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PRIMARY CILIUM LOSS IN ADVANCED MESOTHELIOMA CORRELATES WITH CONSTITUTIVE GLI1 OVEREXPRESSION

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Abstract

Malignant mesothelioma is an aggressive cancer of the membranes covering the lung and chest cavity (pleura), or the abdomen (peritoneum), mainly linked to asbestos exposure. It is characterized by high intrinsic heterogeneity, diagnosis in the late stages and a high immunosuppressive microenvironment. In the past years many agents have been evaluated for use in mesothelioma but with modest results so that the prognosis remains poor. Recently, in light of the promising results achieved in other cancers, the targeting of the Hedgehog-GLI (HH-GLI) pathway has been investigated as possible new therapy for MPM cure. The HH-GLI pathway starts at Primary Cilium (PC), an organelle protruding from the extracellular membrane of the cells that expresses specific receptors for the Hedgehog ligands. In cells lacking PC, the HH-GLI pathway can also be activated by intracellular signaling that make cells resistant to HH-GLI ligand-dependent pathway inhibitors. In MPM the HH-GLI signaling is active but response to targeting agents is poor. Activating mutations in the core components of the pathway, that in other cancers lead to drug resistance, in MPM are rare. Here we studied the presence of PC in mesothelioma and its correlation with HH-GLI pathway activation. We found an heterogeneous presence of PC in MPM and, in the cells loosing PC, GLI1 was overexpressed. Our preliminary results suggested that PI3K/AKT pathway can be, at least in some cells, responsible for the activation of HH-GLI1 pathway.

In summary, we have documented for the first time the loss of PC in mesothelioma and the activation of a non-canonical HH-GLI pathways in this cancer.

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1 INTRODUCTION

1.1 Malignant Pleural Mesothelioma

Malignant pleural mesothelioma (MPM) is an aggressive cancer affecting the pleural membranes covering the lungs. The inner and the outer membranes of the pleura form the pleural space which occupy a crucial role in the respiratory system facilitating the movements of the lungs for respiration.

MPM is principally related to asbestos exposure in the past and, even if it is recognized as a human carcinogen, it is actually banned in only 30% of the world. For this reason, the incidence of mesothelioma is not expected to decrease in the next years. Moreover, the increasing use of carbon nanotubes (CNT), a nano-material used in the field of nanotechnology, has recently posed the attention of the scientific community for its physico-chemical characteristics similar to that of asbestos. CNTs carcinogenic potential is subject of intense research and, even if is not yet recognized as a human carcinogen, there are strong evidences of their dangerous effects on the respiratory system, as we recently reviewed in [1] .

1.1.1 Histopathology and diagnosis

Three histological subtypes of MPM can be distinguished, having different incidence and prognosis. Epithelioid MPM represent the majority of the cases (≃70%) and is correlated to a better prognosis, whilst sarcomatoid (≃10% of cases) and mixed, or biphasic, (≃ 20% of cases) histotypes are the more aggressive with a poor prognosis.

For the lack of specific markers, the diagnosis of MPM can be very challenging. Moreover, the symptoms mainly manifest in the late stages of the disease compromising the tolerance to treatment and limiting the therapeutic options.

1.1.2 Genetics

MPM is characterized by a low tumor mutation burden, uncommon genetic aberrations, and recurrent somatic mutations in tumor suppressor genes, in both asbestos and non-asbestos induced tumors [2] .

The first and most common mutation described in mesothelioma is the deletion of the *CDKN2A* gene on chromosome 9 [3] , encoding for cell cycle regulating proteins $p16^{INK4a}$ and $p14^{ARF}$, accounting for approximately 70% of MPM cases[4] . The deletion of this gene determines the loss of function in the p16 and p14 proteins thus affecting the cell cycle regulating function of pRB and p53. In CDKN2A positive MPM cells have also be described the silencing of CDKN2A by hypermethylation of p16/Ink4a and p19/Arf [5] .

For its proximity to CDKN2A, the methylthioadenosine phosphorylase (MTAP) gene is frequently co-deleted in different cancer types including MPM [6, 7] . The MTAP gene encodes a key enzyme in the adenosine and methionine salvage pathway resulting in alterations in polyamine metabolism and ATP production, and is considered a tumor suppressor gene. MTAP loss determines the accumulation of the MTA substrate, a natural inhibitor of protein arginine methyltransferase 5 (PRMT5). Similar to other cancers, it has been demonstrated in mesothelioma that the loss of *MTAP*, and the subsequent accumulation of its substrate, generates a targetable vulnerability that can be therapeutically exploited by PRMT5 pharmacological inhibition [8, 9] .

Other common mutations in mesothelioma are localized in chromosome 3, involving the loss of the *BAP1* gene, in the chromosome 22 enclosing the Neurofibromin2 (NF2) gene, accounting for approximately 60% of MPM cases, and in *TP53* [2, 10–12] .

BRCA1-associated protein–1 (BAP1) is a member of the ubiquitin C-terminal hydrolases (UCH) subfamily of deubiquitylating enzymes (DUBs) [13] . BAP1 has many biological activities including genome stability, DNA damage repair, modulation of the cellular metabolism, regulation of transcription and cell death, among others. *BAP-1* loss, together with *MTAP/CDKN2A* deletion, has been recently proposed as useful markers to improve the diagnostic sensitivity for MPM [14] . Germline mutation or deletion in BAP1 have also been described in about 1% of mesothelioma [15] .

Mutation in the *NF2* gene, encoding the cell growth-regulating protein Merlin, has been described in about 50% of MPM [16] and has been linked to mesothelioma progression in both Hippo- dependent and independent manner. Alteration in NF2 function has been recently related to tumor immune microenvironment and proposed as biomarker for MPM patients stratification for immune-checkpoint blockade (ICB) therapies [14] .

1.1.3 Current therapies and new approaches under investigation

The intrinsic heterogeneity of MPM, the diagnosis in the late stages and the high immunosuppressive microenvironment are the main mechanisms underlying the poor prognosis for MPM patients.

When the clinical parameters allow the standard treatment with platinum-based chemotherapy combined with an antifolate, and the optional addition of bevacizumab, only a small chance of improvement is achieved for advanced stages [17] [18] .

In conjunction with chemotherapy in patients with a good performance status, radiotherapy can provide local tumor control.

Surgical therapy in patients who can handle the surgical risks and who have a low tumor stage, involves the partial removal of tumor mass and pleura and can significantly improve the quality of life of the patient without creating severe side effects.

Over the last 20 years, many agents have been also evaluated for use in mesothelioma but with modest results [19] . Among these, targeted therapies that have shown benefits in other tumors, such as tyrosine kinase, mTOR and histone deacetylase (HDAC) inhibitors, and monotherapy with immune checkpoint inhibitors anti-PD1 and CTL4-4, have failed to improve survival in MPM patients [20–22] .

Very recently, the US Food and Drug Administration approved the first new treatment in 16 years with anti-PD1 (nivolumab) in combination with anti-CTLA-4 (ipilimumab) [23] . This new treatment have shown promising activity in unresectable MPM or in MPM relapsed after first-line therapy [24] .

Other immunotherapic strategies such as dendritic cells immunotherapy[25] , mesothelin-chimeric antigen -T (mesothelin CAR-T) cells [20] and pluripotent stem cells (iPSC) vaccines [26] are under investigation.

1.2 THE HEDGEHOG-GLI PATHWAY

Recently the targeting of the Hedgehog-GLI (HH-GLI) signaling have been investigated as possible new therapy for MPM cure in light of the promising results achieved in other cancers [27] .

