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Title: ROLE OF HMGB1 (High Mobility Group Box 1) IN MALIGNANT MESOTHELIOMA PATHOGENESIS , PROGNOSIS AND AS PREDICTIVE BIOMARKER

Introduction/ Aims of study

Malignant Mesothelioma

Malignant mesothelioma (MM) is an aggressive tumour originates from the mesothelial cells of the pleura, pericardium and peritoneum cavities.(1) The most frequent site of the disease presentation is the pleural surface (>90%) related mainly to exposure to mineral fibers such as asbestos and erionite (2). Morever other factors, such as simian virus 40 (SV40) infection, genetic predisposition, radiation and exposure to other fibers with similar physical properties to asbestos have been also implicated in the carcinogenesis of MM. Malignant Pleural Mesothelioma (MPM) is characterized by a long latency period that ranges from 10 to 45 years (3).

The incidence of MPM is much lower than lung cancer (2000-3000 new cases per year versus 160 000 new cases of lung cancer patients per year), but it has increased over the past five decades due to the unrestricted use of asbestos into the industrialized countries (4). in Italy its incidence is 2,94/100.000 for men and 1,06/100.000 for women. In the areas in which asbestos production factories are frequent like Casale Monferrato in Piedmont region, the incidence is estimated to be about 43.7/100.000 for men and 27/100.000 for woman (Centro di Riferimento per l'Epidemiologia e la Prevenzione Oncologica in Piemonte). According to the amount of epithelial and spindle cells MPM is divided into four histologic subtypes: epithelial (50-70%), sarcomatoid (10-15%), biphasic (30%) and desmoplastic a quite rare variant of the tumour (5) associated with a different prognosis. The epithelioid subtype is considered the less aggressive and most responsive to treatments, with the best prognosis (6).

The prognosis of malignant pleural mesothelioma is very poor due to a late-stage diagnosis, rapid tumor progression, high invasiveness and resistance to current conventional therapies (7).

Prognostic factors that would suggest a poor outcome for mesothelioma's patients include breathlessness, weight loss at the time of diagnosis, poor performance status, sarcomatoid subtype and, interestingly, the absence of comorbid illness (8).

The median survival of untreated patients is approximately 12 months in stage I, 4 months in stage II and about 3 months in stages III-IV. The disease stage determines whether the surgical resection is an effective therapeutic option providing a long-term survival for patients (9).

There are no therapeutic standards for MPM and the treatment options depend on performance status, pulmonary function, stage and age of the patient. Multimodality treatment including radiation therapy, surgery and chemotherapy is an option for some MPM patients with limited disease extension. Although MM has a low tendency to metastasize, MM grows highly invasive into surrounding tissue. A systemic treatment is the main therapeutic option for most patients due to the invasive behavior and limited efficacy of radiation therapy. Thus the most commonly regimen used now includes: the multitargeted antifolate drug (Pemetrexed) with a platinum drug such as Cisplatinum.

Due to the limited efficacy of conventional therapeutic treatments (chemiotherapy and radiation), (9) many different targeted therapeutic agents have been explored.

Before any therapeutic improvements can be expected, the molecular defects involved in Malignant mesothelioma pathogenesis and progression should be understood [10]. The continuing identification of molecular bases of neoplastic transformation should lead to better disease control and greater therapeutic options in the future.

BIOLOGY AND PATHOGENESIS OF MPM

The clinical evidences of MPM are thought to arise as a result of the accrual of several molecular alterations. Recently studies using standard karyotype analyses and comparative genomic hybridization (CGH) (11) reported multiple chromosomal abnormalities (alterations, deletions and amplifications) into MM cases.

Malignant mesothelioma frequently displays chromosomal losses than gains. The most commonly gained chromosomal regions are 5p, 7p, 7q, 8q, and 17q, whereas the most frequent losses are at 1p, 3p, 6q, 9p, 13q, 14q, 15q, and 22q (12). A particularly high frequency of homodeletion has been detected in the 9p21 region, causing a high frequency of deletion p16INK4a (a critical CDK inhibitor) and p14arf (the p53 regulator) (13). The lack of their expression is responsible of the retinoblastoma gene and p53 gene inactivation in mesothelioma cells, which cause the breakdown of cell cycle control mechanisms (14). Otherwise, mutations in other genes including p53, ras and RB, highly frequent in malignant tumours, are very rare in MPM. Another frequent occurrence (40-50%) in MM cases is the loss of 22 chromosome which causes the lost of NF2 neurofibromatosis type 2 expression.

Some of the most epigenetic changes of chromatin structure include promoter methylation and histone deacetylation causing gene silencing and contribute to neoplastic transformation and progression (15).

A large number of studies have focused on the activity (paracrine and autocrine) of several growth factors and their receptors which promote an increased MPM cell proliferation. Epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-I and II, platelet-derived growth factor

(PDGF), transforming growth factor (TGF)-b, vascular endothelial growth factor (VEGF) and their specific transmembrane receptors are highly expressed in MPM. EGF and TGF-a are the main ligands for EGF receptor (EGFR), a member of the erbB family TK receptors. Upon the specific binding of ligand to receptor, the transphosphorylation of the receptor tail-located TK domains occur. This result in activation of Raf-MEK-ERK1/2 and phosphoinositide-3kinase (PI-3 K)-Akt signalling pathways, associated with cell proliferation, differentiation and survival (Fig. 1) (16). It was also observed that asbestos fibres cause aggregation and autophosphorylation of EGFR in mesothelial cells which lead to the induction of the AP-1 family members, c-fos and c-jun.

The Vascular Endothelial Growth Factor (VEGF) and its receptor are potent inducers of the angiogenesis whose up-regulation is relevant for mesothelial cell transformation. Besides the stimulation of the neovascularization, VEGF may induce activation of its receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), acting as autocrine GF in MPM cell (17). Morever, the importance of this receptor into tumour progression and angiogenesis is established, making it a possible therapeutic target in MPM (18).

The PI3K/AKT/mTOR pathway is altered in MPM, playing a pitoval role into cell proliferation, survival and motility in many cancers (Fig.1).

The matrix metalloproteinases, particularly MMP-2 and MMP-9 contribute to high local invasiveness and distant metastases, which sometimes characterize advanced stage of MPM (19).

Bcl-2 protein involved in apoptosis is strongly expressed in many malignant tumours whereas is weakly expressed in MPM; however, expression of a member of Bcl-2 family (Bcl-XL) and the potent anti-apoptotic Bax are frequently found. Also, survivin and inhibitor of apoptosis protein (IAP) expression, considered resistant factors for chemotherapy, have been observed (20).

