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Malignant pleural mesothelioma with an *EML4-ALK* fusion: expect the unexpected!

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Abstract

Case report of malignant pleural mesothelioma with an *ALK* gene rearrangement, detected by FISH and confirmed by RNA-based next-generation sequencing. The co-occurrence of *ALK* gene fusions with the more common genetic alterations in *CDKN2A*, *NF2* and *BAP1* has, to our best knowledge, not yet been described in malignant mesothelioma. Furthermore, this unexpected finding could suggest a potential target for therapy in this subset of malignant mesotheliomas.

Keywords:

pleural mesothelioma, *ALK* fusion, immunohistochemistry, molecular techniques, copy-number variation sequencing, next-generation sequencing

Introduction

Malignant mesothelioma is a rare aggressive neoplasm, mostly arising from the pleura in patients with a chronic exposure to asbestosis. [1-3] The genomic features of malignant pleural mesothelioma (MPM) include mutations in *BAP1*, *NF2*, *TP53*, *SETD2*, along with deletions of *CDKN2A* and *NF2*. [1,4] Here, we present an exceptional case of an epithelioid MPM with loss of *BAP1*, presence of a *TP53* pathogenic variant, a homozygous deletion of *CDKN2A* and deletions of *NF2*, *LATS2* and *SETD2*. An *ALK* gene rearrangement was

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detected by fluorescence in situ hybridization (FISH) and confirmed by RNA-based next-generation sequencing (RNA-based NGS). The co-occurrence of *ALK* gene fusions with the more common genetic alterations in *CDKN2A*, *NF2* and *BAP1* has, to our best knowledge, not yet been described in malignant mesothelioma. Furthermore, the finding of an *ALK* gene fusion could suggest a potential target for therapy in this subset of malignant mesotheliomas.

Case presentation

The patient was a 78-year-old man, who was admitted to the hospital with increasing dyspnea over the past 14 days. There was no relevant medical history, except for prolonged exposure to asbestos. A chest X-ray scan revealed a complete "white out" of the left hemithorax with a slight deviation of the mediastinal structures to the contralateral right side (Figure 1A). A chest drain was placed and drained 3500cc of serosanguinous fluid over the next 4 days. A computed tomography (CT) scan revealed a heterogenous mass in the upper left lung abutting the pleura (Figure 1B-C) with persistent pleural effusion. As the draining fluid became more sanguineous, single port thoracoscopy was performed during which a large ruptured hilar mass with diffuse bleeding was observed. Hemostasis was achieved with compression and the use of several hemostatic agents. Operative blood loss was 2500cc. Biopsies were taken from the lesion for diagnostic purposes.

Microscopy (Figure 2) of the biopsies showed a cellular, poorly differentiated proliferation composed of nests and sheets of markedly atypical cells with an epithelioid morphology. The tumor cells displayed an enlarged round to oval nucleus with one or more prominent nucleoli and eosinophilic cytoplasm. Some tumor cells showed rhabdoid features with cytoplasmatic eosinophilic globules and an eccentric nucleus. Some pleomorphic multinucleated cells were noted. Focally the tumor cells showed a more discohesive growth pattern with pseudo-alveolar spaces. Co-occurrence of a spindle cell, sarcomatous or desmoplastic tumor component could not be detected. Brisk mitotic activity and marked tumor necrosis were observed.

Immunohistochemistry (IHC) showed a strong and diffuse expression for D2-40 (podoplanin) and ALK in all the tumor cells (Figure 3A-B). There was scattered positivity for calretinin and focal nuclear expression for GATA-3 (Figure 3C-D). IHC for WT1 was negative. All tumor cells showed loss of expression for p16 and BAP1 (Figure 3E-F). There was no expression of TTF-1, Napsin A, Claudin-4, EpCAM, S100, SOX10, SMA, desmin, myogenin, MyoD1, CD34, CD31, ERG, CD45, CD38, MUM1, CD3, CD20, CD79a, CD30, cytokeratin

AE1/AE3, cytokeratin 7, cytokeratin 5, cytokeratin 20, cytokeratin 8-18, p40, p63, CD56, MDM2, NUT, SALL4, SS18-SSX, NKX2.2 and NTRK on IHC. Nuclear expression for SMARCB1, SMARCA2 and SMARCA4 was preserved in the tumor cells. PD-L1 (22C3) showed a weak to moderate cytoplasmatic and membranous staining in 10% of tumor cells, with absence of lymphocytes/macrophages (CPS score 10).