The HH-GLI signaling is a conserved pathway playing a central role in the maintenance of tissue development and homeostasis, regulating important processes such as embryonic development, stem cell maintenance, cellular proliferation and differentiation [28] . It is an intricate but highly regulated signaling involving the binding of extracellular ligands to specific receptor proteins that activate intracellular molecules, transcription factors and target genes. The three ligands, – Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh) – can be secreted in both autocrine and paracrine manner (Figure 1) activating the G-protein-coupled receptor smoothened (SMO), allowing GLI transcription factors to translocate into the nucleus and transcribe the target genes. When ligands are absent, SMO is inhibited by PTCH and the member of GLI family of transcription factors are processed to generate a repressor form. This ligand-mediated activation of the HH pathway is called canonical HH pathway.

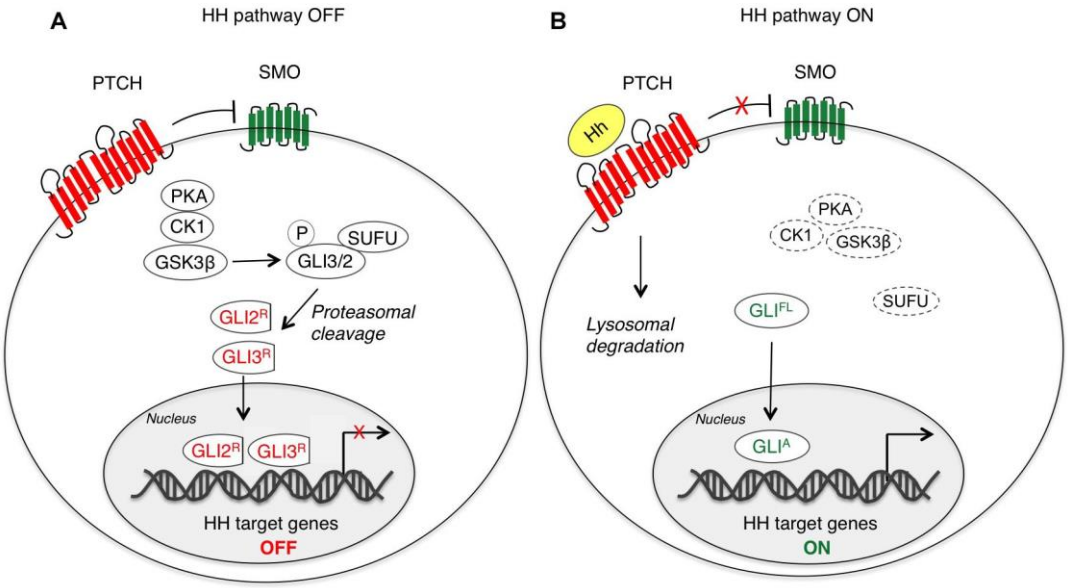

Figure 1. Canonical activation of HH-GLI signaling. A) When ligands are absent, SMO is inhibited by PTCH and therefore the member of GLI family of transcription factors are processed to form a repressor form. B) In presence of HH ligands, SMO is activated and the GLI proteins can translocate into the nucleus to transcribe target genes [29] .

The HH pathway can also be activated through "non-canonical/SMO-independent" signaling involving the contribution of many different pathways. Among others, PI3K/AKT, RAS-RAF-MEK-ERK signaling, KRAS constitutive activation, and TGF-beta have been identified. The intricate network of signals activating HH in non-canonical manner have been recently reviewed by Pietrobono et al. [29] (Table 1).

In physiologic condition the HH pathway is repressed in developed tissue. Deregulation or constitutional activity of the pathway can lead to pathological conditions like cancer.

Table 1. Mechanisms of non-canonical activation of Hedgehog-GLI signaling. [29]

Upstream Regulator	Mechanism of action	Cancer/Cell type	References
RAS-RAF-MEK-ERK			
MEK1/2-ERK1/2	Increases expression of Gli target genes; Gli1 required for KRAS-driven transformation	KRAS-driven PDAC mouse model	Nolan-Stevaux et al., 2009
	Increases GLI1/2 transcriptional activity	NIH3T3	Riobò et al., 2006a
		Melanoma	Stecca et al., 2007
		PDAC	Ji et al., 2007
		Gastric cancer	Seto et al., 2009
		Colon cancer	Mazumdar et al., 2011
		LAC	Po et al., 2017
	Increases GLI1 nuclear localization	Melanoma	Stecca et al., 2007
	Induces GLI1 protein stability	PDAC	Ji et al., 2007
	Induces GLI2 protein stability	BCC	Kasper et al., 2006
MEK1/2-RSK2	Promotes GLI2 nuclear localization and stabilization	Multiple myeloma	Liu et al., 2014
MAPKKK/MEKK			
MEKK1	Inhibits GLI1 transcriptional activity	MB	Antonucci et al., 2019
MEKK2/3	Inhibits GLI1 transcriptional activity and protein stability through SUFU	MB	Lu et al., 2018
PI3K/AKT/mTOR			
AKT	Increases Gli2 transcriptional activity	NIH3T3	Riobò et al., 2006b
	Increases GLI1 transcriptional activity and nuclear translocation	Melanoma	Stecca et al., 2007
	Enhances GLI1 protein stability	PDAC, ovarian cancer	Singh et al., 2017
	Prevents GLI degradation (GSK3β-dep.)	ALCL	Singh et al., 2009
mTOR/S6K1	Enhances GLI1 activation preventing SUFU association	EAC	Wang et al., 2012
p70S6K2	Prevents GLI1 degradation (GSK3β-dep.)	NSCLC	Mizuarai et al., 2009
		EAC	Kebenko et al., 2015
TGFβ	Increases GLI2 transcription (SMAD3-dep.)	PDAC, BC	Dennler et al., 2007, 2009
	Increases GLI2 expression	Colon CSC	Tang et al., 2018
	Stimulates GLI1 transcriptional activity (PCAF-dep.)	PDAC	Nye et al., 2014
PKC signaling			
PKCα	Reduces GLI1 transcriptional activity	HEK-293T	Neill et al., 2003
	Increases GLI1 transcriptional activity	Hep3B, NIH3T3	Cai et al., 2009
PKCδ	Increases GLI1 transcriptional activity	HEK-293T	Neill et al., 2003
	Reduces GLI1 transcriptional activity	Hep3B, NIH3T3	Cai et al., 2009
aPKCι/λ	Enhances DNA binding and GLI1 transcriptional activity	BCC	Atwood et al., 2013
DYRK family			
DYRK1A	Promotes GLI1 nuclear translocation	NIH3T3, HEK-293T	Mao et al., 2002; Shimokawa et al., 2008; Ehe et al., 2017
	Induces GLI1 degradation, mediated by F-actin and MKL1	Lung carcinoma, rhabdomyosarcoma	Schneider et al., 2015
DYRK1B	Enhances GLI1 transcriptional activity	PDAC, MB	Gruber et al., 2016
DYRK2	Induces GLI2 protein degradation	NIH3T3	Varjosalo et al., 2008
Oncogenic drivers			
EWS/FLI1	Induces GLI1 transcription	Ewing sarcoma	Zwerner et al., 2008; Beauchamp et al., 2009
SOX9	Prevents βTrCP-mediated GLI1 degradation	Pancreatic CSC	Deng et al., 2015
FOXC1	Enhances GLI2 transcriptional activity	Basal-like BC	Han B.C. et al., 2015
c-MYC	Enhances GLI1 transcription	Burkitt lymphoma	Yoon et al., 2013
IKKβ	Promotes GLI1 stability	DLBCL	Agarwal et al., 2016
SRF-MKL1	Induces GLI transcription and enhances DNA binding	BCC	Whitson et al., 2018
WIP1	Enhances GLI1 transcriptional activity, nuclear localization and protein stability	Melanoma	Pandolfi et al., 2013
Tumor suppressors			
p53	Inhibits GLI1 transcriptional activity, nuclear translocation and protein stability	Glioblastoma	Stecca and Ruiz i Altaba, 2009
	Promotes proteasome-dependent degradation of GLI1 (PCAF-dep.)	MB	Mazzà et al., 2013
	Interferes with DNA binding ability of GLI1 (TAF9-dep.)	Rhabdomyosarcoma, Osteosarcoma	Yoon et al., 2015
NUMB	Induces GLI1 ubiquitination and proteasome degradation (ITCH-dep.)	MB	Di Marcotullio et al., 2006, 2011
SNF5	Interferes with promoter occupancy of GLI1	Rhabdoid Tumors	Jagani et al., 2010
miRNAs			
miR-324-5p	Represses GLI1 expression	CGCPs	Ferretti et al., 2008
miR-361	Represses GLI1 expression	Prostate cancer	Chen et al., 2017
	Represses GLI1 and GLI3 expression	Retinoblastoma and CSC	Zhao and Cui, 2019
miR-326	Represses GLI2 expression	Ptch+/− MB CSC	Miele et al., 2017
BET proteins			
BRD4	Increases GLI1/2 transcription	BCC	Tang et al., 2014
		MB	Long et al., 2014
BET	Upregulates Gli1 in murine CAFs	PDAC	Yamamoto et al., 2016
BET	Promotes GLI occupancy on target promoters	PDAC	Huang et al., 2016
HDAC			
HDAC	Stimulates GLI1 nuclear localization and transcriptional activity	Multiple Myeloma	Geng et al., 2018
HDAC class I	Increases DNA binding ability of GLI1 (HDAC1)	MB MB, murine BCC	Canettieri et al., 2010 Gruber et al., 2018
HDAC class II	Transcriptional control of GLI2 (HDAC6)	MB	Dhanyamraju et al., 2015
HAT			
p300	Prevents GLI2 recruitment to chromatin	HEK-293T, NIH3T3	Coni et al., 2013
PCAF	Acts as GLI1 transcriptional cofactor	Glioblastoma, MB	Malatesta et al., 2013
	Promotes GLI1 ubiquitination and proteolysis	MB	Mazzà et al., 2013
PRMTs			
PRMT1	Enhances DNA binding ability of GLI1	PDAC	Wang et al., 2016
PRMT5	Enhances GLI1 protein stabilization and nuclear translocation	C3H10T1/2, HEK-293T, SCLC	Abe et al., 2019
	Inhibits GLI1 expression through Menin1	Neuroendocrine tumors	Gurung et al., 2013