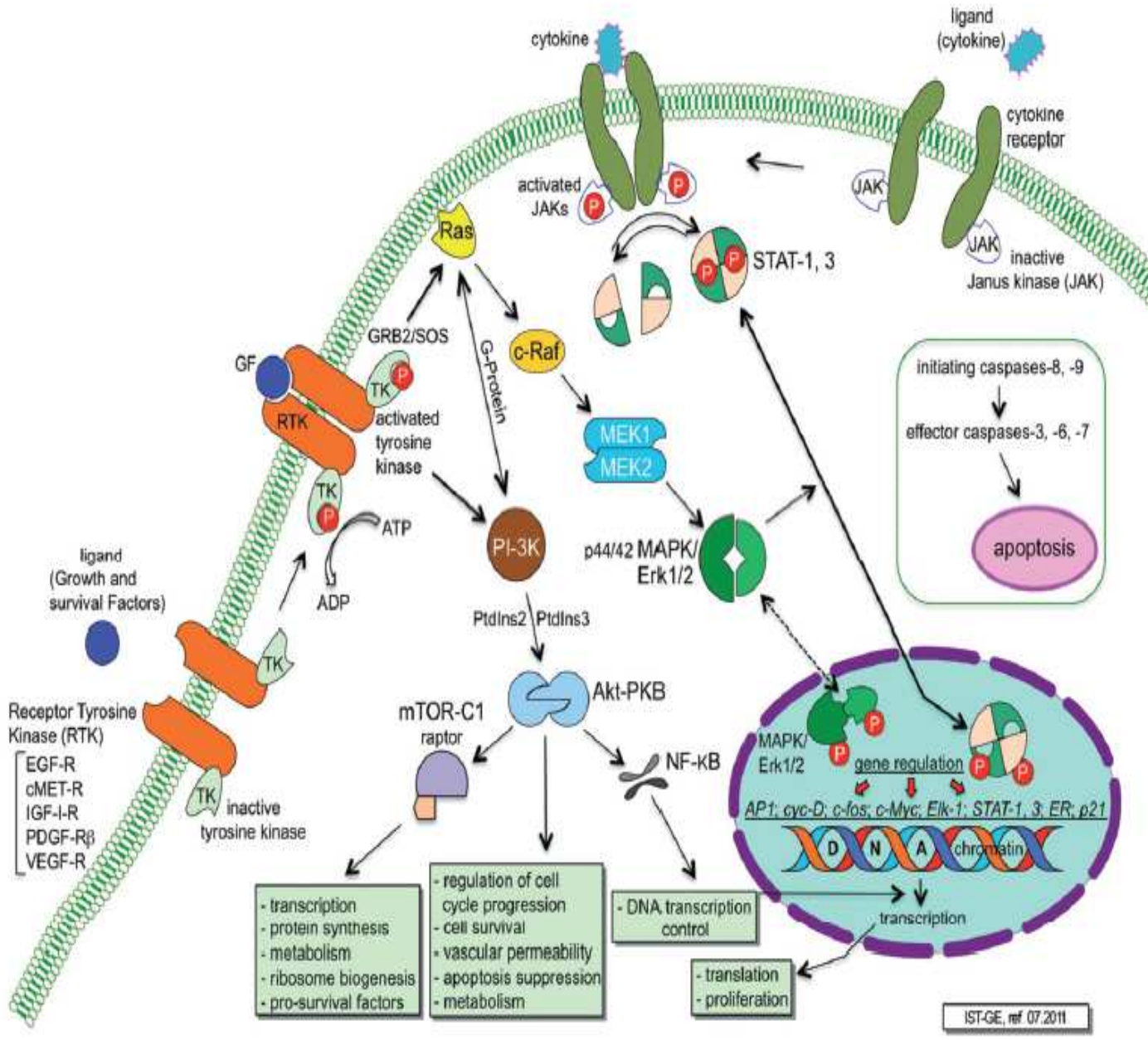


Figure 1: Biology and important pathway in malignant mesothelioma

Exposition to asbestos fibres induce the expression of the nuclear proto-oncogenes c-fos and c-jun, which result in cell proliferation and gene transcription. Furthermore, asbestos fibers promotes secretion of the pro-inflammatory cytokine TNF-a by mesothelial cells and macrophages leading to activation of NF-kB, which plays a role in cell proliferation and antiapoptosis. Recently, a key mechanism by which asbestos causes the transformation of mesothelial cells has been elucidated: working with primary human mesothelial (HM) cells, Yang et al discovered that asbestos induces necrotic cell death with resultant release of HMGB-1 in the extra cellular space (Fig.2). Thus, High-mobility group box 1 (HMGB 1) release has been identified as a critical initial step in the pathogenesis of asbestos-related MPM. Mesothelial cells exposed to asbestos translocate HMGB 1 from the nucleus across the cytoplasm, into the extracellular space. Macrophages receive 'HMGB1' stimuli and release TNF-a, which increased the survival of asbestos-damaged human mesothelial cells triggering a chronic inflammatory response (21). The activation of TNF-a pathway allow the neoplastic transformation of mesothelial cells (22).

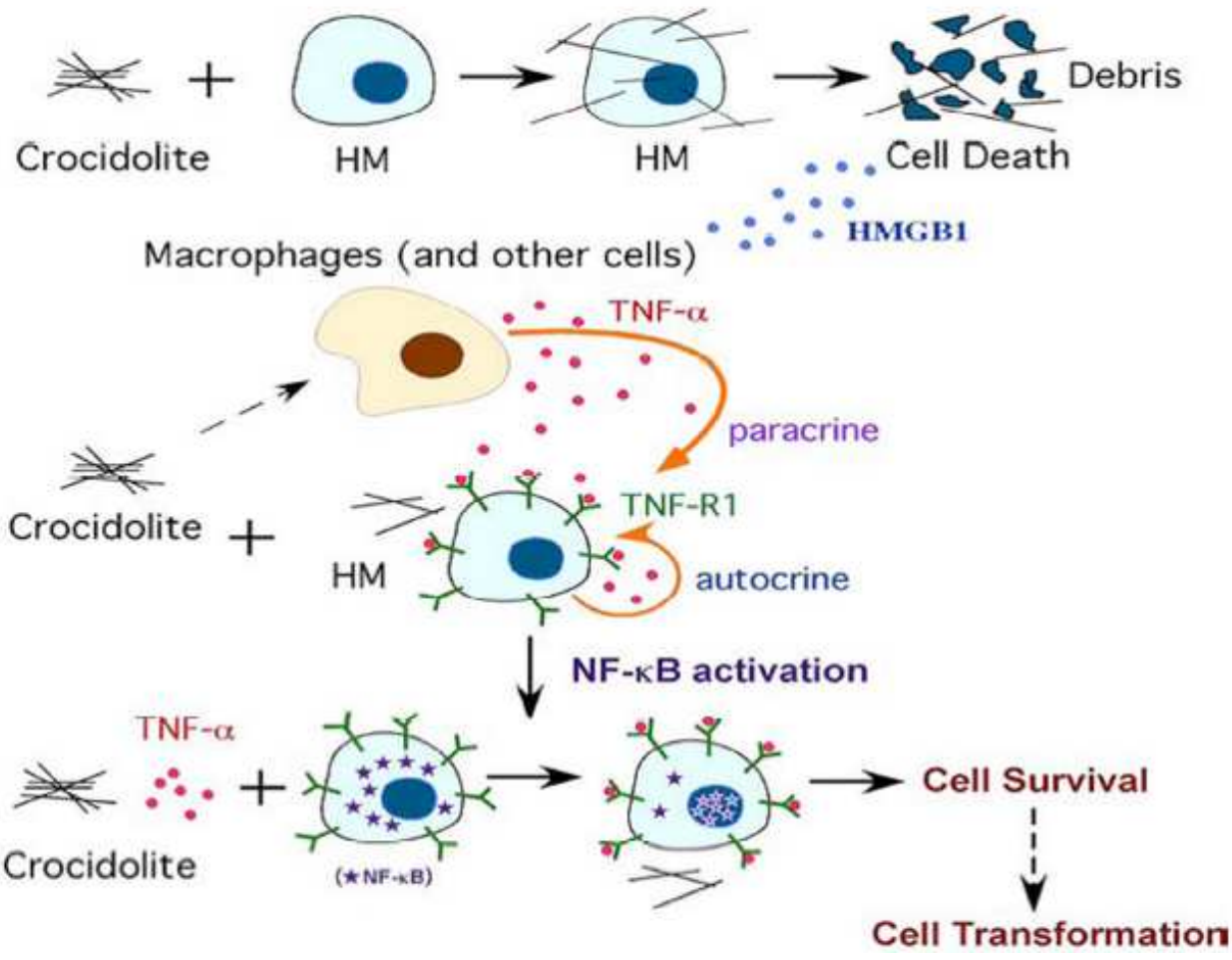


Figure 2: Role of HMGB1 in the pathogenesis of asbestos-related MPM

HMGB1 and its dual role in cancer

HMGB1 High mobility group box 1 is a highly conserved protein which has many biological and pathological functions inside as well as outside the cell (Fig. 1B) (23). HMGB1 contains two DNA-binding HMG-box domains (N-terminal A and central B, termed A box and B box) and a highly negatively charged C-terminal tail (Goodwin GH et al, 1977; Bustin M et al, 1990). HMGB1 is a nuclear protein, acting as a chromatin-binding factor that bends DNA and help maintain nuclear homeostasis (24). The HMG boxes enable HMGB1 to bind in a non-specific manner to different DNA structures. HMGB1 also facilitates the assembly of site-specific transcription factors such as p53, p73, the retinoblastoma protein (Rb), NF-kB, and the estrogen receptor to their cognate binding sites within chromatin (25).

Loss of HMGB1 increases DNA damage and decreases the efficiency of DNA repair in response to chemotherapy, irradiation, and oxidative stress. HMGB1 plays a critical role in DNA repair because of its ability to bind directly a variety of bulky DNA lesions and to participate in various DNA repair pathways (26).

In addition to its nuclear role, HMGB1 functions also as an extracellular signaling molecule promoting both cell survival and cell death during inflammation, cell differentiation, cell migration and tumor metastasis (27). HMGB1 is passively released from necrotic cells or actively secreted from immune cells or cancer cells in response to exogenous and endogenous stimuli such as endotoxin, double-stranded RNA (dsRNA), TNF-a, interleukin (IL)-1, IFN-g, hydrogen peroxide, ATP, and hypoxia. Depending on the inducing stimulus, the mechanism of HMGB1 secretion can vary and recently several mechanisms have been proposed to explain its release. The presence of HMGB1 in the extracellular medium indicates that some cells are stressed or have died and alert the other cells to the clear and immediate danger (28).

