Molecular and Histopathologic Prognostic Factors in Malignant Pleural Mesothelioma

Synopsis of PhD thesis

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1. INTRODUCTION

Malignant pleural mesothelioma is a rare tumor of dismal prognosis. The current therapeutic modalities available to MPM patients fail to achieve a significant improvement in long-term outcomes.

There are three main histologic types of MPM – epithelioid, biphasic and sarcomatoid – that are established prognostic factors and are routinely used in selecting candidates for radical multimodality treatment consisting of surgery, chemotherapy and radiation therapy. Resection of the tumor is, however, only feasible in a minority of cases, and most patients receive platinum-based combination chemotherapy. There is a lack of established histologic prognostic factors that would help further stratify patients with epithelioid MPM. The prognostic and predictive impact of tumor morphology is well known in lung adenocarcinomas. Among subtypes of EMM, the pleomorphic variant is known to confer dismal prognosis, however, only limited data is available on the differential behavior of other morphologic subtypes of EMM in the literature. Grading of EMM is not routinely included in pathology reporting, there are, however grading systems recently proposed for risk stratification of EMM patients.

The genomic landscape of MPM is defined mostly by alterations leading to lost function of tumor suppressors, such as p53, CDKN2A and BAP1. The reactivation of telomere activity is a hallmark of cancer. One important and recently discovered mechanism of

telomere reactivation is the creation of de novo binding site for activating transcription factors through point mutations in the encoding gene's core promoter region. The mutations of the TERT promoter confer dismal prognosis in a variety of solid malignancies, such as thyroid cancers, diffuse gliomas and bladder cancer, but there is limited data on its role in MPM.

2. OBJECTIVES

We investigated histopathologic and molecular features of prognostic and potentially predictive significance in malignant pleural mesothelioma.

We have focused on the following questions:

1. The prognostic impact of nuclear grading, mitosis-necrosis score and the predominant growth patterns of epithelioid malignant pleural mesothelioma, as well as associations between these variables.
2. Histomorphologic parameters for identification of patients who might benefit from a more aggressive, multimodal treatment, and those who do not benefit from such relatively high-risk therapies.
3. Frequency of TERT promoter mutations in malignant pleural mesothelioma, their correlation with other clinicopathologic features and their potential prognostic role.
4. Interaction between the common polymorphism rs2853669 and TERT promoter mutation in MPM.
5. Mechanisms underlying the aggressive clinical behavior of TERT promoter mutant MPMs, such as cell line forming ability, TERT mRNA expression and in vitro cisplatin sensitivity in cell lines with wild-type or mutant TERT core promoter region.
6. Association between the TERT promoter mutant genotype and BAP1 status.

3. METHODS

3.1. Patient collective

Our patients were diagnosed with MPM in five large European centers, Division of Thoracic Surgery, Medical University of Vienna, Austria; Department for Respiratory Diseases Jordanovac, School of Medicine, University of Zagreb, Croatia; University Medicine Essen - Ruhrlandklinik, Essen, Germany; National Koranyi Institute of Pulmonology, Budapest, Hungary and Department for Pulmonology, University Clinic Golnik, Slovenia. The study was conducted in line line with the Declaration of Helsinki and was approved by the ethics committees of each participating institution. Clinical data included IMIG stage of the tumor, patients' treatment information, ECOG prognostic score, Karnofsky performance score, date of diagnosis and date of death or last contact. As a validation cohort for the histologic subtype analyses, we also evaluated 55 virtual slides of epithelioid MPMs openly available at the Cancer Digital Slide Archive which are digitalized diagnostic sections of specimens collected by The Cancer Genome Atlas. Corresponding variables collected by TCGA were downloaded from the cBioPortal.

3.2 Samples

In our investigation on histologic subtypes and grading of epithelioid MPM we included FFPE samples of 192 epithelioid MPMs. These were obtained through video-assisted thoracoscopy (n=106), pleurectomy (n=28) or percutaneous pleural needle core biopsy

(n=28). In 30 cases the diagnostic procedure was not specified. TERT promoter mutations were evaluated in a partially overlapping cohort. For this study a total of 182 FFPE samples were included, consisting of 127 epithelioid and 54 non-epithelioid MPMs. We analyzed patient outcomes separately in the Austrian test cohort (n=83) and Croatian-Slovenian validation cohort (n=99) to test if the prognostic impact of the TERT mutations was independent of geographical location, socioeconomic status and health care systems.

3.3 Histologic subtype analysis

We reviewed 192 HE stained slides of epithelioid MPMs and categorized them according to predominant growth patterns using the categories and definitions according to the latest WHO classification of tumors of the lung, pleura, thymus and heart. Tumors were classified as predominant tubulopapillary, microcystic, solid, trabecular, micropapillary, pleomorphic.