ALCL, anaplastic large cell lymphoma; β-TrCP, β-transducing repeat-containing protein; BC, breast cancer; BCC, basal cell carcinoma; BET, bromo- and extra-terminal domain; CAF, cancer-associated fibroblats; CSC, cancer stem cells; DLBCL, diffuse large B-cell lymphoma; DYRK, dual-specificity tyrosine phosphorylation-regulated kinase; EAC, esophageal adenocarcinoma; CGCPs, cerebellar granule cell precursors; GSK3β, glycogen synthase kinase 3β; HAT, histone acetyltransferase; HDAC, histone deacetylase; LAC, lung adenocarcinoma; MEF, mouse embryonic fibroblasts; NSCLC, non-small cell lung cancer; PCAF, p300/CREB-binding protein-associated factor; PDAC, pancreatic ductal adenocarcinoma; PKC, protein kinase C; PRMT, protein arginine methyltransferases; SCLC, small cell lung cancer; SUFU, Suppressor of Fused.

1.2.1 HH-GLI pathway deregulation and cancer

In cancer the aberrant activation of the HH pathway is mainly due to loss of function of PTCH1 negative regulatory functions or activating mutation of SMO, as well as overproduction of the HH ligands. Mutations in the canonical signaling has been described in cancers of the skin, brain, liver, gallbladder, pancreas, stomach, colon, breast, lung, prostate, and hematological malignancies [30] .

Among these tumors, called SHH-dependent, for basal cell carcinoma (BCC) and acute myeloid leukemia (AML) patients who are not candidates for chemotherapy, the use of SMO inhibitors has been approved by the USA Food and Drugs Administration and European Medicines Agency [31–33] .

Many clinical trials are also ongoing, with Vismodegib or other SMO inhibitors (Sonidegib, Glasdegib, Saridegib, Taladegib), on other SHH-dependent cancers such as pancreatic, multiple myeloma, triple-negative advanced breast, ovarian cancer and other solid tumors, as recently reviewed in [34] .

However, resistance to therapy with SMO inhibitors can occur due, among other mechanisms, to SMO mutations, amplification of downstream HH pathway components and intracellular activating pathways including phosphatidylinositol 3-kinase (PI3K) kinase [35] .

1.2.2 The HH-GLI pathway in MPM

The HH-GLI pathway is activated in the mesothelium during the development and inactivated in the adult tissue [36] [37] . It has been hypothesized that in MPM the HH-GLI pathway can be activated in a ligand-dependent manner during the repair of the

damaged tissue after mineral fibers exposure [38] . Despite the activation of HH signaling has been described in MPM [36] and SMO levels has been correlated to a poor prognosis [39, 40] , the involvement of HH-GLI signaling in mesothelioma is still subject of debate [41] .

Most of the studies on the effect of ligand-mediated HH-GLI pathway inhibition by SMO antagonists are from in vitro and in vivo studies. Indeed, in phase I trials in solid tumors with SMO antagonists Vismodegib and Sonidegib, which includes 5 MPM, responses were observed only in BCCs and medulloblastoma cases [42, 43] .

However, in this study, activation of the HH-GLI pathway was not investigated.

Differently from other tumor types, mutations in the component of the pathway are rare in mesothelioma and mutation in only one member of the pathway, SMO, has been found in 15.6% of patients [39] .

As far, the activation of HH signaling in MPM has been demonstrated to be ligand-dependent and only mediated by DHH, in both autocrine and paracrine manner [36] .

However, in light of limited response to SMO-inhibitors in vitro and few data from clinical studies, biomarkers for selection of MPM patients who can benefit from Hedgehog signaling inhibition, as well as mechanisms of resistance to SMO inhibitors, are far to be identified.

1.3 THE PRIMARY CILIUM

Primary Cilia (PC) are microtubule-based organelle protruding from the extracellular membrane containing different transmembrane receptors [44] . Differently from cilia, primary cilia are non-motile and are present in a single copy on the extracellular membrane of vertebrate cells.

From the PC start signals from soluble factors (including Sonic hedgehog (SHH), platelet-derived growth factor (PDGF-AA), WNT, TGFβ, NOTCH, G-protein) as well as mechanosensory stimuli provided by flow or extracellular matrix [45, 46] . Development of PC is regulated by several pathways and ciliary assembly and disassembly depend upon the cell cycle progression. PC regulates in turn the activation of intracellular pathways and cell proliferation, therefore is considered a tumor suppressor organelle.

The exhaustive comprehension of all its functions is still an active field of research and, in cancers, seems to have tissue-specific functions (Fig. 2).