Once secreted, extracellular HMGB1 binds to several cell surface receptors such as the receptor for advanced glycation end products (RAGE), Toll-like receptors (TLRs; such as TLR2, TLR4, and TLR9), Mac-1, syndecan-1 (CD138), phosphacan proteintyrosine phosphatase (PPTP)-z/b, CD24, chemokine (C–X–C motif) ligand 4 (CXCL4), T-cell immunoglobulin mucin-3 (TIM-3), and possibly others (Fig. 3). The specific binding of HMGB1 to its receptors promotes the activation of the downstream signaling pathway [e.g., NF-kB, IFN regulatory factor-3 (IRF3), and phosphoinositide 3-kinase (PI3K)]. The activity of these signaling pathways produce a functional response, such as activation of innate immune cells, induction of proinflammatory cytokines and type I IFNs, stimulation of cell adhesion and migration, inhibition of phagocytosis, promotion of cell proliferation and angiogenesis, and induction of autophagy (29, 30). Whereas, CD24 and TIM-3 act as negative receptors and inhibit immune activity of HMGB1 in macrophages and tumor-associated

dendritic cells (TADC), respectively (31, 32). HMGB1 may also form heterocomplexes with other immune coactivators such as IL-1, CXCL12 or LPS and produce synergistic responses in inflammation and immunity.

Experimental evidences indicate that disfunction of HMGB1 is associated with tumorigenesis and contributes to cancer development and therapy (Fig.3) (33). For this reason it will be important to understand HMGB1 regulation and its function in the mechanism of cancer biology. Furthermore, the understanding of its role into tumorigenesis will influences the strategies of a HMGB1 tageted therapy for prevention and treatment.

The neoplastic transformation and tumor growth, invasion, and metastases was supported by the inflammatory tumour microenvironment (TME). The development of the inflammatory tumour microenvironment is associated with the Tumor-infiltrating leukocytes and the cytokine-related signaling pathways. Infiltrating leukocytes and cancer cells themselves secrete HMGB1 under hypoxia, injury, inflammatory stimuli, or environmental factors (34). In turn, extracellular HMGB1 can activate proinflammatory signaling pathways, such as the NF-kB and inflammasome pathways, to induce proinflammatory cytokine release. This loop accelerate inflammatory responses and induce tumor formation and metastasis.

Another of the most common cancer's phenotype is a high energy request by cancer cells in order to allow a rapid, invasive and metastatic growth of tumour. HMGB1 has been implicated in tumor energy metabolism (35). Recently it was demonstrated that extracellular HMGB1 increases mitochondrial RAGE expression and translocation, which in turn increases mitochondrial complex I activity and ATP production (36).

Different clinical, pre-clinical and in vitro studies demonstrate that inhibition of RAGE–HMGB1 interaction suppresses tumor growth and metastasis by activation of mitogen-activated protein kinases and the NF-kB pathway. The NF-kB activation results in the expression of matrix metalloproteinases (MMP), such as MMP2 and MMP9 (37), which degrade extracellular matrix proteins and play a major role in tumor invasion and metastasis. Thus, HMGB1–RAGE signaling pathway is pitoval in tumor invasion and metastasis.

Recent findings suggest that endogenous intracellular HMGB1, as a Rb-associated protein, suppresses breast tumorigenesis, behaiving as a tumour suppressor gene. In addition, HMGB1 is also an important regulator of autophagy and its lost inhibits autophagy and increases apoptosis. Suppression of autophagy promotes tumorigenesis and increases the effectiveness of anticancer therapy.

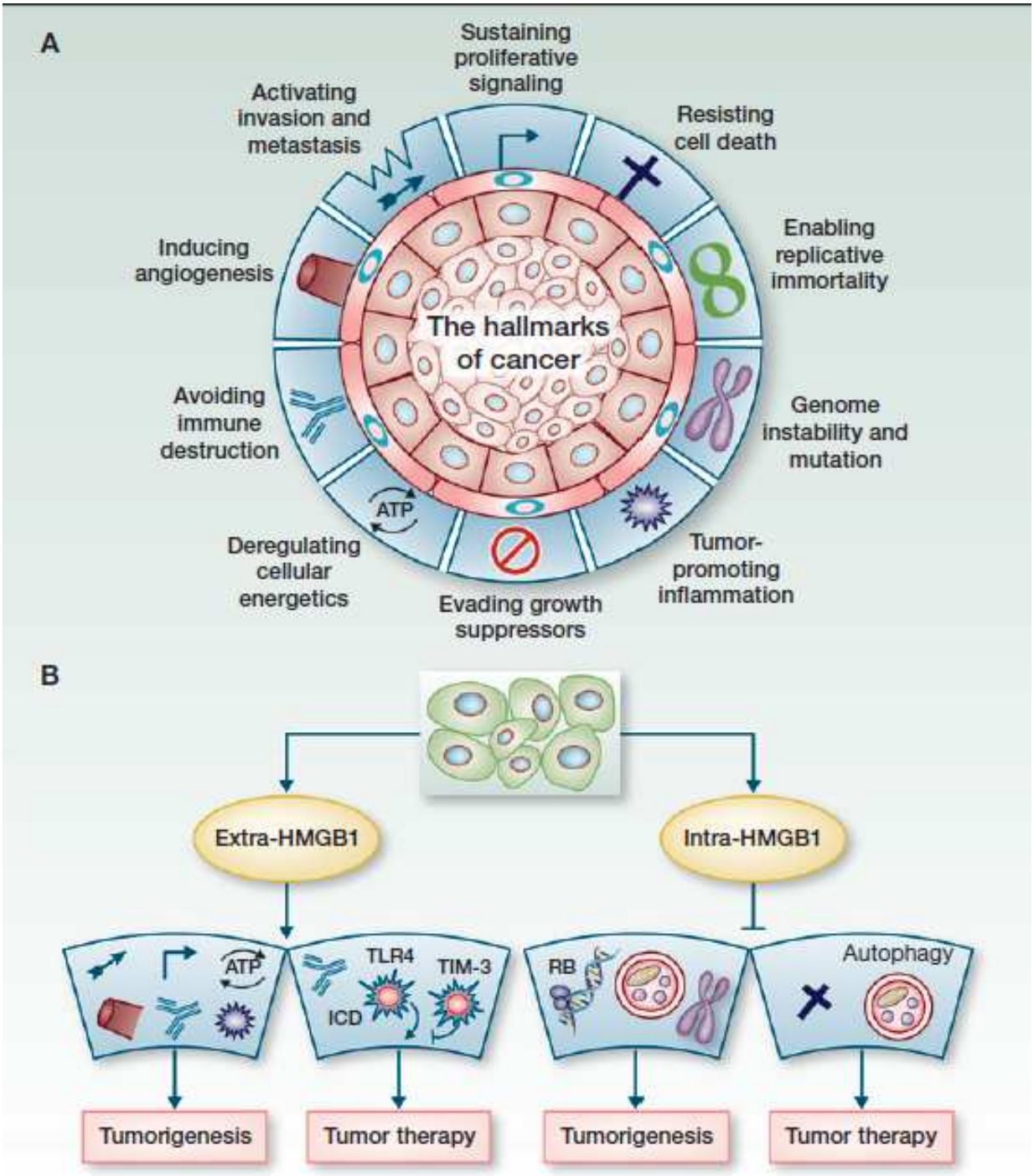


Figure 3: HMGB1 and its relationship with the hallmarks of cancer

Generation of rapresentative murine cell lines and murine model as an experimental study system for malignant mesothelioma

MM is resistant to the conventional forms of treatment, and adequate scientific and clinical assessment of this disease has been severely limited by the lack of representative cell lines and animal models and by the limited number of patients treated in a single institution. Thus, the establishment of representative in vitro cell lines and animal models is important for the development of potentially effective forms of diagnosis and therapy and for the study of basic biology. These models have been used primarily to study the pathogenesis and karyotypic changes found in mesothelioma, and have shown that the animal models mimic the human disease in several aspects including morphology, histopathology and the presence of chromosomal abnormalities.

Murine mesothelioma cells have been obtained by tumour masses which were developed into BALB/c mice induced by asbestos intraperitoneally injection (38). The AB1, AB12 and AB22 cell lines are used as model systems for various *in vitro* and *in vivo* studies of mesothelioma basic biology (39,40). These cell lines have been shown to be mesothelial in origin, tumorigenic in syngeneic mice and to have many features in parallel with human MM cell line. So, they will provide a valid experimental model for human MM. Furthermore, the murine MM lines offer the opportunity to set up a syngeneic model system providing a full immunological tumour response. However, the characterization of murine MM cell lines is partial and need the elucidating of other features. To complete their characterization, I in collaboration with the Division of Genetics and Cell Biology, San Raffaele Hospital provide the elucidating of phenotypical and molecular features of AB cell lines and tumor masses derived thereof in BALB/c mice. First, we characterize their surface markers, explored their migration and invasion potential in vitro and their response to exogenous HMGB1. Upon the generation of tumor masses into BALB/c mices by intraperitoneally AB1, AB12 and AB22 injection, we study their growth and vascularization employing different imaging techniques. Whereas their surface markers were analyzed by immunohistochemistry. Finally, we provide evidence that murine MM masses respond to treatment with chemotherapeutics routinely used to treat mesothelioma patients. The syngeneic system recapitulate the features of human mesothelioma, providing an experimental system for preclinical studies.

