

M14K and M38K Malignant Pleural Mesothelioma Cell Lines Preserve the Same Claudin-based Phenotype *In Vivo*

SIHAM CHAOUICHE-MAZOUNI^{1,3}, MARIE-CHRISTINE COPIN^{2,4}, PHILIPPE LASSALLE³,
NEMCHA LEBAILI¹, ALEXIS CORTOT^{3,4,5} and ARNAUD SCHERPHEREEL^{3,4,5}

¹Department of Biology, Kouba High School, Algiers, Algeria;

²Department of Pathology, Lille University Hospital, Lille, France;

³Lille Center for Infection and Immunity, INSERM U1019-CNRS 8204, Pasteur Institute, Lille, France;

⁴Lille Nord de France University, Lille, France;

⁵Department of Pulmonary and Thoracic Oncology, Lille University Hospital, Lille, France

Abstract. *Background: Malignant pleural mesothelioma (MPM) is an aggressive neoplasm with few treatment options. Reliable tumour cell lines for mesothelioma research are rare. Claudins seem to be attractive targets for cancer therapy. Aim: To establish a claudin-based MPM phenotype and to verify whether it is preserved in vitro and in vivo. Materials and Methods: Claudin-3 and -4 expression was examined by immunohistochemistry and immunoblotting in MPM (n=15) and lung adenocarcinoma (n=5) specimens. Claudin-3, -4 and -15 expression was also assessed in MPM versus adenocarcinoma cell lines and in MPM versus adenocarcinoma-derived tumour xenografts mouse models. Results: A defined MPM phenotype was established: M14K and M38K cell lines highly express Claudin-15 and calretinin but not claudin-3 nor claudin-4. Similar results were obtained in xenograft mouse models. Conclusion: M14K and M38K cell lines, whether in vitro or in an animal model are representative models and appropriate in exploring new therapeutic strategies in MPM that may target claudins.*

Malignant pleural mesothelioma (MPM) is a highly aggressive neoplasm with rising incidence in many countries worldwide. Because of its difficult diagnosis, poor prognosis and few treatment options without curative intent, new diagnostic and therapeutic strategies are needed (1, 2). However, mesothelioma cell lines available for such research are rare and often heterogeneous (3, 4). Therefore, characterization of MPM cell

lines expressing the same phenotype *in vitro* and *in vivo* would be of great interest.

Emerging studies suggest the interest of claudins in cancer. Claudins are a family of 27 protein members (5, 6). The second extracellular loop in claudin-3 and claudin-4 serves as a binding site for *Clostridium perfringens* enterotoxin (CPE) (7).

Members of the claudin gene family participate in the pathogenesis of human cancer (8). The expression of claudins in a tissue-specific manner, their differential expression between tumour and normal cells (9) and their membrane localisation, make them candidates for cancer therapy (10). Pre-clinical studies showed that tumour cells overexpressing claudins can be targeted *via* several approaches. Examples include the therapeutic antitumor efficacy of anti-claudin-4 monoclonal antibody for pancreatic and ovarian cancer (11), the therapy of experimental hepatocellular carcinoma using siRNA-based knockdown of claudin-10 (12) and the prevention of tumour growth by pre-treatment of mice with chimeric virus-like particles containing claudin-18 isoform-2 before challenge with syngeneic cell lines overexpressing this claudin (13).

In human mesothelioma, two studies reported claudin-3 and -4 to be absent from mesothelioma but overexpressed in adenocarcinoma (14, 15). In the other hand, Gordon reported a list of genes overexpressed in epithelioid MPM including claudin-15 (16), and Davidson found claudin-15 gene to be overexpressed in peritoneal malignant mesothelioma (17). Taken together, these data raise the interest of claudins in MPM.

In human MPM cell lines, little is known about claudin expression. Since claudins are targets for cancer therapy, a defined claudin-based MPM phenotype would be interesting for use in exploring new therapeutic strategies. In this work, we evaluated claudin-3, -4, -15 and calretinin expressions.