Additional molecular testing was performed. FISH analyses showed an *ALK* rearrangement in 46% of the tumor cells (Figure 4) and a homozygous deletion of *CDKN2A* in 60% of the tumor cells. There was no rearrangement of *MYC*. Chromogenic in situ hybridization for EBV was negative. Further, DNA and RNA extraction from formalin-fixed paraffin-embedded tissue was performed and revealed a *TP53* pathogenic variant by DNA-based next-generation sequencing (DNA-based targeted NGS) and an *EML4* (exon 6)-*ALK* (exon 20) gene fusion by RNA-based targeted NGS. Supplementary, copy number variation (CNV) sequencing was carried out. This showed a complex molecular karyotype with loss of *BAP1* and a homozygous deletion for *CDKN2A*. Deletions for *NF2* (chromosome 22), *LATS2* (13q12.11) and *SETD2* (3p21.13) were observed (Footnote).

Based on the clinical presentation of a mass arising from the pleura, the tumor morphology, the immunohistochemical (loss of expression of BAP1, patchy calretinin expression, no expression for other markers like TTF-1, Napsin A, Claudin-4 and EpCAM) and molecular profile (homozygous deletion of *CDKN2A*) the diagnosis of an epithelioid MPM was made.

Unfortunately, after the diagnosis the patient refused all medical treatment and died a few weeks later. Time between surgical biopsy and death was 54 days.

Discussion

We describe a case of an epithelioid MPM with a peculiar genetic profile, detected and confirmed by the use of different molecular techniques. Loss of BAP1 seen on IHC was confirmed by CNV-sequencing. There was no positivity for p16 on IHC, confirmed by FISH and CNV-sequencing showing a homozygous deletion of *CDKN2A*. CNV-sequencing also revealed deletion of *NF2*, *LATS2*, and *SETD2*. A *TP53* pathogenic variant was detected by DNA-based targeted NGS. All these genetic alterations are known to be present in malignant mesothelioma. [1-2,4-6] The finding of a *TP53* pathogenic variant (present in 8% of mesotheliomas) and homozygous deletion for *CDKN2A* (present in 67-83% of mesotheliomas) has been reported to be associated with a poor survival. [1,7]

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Noteworthy, our case showed overexpression of ALK on IHC leading to the detection of an *ALK* gene rearrangement by FISH and confirmation of an *EML4* (exon 6)-*ALK* (exon 20) fusion with RNA-based targeted NGS. The presence of *ALK* rearrangements can be detected in lung adenocarcinoma, with *EML4* as common fusion partner. Although *ALK* rearrangements are heterogeneous, the breakpoint mostly lies in intron 19 in lung adenocarcinoma. The breakpoint within *EML4* is also variable, but occurs most commonly in intron 13, 20, and 6 in lung adenocarcinoma. [8] As our case showed an *EML4* (exon 6)-*ALK* (exon 20) the main differential diagnosis therefore includes a non-small lung carcinoma, type adenocarcinoma. However, we observed features that strongly favor the diagnosis of a MPM over that of an adenocarcinoma in this case. First, the clinical and radiological presentation, with exposure to asbestosis and a large lesion abutting the pleura with an aggressive nature and pleural effusion, leads more in the direction of a MPM. Next, immunohistochemically the tumor showed no expression of TTF-1, Napsin A, Claudin-4, EpCAM, cytokeratin AE1/AE3 and cytokeratin 7, while calretinin and D2-40 were positive in the tumor. Loss of BAP1 expression is more frequently seen in mesothelioma, although it can very rarely be detected in lung adenocarcinoma. [9, 10]

Further, the molecular CNV-sequencing profile, in which deletions predominate over gains with deletion of *NF2*, *LATS2*, *SETD2* and *CDKN2A*, strongly favors the diagnosis of a mesothelioma [6, 11-13].