Materials and methods

Cell lines, culture conditions and manipulations

Murine malignant mesothelioma (MM) AB1, AB12 and AB22 cells were obtained from Cell Bank Australia and cultured in RPMI 1640 (Life Technologies) supplemented with 5% (AB1 and AB12) or 10% (AB22) v/v fetal bovine serum (Life Technologies), 2 mM L-glutamine and 100U/ml penicillin/streptomycin. The cell-culture flasks were incubated in a humidified, 5% CO2 atmosphere at 37°C and the medium was change every 2 days.

Each cell line was intraperitoneally injected in BALB/c mice to obtain tumors (Fig.4). The masses were explanted and disaggregated; the resulting cells were cultured as above and named AB1-B/c, AB12-B/c and AB22-B/c. Luciferase-expressing cells were obtained by infecting MM cells with a lentiviral vector carrying the luciferase gene (pLenti PGK V5-LUC Neo (w623-2); Addgene). Infected cells were selected with geneticin and maintained in culture as above. Cells generated from the original strains were named: AB1-LUC, AB12-LUC and AB22-LUC. Cells generated from the masses in BALB/c mice were named: AB1-B/c-LUC, AB12-B/c-LUC and AB22-B/c-LUC.

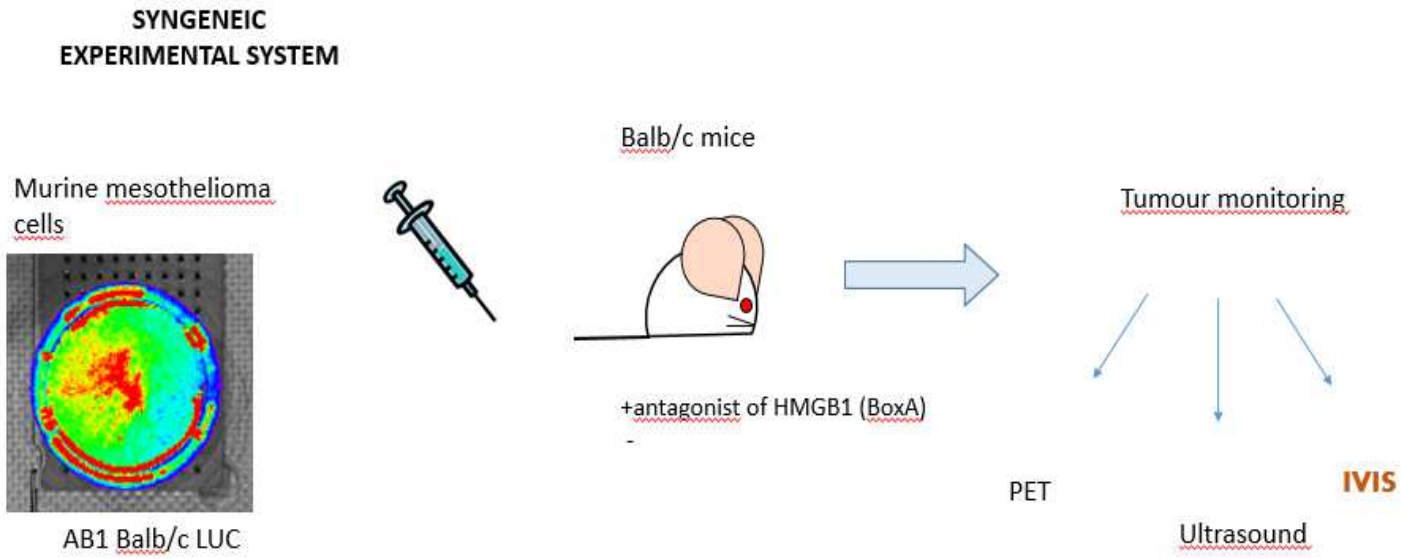


Figure 4: Description of a syngeneic experimental system

Mice

Animal experiments have been reviewed and approved by the Animal Care and Use Committees (IACUC) of both the Ospedale S. Raffaele that include members for ethical issues.

The animals were monitored and supervised by a certified veterinarian. He also checked the experimental protocols and procedures revision. Animals were housed in the Institutes' Animal Care Facilities, which meet international standards.

***In* vivo BioLuminescence optical Imaging (BLI)**

We injected intraperitoneally 7 x 104 AB1-B/c-LUC cells on mice and monitored tumour growth using an IVIS SpectrumCT System (Perkin Elmer).The system is equipped with a low noise, back-thinned, back-illuminated CCD camera cooled at -90 C (quantum efficiency in the visible range above 85%). The mices received intraperitoneally injections of 6 g of luciferin/kg body weight about 20 minutes before image acquisition. During image acquisition, the animals were kept at 37o C and under gaseous anesthesia (2–3% isoflurane and 1 lt/min O2).

After luciferin injection dynamic BLI was performed from 0 to 30 minutes by acquiring an image every 2 minutes (exposure time = auto, binning = 8, f = 1 and a field of view equal to 13 cm (field C)) in order to detect the highest BLI signal. BLI image analysis was performed by measuring the total light flux (photons/seconds) in a Region of Interest (ROI) placed over the animal abdomen. Images were acquired and analyzed using Living Image 4.4 (Perkin Elmer);

Ultrasound scans

First we anesthetized the mices by gaseous isoflurane. We use a Vevo 2100 equipment (FUJIFILM VisualSonics Inc.) especially designed for the examination throught ultrasound of small experimental animals to examine the tumor masses. Ultrasound images in B-mode (Brightness mode) were performed using a Vevo 2100 linear array transducer with a center frequency of 40 MHz (MicroScan MS 550D; 22–55 MHz; FUJIFILM VisualSonics Inc.).

Clear field microscopy

The Cell lines maintained in culture (AB1, AB12 and AB22) in cell dishes were visualized with a Zeiss Observer Z1 microscope.

Electron microscopy

Cells were grown on coverslips and previously prepared to be mounted on a Leica Ultracut UCT ultramicrotome. Ultrathin (70-90nm) sections were collected on copper grids and stained with uranyl acetate and Sato's lead citrate before imaging with a ZEISS Leo AB 912 Omega transmission microscope. Images were acquired by a 2k x 2k bottom-mounted slowscan Proscan camera controlled by the EsivisionPro 3.2 software.

Hematoxylin and eosin stain

The staining of tumor masses were visualized with an Olympus BX51 Light Microscope.

Histopathology and Immunohistochemistry

The samples that will be processed for histopathological examination were fixed in formalin at 10% for at least 24-48 hours and paraffin embedded (Embedding Center Leica EG1160).
4 microm sections were routinely stained with Hematoxylin-Eosin (HE) and evaluated under a light microscope (Leica DM 2500). The images were captured with a digital camera (Leica DFC310 FX).
For immunohistochemistry 4 □m serial sections from each sample were immunostained with the primary antibodies l'Anti-CD31 specific for endothelial cells to monitor the vascularization into the tumor masses. After the incubation with appropriate biotinylated secondary antibody (goat antirabbit VC-BA-1000-MM15 or rabbit anti-goat VC-BA-5000-MM15, Vector Laboratories, USA) will occur. Sections were labelled by the avidin-biotin-peroxidase (ABC) procedure using the VECTASTAIN® Elite ABC-Peroxidase Kit Standard, VC-PK-6100-KI01 kit (Vector Laboratories). The immunoreaction was visualized with 3,3'-diaminobenzidine (Peroxidase DAB Substrate Kit, VC-SK-4100-KI01, Vector Laboratories) substrate and sections were counterstained with Mayer's haematoxylin.

Results:

AB1, AB12 and AB22 murine MM cell lines were previously generated in female BALB/c mice by IP injection of asbestos fibers and we cultered them in vitro. We observed their aspect in microscope and detected that they have similar phenotypes to the sarcomatoid, biphasic and epithelioid cells of human mesothelioma, respectively (Figure 5a-f).