Correspondence to: Arnaud Scherpereel, Pulmonary and Thoracic Oncology Department, Hôpital Calmette, CHRU of Lille, 59037 Lille, Cedex, France. Tel: + 33 320444998, Fax: + 33 320445611, e-mail: arnaud.scherpereel@chru-lille.fr

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We selected claudin-3 and -4 because of the absence of their expression in mesothelioma. Claudin-15 was selected on the basis of its gene overexpression in mesothelioma (16, 17). The purpose was to verify whether the claudin-based MPM phenotype is preserved *in vitro* and *in vivo*, to select the most representative MPM cell lines that could serve as a model to improve knowledge and to explore new therapeutic strategies in MPM using claudins.

Materials and Methods

Twenty-four human specimens including MPM (n=15), lung adenocarcinoma (n=5) and normal lung (n=4) were used to examine the expression of claudin-3, -4 and calretinin. Specimens were provided by the Biological Center research (CRB) of Lille.

In addition to claudin-15 expression, the expression of these proteins in pathological tissues was compared with that of human MPM-derived cell lines (namely MSTO-211, NCI-H28, M38K and M14K), of human ADC-derived cell lines (namely A549, HT29, MCF7 and MDA-MB-231), of a primary culture of human bronchial epithelium (16HBE) and in human MPM or ADC-derived subcutaneous tumour xenografts obtained in severe combined immunodeficient (SCID) mice.

Antibodies used for immunohistochemistry and immunoblotting were purchased from Invitrogen Life Technologies (California, USA). They consisted of rabbit polyclonal antibodies against claudin-3, claudin-4, claudin-15 and calretinin diluted 1:100 in blocking buffer and the mouse monoclonal antibody against β -actin (Clone AC-15, Sigma Aldrich, Germany) diluted 1:5000.

Immunohistochemistry (IHC). The samples, fixed in formalin and paraffin embedded, were cut (3 μ m thick sections) and placed on silan covered slides. Morphological assessment of the same blocks was obtained by examining sections stained with haematoxylin-eosin-safran. Slides were stained with anti-claudin-3 and anti-claudin-4 (dilution 1:100), using a Ventana Ultraview DAB detection kit in a Ventana BenchMark XT processor (Ventana, Tucson, AZ, USA).

Antigen retrieval in EDTA buffer was carried out as a standard automated process on the Ventana BenchMark XT at 37°C for 30 min.

Cell culture samples. Four human MPM cell lines (MSTO-211H: mixed type, NCI-H28: sarcomatoid type, M38K: mixed type, and M14K: epithelial type) and four human ADC cell lines (A549: lung ADC, HT29: colorectal ADC, MCF7: non-invasive breast carcinoma and MDA-MB-231: invasive breast carcinoma) in addition to one primary culture of human bronchiolar epithelium (16HBE) were purchased from the American Type Culture Collection (USA), except for M38K and M14K cell lines, which were kindly provided by Dr Sakari Knuutila (Haartman Institute, Laboratory of cytomolecular genetics, University of Helsinki, Finland). MPM cell lines were grown in GIBCO RPMI-1640 supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine and Ticarcillin (200 mg/ml). ADC cell lines and the primary culture were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 2 mM glutamine and Ticarcillin (200 mg/ml).

All cells were grown in a humidified incubator at 37°C and with 5% CO₂ atmosphere. The cells were grown to confluency, detached with trypsin, centrifuged, and washed with phosphate-buffered saline (PBS).

Human cell line-derived tumour xenografts. To investigate the *in vivo* expression of claudin-3, -4, -15 and calretinin in MPM and ADC cell lines, 24 SCID male mice, 6 weeks old (Charles River laboratories, France) were divided into eight groups (n=3 each). Each group was inoculated with a subcutaneous injection of one of the eight cell lines. Groups inoculated with MPM cell lines received 2×10⁶ cells /200 μ l phosphate-buffered saline (PBS) and those inoculated with ADC cell lines received 1×10⁶ cells /200 μ l PBS, except the group for HT29 which received only 2.5×10⁵ cells.

Animal experiments were approved by the regional Ethical Committee. Mice were maintained and handled under aseptic conditions and were allowed free access to food and water. Tumour growth was assessed twice a week. The mice were sacrificed when the tumour size reached 1 cm³. Xenografts were harvested and snap-frozen for protein extraction and analysis.

