested for differences with Wilcoxon's rank sums test. Correlation coefficients were calculated using Spearman's rank correlation coefficient of the product score. Tests of differences between correlation coefficients of LTS and STS were based on differences between z-transformed Spearman correlation coefficients. P-values were calculated from simulated permutation tests.

The statistically relevant correlations and differences between correlations were displayed as network graphics with antibodies as nodes and correlation as edges. P-values below 0.05 were considered to be statistically significant. All permutation tests comprised 10000 replications. Correlations between immunohistochemical parameters were displayed as network graphic using Graphviz graph visualization software (url http://www.graphviz.org). The absolute values of correlations determined the strength of springs between nodes. The neato algorithm placed the nodes such that the energy of the system was minimized.

RESULTS
Clinical features
48 MPM cases used for the TMA construction were histologically classified as epithelioid mesotheliomas. The survival of the 26 patients within LTS (survival >36 months) ranged from 36 to 116 months with a mean of 50.7 months, whereas the 22 patients in STS (<36 months) ranged from <1 to 31 months with a mean of 9.4 months. The mean survival time in the LTS group is 5.4 times longer than in the STS group (tab 1). Sex was neither significantly correlated to survival nor to immunohistochemical reactions.

Immunohistochemistry and statistical evaluation
MIB-1 immunostaining revealed diffuse nuclear staining. In line with published data the proliferative activity, detected by MIB-1 immunoreactivity was significantly different between LTS and STS (p 0.014, Fisher’s exact test; tab 1).
Many of the proteins analysed on the TMA were characterised by low expression in the parenchyma and increased expression in the tumour tissue. The comparison of protein expression profiles between LTS and STS shows a significant difference (p<0.009, Goeman’s global test).

In the comparison of expression levels of single proteins detected by the corresponding antibodies significantly higher values were found in LTS only for STAT3, IGF1Ra, pAKT, c-Met, TGF-β, c-Jun, IGF1R, and paxillin (fig 1, p<0.05, Wilcoxon’s rank sum test).
Representative immunohistochemical stains for EGFR, PDGFR, TIE2, STAT1, STAT3, and Survivin are shown in fig 2a-f.

Correlations and correlation differences between LTS and STS are displayed in the network graphic (fig 3), the corresponding correlation differences are summarised in tab 2. Differences in correlations indicate differences in the underlying regulatory network.[15] The complete statistical data set including correlation- and p-values is given in the Supplementary tab 2a-c for the LTS, the STS, and the differences in correlations between LTS and STS (z-transformed correlations).
Table 2. Differentially correlated proteins in STS and LTS with p<0.05. The resulting differences between correlations are given either in the left or right section of the table depending on whether correlations were higher in STS or LTS respectively.