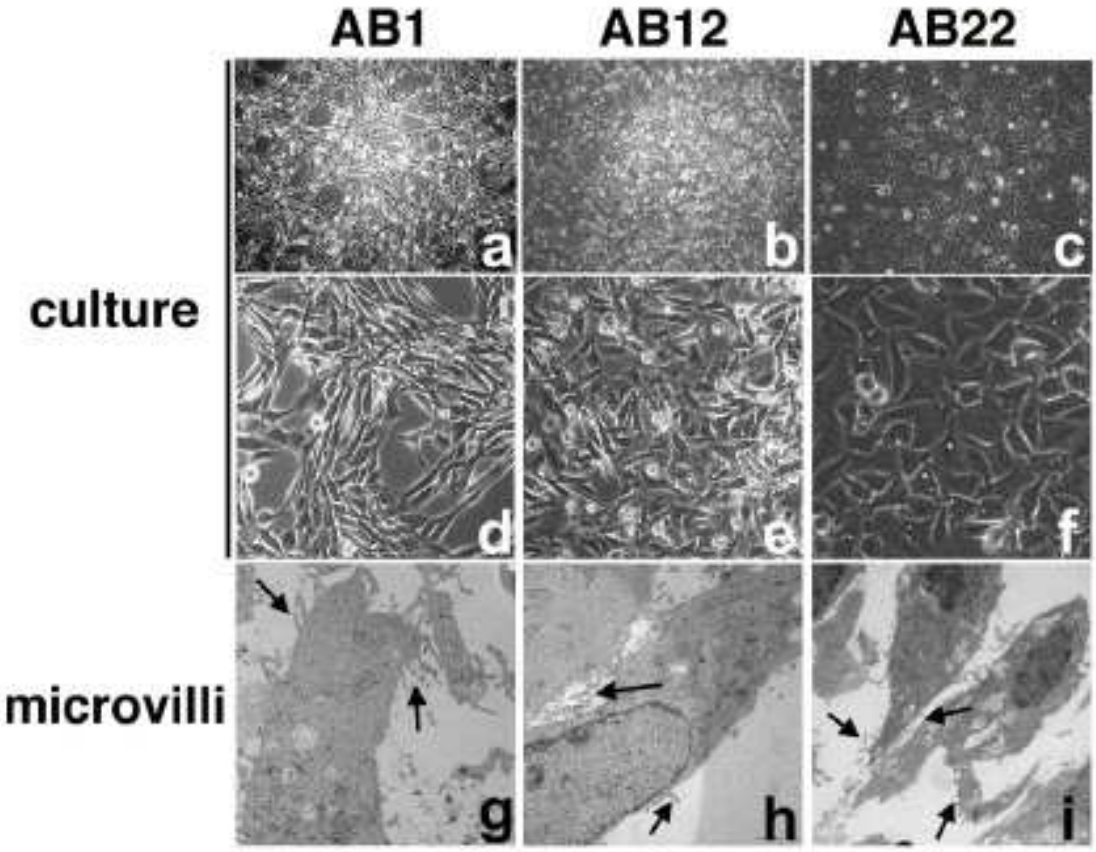
The detection of microvilli on the surface of such cells by electron microscopy (Figure 5 g-i), indicate similiraties with the electron micrograph of cultured MM cells with a microvillous-rich surface.

Thus murine MM cell lines accurately recapitulate the morphologic features of human MM cell lines.

The original cell lines were: 1) re-injected in male BALB/c mice (and named AB1-B/c; AB12-B/c; AB22-B/c); 2) stably infected with a lentiviral vector which express constitutively the luciferase gene (and named AB1-LUC; AB12- LUC; AB22-LUC) or

3) sequentially underwent both manipulations (and named AB1-B/c-LUC; AB12-B/c-LUC; AB22-B/c-LUC).

The manipulated cell lines displayed the same features of the original strains. For this reason they were interchangeably used in the experiments.



, **Figure 5– Morphology of murine MM cell lines –** Cultured AB1, AB12 and AB22 cells were visualized with a Zeiss Imager M2 microscope and photographed with a 10x (a-c) and 20x (d-f) objective; cell lines (g-i) in electron microscopy

Characterization of tumor masses generated by murine MM cell lines

IP injection of murine MM cells, whether manipulated or not, in BALB/c mice generated sizable tumor masses in approximately 2 – 3 weeks after injection. Their growth was followed by ultrasound scans in parallel with the detection of bioluminescence (IVIS), as shown in Figure 6. The combination of the two techniques allows the evaluation of 2 important parameters: the size and the location of masses, relative to the other organs in the abdomen. In particular, time-wise increases of the bioluminescence signal allow following the growth of masses and assign a rough abdominal location. Strong vs. weak IVIS signals can be due to the location of tumors in the abdomen (superficial vs. deep) or to their size (large vs. small). Such quandaries can be clarified by the use of ultrasound scans that yield more precise measurements of tumor size, pinpoint their spatial location and reveal their relationship with other organs of the abdominal cavity.

Figure 7 show a haematoxylin and eosin stain of explanted and formaldehyde-fixed tumors form AB1 cells. Another important features of tumor development is the neoangiogenesis ant its ability to support tumor invasiveness and growth. We also study the vascularization and observed it using anti-CD31 antibodies, specific for endothelial cells. We did this evaluation in both human MM tumor embedded samples and tumors derived from murine MM cells. In the figure 4/a we observed the vascularization only at the periphery of the murine masses. Although their inner portions do not show identifiable vases, they do not show any sign of necrosis either. It was shown that indeed small ectopic vases are present inside the masses (Figure 8a), providing a sufficient vascularization to support tumor growth and prevent necrosis. .In the figure 8b we can observe the vascularization into a human MM samples stained with CD-31. We can see the similarity of vascularization process between human and murine tumors.

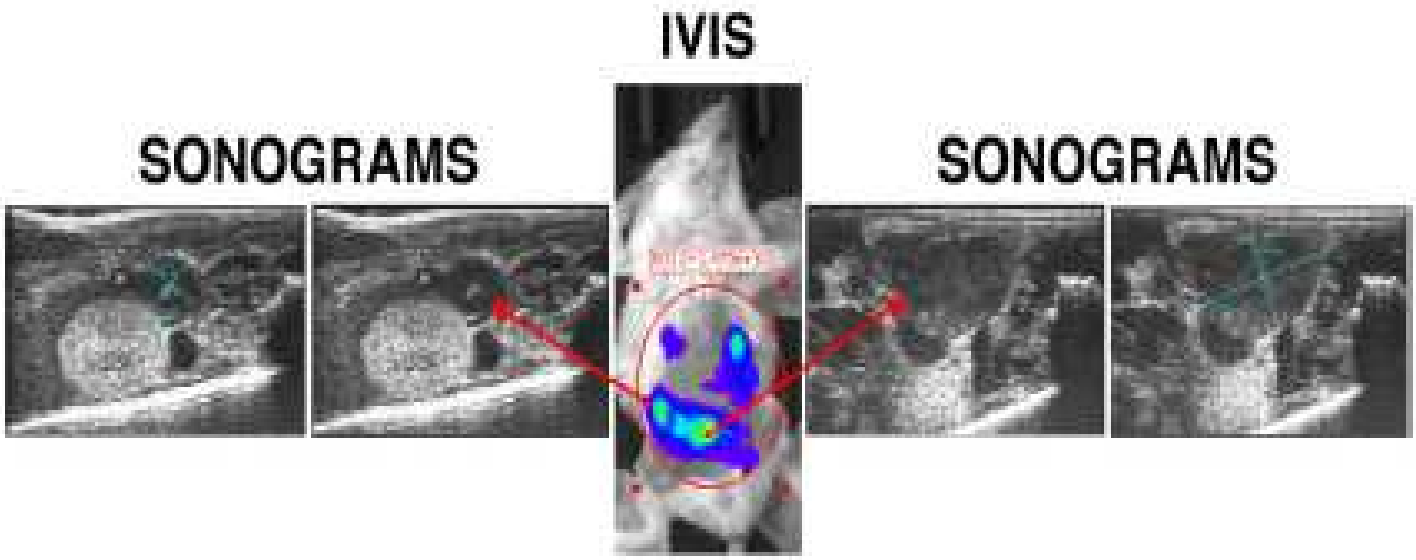
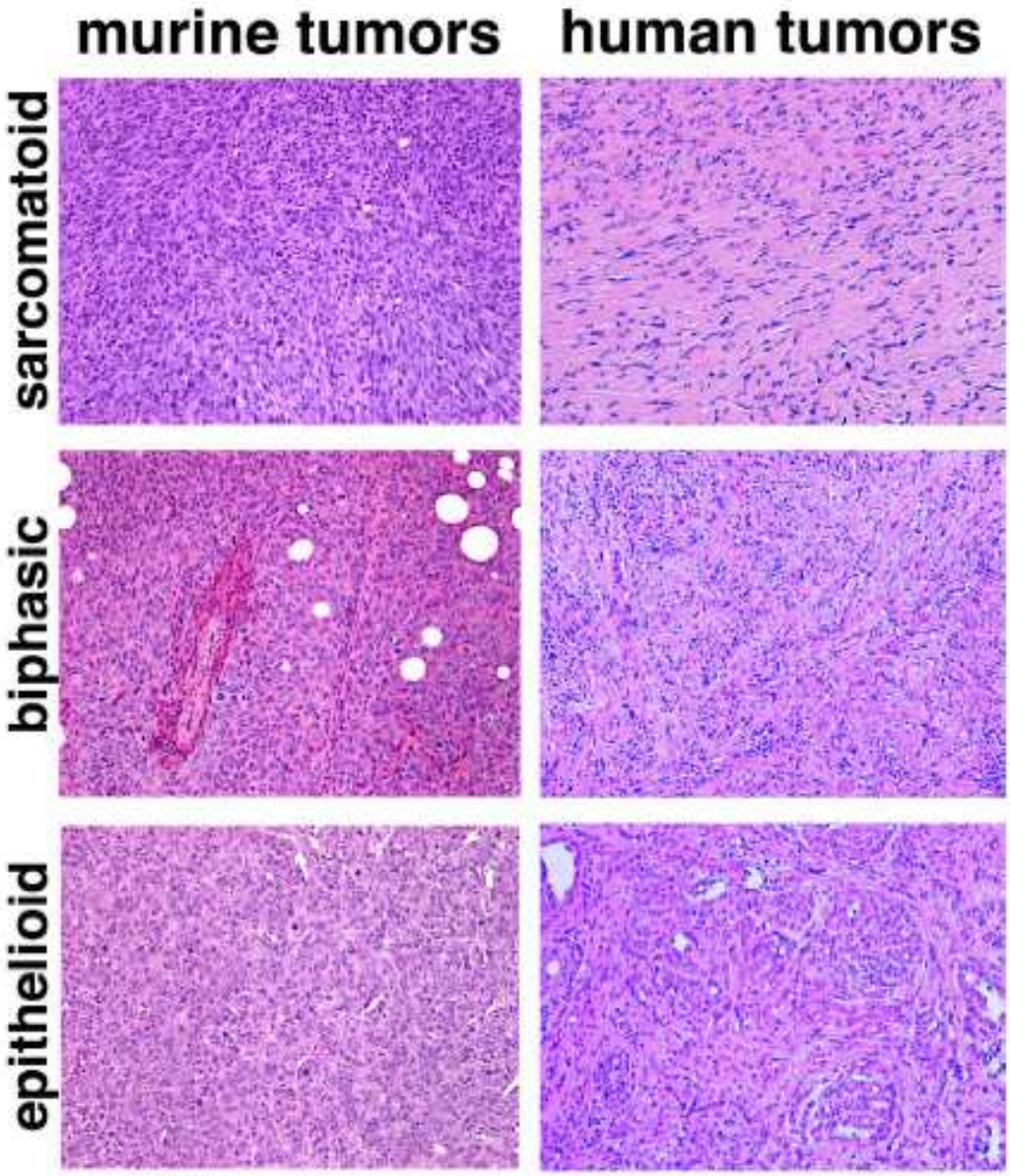