Immunoblot analysis. Human fresh-frozen specimens, as well as tumour xenografts and cell pellets, were lysed in a lysis buffer containing PBS, 0.5% Nonidet P-40 and EDTA-free protease inhibitor cocktail (Complete, Roche Mannheim, Germany) for 1 h at +4°C. The lysed materials were then centrifuged at 12 000 \times g for 15 min at +4°C.

Total proteins were estimated using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA). Aliquots of cell lysates were combined with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, containing 0.1 M dithiothreitol (DTT) and denatured by boiling for 3 min at 100°C.

Samples containing 40 μ g of protein per lane were separated on 15% SDS-PAGE and transferred onto Hybond ECL nitrocellulose membranes (Amersham, Arlington Heights, IL, USA), according to standard procedures. The blotted membranes were incubated for 1 h at room temperature in a blocking buffer consisting of 5% nonfat dry milk in PBS and 0.1% Tween-20.

Membranes were then incubated by agitation overnight at 4°C with anti-claudin-3, anti-claudin-4, anti-claudin-15 and anti-calretinin diluted at 1:100 in blocking buffer and anti- β -actin mouse monoclonal antibody as control diluted 1:5000.

Blotted membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies diluted at 1:10,000 in blocking buffer for 1 h at room temperature. Membranes were washed after the primary and secondary antibodies in three changes of 0.1% Tween 20 in PBS for 10 min each. Chemiluminescent detection was performed using ECL reagents (Amersham) and luminol excitation was imaged on X-ray film. The size of protein bands was estimated by comparison to Full Range Rainbow molecular marker standard (Invitrogen, Life Technologies, CA, USA). For each case, immunoblot analyses were performed twice and the same results were obtained.

Results

MPM specimens lack claudin-3 and claudin-4 expression. To confirm the differential expression reported by others (15, 16), immunohistochemical detection of claudin-3 and -4 was performed on specimens of MPM (n=15), lung ADC (n=5), and normal lung tissue.

Claudin-3 and -4 were expressed in normal bronchus as well as in lung ADC. Claudin-3 was expressed at the cell membrane of normal bronchiolar epithelial cells (Figure 1C),