Additionally, electron microscopy (EM) was executed, which showed no further pathognomonic features leading into the direction of a mesothelioma (no long thin apical microvilli, no intracellular canaliculi) nor an adenocarcinoma (no perinuclear tonofilament bundles, no basal lamina, and no long desmosomes). However, it is known that the majority of poorly differentiated MPM cases lack the specific features at EM. [14-15]

Next, DNA methylation profiling was performed using the cfRRBS method. [16] This novel technique is based on Reduced Representation Bisulfite Sequencing (RRBS) but has some adjustments to work with highly fragmented DNA, such as cell-free DNA or FFPE-DNA. After sequencing, methylation calling was performed with Bismark [17] and neighboring CpGs (within 100bp distance) are clustered together. As every tissue sample is a mixture of different cell types, a deconvolution algorithm called meth_atlas [18] was used to estimate the relative contribution of each cell type. The algorithm was trained on a reference dataset composed of public methylation. DNA methylation profiling and subsequent deconvolution

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pointed to mesothelioma as the best fitting diagnosis out of the 13 included tumor types (Supplementary Data).

Moreover, the presence of an *EML4-ALK* fusion has been described in malignant peritoneal mesotheliomas occurring in young woman, without a history of exposure to asbestosis. [2,19-21] To the best of our knowledge, there are 2 reported MPM harboring an *EML4-ALK* fusion. Leal et al. described the presence of an *EML4-ALK* fusion in 1 MPM, without mentioning other molecular alterations or clinical information. The described fusion by Leal et al. involved exon 13 of the *EML4* gene and exon 20 of the *ALK* gene.[5] This is different from our case. Bronte et al. reported a case of a 45-year-old woman with an epithelioid MPM harboring an *EML4-ALK* fusion detected by IHC and FISH. This fusion however could not be detected in the brain metastasis, suggesting a potentially false positive *ALK* fusion in the pleural biopsy. [22-23] Hung et al. showed that *ALK*-rearranged malignant peritoneal mesotheliomas harbored no loss of chromosomal region 9p (including *CDKN2A*) or 22q (including *NF2*) or genetic alterations in *BAP1* or *SETD2*, which are typically present in peritoneal and pleural mesotheliomas. They also studied 205 patients with pleural mesothelioma but did not find ALK positivity by IHC. [2] In another cohort, Hung et al. characterized 3 different subgroups of localized pleural mesothelioma including *BAP1*-mutated, *TRAF7*-mutated, and near-haploid subgroups. However, no *ALK* gene rearrangements were found in these groups. [4]

Finally, histology of the tumor showed no different cell populations, nor intratumoral immunohistochemical heterogeneity. Therefore, we have, based on histomorphology of the received biopsies, no indications for a collision tumor or tumor heterogeneity. Still, we must always keep in mind that de diagnosis was made on biopsies. However, it concerned rather large surgical, no true-cut biopsies, in this case. This greatly reduces the chance of sample error.

The finding of an *ALK* fusion in a malignant mesothelioma suggests a valuable asset for application of targeted therapy. This finding could be crucial in these aggressive neoplasms since current treatment with chemotherapy is only effective in 25-30% with no salvage therapy after failure of first-line treatment. [2-3] The group of Mönch et al. showed already that pleural mesotheliomas with ALK overexpression could benefit from therapy with ALK inhibitors *in vivo*. [3] Also, Rüschoff et al. described a 13-year-old girl, with peritoneal mesothelioma harboring a *STRN-ALK* gene fusion, who showed a substantial tumor regression after treatment with the ALK-targeting drug crizotinib. [24] Another possible

therapy in adults with pleural mesothelioma is the use of PD-L1 inhibitors. [25] A recent study showed that pleural mesothelioma with loss of *BAP1* tend to show a higher expression of PD-L1 and have a prominent mRNA signature of activated dendritic cells, suggesting that loss of *BAP1* could be a predictive marker for immunotherapy. [26] The case of the MPM described by Bronte et al. showed PD-L1 positivity, in 10% of the tumor cells in the pleural biopsy and in all the tumor cells of the brain metastasis, with a good clinical response to pembrolizumab. However, the authors did not describe BAP1-loss in this report. [23] PD-L1 IHC in our case showed a weak to moderate cytoplasmatic and membranous staining in 10% of tumor cells. Unfortunately, our patient refused all medical treatment and died several weeks later, emphasizing the aggressive nature of these tumors.