FIGURE 6–Tumor detection *in vivo* by IVIS and ultrasound



Figures 7: Murine and human tumors have similar phenotypes – Slices of explanted tumor masses generated by injection of AB1, AB12 and AB22 cells in BALB/c mice were stained with hematoxylin and eosin (H&E), as were slices from human sarcomatoid, biphasic and epithelioid mesotheliomas. The architecture of murine tumors appears similar to that of the corresponding (sarcomatoid, biphasic, epithelioid) human masses.

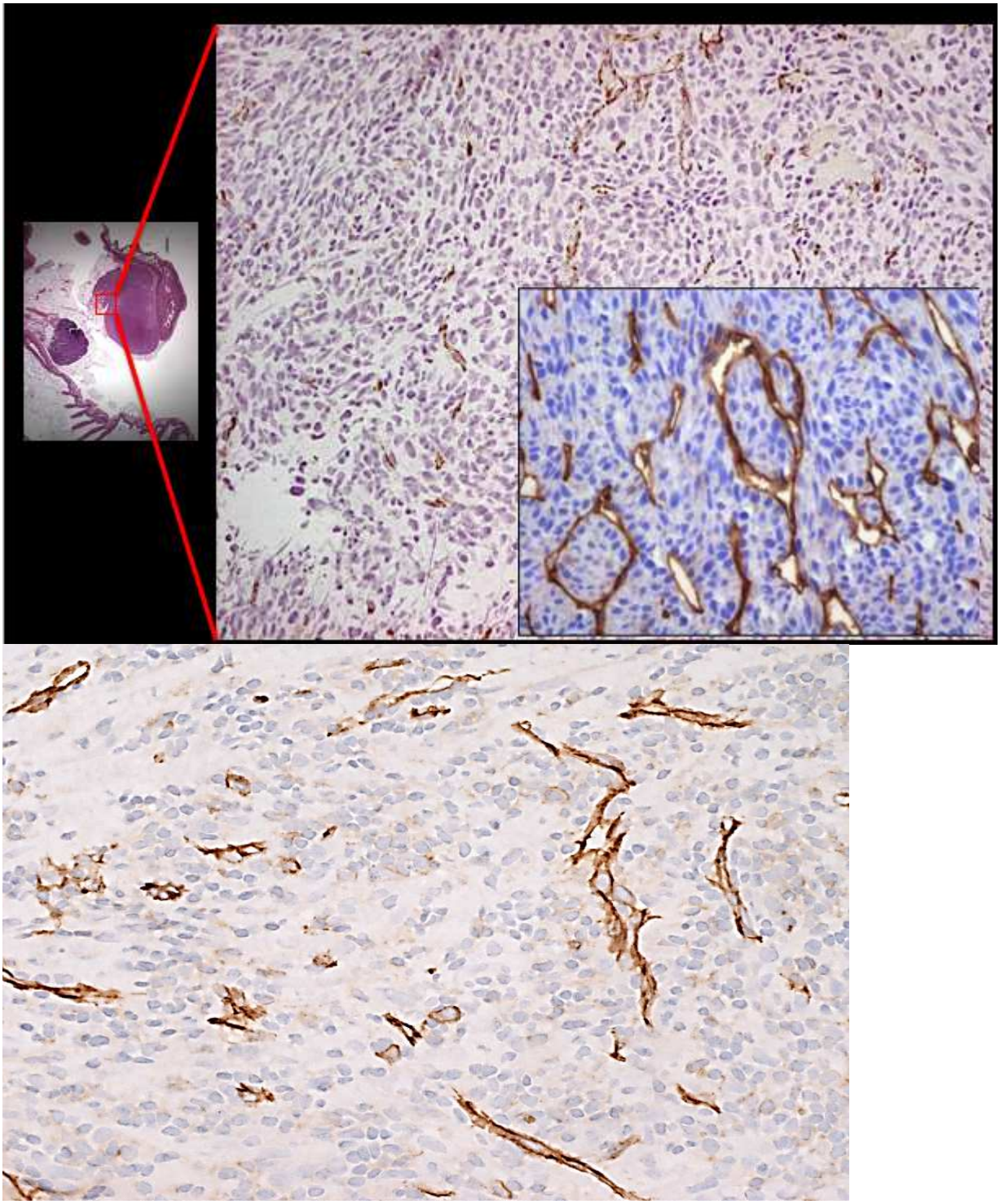


Figure 8a: vascularization of mice tumor masses generated after IP injection of AB1 cell lines;
Figure 8b: vascularization of human MPM

Explanted murine sarcomatoid, biphasic and epithelioid tumors bear a significant similarity with samples of human biopsies of the same tumor subtype (Figure 9). The murine MM cells and the tumors generated by them were immunostained by a panel of antibodies used for the diagnosis of human malignant mesothelioma.

The results of staining done by the other members which collaborate in this project indicate that only vimentin yielded a positive signal on all cells and tumors, whereas only AB22 cells were positive for WT1. Nevertheless, both cells and tumors were positive for other epithelial markers, such as E-cadherin and b-catenin, and also yielded a signal for SMA (Figure 9).

IHC characterization of MM is still ambiguous: a high variability of surface markers has been observed and is not reached a consensus on which and how many markers should be considerate for a positive identification. These data are the first IHC characterization of these
murine MM lines and tumors generated. The expression of their surface marker stained by IHC
The data reported here are the first IHC characterization of these display discrepancies in the expression of surface markers. But this different expression has been reported previously for human MM cell lines (mantained in vitro) and tumors.

Primary Antibodies *(abbreviation)*	Cell culture			Tumors		
	AB1	AB12	AB22	AB1-B/c	AB12-B/c	AB22-B/c
Vimentin *(Vim)*	+	+	+	+	+	+
Wide Spectrum Cytokeratin *(WSCK)*	-	-	-	-	-	-
E-cadherin phospho *(E-cad)*	+	+	+/-	+	+/-	+
Smooth Muscle Actin *(SMA)*	+	+*	+*	+/-	+/-	+/-
Wilm's Tumor Antigen 1 *(WT1)*	-	?	+	-	-	-
β-catenin *(βcat)*	+	+	+	+	+	+

(-) = absence of staining; (+/-) = weak staining; (+) = positive staining; (*) = some cells do not express the protein; (?) = uncertain.

Figure 9: Immunohistochemical characterization of the surface markers of MM cell lines and tumors derived thereof. – (a) Following detachment from culture dishes, cells were fixed and centrifuged; the pellets were then slices with a microtome and stained with the indicated antibodies as described. (b) Explanted tumor masses were fixed, sliced and stained with the same antibodies as in (a), as described.(data provide not by other collaborators of the project).