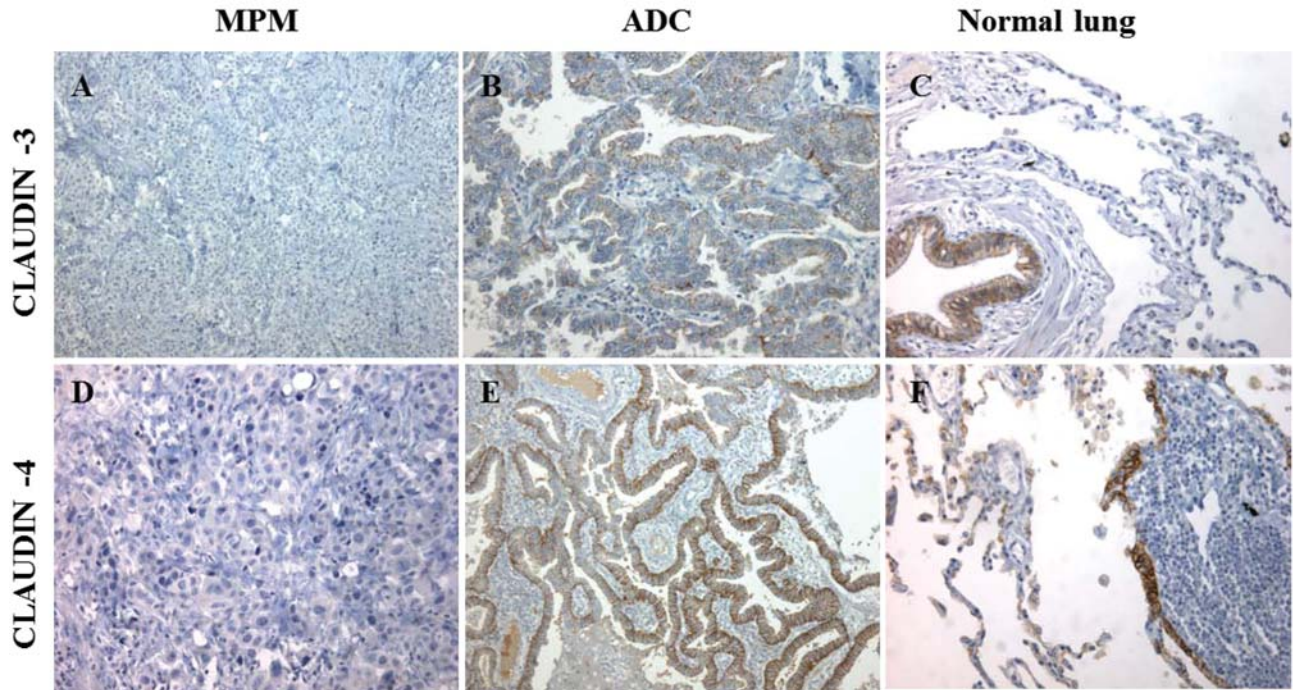


Figure 1. Claudin-3 and -4 expression in malignant pleural mesothelioma (MPM), lung adenocarcinoma and normal bronchus. Claudin-3 immunostaining: (A) in MPM, $\times 10$; (B) in lung adenocarcinoma (ADC), $\times 20$; and (C) in normal bronchus, $\times 20$. Claudin-4 immunostaining: (D) in MPM, $\times 20$; (E) in lung ADC, $\times 20$ and (F) in normal bronchus and pneumocytes, $\times 20$.

and at the cell membrane of ADC cells (Figure 1B). Claudin-4 expression and distribution were similar to those of claudin-3: intense expression at the cell membrane of normal bronchiolar cells (Figure 1F) and ADC cells (Figure 1E), but a weak expression in pneumocytes (Figure 1F). In contrast, both claudins were completely absent from MPM specimens (Figure 1A, D).

Human fresh-frozen tissues of each specimen were examined by immunoblots to verify whether claudin-3 and -4 expressions were similar to those observed by IHC. Claudin-3 (Figure 2A) and -4 (Figure 2B) were completely absent from MPM samples while present in lung ADC samples and their corresponding normal tissue, except sample 3 (IDC3, Figure 2A), where the normalization with β -actin indicated possible degradation of the sample. Calretinin (Figure 2C) was present in MPM specimens and absent from ADC. These results confirmed those obtained by IHC.

The results suggest an interesting claudin-based MPM phenotype, defined as the lack of expression of claudin-3 and -4 and the presence of that of calretinin.

Preservation of claudin-based MPM phenotype in human MPM-derived cell lines. We examined the expression of claudin-3, claudin-4 and calretinin in human cell lines of MPM and ADC and in 16HBE (Figure 3), in order to compare

them with the cell phenotypes observed in tumour and normal bronchial specimens.

Claudin-3 was absent from three MPM cell lines (NCI-H28, M14K and M38K) and was only present in MSTO-211. In ADC cell lines, claudin-3 was expressed in HT29 and MCF7, and was absent from A549 and MDA-MB-231.

Claudin-4 was absent from M14K and M38K but expressed in MSTO-211 and NCI-H28, and was present in all ADC cell lines except A549.

All MPM cell lines, except MSTO-211, exhibited a very strong signal for calretinin, contrasting with a very low signal observed in MDA-MB-231, MCF7 and A549. HT29, as well as 16 HBE, exhibited a strong signal for calretinin.

The expression of claudin-15 was also examined in MPM cell lines. The results indicated the expression of this protein at a 24-kDa band in all MPM cell lines except MSTO-211 (Figure 4).