<table>
<thead>
<tr>
<th>Protein 1</th>
<th>Protein 2</th>
<th>correlation difference</th>
<th>p</th>
<th>Protein 1</th>
<th>Protein 2</th>
<th>correlation difference</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGFRa</td>
<td>Tie2</td>
<td>0.77</td>
<td>0.036</td>
<td>EGFR</td>
<td>PCNA</td>
<td>1.01</td>
<td>0.004</td>
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<tr>
<td>PDGFRb</td>
<td>Tie2</td>
<td>0.78</td>
<td>0.045</td>
<td>EGFR</td>
<td>STAT3</td>
<td>0.80</td>
<td>0.027</td>
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<tr>
<td>PDGFRa</td>
<td>GSK3</td>
<td>0.73</td>
<td>0.039</td>
<td>EGFR</td>
<td>c-jun</td>
<td>0.70</td>
<td>0.038</td>
</tr>
<tr>
<td>PDGFRa</td>
<td>c-fos</td>
<td>0.73</td>
<td>0.035</td>
<td>EGFR</td>
<td>Tie2</td>
<td>0.86</td>
<td>0.013</td>
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<tr>
<td>PDGFRa</td>
<td>PCNA</td>
<td>0.70</td>
<td>0.045</td>
<td>EGFR</td>
<td>c-fos</td>
<td>0.65</td>
<td>0.046</td>
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<td>PDGFRb</td>
<td>cyclinD1</td>
<td>0.67</td>
<td>0.049</td>
<td>Erk</td>
<td>pAkt</td>
<td>0.84</td>
<td>0.027</td>
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<tr>
<td>STAT1</td>
<td>p27</td>
<td>0.96</td>
<td>0.013</td>
<td>pELK</td>
<td>pAkt</td>
<td>0.82</td>
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<td>STAT1</td>
<td>Survivin</td>
<td>0.76</td>
<td>0.025</td>
<td>Erk</td>
<td>pELK</td>
<td>0.81</td>
<td>0.028</td>
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<tr>
<td>STAT1</td>
<td>GRB2</td>
<td>0.77</td>
<td>0.029</td>
<td>GAB1</td>
<td>Jak1</td>
<td>0.67</td>
<td>0.047</td>
</tr>
<tr>
<td>Survivin</td>
<td>Erk</td>
<td>0.91</td>
<td>0.012</td>
<td>IGF1R</td>
<td>TGFb</td>
<td>0.87</td>
<td>0.005</td>
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<tr>
<td>Survivin</td>
<td>c-jun</td>
<td>0.85</td>
<td>0.022</td>
<td>PCNA</td>
<td>TGFb</td>
<td>0.87</td>
<td>0.007</td>
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<td>Survivin</td>
<td>PCNA</td>
<td>0.73</td>
<td>0.032</td>
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<tr>
<td>IGF1R</td>
<td>MECP2</td>
<td>0.87</td>
<td>0.017</td>
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<tr>
<td>MAP4K1</td>
<td>SRC</td>
<td>0.69</td>
<td>0.042</td>
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</table>
DISCUSSION

We explore differences in signalling pathways by defining correlation differences in the protein expression between LTS and STS (tab 2). Only those differences for which a rationale to signalling pathways exists were included in the discussion below. These differences indicate differences in the underlying regulatory pathway, including proliferation (EGFR or PDGFR), angiogenesis (TIE2), and anti-apoptosis (STAT1, survivin), which seem differentially regulated in both groups. A simplified schema showing interactions of EGFR and PDGFR signalling is given in fig 4.

**EGFR signalling in LTS**

In the LTS EGFR was correlated with downstream acting signal transducer and transcription factor 3 (STAT3), the proto-oncogenes c-Fos and c-Jun, and proliferating cell nuclear antigen (PCNA). Further EGFR-downstream acting proteins including GAB1, janus kinase 1 (JAK1, plays a pivotal role in phosphorylating STAT3), extracellular signal regulated kinase 2 (ERK2), pAKT, and pELK were highly correlated in LTS but not detected in STS. These results indicate that cell survival pathways in LTS were preferentially driven through EGFR. ERK, Akt and STAT3 are on signal transduction pathways triggered by EGFR [16] and the subsequent activation of c-Fos, c-Jun, and PCNA is caused by EGFR activation too.[17] Blocking EGFR signalling with the EGFR inhibitors gefitinib and erlotinib in early phase II trials of MPM patients had limited or no effect [18], suggesting that EGFR is a therapeutic target only for a minority of mesothelioma patients.[16] From our findings we suppose that this minority belongs to the LTS.

Our data demonstrate that transforming growth factor-ß (TGF-ß) promotes EGFR signalling in the LTS. It is known that TGF-ß activates EGFR by inducing the expression of the EGFR ligands, TGF-α and HB-EGF (heparin-binding EGF like growth factor).[19] The ligands form dimers and after receptor binding EGFR will be activated for further downstream signalling. TGF-ß might be responsible for EGFR activation in LTS because of the higher expression value in LTS (p 0.02, fig 1) compared with STS as well as TGF-ß was correlated with PCNA and IGF1R. The latter was also higher expressed in LTS (p 0.022, fig 1).