Conclusions and future research

The previous study indicates the description of a murine syngeneic system in order to have an experimental model of study for MPM. Our results indicates that the MM cell lines (AB1, AB12) generated by Davis M.R. et al 1992 exhibit some of the main features at the morphological level, functional and phenotypical of human MM in vitro and in vivo; whereas AB22 cells do not fully summarize epithelioid mesotheliomas. Such a model study would be used for the pre-clinical evaluation of potential therapeutic agents because it 'mimic' the phenotipic characteristics of mesothelioma.

However, this system limit us to better understand the spectrum of genetic, epigenetic changes and the role of tumor microenvironment in tumorigenesis of mesothelioma. Recently, xenografts using well-established human tumor have become popular because they accurately recapitulate the features of patient tumors and the complex factors that promote tumor progression and metastasis.
Establishing a PDX Patient derived xenograft model would allow us to significantly broaden the studies on MM, ranging from sequencing to drug testing. More importantly, these studies can be carried out on the same "lesion" originally transplanted in mice (and expanded in other mice of the same strain), thus maintaining a high degree of homogeneity. Although a PDX model does not fully recapitulate the patient's tumor, it is a system resulting "less different" from the original lesion and therefore providing highly translational results.

My PhD project this year will give me the opportunity to provide the development of a direct xenograft models generated directly from MPM tumor samples of patients without a cell line intermediary. This approach may better preserve tumor heterogeneity and limit the *ex vivo* manipulation inherent in the culturing of cancer cell lines. Most direct xenograft tumors grow with considerable stromal elements and recapitulate the histological appearance of the original patient tumor over multiple passages in mice. The value of such a program is reflected in its inherent versatility; direct xenograft models may be used to study diverse aspects of cancer biology including drug resistance, angiogenesis, tumor microenvironment, cancer stem cells and experimental therapeutics.

To develop this effective system, I will use tumor fragments obtained by patients diagnosed with MPM and will directly implant them in NSG (NOD scid gamma) mice in heterotopic sites. Tumor take and growth will be monitored by small animal imaging (sonogram, PET, IVIS) and analysed by immunohistochemistry and molecular biology experiments. Grown tumors will be then transplanted in recipient NSG mice for further studies, such as, for instance, treatment with HMGB1 inhibitors. Recent published results sustain that inhibition of HMGB1 impaired in vitro tumorigenesis of malignant mesothelioma (MM) cells and reduce

tumour growth in xenografted SCID mice. The treatment with BoxA (HMGB1 antagonist) will allow to determine how many and which patients are potentially responsive, providing both quantitative and qualitative pre-clinical results.

MPM tumor samples and tumor xenografts into immunodeficient mices

There were collected 10 resected tissues of Malignant Pleural mesothelioma biopsies after extrapleural pneuctomeny at the Unit of Thoracic Surgery at Hospital of Novara for the period 2014-2015. Fragments for each tumor samples was processed for Hematoxylin and eosin stain and Immunohistochemistry in order to obtain a diagnosis and histologic classification analyzing the expression of specific markers by the pathologist. The tumor samples characterized by a adequate immunohistochemical pattern was named MN1-MN10. The samples were frozen in 10% DMSO/90% FCS and stored at -135 °C for future use. These frozen stocks should maintain their capacity to grow both in vitro and in vivo. The human MPM samples from surgical specimens should be acquired under the strict supervision of staff pathologist(s) to maintain patient care and to ensure adequate tissue diagnosis.

Generating of a xenograft model

Immunodeficient mice NSG aged 6 to 8 weeks should be housed under specific pathogen-free conditions to prevent sickness and infectious outbreak. Direct transfer of human pancreatic tumors into immunodeficient mice requires institutional review board as well as Institutional Animal Care and Use Committee (IACUC) approval and must be conducted in accordance with institutional and national regulations. The MPM samples conserved as decrived previously may be implanted in an heterotopic site (subcutaneously) which permit us an accurate monitoring and measurement of tumor size. The tumor implantation will performed as described into the protocol (41).

After tumor implantation, mice should be monitored daily for signs of illness and surgical wounds assessed for infection. To maintain and expand tumor derived from a specific xenograft 'line', we routinely propagate heterotopic tumors. Furthermore the NSG mices which have developed MPM will be treated with BoxA by intraperitoneal injections. Box A, inhibitor of HMGB1 will be test in a preclinical level and will provide us further information about the role of HMGB1 in the MPM progression and its potential role as therapeutic target.

Consistently,

Different experimental results indicate that the sustained release of HMGB1 by malignant mesothelioma cells, along with its secretion by surrounding inflammatory cells, support the malignant phenotype of mesothelioma, suggesting its role as a novel molecular target. Furthermore , mesothelioma patients have elevated HMGB1 serum levels, suggesting that HMGB1 may be a novel biomarker for malignant mesothelioma.

Different clinical and in vitro studies revealed that overexpression of HMGB1 in many tumour types, including breast cancer], hepatocellular carcinoma, melanoma , gastric cancer, and colorectal cancer and its association eith tumour growth and metastasis (41). There are yet present clinical studies which evaluate the levels of HMGB1 in a 'important' number of MPM paraffin-embedded tissues and its possible correlation with the tumor progression and its role in treatment resistance. For this reason during my PhD period, I would be very interested to Investigate the HMGB1 expression in MPM cases and its potential prognostic/predictive significance.

The present study will be carried out in a larger number of archival MPM tissue samples and cell lines and will assess whether HMGB1 expression was correlated with clinicopathological parameters and prognosis in patients.

Patients and MPM tissue samples:

We hipothesize that our study will involve at least 50 consecutive MPM patients admitted to the Thoracic Unit of the University Hospital of Novara between January 2008 and December 2010, all of whom were diagnosed as having MPM on the basis of multiple pleural biopsies taken by means of video-assisted thoracoscopy. We obtained by surgery tissue samples and after their routinely processing for histology and immunohistochemistry staining, the pathologists of Pathology Unit at Novara's Hospital diagnose the MPM cases. The diagnosis and their histological classification (epithelioid, biphasic and sarcomatoid) was based on standard histological and immunohistochemical criteria, including positivity to calretinin, vimentin, and cytokeratins 5 and 6, and negativity to carcinoembryonic antigen, thyroid transcription factor 1, and Ber Epy 4. For this study we will analyze the 70 paraffin-embedded MPM tissue samples previously involved in a study done at the Laboratory of Molecular biology, Unit of Pathology (Mezzapelle R et al, 2012). Thus we have the patient clinicopathological data (asbestos exposition, histological subtypes, tumor stage, PS, DSS, therapy and treatment), their follow-up and the molecular analysis (mutation analysis of EGFR downstream pathway).

To investigate whether HMGB1 abnormalities are linked to MPM, we first analysed HMGB1 mRNA expression using RT-PCR and evaluate the protein expression by Immunohistochemistry into the paraffin-embedded sections of mesothelioma tissues.

Immunohistochemistry for HMGB1 staining

Immunohistochemistry will carried out on MPM tissues and normal pleura paraffin-embedded with rabbit polyclonal anti-HMGB1 (Abcam). Goat anti-rabbit secondary antibody and Vectastain Elite ABC kit (Rabbit IgG; Vector Labs) will be used according to the manifacture's instruction. The IHC results should be analyzed by a board of pathologist.

mRNA extraction, cDNA synthesis and evaluation of HMGB1 mRNA by real-time PCR

First, the collection of FFPE samples and their Hematoxylin-eosin staining will be analyzed by a pathologist to select the neoplastic area of tissue. Then, the total RNA will be extracted by Total Recover RNA following manufacture's instruction. We will quantify the RNA extracted by spectrophotometry, using NanoDrop (ThermoScientific). Then, the reverse transcription to complementary DNA (cDNA) by means of TaqMan® reverse-transcription kit (Applied
Biosystems). TaqMan® miRNA Assays (Applied Biosystems) will be used to quantify mature mRNA expression for HMGB1 and the reference gene GAPDH. Quantitative Real Time PCR will be carried out in triplicate on 7500 Fast Real-Time PCR Systems (Applied Biosystems) using a primer specific TaqMan® gene expression Assays (Applied Biosystems) for profiling HMGB1. Quantification of miRNA expression will be performed with the 2-ΔΔCt method using normal lung tissue as calibrator

microRNAs and Mesothelioma

The study of MM basic biology help me to better understand the molecular mechanisms which contribute its tumorigenesis and to enhance my knowledges in the new potential clinical markers.

Recent research revealed that microRNAs play important roles in the biology of MPM, and could be potential biomarkers and therapeutic targets. These research findings also suggested that miRNAs changes affect a variety of the phenotypes characteristic of the tumour, including reduced response to apoptotic signals, elevated rates of metabolism and proliferation, enhanced migration and invasion, and resistance to chemotherapy and radiation.