3.4 Histologic grading

We investigated two histologic grading systems recently established for epithelioid MPM. The nuclear grading system is based on a three-tier (1-3) scoring of nuclear atypia and mitotic figures in 10 HPF. Based on the combination of the scores the tumors are then grouped into three nuclear grade groups (1-3). The mitosis-necrosis score combines the two-tier assessment of mitotic count per 10 HPF and

the presence or absence of necrosis, and results in a mitosis-necrosis score of 0, 1 or 2.

3.5 BAP1 staining

BAP1 immunohistochemistry was carried out on 4 μm sections using the primary antibody BAP-1, Clone C-4 (sc-28383, Santa Cruz Biotechnology) at a dilution of 1:200. A sample was considered negative for BAP1 expression in the absence of nuclear reactivity, regardless of the presence or absence of cytoplasmic staining.

3.6 Mesothelioma cell lines

We used 22 primary cell lines established from surgical MPM samples and an additional 5 international MPM cell lines.

3.7 DNA extraction and TERT promoter status analysis

Genomic DNA from FFPE samples was isolated using High Pure FFPE DNA Isolation Kit (Roche Diagnostics), while from MPM cell lines extraction was carried out using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturers' protocol. The core TERT promoter region between the +65 and -278 bp from the ATG start site was amplified by PCR and screened using Sanger sequencing by Prof. Rajiv Kumar's group at the German Cancer Research Center, Heidelberg, Germany.

3.8 Quantitative PCR

Total RNA was extracted from 22 MPM cell lines using TRIzol Reagent (Invitrogen) and purified with Turbo DNase Kit (Ambion).

Reverse transcription of RNA was carried out using High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative PCR was performed using Maxima SYBR Green qPCR master mix (Thermo Scientific) and gene specific probes for hTERT and RPL41 in a C1000 Touch Thermal Cycler.

3.9 Characterization of cisplatin sensitivity

24 MPM cell lines were treated with different concentrations of cisplatin (0, 0.5, 1, 3, 5, and 10 uM) for 24 hours. Cells were then fixed and stained with SRB. The amount of protein-bound SRB was determined photometrically.

3.9 Statistical analyses

Associations between two categorical variables such as histopathologic characteristics, clinical parameters and TERT promoter status were calculated by Fisher's exact test. Median survival was calculated through Kaplan-Meier method, and differences in median OS were analyzed through log-rank test. Differences in mRNA expression and in cisplatin sensitivity were analysed by two-tailed Student`s *t* test. To identify independent prognostic factors, multivariate Cox regression tests were performed and hazard ratios and corresponding 95% confidence intervals were calculated. Results were considered statistically significant if $p<0.05$, two-sided. All statistical analyses were performed in GraphPad Prism 5 (GraphPad Software Inc) and in SPSS Statistics 23.0 package.

4. RESULTS

4.1 Histologic subtypes, nuclear grade and mitosis-necrosis score in epithelioid MPM

Among the samples solid pattern was predominant in 52.1%, tubulopapillary in 28.6%, trabecular in 10.4% of the samples. Microcystic, pleomorphic and micropapillary subtypes were rare, accounting for 4.7, 3.1 and 1.0%, respectively. After assessing nuclear atypia and mitotic counts, 54.7% of the samples resulted nuclear grade 1, 32.3% nuclear grade 2 and 13.0% nuclear grade 3. Necrosis was present in 49.0% of the samples. A mitosis-necrosis score of 0 was assigned in 45.8% of the cases, while 40.1% of the cases was given mitosis-necrosis score 1 and 14.1% mitosis-necrosis score 2.

We found a significant association between solid and trabecular histologic subtypes and higher nuclear grades ($p=0.0008$, Chi-squared test) and mitosis-necrosis scores ($p<0.0001$, Chi-squared test).

Tubulopapillary and microcystic subtypes were associated with longer survivals (median OS=727 and 936 days, respectively), while patients with a predominantly solid or trabecular pattern EMM had shorter median OS (397 and 394 days, respectively). The shortest OS (173 days) was observed among patients with pleomorphic subtype which was significantly worse compared to tubulopapillary,

microcystic and solid subtypes ($p<0.0001$, 0.0085 and 0.0277). Based on their overlapping survival curves we merged microcystic and tubulopapillary variants, as well as trabecular and solid patterns for further survival analyses. Lumped solid/trabecular and tubulopapillary/microcystic subtype groups showed significantly different outcomes (median OS: 397 vs. 732 days, respectively, $p=0.003$).

We found that the composite histologic grading systems were able to predict patient outcomes. Median OS in subgroups with mitosis-necrosis scores 0, 1 and 2, was 720 days, 383 days ($p<0.0001$) and 165 days ($p<0.0001$), respectively. There was no significant difference between nuclear grade 1 and 2 patients median OS (555 days and 486 days, respectively, $p=0.531$), however, nuclear grade 3 was associated with significantly shorter median OS (123 days, $p=0.0002$).