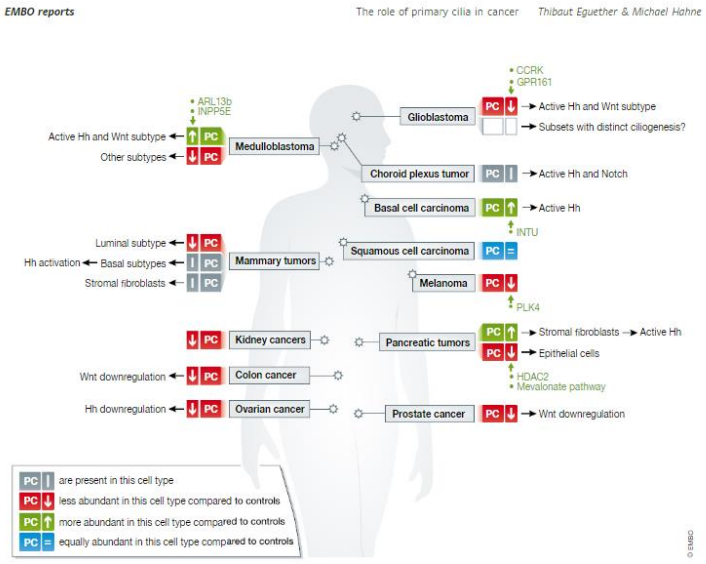

Figure 2. Overview on reported functions of PC in different cancer type [46]
ARL13b: ADP ribosylation factor like GTPase 13B; CCRK: cell cycle-related kinase; Gpr161: G-protein-coupled receptor 161; HDAC2: histone deacetylase 2; INPP5E: inositol-polyphosphate-5-phosphatase E; Intu: inturned planar cell polarity protein; PLK4: Polo-like kinase 4

1.3.1 The Primary Cilium and the HH-GLI pathway in cancer

Due to its important role in several tumors, the most studied PC-dependent signaling in cancer is the HH-GLI pathway [47–49] .

On the PC are located the core components for the HH-GLI signaling transduction in response to the presence of HH ligands in the extracellular space. The binding of the these ligands to specific receptors localized in the ciliary membranes activated downstream events in cells leading to transcription of target genes. Thus, Canonical HH-GLI pathway depends on the presence of functional PC (Fig. 3).

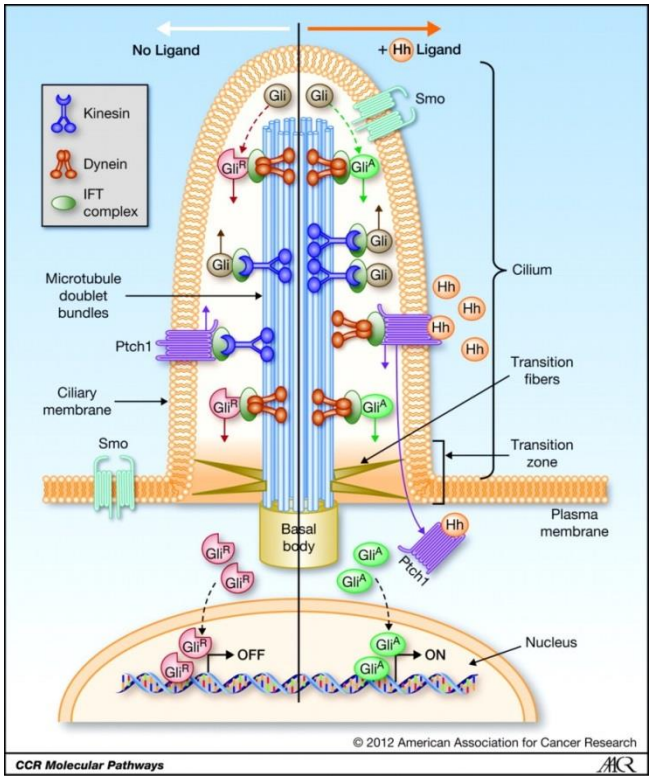

Figure 3. Canonical activation of the HH pathway through Primary Cilium.[50]

The best described receptor is PTCH1 which, in presence of HH ligands, exits from the cilium membrane releasing SMO from its inhibition. The entry of SMO in the cilium starts the signaling cascade leading to GLI-mediated target genes expression. Among these, GLI itself, and the repressors PTCH1 and HHIP, are used as markers for HH-GLI pathway activation.

When mutations in the component of HH-GLI signaling cascade are present, cilia can function both as activator or repressor of the pathway.

Many cancers loss PC and only a minority retains functional PC [46] . In tumors exhibiting activating mutation in SMO, removal of cilia has been demonstrated to inhibit cancer growth. On the contrary, in tumors with constitutional activation of GLI proteins, ciliary ablation accelerates tumors. These dual role of cilia in promoting or repressing the HH-GLI pathway made the PC a mediator and a repressor of tumorigenesis [51] .

From a therapeutic point of view, the presence of cilia can be a useful marker for the identification of a subset of oncologic patients that can respond to SMO antagonists.

As well, in SMO-independent tumors with activated GLI proteins, restoring PC may be a therapeutic strategy.

1.3.2 The Primary Cilium in mesothelioma

Differently for other cancers, there are few information available about the presence and the role of PC in MPM. As far, only a study has documented the presence of PC in the stem cell population of one primary mesothelioma cell line [52] .

Interestingly, a recent in silico transcriptomic analysis of PC-related components in mesothelioma patients, revealed that the expression of BBS2 and BBS12 gene encoding

for two proteins involved respectively in cilia formation and function, and in ciliogenesis regulating transports vesicles to the cilia [53] , favors survival in mesothelioma patients. Moreover in this study the expression of these genes was found to be increased in the epithelioid histological subtype compared to the biphasic phenotype [54] .

2. AIMS

The therapeutic potential of targeting of the Hedgehog-GLI pathway is becoming clear in many types of cancer. Many inhibitors of the pathway are under clinical investigation and different SMO-inhibitors are already approved for the clinical use in some cancer.
However the lack of specific markers that allow the stratification of patients that could benefit from the treatment with SMO-inhibitors, as well as the development of resistance due to the activation of non-canonical HH-GLI pathways, limit the therapeutic successes.
Primary cilia have a major role in the regulation of the HH-GLI pathway, both activating or repressing tumors, depending on the presence of specific mutations in the component of the HH-GLI pathway. Therefore, inhibiting or promoting the disruption of PC can be a valuable strategy to improve the pharmacologic targeting of the HH-GLI signaling.
MPM is a cancer with limited therapeutic options. Recently it has been demonstrated that HH-GLI pathway is activated in mesothelioma, but the precise molecular mechanisms, the contribution of the canonical and non-canonical activating pathway, and the presence and the role of PC in MPM tumorigenesis has not thoroughly investigated.
Here we investigated the presence of PC in formalin fixed- paraffin embedded (FFPE) MPM specimens and in primary MPM cell lines, together with the expression of GLI1 and PTCH1 as markers of HH-GLI pathway activation. The aim of this work is to provide novel insight into the molecular mechanisms underlying MPM tumorigenesis. We also aim to help the understanding of the role of PC in mesothelioma, its correlation with HH-GLI pathway activation in MPM, and the contribution of non-canonical activating pathways. This work could provide new target to investigate for MPM treatment.

3. MATERIALS AND METHODS

3.1 Immunohistochemical analysis

Formalin-fixed, paraffin-embedded tumor specimens were obtained from the Section of Pathology, Siena Hospital, Siena, Italy. From each tissue, 4-µm-thick paraffin sections were prepared for immunohistochemistry. The primary rabbit polyclonal anti ADP-ribosylation factor-like protein 13b (Arl-13b) antibody (Proteintech) was used according to the manufacturer's instructions. Age, gender, histotype, and asbestos exposure of patients were summarized in Table 2.