**PDGFR signalling in STS**

Nonneoplastic mesothelial cells express predominantly PDGFR-α subunit and less PDGFR-ß, while MPM prefers PDGFR-ß expression.[20] In our cases both, PDGFR-α and PDGFR-ß, were expressed equally in LTS and STS. PDGFR-α expression in MPM could be induced by interferon gamma (IFNγ). In THP1 cells it was shown that IFNγ-stimulation resulted in an augmented expression of PDGFR-α through transient STAT1 promoter binding [21], and IFNγ was described to induce STAT1 overexpression.[22]

Only in STS we found PDGFR-α and PDGFR-ß associated with downstream glycogen synthase kinase 3 (GSK3), TIE2, c-Fos, Cyclin D1, and PCNA. Some of these downstream acting proteins (TIE2, c-Fos, and PCNA) were also important in LTS. But in LTS these three proteins were associated with EGFR expression. The analysis of correlation differences implicates that PDGFR and EGFR pathways were differentially activated in both groups. Although STS and LTS show similar expression levels of EGFR and PDGFR, correlation analysis showed that LTS prefer EGFR whereas STS prefer PDGFR signalling pathway with different downstream proteins.

PCNA could be a key-player in MPM proliferation, in both STS and LTS. Interestingly, it is known that binding of EGF, PDGF, and IGF1 to their corresponding receptors, induces
PCNA expression and stabilisation of the resulting mRNA.[23] Taking this into account, our results imply that PCNA expression benefits from EGFR and/or PDGFR activation in LTS and STS. Blocking key-proteins acting within several signalling pathways might be potential targets for further translational approaches in MPM. PCNA alone or in combination with PDGFR could be one of these targets.

**TIE2 and angiogenesis in MPM**
TIE2/Tek a recently identified RTK principally expressed on vascular endothelium induces angiogenesis upon stimulation through one of the four angiopoetin ligands (Ang1-4). So far TIE2 protein expression was not studied in MPM, but TIE2 involvement was already reported for breast [24], lung [25], and liver tumours.[26] In STS we found TIE2 highly associated with PDGFR-α and PDGFR-β. In contrast to LTS, where TIE2 was associated with EGFR. Hence, we conclude that PDGFR expression in STS and EGFR in LTS leads to a subsequent upregulation of TIE2 in MPM.

**Anti-Apoptosis induced by Survivin and STAT1 in STS**
Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is specifically upregulated in a variety of human cancers and undetectable in normal tissue.[27] In our cases survivin was also exclusively expressed in the tumour tissue whereas it was not detected in adjacent normal pleura. Survivin was lower expressed in the LTS than in STS (fig 1). Xia and colleagues previously reported that overexpression of survivin in mesothelioma cell lines and fresh tumour samples contribute to the poor response of MPM cells to chemotherapy and radiation therapy.

Survivin in STS was highly correlated with ERK2, STAT1, c-Jun, and PCNA. The correlation between survivin and STAT1 indicates a novel role for STAT1 promoting anti-apoptosis through survivin upregulation. For chemotherapy-resistant breast carcinomas it was reported that survivin was induced by STAT3. In addition the authors could show that direct inhibition of STAT3 signalling blocked survivin expression.[28] Contrary to breast carcinomas our findings provide a strong argument that in STS of MPM survivin upregulation is induced by STAT1, instead of STAT3. Survivin disrupt the intrinsic apoptosis pathway most probably by blocking the activation of caspase 9.[29] Enhanced STAT1-survivin and PCNA-survivin interactions imply that in the STS proliferation is promoted and apoptosis is blocked via survivin expression, while in LTS survivin plays no significant role.

**STAT1 and MECP2, a novel role in MPM pathogenesis**
STAT’s are a family of latent cytoplasmic signal transducers and activators of transcription with known opposing actions. While STAT3 and STAT5 are referred as the oncogenic STAT’s, STAT1 is regarded as tumour suppressor.[30] Curiously, we found STAT1 and STAT5 equally expressed in LTS and STS, whereas STAT3 was higher expressed in LTS (p 0.002, fig 1). In LTS we found STAT3 further associated with EGFR.

It looks like that in MPM the tumour suppressor STAT1 acts similar to an oncogene. Exclusively in STS, STAT1 was correlated with survivin, p27, and Grb2. STAT1 overexpression has been also reported in breast-, head and neck cancers, and in some hematologic tumours again suggesting a prosurvival mode of action [31], but its role has not been clarified. Only one report describes the role of STAT1 in mesothelioma. [22] Buard et al. reports that INFγ induces STAT1 overexpression in mesothelioma cell lines.