Preservation of claudin-based MPM phenotype in human MPM cell line-derived tumour xenografts. When inoculated in SCID mice, only five out of the eight cell lines induced tumours. Subcutaneous tumour reaching 1 cm³ in volume was observed between five to eight weeks after initial inoculation using HT29 (weeks 5-6), A549 (weeks 5-8), MSTO-211 (weeks 6-8), M14K and M38K cells (both weeks 7-8),

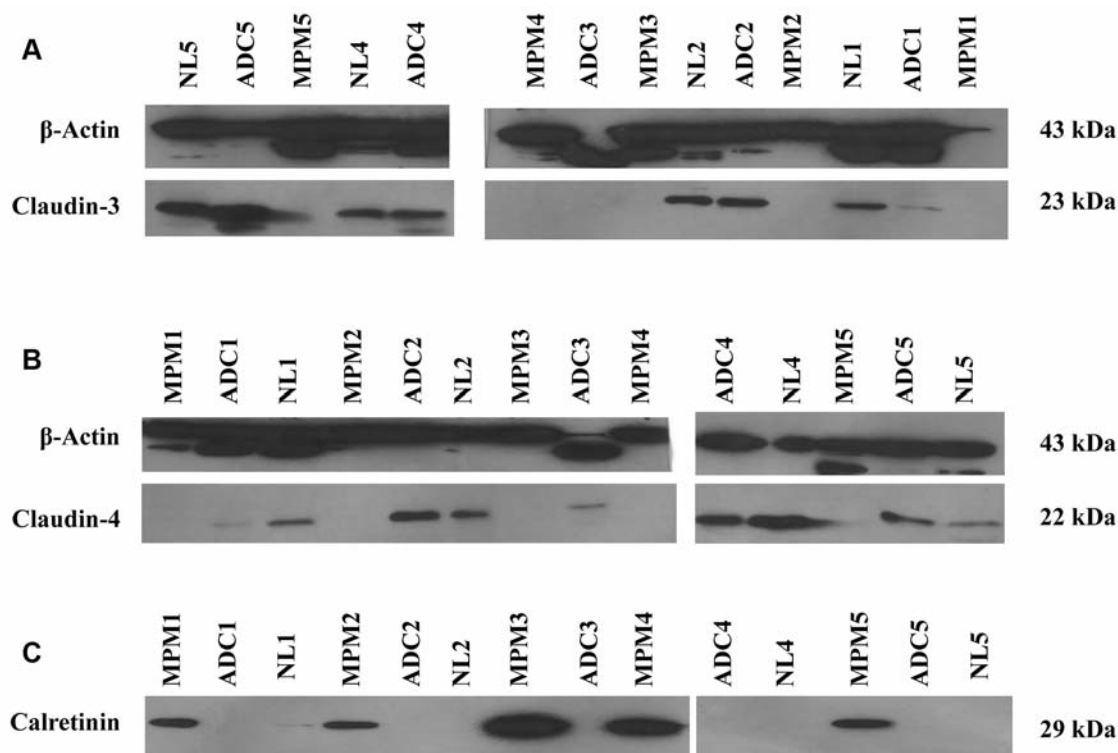


Figure 2. Immunoblot analysis of claudin-3 (A), claudin-4 (B) and calretinin (C) proteins in human biopsies of malignant pleural mesothelioma (MPM) and lung adenocarcinoma (ADC). Forty micrograms of total proteins extracted from each tumour specimen were subjected to immunoblot. The bands corresponding to claudin-3, claudin-4, and calretinin were identified by their molecular size (23 kDa, 22 kDa, and 29 kDa, respectively). ADC: Lung Adenocarcinoma; NL: Normal lung tissue.

whereas mice injected with NCI-H28, MCF7, and MDA-MB-231 cells did not yield any tumours.

Finally, we evaluated each protein in xenografts SCID mice to verify whether the claudin-based MPM phenotype observed *in vitro* was preserved *in vivo*. Interestingly, there was no significant change in this phenotype between the cell lines *in vitro* and those of xenografts in SCID mice, except for MSTO-211, which exhibited a signal for calretinin and claudin-15 *in vivo*-only (Figure 5).