3.2 Cell lines and culture conditions

Mesothelioma cell lines were isolated from patients' who underwent surgery at the Thoracic Surgery Unit (Siena, Italy) for decortication, without prior chemotherapy or radiotherapy. All specimens were collected from patients diagnosed for pleural mesothelioma selected for surgery based on the pre-operative staging and with their written consensus. Human investigations were performed after Research Ethics Committee (Comitato Etico Regione Toscana-Area Vasta Sud Est) approval (#CCMESOLUNG). The study is conformed to the standards of the Declaration of Helsinki. The original pathologic materials were analyzed by light microscopic analysis, followed by extensive immunocytochemical analysis using a battery of markers.

Tissues were transported to the laboratory for primary cell culturing within 30 min of collection. Solid tissue was minced into small pieces, 1 to 3 mm, and then incubated in complete medium supplemented with collagenase type I from Clostridium histolyticum (Thermo Fisher Scientific, Waltham, Massachusetts, USA, Cat #17100017) at 200 U/mL concentration for 1 hour to digest collagen and release tumor cells. Macrophages, red

blood cells and lymphocytes were the main contaminants; to avoid their interference in the analysis, all the primary cells were used after the 6^{th} passage.

The mesothelial origin of patients-derived cultures were assessed by IHC for a panel of mesothelial markers (WT-1, α-SMA, CD31, CD34), and by Transmission Electron Microscopy (TEM) for the presence of microvilli on the cellular membrane.

Age, gender, histotype, and asbestos exposure of patients were summarized in Table 1.

Non-malignant mesothelial cells LP-9 were from Coriell Institute.

All cell lines were cultured in Medium 199 (Euroclone), supplemented with 2 mM L-Glutamine (Euroclone), 100 U/ml Penicillin, 100 µg/ml Streptomicin (Euroclone), 10% FBS (Euroclone) at 37 °C and 5% CO2. LP-9 cells were growth with the addition of 20 ng/ml hEGF (Sigma-Aldrich).

All cell lines were routinely passaged every 3-5 days.

3.3 Cell treatments

Akt1/2/3 Inhibitor VII was obtained from Chem Cruz. Stock solutions of the drug were prepared in dimethyl sulfoxide cell culture grade (DMSO) (Euroclone) and stored at −20°C.

GLI1 inhibitor Arsenic (III) oxide (ATO) was obtained from Sigma-Aldrich. Stock solutions were prepared in distilled water, the pH adjusted at … and stored at −20°C.

Cells were seeded in 96-well plates 24 hours before treatments and incubated for further 72 hours. Control cells were treated with vehicle at the same amount used to deliver the molecule. Each experiment was conducted in triplicate. Cell viability was evaluated by sulforhodamine B (SRB) assay (Sigma-Aldrich), as described by Skehan et al. [55] . Absorbance values were measured with a microplate reader (Euroclone) at 540 nm. The half maximal inhibitory concentration (IC50) values were calculated using

GraphPad Prism 6 (GraphPad Software Inc; http://www.graphpad.com/scientific/software/prism/).

3.4Immunofluorescence

For the detection of primary cilia, the cells were seeded on a glass coverslip, fixed for 10 minutes in 4% paraformaldehyde, washed and then incubated with the anti-acetyl tubulin antibody and anti-IFT88 antibodies (Sigma).

Then the cells were incubated with the appropriate secondary antibody for 45 minutes at room temperature. Unspecific signal was evaluated for each antibody using a control condition without antibody and a non-specific antibody. Images were taken on a Zeiss Axio microscope.

3.5 Real-time Reverse Transcriptase-Quantitative PCR (RT-qPCR)

Total RNA was isolated from cell lines using the RNeasy Mini kit (Qiagen). RNA concentration was determined using a NanoDrop™ ND-1000 (Thermo Fisher). Complementary DNA (cDNA) was synthesized from 500 ng of RNA using the iScript cDNA Synthesis Kit (Bio-rad) and amplified in the LightCycler™ instrument (Roche Applied Sciences) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-rad,) according to the manufacturer's instruction. The primers used were from Bio-Rad: GLI1 Assay ID qHsaCED0043346, PTCH1 Assay ID qHsaCED0001809, GAPDH Assay ID qHsaCED0038674. The housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used to normalize the expression of genes of interest. Gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method [56] .

3.6 Western blot analysis

For Western blotting analysis, cells were harvested on ice and lysed as previously described [9] . Equal amounts of proteins (30 μg) per sample were electrophoresed and, after transferring to nitrocellulose membranes (Bio-Rad), were incubated overnight at 4°C with the following antibodies: p-AktSer473 (Cell Signaling), Akt (Cell Signaling) and β-actin (Sigma).

Membranes were washed with TBS with 0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Membranes were washed before chemiluminescence detection using Clarity ECL reagents (Bio-Rad).

3.7 Statistical analysis

Statistical analyses were performed using the GraphPad Prism Software, version 5.01 for Windows. Statistically significant differences were evaluated by one-way repeated measures ANOVA. To compare the means of 2 unmatched groups, we used the two-sided unpaired Student's t test. $P < 0.05$ was considered statistically significant.

4. RESULTS

4.1 Pc is heterogeneously present in MPM cell lines and in MPM tissue specimens

We analyzed the presence of PC in six primary mesothelioma cell lines, and in the non-malignant mesothelial cells LP-9.

The characteristics of the tumors of origin were summarized in Table 1.

Table 1. Clinical data of patients-derived cell lines. All patients have Italian nationality.

Cell Line	Gender	Histotype	Age	Abestos exposure
MMP1	Male	Epithelioid	81	no
MMP4	Male	Epithelioid	74	no
MMP14	Male	Biphasic	80	yes
MMP18	Female	Early stage epithelioid	73	no
MMP21	Male	Early stage epithelioid	74	no
MMP23	Female	Epithelioid	68	no

We observed the expression of the PC in MPMP18 and MPMP21 cells with length and structure similar to that of normal peritoneal mesothelial cell line LP-9 (Fig. 1A). In the other cell lines analyzed (MPMP1, MPMP4, MPMP14 and MPMP23) we did not detect PC (Fig. 1B).

Some MMP1 cells, as well as the corresponding tissue specimen, showed a PC with evident alterations in length and structure (Fig. 2).