STAT1 overexpression confers some tumours resistance against radiation and cisplatin treatment.[32] Resistance against cisplatin- and radiation-based therapies is also frequent in MPMs.
Does increased STAT1 protein induce resistance in MPM too? Our data indicate that the axis STAT1-survivin can be associated with anti-apoptotic and therapy resistant mechanisms in MPM, especially in patients with a poor prognosis, like STS. Although similarly expressed in LTS no similar association for STAT1 was found.

We found high levels of MECP2 (methyl-CpG binding protein 2) protein in LTS and STS, as well. MECP2 specially binds to methylated CpG islands in the genome and can be linked to DNA methylation and histone deacetylation. The implication of MECP2 in neoplasms was already reported for a variety of human tumours.[33] The prominent interaction between MECP2 and IGFR1 was detected in the STS, not in LTS. Presently, we are not able to explain the role of MECP2 in STS or in MPM generally. Interestingly Gordon et al. detected high levels of FMR1 mRNA (fragile X mental retardation 1) another x-linked gene in MPM [34]. The role of these X-linked genes, MECP2 and FMR1, in MPM is unknown, but indicates involvement in the epigenetic silencing network.

Our study provides novel insights into the regulatory mechanisms of signalling pathways in MPM, which differentially promote tumour growth in LTS and STS. As shown, EGFR signalling is stronger activated in LTS, whereas PDGFR signalling is more abundant in STS. Anti-apoptosis is upregulated in STS by STAT1-survivin and related molecules, but not in LTS. We have demonstrated that small scale proteomics can be carried out by a powerful linkage of TMA, immunohistochemistry and statistical methods, to identify differences in protein expression. Nevertheless, these tools were used to generate a hypothesis. Additional in-vitro studies are needed to evaluate multiple targeting strategies, including EGFR, PDGFR, TIE2, STAT1, and PCNA. This might open new options of treatment in these aggressive neoplasms. To the best of our knowledge, we provide the first evidence of an immunohistochemistry based comparison in regard to signalling pathways in MPM between STS and LTS in a large series of LTS.
ACKNOWLEDGEMENT

This work was supported by a research grant from the Austrian Cancer Aid/Styria 04/2004 to HH.P.
We gratefully thank Margit Gogg-Kamerer and Elisabeth Grygar, Institute of Pathology, Medical University of Graz, Austria, for technical support.

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FIGURE LEGENDS

Fig. 1 - Box- and Whiskers plots of all antibodies measurements for LTS (orange boxes) and STS (yellow boxes).
The log2(x+20) - transformed product score is shown on the horizontal axis. On the vertical axis for each antibody the p-value of the Wilcoxon rank sum test which compares the median of the product score in LTS and STS. In LTS significantly higher values (p<0.05), were found for STAT3, IGF1Ra, pAKT, c-Met, TGF-β, c-Jun, IGF1R, and paxillin.

Fig. 2 – Immunohistochemical stains.
Immunohistochemical stainings for EGFR (a), PDGFR (b), TIE2 (c), STAT1 (d), STAT3 (e), and Survivin (f) of different cores from the epithelioid MPM tissue microarray (original magnification x200). Small bars represent 20µm, large bars 50µm.

Fig. 3 – Network graphic.
The network graphic shows the correlations between antibodies using the Spearman rank correlation of the product score. There are 666 possible correlations between the antibodies, only correlations higher than 0.7 are shown. Blue edges indicate correlations within LTS, red edges indicate correlations within STS. Black edges represent differences in correlations between LTS and STS with a p-value below 0.05. Nodes represent the proteins detected by the antibody. The neato algorithm placed correlated antibodies close to each other.

Fig. 4 – Schema showing the different interactions of EGFR and PDGFR signalling with respect to LTS and STS in MPM.
All molecules shaded in gray have been tested on the tissue microarray, molecules not tested in white; arrows symbolise activation, P symbolise phosphorylation. For simplicity only major key proteins are included.
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