Discussion

MPM, a quite rare and highly aggressive tumour, represents a great challenge for pathologists, clinicians and researchers because of its difficult diagnosis and limited treatment options, without curative intent as yet. Therefore, new diagnostic and therapeutic strategies are urgently needed, but frequent discrepancies between MPM cell line phenotypes and human tumour specimens hamper studies with this goal. In this regard, characterization of MPM cell lines maintaining the same phenotype *in vitro* and *in vivo* would be of great interest. We evaluated the expression of claudin-3 and claudin-4 in specimens of MPM and ADC. The expression of these

proteins in addition to claudin-15 and calretinin was also evaluated in MPM cell lines and in MPM-derived tumour xenografts to establish a phenotype of MPM based on the absence of claudin-3 and claudin-4 and on the presence of claudin-15 and calretinin.

Our results highlight a new claudin-based phenotype of MPM. We confirmed the absence of claudin-3 and -4 in specimens of MPM contrasting with their presence in lung ADC and normal lung. The absence of these proteins was also observed in the cell lines M14K and M38K and their corresponding xenografts, but not in MSTO-211. NCI-H28 was not tumorigenic and, thus, was ruled out from the selection.

Claudin-15 gene is overexpressed in epithelioid MPM (16) and in diffuse peritoneal malignant mesothelioma (17). At the protein level, to our knowledge claudin-15 expression has not been previously studied in MPM. We found claudin-15 and calretinin to be overexpressed in MPM cell lines, except in MSTO-211. Interestingly, the latter cell line is negative for tenascin-X expression (18). These findings suggest that the phenotype of MSTO-211 is not that of MPM since this cell line expresses both claudin-3 and -4 and expresses neither claudin-15, calretinin nor tenascin-X.

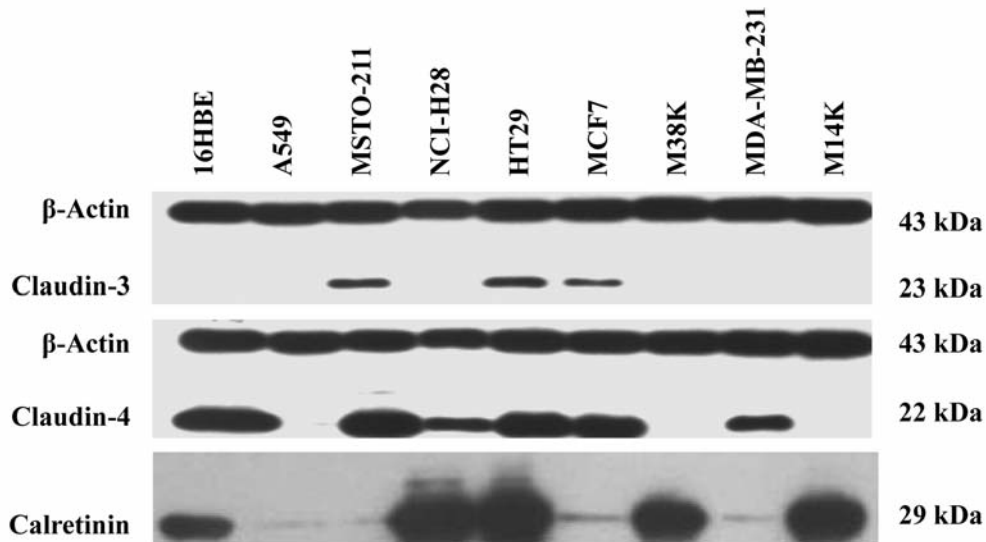


Figure 3. Immunoblot analysis of claudin-3, -4, and calretinin proteins in human cell lines derived from human adenocarcinoma or human malignant pleural mesothelioma (MPM). Forty micrograms of total proteins extracted from each cell pellet were subjected to immunoblot. The bands corresponding to claudin-3, claudin-4, and calretinin were identified by their molecular size (23 kDa, 22 kDa, and 29 kDa, respectively).

Results reported for cultivated cells were identical to those obtained in tumour xenografts induced by M14K and M38K but not in those induced by MSTO-211. This cell line exhibited a heterogeneous behaviour when comparing the expression of claudin-15 and calretinin *in vitro* and *in vivo*.

This highlights the heterogeneity of some cell lines and encourages the characterization of those preserving the same phenotype *in vitro* and *in vivo*.