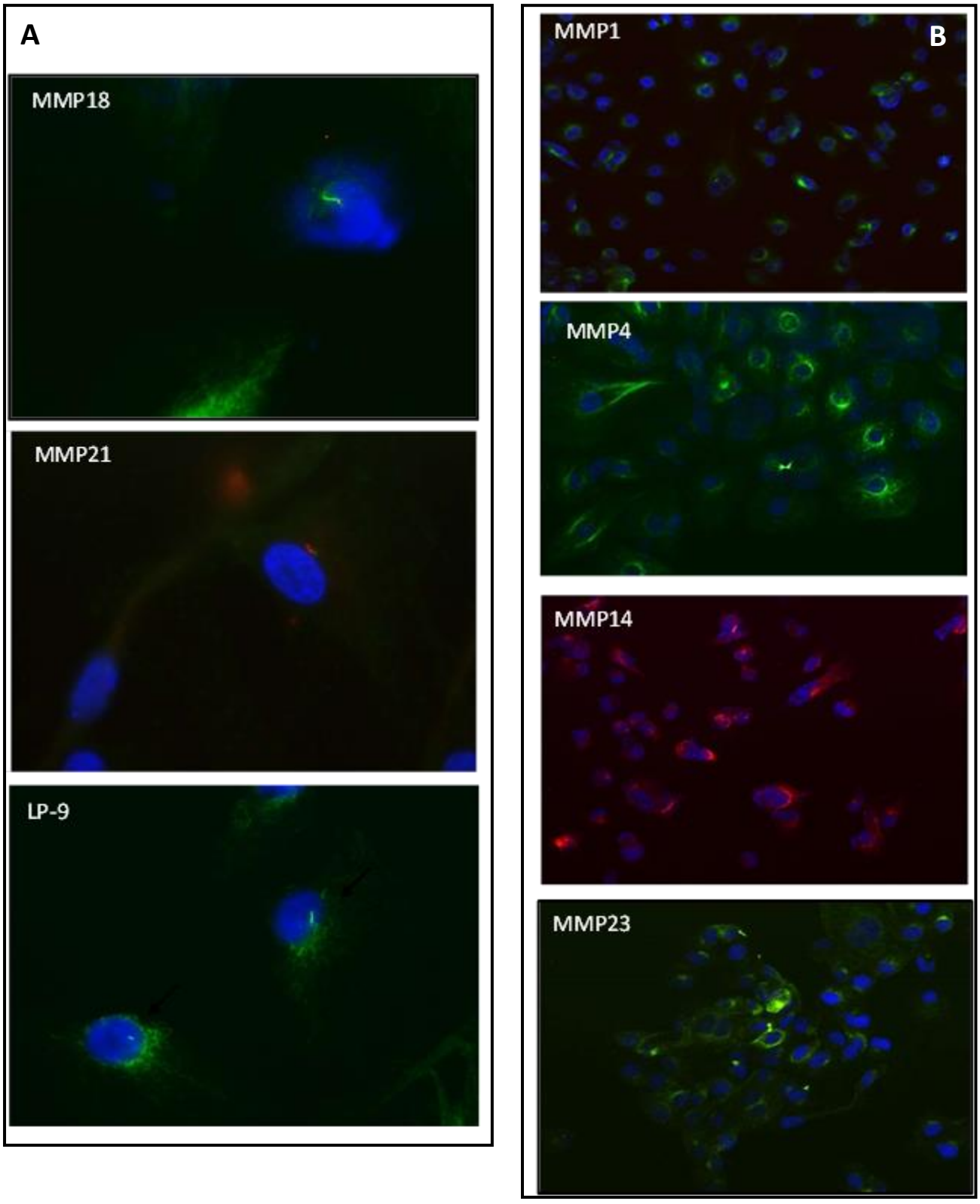

Figure 1. The presence of PC were analyzed in MPM primary cells and in normal mesothelial cells by IF. A) PC was presents in MPMP18, MPMP21 cell lines and in normal mesothelial cell LP9. B) PC is lost in MPMP1, MPMP4, MPMP14 and MPMP23 mesothelioma cell lines.

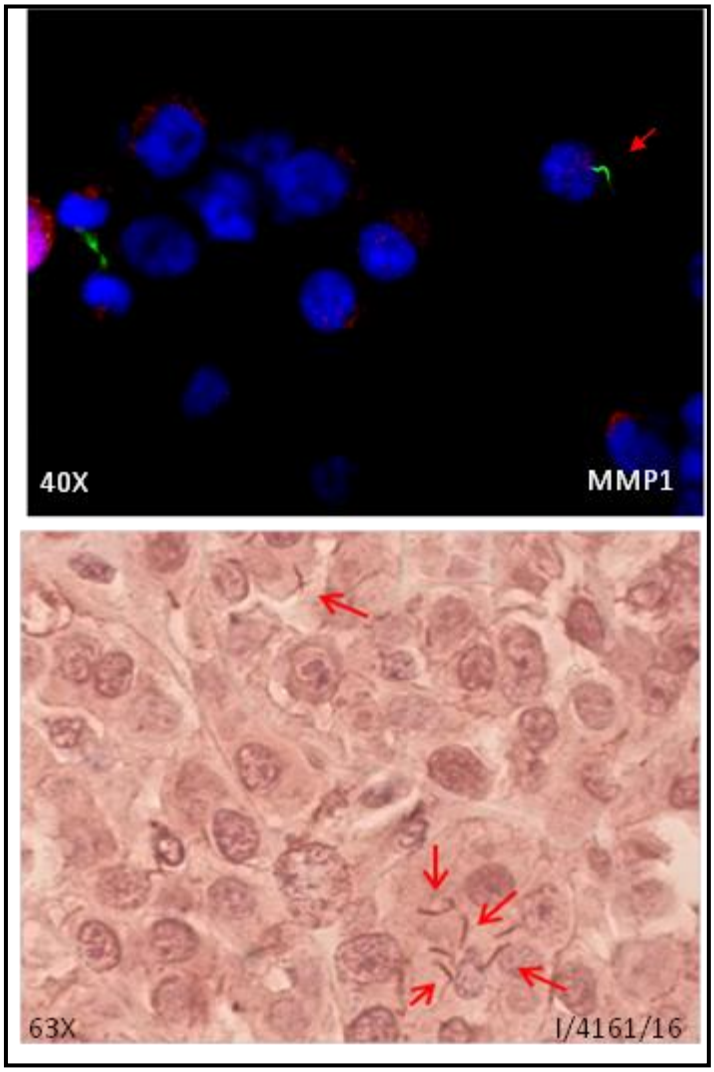

Figure 2. Abnormal PC observed in MPMP1 cell line and in the corresponding tissue specimen.

An exploratory IHC analysis of PC in ten FFPE specimens showed the presence of PC in an early stage epithelioid mesothelioma (Fig 3A). Conversely, in a sarcomatoid MPM the PC is loss (Fig. 3B). Similarly, tumor tissues from the same patient, evolving from epithelioid (I/7225.2/16) to biphasic phenotype (I/11362/16), retain the expression of PC only in epithelioid component (Fig. 3C).

Age, gender, histotype and asbestos exposure of patients were summarized in Table 2.