Conclusion

We described a case of an aggressive epithelioid MPM showing loss of *BAP1* and a *TP53* pathogenic variant, a homozygous deletion of *CDKN2A* and deletions of *NF2*, *LATS2* and *SETD2*. An *EML4-ALK* fusion was identified and confirmed by the use of different (molecular) techniques. We showed that *ALK* gene fusions, although a very rare phenomenon in MPM, can occur and that the finding of an *ALK* gene fusion and other more common genetic alterations seen in malignant mesothelioma (including *CDKN2A*, *BAP1*, *NF2* and *SETD2*) are not mutual exclusive, in contrast to what has been described in the literature. More studies are necessary to explore the possible advantage of the use of ALK targeted therapy in MPM cases with an *ALK* rearrangement.

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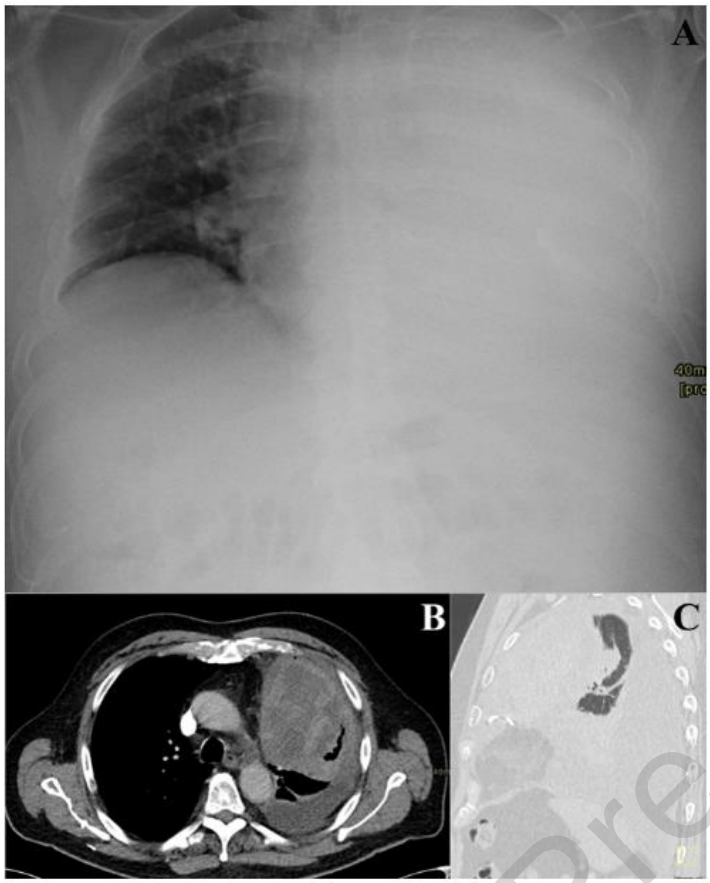

Figure 1: A Anteroposterior chest X-ray at hospital admission shows a complete "white out" of the left hemithorax. There is a slight deviation of the mediastinal structures to the contralateral right side. **B-C** Chest CT 4 days later, after placement of a chest tube. B Axial CT image (soft tissue window) shows a heterogenous mass in the upper left lung abutting the pleura. Note also a left pleural effusion. C Sagittal reformatted CT (lung window) shows almost complete collapse of the left lung.