In this context, A549 is well-known to be heterogeneous. Our results confirm the phenotypic heterogeneity of this cell line, widely used in cancer research. Indeed, we did not find any expression of claudin-3 and claudin-4 in A549. When comparing the expression of the two proteins in specimens, we observed a positivity for claudin-3 (Figure 1B) and claudin-4 (Figure 1E) in lung ADC. Thus, the lack of these proteins in A549 and their presence in ADC specimens appear to be rather due to the cellular heterogeneity of this cell line.

Conclusion

Two human MPM cell lines, M14K and M38K, preserve their phenotype *in vitro* and *in vivo* based on the claudin expression pattern. Therefore these two cell lines may represent useful models for basic research in MPM, for developing new therapeutic strategies, in particular targeting claudins. Thus, future directions may include, for example, the possible antiproliferative role of claudin-15 by the evaluation of cell proliferation of M14K and M38K cell lines overexpressing this protein after gene silencing by siRNA claudin-15.

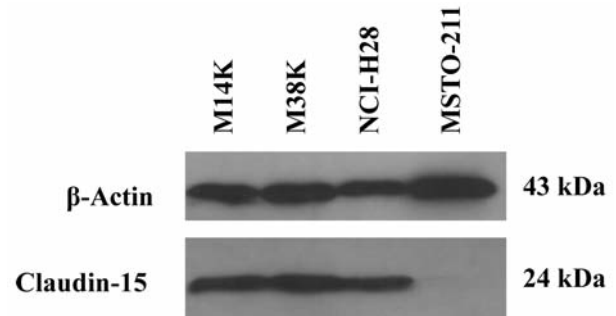


Figure 4. Immunoblot analysis of claudin-15 protein in human malignant pleural mesothelioma (MPM)-derived cell lines. Forty micrograms of total proteins extracted from each cell pellet were subjected to immunoblot. The band corresponding to claudin-15 was identified by its molecular size of 24 kDa.

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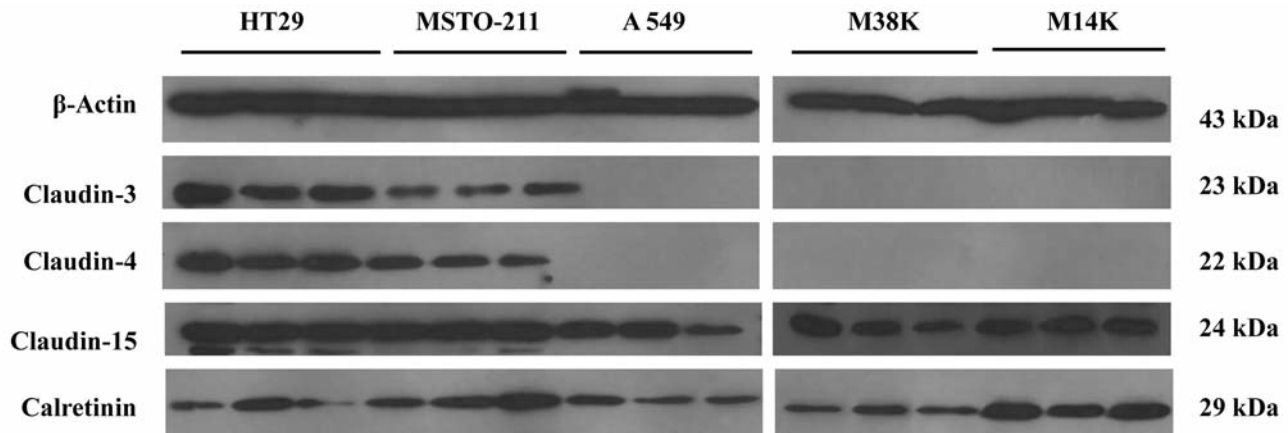


Figure 5. Immunoblot analysis of claudin-3, -4, -15 and calretinin in mouse xenografts. Forty micrograms of total proteins extracted from each tumour xenograft were subjected to immunoblotting. The bands corresponding to claudin-3, claudin-4, claudin-15 and calretinin were identified by their molecular size (23 kDa, 22 kDa, 24 kDa and 29 kDa, respectively).

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