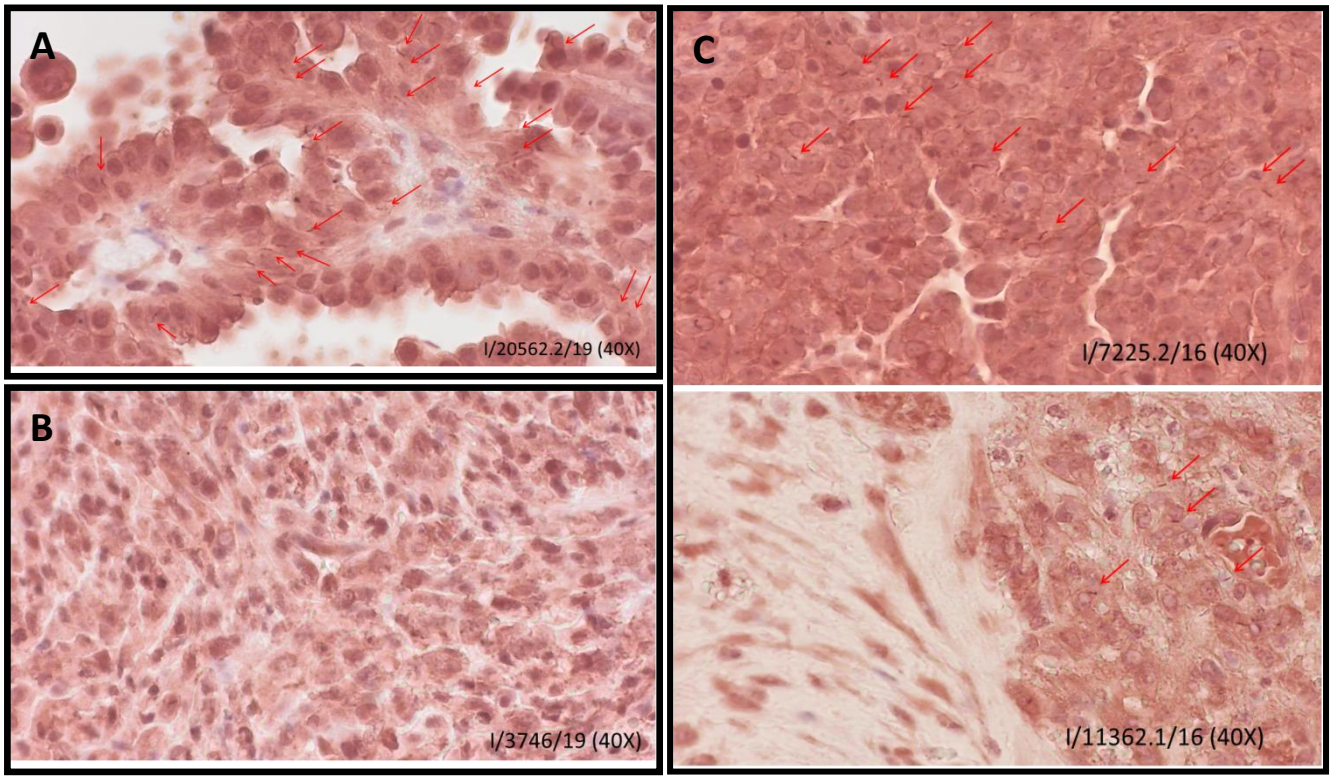

Figure 3. IHC analysis of Arl13B as a marker for PC. A) Early stage mesothelioma retained PC. B) Sarcomatoid mesothelioma C) Tumor specimens from the same patient evolving from epithelioid mesothelioma to mixed histotype.

Table 2. Clinical data of patients-derived cell lines. All patients have Italian nationality.

Sample	Gender	Histotype	Age	Asbestos fibers
I/3746.1/2016	Male	Biphasic post-chemio	79	yes
I/4161.2/2016	Male	Epithelioid	77	no
I/4280.7/2020	Female	Epithelioid post-chemio	73	no
I/4939.3/2020	Male	Early stage epithelioid	74	no
I/7225.2/2016	Male	Epithelioid	77	no
I/9272.6/2018	Male	Epithelioid	72	no
I/11362.1/2016	Male	Biphasic	77	no
I/20513.5/2018	Male	Epithelioid	78	yes
I/20562.2/2019	Female	Early stage epithelioid	72	no
I/25227/2019	Female	Epithelioid	67	no

4.2 GLI1 pathway is activated in cells lacking PC

As PC transduces HH/GLI signaling through canonical pathway, we aimed to explore if it presence in MPM could be a marker for selection of MPM patients eligible for therapy with SMO antagonist. We analyzed the expression of well-known markers of HH pathway activation GLI1, a transcriptional activator, and PTCH1, a transcriptional target of GLI1 and an inhibitor of the pathway.

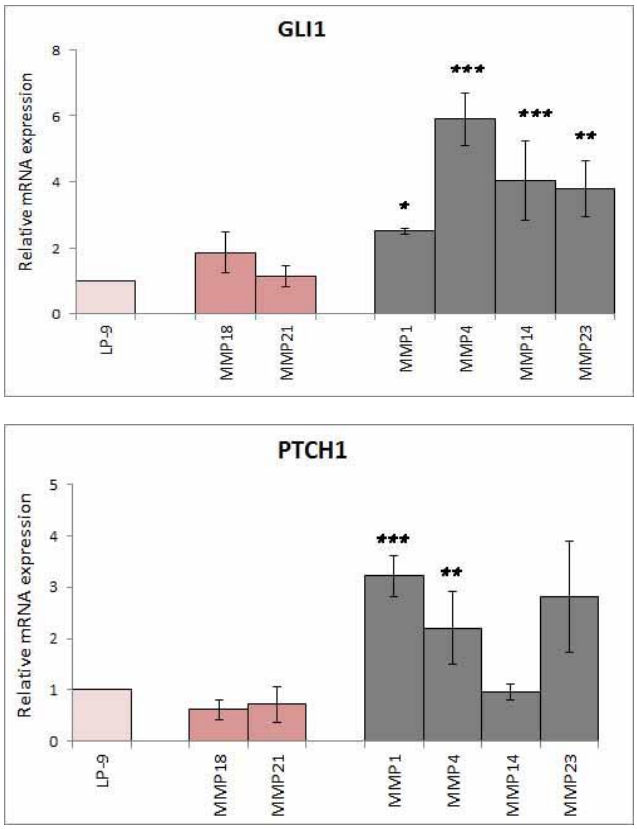

Figure 4. Loss of PC in MPM cell lines correlated with high levels of *GLI*1 and *PTCH*1 (gray bars). PC expressing cells (dark pink bars) showed levels of HH related genes similar to normal mesothelial cells LP-9 (light pink bars). Statistical analysis was performed by subjecting the ΔCt values to one-way repeated measures ANOVA with Tukey's post-test. Statistically significant differences are indicated with: * significant (P< .05) ** very significant (P < .01) and *** extremely significant (P < .001)

We observed an overexpression of GLI1 and PTCH1 in all the cell lines lacking PC, indicating a constitutive activation of HH-GLI pathway. Conversely, in cells with normal

PC we observed a GLI1 expression levels in MMP21 similar to normal cells LP-9, and about 2 fold increase in MPMP18. However, in these cell lines, the expression of HH pathway inhibitor PTCH1 is comparable to normal mesothelial cell indicating that, in MMP18, the HH pathway should be active. Notably, PTCH1 overexpression in PC negative cells cannot exerts its inhibitory function due to the lack of its substrate SMO at PC (Fig. 4).

4.3 HH/Gli1 signaling is activated through non-canonical signaling

To explore the molecular mechanism underling GLI1/HH pathway activation in PC negative cells, we focused the attention on PI3K/Akt signaling that, among others, is under investigation as possible therapeutic target in MPM [57, 58] . First, we analyzed the Akt activation (phospho-AktSer 473) in our primary MPM cells. Among PC negative cells we selected those with a statistically significant upregulation of GLI1 and PTCH1, MMP1, MMP4 and MMP23 and, among PC positive cells, we analyzed MMP18 which has low levels of the inhibitor PTCH1, the normal mesothelial cell LP-9 and NCI-H2052 as positive control for Akt activation (pAKT) [59] (Fig.5A).

Then, we selected MMP1 cell line showing the highest levels of phospho-Akt (Fig.5A) and the better response to Akt-inhibitor treatment (Fig.5B) to investigate the effects of Akt inhibition on GLi1 expression levels. Since Akt is also described a downstream target of Gli/HH pathway, we also investigated the effects of GLI1-inhibitor ATO on Akt activation. Whilst Akt inhibiton decreased GLI1 and PTCH1 expression (Fig. 5C), GLI1 inhibition did not change the phosphorylation of Akt (Fig. 5D), suggesting that Akt is upstream non-canonical HH-GLI signaling in this cell line and its upregulation is not a consequence of high basal GLI1 levels.