Our findings were confirmed in the validation cohort. Median OS associated with solid/trabecular growth patterns was significantly shorter to tubulopapillary/microcystic tumors (406 vs. 795 days, respectively, $p=0.01$). Nuclear grade 3 tumors had a significantly shorter median OS, than those with lower grade tumors (232 days vs. 823 and 459 days, respectively), as well as mitosis-necrosis score 2 tumors compared to M/N scores 0 and 1 (330 days vs. 795 and 511 days).

4.2 Differences in response to MMT among EMM subtypes

MMT provided a significant benefit within both tubulopapillary/microcystic and solid/trabecular patterns, compared to a non-MMT approach, however, the difference was more pronounced in the tubulopapillary/microcystic predominant subtype. We were able to identify a tendency for better patient outcomes associated with tubulopapillary/microcystic patterns in contrast to solid/trabecular subtypes within the MMT subgroup (1068 versus 580 days, HR: 2.29 [95% CI: 0.95-5.12], p=0.066).

4.3 TERT promoter mutation, clinicopathological characteristics and patient outcomes

We identified TERT promoter mutations in 10.4% of the samples in our cohort of 182 MPM cases including 69.8% epithelioid, 24.2% biphasic and 4.9% sarcomatoid type tumors. TERT promoter mutations were associated with non-epithelioid histologic type ($p<0.001$), the pleomorphic subtype of epithelioid MPM ($p= 0.035$) and advanced stage ($p=0.002$). TERT promoter mutant status was associated with a significantly worse median OS when compared to TERT promoter wild-type samples (262 vs. 469 days, $p<0.0001$). The prognostic impact was also found when analyzing the Austrian and the Croatian-Slovenian patient collectives separately. The significant negative prognostic effect of TERT promoter mutation

was identified within both epithelioid and non-epithelioid histologic subgroups, median OS of mutant and wild-type tumors was 340 and 510 days among the epithelioid, and 199 days vs. 412 days among non-epithelioid tumors. In multivariate analyses we found that TERT promoter status and main histologic types were independent prognostic factors (p=0.011 and p=0.009, respectively).

4.4 Interaction between the common polymorphism rs2853669 and TERT promoter mutation in MPM

There was no significant difference in median OS between carriers and non-carriers of the SNP among TERT promoter mutants (p=0.935). Thus, the presence of the common polymorphism did not eliminate the negative prognostic impact of the promoter mutations

4.5 TERT mRNA expression and TERT promoter status

TERT mRNA expression relative to expression of housekeeping gene RPL41 was significantly higher in cell lines harboring TERT promoter mutations (n=9) compared to TERT promoter wild-type cell lines (n=13).

4.6 TERT promoter status and cell line formation

We attempted to establish a de novo cell line from surgical MPM samples in 45 cases. Of the 22 successfully immortalized tumor cell lines 9 harbored TERT promoter mutations, while neither of the 23 cultures that failed to undergo immortalization did harbor any of

these non-coding mutations. Thus, TERT promoter mutant status was significantly associated with cell line formation ($p<0.001$), while IMIG stage of the original tumor or non-epithelioid histology did not confer a pro-immortalization effect ($p=0.539$ and $p=0.206$, respectively).

4.7 TERT promoter status and in vitro cisplatin sensitivity

We carried out SRB assays in 24 cell MPM cell lines to determine their IC50 values for cisplatin. 7 of the cell lines harbored a TERT promoter mutation, while 17 were TERT promoter wild-type. We did not find a statistically significant difference in cisplatin sensitivity, however we observed that cell lines harboring TERT promoter mutations showed a tendency to have lower IC50 values ($p=0.097$).

4.8 Association between the TERT promoter mutant genotype and BAP1 status.

We found a strong correlation between TERT promoter mutant status and retained BAP1 expression ($p=0.0002$). Among all TERT promoter mutant samples, each exhibited a retained nuclear BAP1 expression, thus TERT promoter mutations and the loss of BAP1 were mutually exclusive in our cohort.

5. CONCLUSIONS

1. Histologic subtypes, nuclear grade and mitosis-necrosis score have a prognostic impact in epithelioid MPM.

2. Higher histological grades and solid/trabecular subtypes show a significant association.

3. Patients with tubulopapillary/microcystic subtype tumors compared to solid/trabecular tumors show a more pronounced benefit from a multimodal treatment approach.

4. TERT promoter mutant status is a strong, independent predictor of poor prognosis in MPM.

5. The TERT promoter mutant status is strongly associated with non-epithelioid histological type and pleomorphic subtype EMM.

6. The common polymorphism rs2853669 C>T – in contrast to literature data on bladder cancer and primary glioblastoma – did not modify the effect of TERT promoter mutation on patient outcomes.

7. TERT promoter mutant tumors exhibited significantly higher probability of de novo cell line formation.

8. TERT mRNA expression was significantly higher among cell lines harboring a TERT promoter mutation.

9. Cell lines with mutant TERT core promoter region show a tendency of higher in vitro cisplatin sensitivity in comparison to TERT promoter wild-type cell lines.

10. Mutations of the TERT core promoter region and genetic alterations of the BAP1 locus are mutually exclusive in MPM.

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