MicroRNAs (miRNAs) are a class of evolutionarily conserved short non-coding RNAs of 18-22 nucleotides in length that regulate gene expression at the posttranscriptional level (Bartel et al. 2009). MiRNAs negatively regulate gene expression (~30% of protein-coding genes) by modulating target mRNA translation efficiency and are pitoval to many cellular processes including development, differentiation, proliferation, apoptosis, and stress response. As such, dysregulated microRNA expression within the cell can induce abnormal cell behaviour and is common feature in human diseases, especially cancer. Dysregulated miRNAs levels have been observed in many human cancers (Cortez et al. 2011), suggesting that they could be promising alternative biomarkers for cancer diagnosis, informing prognosis and monitoring treatment response.

We will start the studying of a panel of miRNA expression targeting HMGB1 and other proteins involved into MPM carcinogenesis pathways as potential important prognostic and predictive biomarkers.

1- miR-325

In a recent study, has been investigated the potential role of miR-325 in patients with Hepatocellular carcinoma. They verified that miR-325 could regulate cells invasion and proliferation of HCC by targeting HMGB1, as a potential prognostic marker for HCC. Moreover, miR-325 was frequently downregulated in HCC and the decrease of miR-325 levels was significantly correlated with poor prognosis of patients with HCC. HMGB1 overexpression has a significant role in malignant mesothelioma progression. Thus determining the levels of miR325 into mesothelioma cancer cells or tissues in relationship with clinical outcome will be very significant for mesothelioma prognosis.

2- miR-34a

miR- 34A has been implicated in multiple cancer types, including retinoblastoma, and its expression is a prognostic parameter. Numerous studies demonstrate that *MIR34A* is critically involved in regulating diverse tumour cellular processes such as apoptosis and the cell cycle.

It was previously reported that microRNA-34s (miR-34s) are downregulated in MPM. In a recent study, the downregulation of miR-34a, -34b and -34c in all the examined mesothelial cell lines, increased cell proliferation and invasivity. This strongly suggest that miR-34s play an important role in the early carcinogenic process involved in the transformation of human mesothelial cells to MM. *MIR34A* (*microRNA 34a*) is a tumor suppressor gene.

It was shown that *MIR34A*-dependent high mobility group box 1 (*HMGB1*) downregulation inhibits autophagy and enhances chemotherapy-induced apoptosis in the retinoblastoma cell.

MIR34A inhibition of *HMGB1* leads to a decrease in autophagy under starvation conditions or chemotherapy treatment. Inhibition of autophagy promotes oxidative injury and DNA damage and increases the apoptotic process.

Finally, upregulation of *MIR34A* restores chemosensitivity and enhances tumor cell death in the retinoblastoma cell, inhibiting autophagy in a HMGB1 dependent manner. One of the main problems into MPM treatment is the gain of chemotherapy resistance **(**combination of Alimta and cisplatin**.** It will be interesting to study the levels of miR34a into Mesothelioma samples and how their expression contribute into chemotherapeutic response of patients and prognosis.

3- miR-218

Functional studies showed that miR-218 overexpression inhibited cell migration and invasion in non-small cell lung cancer, inhibiting the expression of high mobility group box-1 (HMGB1) by directly targeting its 3 '-Untranslated region. It was previously validated the role of HMGB1 into the tumorigenesis of MPM in vitro and in vivo. This suggest its potential function in inhibition of tumor invading and metastasis in its late

stage. One of the causes of chemoresistance leading to poor prognosis is the increased ability to undergo autophagy. It was clinically proved that miR-218 was significantly downregulated into endothelial cancer cells resistant to chemotherapy compared to the non-drug resistant cell lines. HMGB1 was upregulated in resistant EC cells and mediates autophagy, contributing to chemotherapy resistance. Whereas miR-218 overexpression sensitized the resistant cells to chemotherapy. These results reveal novel potential role of miR-218 against chemotherapy resistance during the treatment of endometrial carcinoma. It can be an important predictive factor for malignant mesothelioma. A study into glioma cell lines and tumor glioma samples has done. It indicates that the downregulation of miR-218 activates the NF-KB pathway (by targeting IKK-b) resulting in reduced migratory speed and invasive ability. Aberrant activation of nuclear factor-kappa B (NF-kB) pathway has been proven to play important role
in the development and progression of MPM. So, it will be interesting to study the expression level of miR-218 and its impact into clinical outcome of MPM's patients.

miRNA Extraction, cDNA synthesis and miRNA expression analysis by real-time PCR.

Total RNA will be extracted from histological and cytological FFPE samples by miRNeasy FFPE Mini Kit (Qiagen) following manufactures instruction. RNA quantity will be tested by spectrophotometry, using NanoDrop (ThermoScientific), and then reverse transcribed to complementary DNA (cDNA) by means of TaqMan®miRNA reverse-transcription kit (Applied Biosystems) and using miRNA specific primer. TaqMan® miRNA Assays (Applied Biosystems) will be used to quantify mature mRNA expression for the chosen candidate miRNAs: hsa-miR-325, hsa-miR-34a, hsa-miR-218 and the reference non-coding RNAs RNU6B.
Quantitative Real Time PCR will be carried out in triplicate on 7500 Fast Real-Time PCR Systems (Applied Biosystems) using a primer specific TaqMan® miRNA Assays (Applied Biosystems) for profiling miRNAs identified. Quantification of miRNA expression will be performed with the 2-ΔΔCt method using normal lung tissue as calibrator.

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SEMINARS:

1) 25/11/2014 at 14.00, Dr.ssa Roberta Arcidiacono e Dr.ssa Marta Ruspa
"La scoperta del bosone di Higgs"

2) 27/11/2014 at 14.00, Prof. Laura Baglietto (Inserm - Centre for Research in Epidemiology and Population Health, Paris) "Nuove sfide ed opportunità dell'epidemiologia molecolare per lo studio dei tumori"

3) 28/11/2014 at 12:00 “Humoral responses to HCV infection and clinical outcomes ”

4) 04/12/2014 at 15.00, Dr. Girish Patel (European Cancer Stem Cell Research Institute, Cardiff) “Uncovering the role of β-HPV in field cancerization: a collaboration in progress ”

5) 5/12/2014 at 14:00, Prof. Rifaat Safadi (University Medical Center, Jerusalem)
“Focus on the liver: from basics of NAFLD to hot topics in HBV & HCV infections ”

6) 16/12/2014 at 11.30, Prof. Antonio Musarò (Unit of Histology and Medical Embryology, Sapienza University of Rome) "From the legend of Prometheus to regenerative medicine"

7) 17/12/2014 at 13.50 , Dr. Roberto Furlan (Università San Raffaele, Milano)
“Microglia microvesicles: messengers from the diseased brain ”

8) 19/01/2015 at 14.10 Prof. Dr Yong-Sang Song (Seoul National University) “Anticancer strategy Targeting cancer cell metabolism in ovarian cancer”

9) 20/01/2015 at 14.00 Dr Tonino Alonzi (Istituto Nazionale per le Malattie Infettive "L. Spallanzani" IRCCS) “Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states”

10) 21/01/2015 at 14.00 Prof. Valeria Poli “Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis”

11) 27/01/2015 at 14.00 Prof. Antonio Sica (UPO Novara) “Myeloid cells as therapeutic target in cancer”

12) 11/03/2015 at 14.00 Prof. Darko Bosnakovski, PhD (Faculty of Medical Sciences Krste Misirkov bb, 2000 Stip R. Macedonia) "Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells "

13) 09/04/2015 at 12.00, Dr. Xiaoping Zhong , MD, PhD "Signal control in iNKT cell development and function"

14) 21/04/2015 at 14.00 Prof. Piergiorgio Percipalle, MD, PhD (Karolinska Institute) "Actin-based mechanisms in the control of gene expression and cell fate "

15) 07/05/2015 at 15.00 Prof. John McDonald, MD, PhD Georgia Tech University, Georgia (Atlanta, USA) "An Integrated Approach to the Diagnosis and Treatment of Ovarian Cancer "

16) 12/05/2015 at 12.00 Prof. Barone Adesi, "Good time for a change: bridging the gap between epidemiology, public health and basic sciences"

17) 14/05/2015 at 11:00 Kathleen Ruff, "Conflicting interests and scientific communication "

18) 25/05/2015 at 14:00 Steven R. Ellis (University of Louisville Kentucky, USA) "Ribosomopathies "

19) 05 /06/2015 at 12:00 Mariet C.W. Feltkamp (Leiden University Medical Center Leiden, The Netherlands) "Recent Developments in (cutaneous) Human Polyomavirus Research "

20) 15/07/2015 at 12.00 Ing. Marco Fadda "High- tech product preservation and operator protection: two apparently opposite requirements in different fields of medicine and biotechnology: the emerging glove box approach.

21) 03/09/2015 : Prof. Darko Boshnakovski (Faculty of Medical Sciences, Stip, R. Macedonia)
at 11.00 – 12.00 Cell based models for studying molecular mechanism of Facioscapulohumeral Muscular Dystrophy (FSHD)

at 12.00-13.00 Toward animal model for Facioscapulohumeral Muscular Dystrophy (FSHD)

Pubblications

None