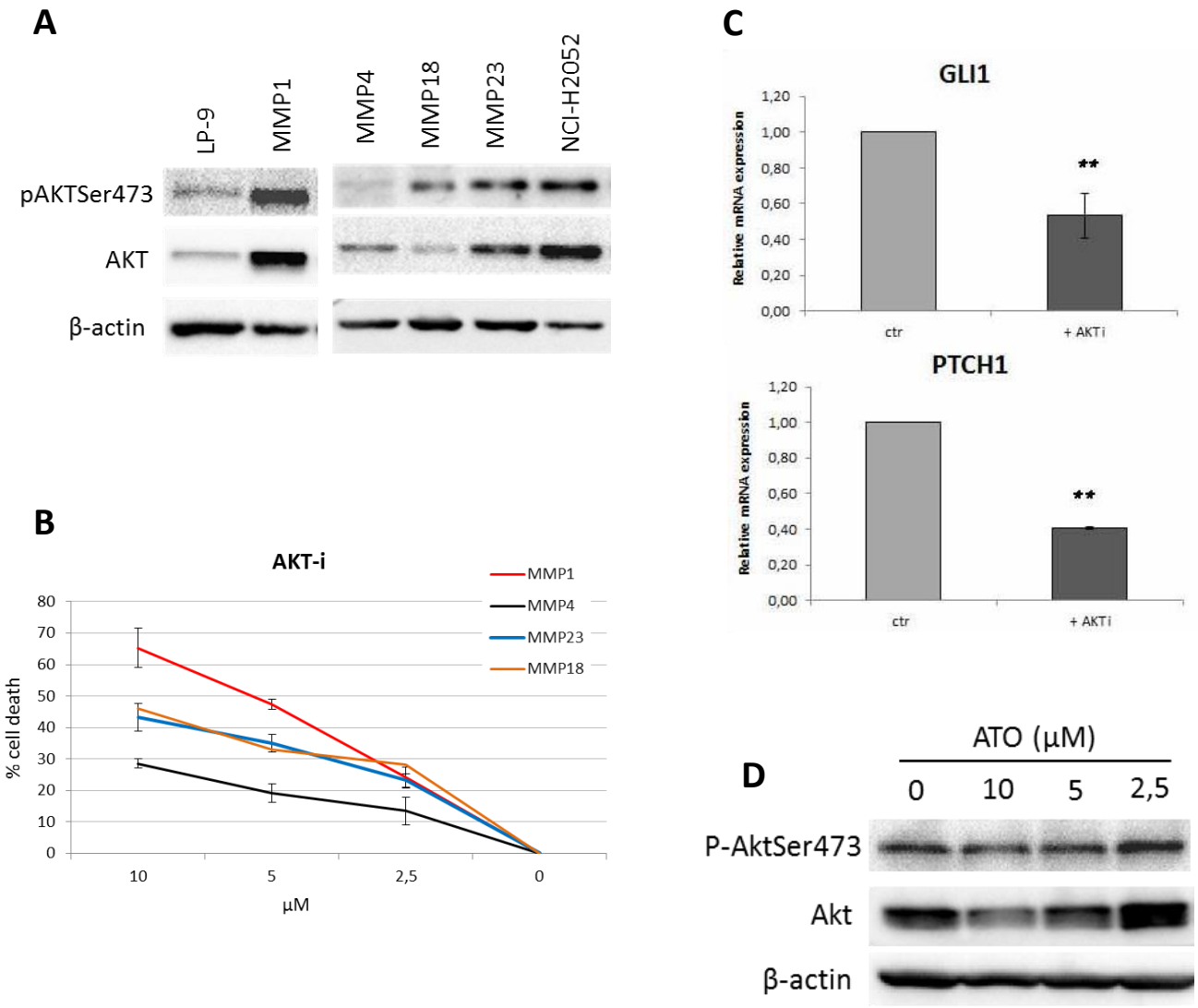

Figure 5. Akt inhibition down-regulates HH-GL1 pathway and is not a target of Gli1 inhibition. A) Western blot analysis of p-Akt/Akt expression in MPM cell lines. LP-9 cells were used as negative control, NCI-H2052 as positive control. B) Effect of Akt inhibition through AKTiVIII on selected primary mesothelioma cells. C) Inhibition of Akt decrease GLI1 and PTCH1 expression. D). GLI1 inhibition with ATO did not affect the levels of phospho-Akt. Statistical analysis was performed by subjecting the ΔCt values to one-way repeated measures ANOVA with Tukey's post-test. Statistically significant differences are indicated with: **very significant ($P < .01$)

5. DISCUSSION

Despite the effort to introduce new therapies in the clinical practice, malignant pleural mesothelioma remains a fatal cancer.

The targeting of the HH-GLI pathway is giving promising results in different cancer types, with the approval of SMO-inhibitors for BCC and AML, and many clinical trials ongoing. However, its therapeutic potential in mesothelioma is not yet thoroughly investigated.

Few data on HH-GLI pathway activation and its role in MPM are available. An exploratory analysis on 45 MPM specimens have shown that GLI1 is significantly overexpressed in the tumor tissues compared to normal pleura, and an high expression of GLI1, SMO and SHH genes are associated with a poor survival [36, 40] .

Nevertheless data from the cancer Genome Atlas, list the signaling events mediated by the HH family among the top 10 pathways deregulated in MPM [60] . Differently from other cancers, mutation in the core components of the pathway has not been documented in MPM [36, 61] .

One study has documented a mutation in SMO only in tissues from MPM normo-survivors patients and not in longo-survivors group. However, the functional impact of this mutation on the protein function has not be investigated. Non-mutated SMO appeared to be overexpressed also in long-survivors group but the relation between SMO expression and HH-GLI pathway activation was not investigated in this study [39] .

In a case report study a PTCH1 variant (F1147fs) was identified in a MPM patient and correlated with a durable and near-complete response to SMO-inhibitor Vismodegib [41] .

Because the presence of PC is necessary for the canonical activation of the HH-GLI pathway, and therefore for SMO function, in this study we conducted an exploratory

analysis of the presence of PC in ten MPM tissue specimens and in a panel of primary MPM cell cultures, investigating its correlation with the activation of the HH-GLI pathway. We aimed to individuate a subset of MPM dependent from the HH-GLI pathway and then eligible for therapy with SMO inhibitors.

We found an heterogeneous presence of PC in MPM tumor tissues and in MPM patients' derived cell lines. In primary mesothelioma cells without PC, the HH-GLI pathway related genes, GLI1 and PTCH1, are overexpressed compared to both non-malignant mesothelial and mesothelioma cells carrying normal PC, indicating a non-canonical activation of HH pathway.

Our preliminary results suggested that PI3K/AKT pathway can be, at least in some cells, responsible for the activation of HH-GLI1 pathway.

In summary, we have documented for the first time the loss of PC in mesothelioma and the activation of a non-canonical HH-GLI pathways in this cancer cells.

We are aware that this study has some limitations, especially due to the limited number of cases analyzed. However, this exploratory analysis has allowed us to hypothesize new and interesting molecular mechanisms in mesothelioma that we hope could translate into new therapeutic targets.

In the light of the data obtained, we are already selecting new archival mesothelioma cases, in collaboration with the Pathological Anatomy of Siena, for the analysis of PC expression.

We hypothesize that PC in MPM has a negative regulatory function on GLI1. To address this question, we will study the expression of GLI1 in this tissues and correlate its activation with the presence or the absence of PC.

Moreover, in cells carrying PC and with low GLI1 levels, we will investigate the effect on GLI1 expression using primary cilia disrupting agents.

If our hypothesis will be demonstrated, we will contribute to the understanding of PC regulating function. We believe that our studies on of the effects of restoring primary cilia in MPM could become a valuable new therapeutic strategy to investigate.

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