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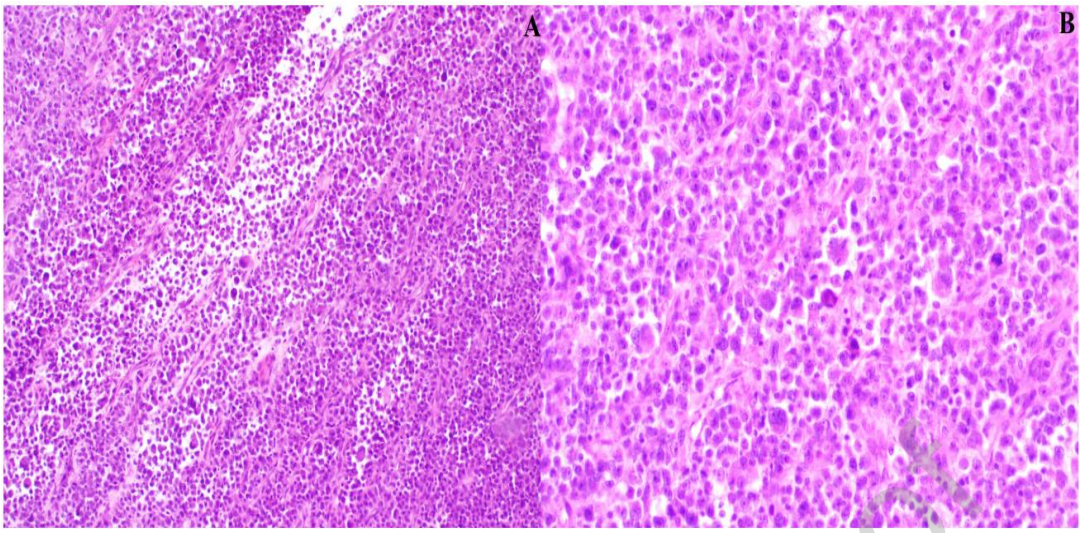

Figure 2: A Cellular, poorly differentiated proliferation composed of sheets of epithelioid cells with focal discohesive growth (original magnification 100x). **B** Higher magnification of atypical epithelioid cells, often with a pleomorphic aspect (original magnification 200x).

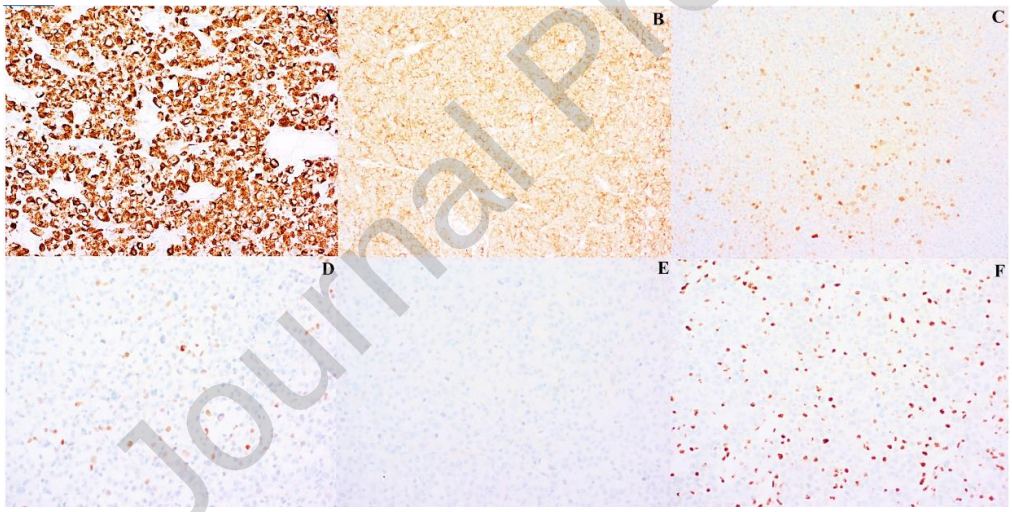

Figure 3: Immunohistochemistry: tumor cells show a strong and diffuse expression for ALK (A) and D2-40 (B). There was scattered positivity for calretinin (C) and focal nuclear expression for GATA-3 (D). There was loss of p16 (E) and BAP1 (F). (Original magnification 200x)

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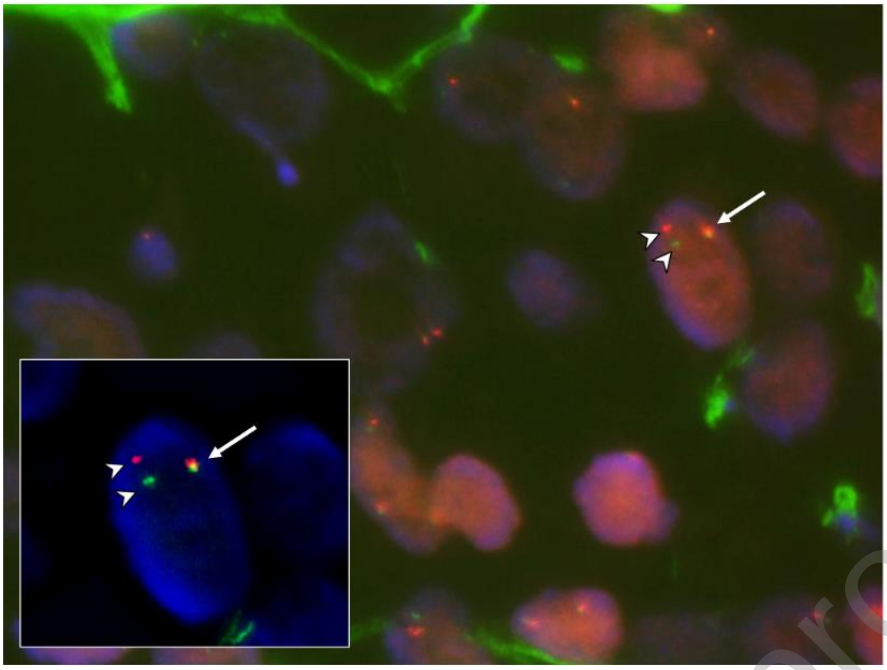

Figure 4: Photomicrograph of the dual-color fluorescence in situ hybridization (FISH) used for the detection of *ALK* translocation: tumor cel with one fusion (yellow, yellow arrow), one green and one red split (arrowhead) signal pattern, indicating the presence of rearrangement.

Footnote: result of CNV-sequencing

del(1)(p31.1p12),dup(1)(q25.2q31.3),del(1)(q31.3q41),dup(1)(q41q44),del(3)(p24.3p12.1,del(4)(q12q35.2),del(6)(q14.1q16.1),del(7)(q31.31q36.2),del(9)(p24.1p11.2),hom del(9)(p21.3),-13,del(14)(q12q32.33),del(15)(q13.1q15.3),del(16)(q12.1q24.3),del(17)(p13.3p11.2),-19,-22

Supplementary data:

Reference dataset used for methylation profiling:

TCGA Infinium HumanMethylation450 BeadChip data from 13 tumor types: melanoma (SKCM), lung adenocarcinoma (LUAD) and squamous cell carcinoma (LUSC), esophagus adenocarcinoma and squamous cell carcinoma (ESCA), breast (BRCA), stomach (STAD), pancreas (PAAD), bile duct (CHOL), prostate (PRAD), ovarian (OV), colon carcinoma (COAD), mesothelioma (MESO) and 12 types of healthy tissue: breast, gallbladder, colon, esophagus-glandular, esophagus-squamous, head&neck-squamous, lung-glandular, lung-squamous, pancreas, prostate, skin and urothelium. Small cell lung cancer Infinium BeadChip data was obtained from Poirier et al.[27] and healthy white blood cell WGBS data from Sun et al.[28]

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Compliance with Ethical standards: Ethical approval for this study was obtained from the ethical committee of Ghent University Hospital (EC/0092-2021).

Informed Consent: Informed consent was obtained from the patient.

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Contributions: Fleur Cordier performed the writing of the paper. Joni Van der Meulen, Nadine van Roy, Jilke De Wilde, Herwig van Dijck, Filip Vanhoenacker, Marc Lambrechts, Valentin Noyez, Koen Van de Vijver, Liesbeth Ferdinande, Amélie Dendooven, Jo Van Dorpe and David Creytens performed the study concept, design and review of the paper. All authors read and approved the final paper.

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