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Tumori, 100: 559-567, 2014

# Expression of thrombomodulin, calretinin, cytokeratin 5/6, D2-40 and WT-1 in a series of primary carcinomas of the lung: an immunohistochemical study in comparison with epithelioid pleural mesothelioma

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ABSTRACT

**Aims and background.** A number of immunohistochemical markers have been suggested as useful in the positive diagnosis of epithelioid mesothelioma. The most widely used mesothelioma markers are thrombomodulin, calretinin, cytokeratin 5/6, D2-40 and WT-1. Numerous investigations have demonstrated their variable sensitivity and specificity in differentiating epithelioid mesothelioma from lung adenocarcinoma. However, data on the expression of these markers in other types of lung carcinomas are very limited. We evaluated the expression of these markers in a series of 172 primary carcinomas of the lung and in 75 epithelioid pleural mesotheliomas.

**Results.** Thrombomodulin expression was found in squamous cell carcinomas (71%), small cell lung carcinomas (11%), adenocarcinomas (4%), large cell carcinomas (50%), large cell neuroendocrine carcinomas (25%) and in sarcomatoid carcinomas (10%). Calretinin expression was common in small cell lung carcinomas (44%) and large cell neuroendocrine carcinomas (25%), less common in squamous cell carcinomas (20%), rare and focal in adenocarcinomas (4%) and sarcomatoid carcinomas (10%). Cytokeratin 5/6 was expressed in most of the squamous cell carcinomas (94.5%). Immunoreactivity was also found in large cell carcinomas (50%), sarcomatoid carcinomas (30%) and rarely in adenocarcinomas (4%). D2-40 was consistently expressed in squamous cell carcinomas (42%). Focal immunoreactivity was found in adenocarcinomas (3%). WT-1 was focally present in one (2%) squamous cell carcinoma.

**Conclusions.** These results indicate that some of the most commonly used mesothelioma markers may react with different types of primary lung carcinomas. These data should be taken into consideration especially when dealing with small biopsy fragments and poorly differentiated tumors.

***Key words:*** differential diagnosis, epithelioid mesothelioma, immunohistochemistry, primary lung carcinoma.

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Received June 3, 2013;
accepted November 27, 2013.

## Introduction

Several immunohistochemical markers have proven to be valuable in the positive identification of mesothelioma[1-4]. Most investigations on this topic have concentrated principally on the differential diagnosis between epithelioid pleural mesothelioma and lung adenocarcinoma. However, the pleura may be involved by other tumors, primary or metastatic, epithelial or epithelioid. Moreover, less differentiated and solid epithelioid mesotheliomas may mimic a variety of tumors other than lung adenocarcinoma. An additional important issue regards the examination of small biopsy fragments that may prove to be considerably challenging, as both morphology and immunohistochemical results may be ambiguous.

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Within positive mesothelial markers, thrombomodulin, calretinin, cytokeratin 5/6 (CK 5/6), and more recently Wilms' tumor 1 (WT-1) protein and D2-40 monoclonal antibody seem to have high sensitivity and/or specificity in the positive diagnosis of epithelioid mesothelioma. However, data on the expression of these markers in tumors other than lung adenocarcinoma are very few.

The purpose of the present study was to evaluate the expression of the most commonly used mesothelial positive markers in a series of primary epithelial lung tumors.

## Materials and methods

Cases were retrieved from the files of the Division of Pathological Anatomy of the Department of Medical and Surgical Critical Care of the University of Florence. The study group consisted of 172 cases of primary pulmonary carcinomas, including 55 squamous cell carcinomas, 71 adenocarcinomas, 18 small cell carcinomas, 4 large cell carcinomas, 4 large cell neuroendocrine carcinomas, 10 sarcomatoid carcinomas and 10 carcinoid tumors. Seventy-five unequivocal epithelioid pleural mesotheliomas from the same source were studied for comparison. Non-small cell carcinomas and 12 small cell carcinomas derived from surgically resected material; 6 small cell carcinomas derived from biopsy specimens. Each tumor was reviewed and classified according to the World Health Organization's criteria[5]. Pulmonary adenocarcinomas were subtyped as follows: 56 were mixed subtype, 12 were acinar, 2 were papillary and 1 was a solid subtype with mucin production. Carcinoid tumors were all categorized as typical. All mesothelioma cases were diagnosed by currently accepted histologic criteria on hematoxylin and eosin-stained sections combined with immunohistochemistry[5]. Most of them showed a tubulo-papillary pattern or a sheet-like, solid pattern.

Staining procedures were conducted on 4-µm-thick sections of paraffin-embedded tissue using an automated immunostainer (Ventana BenchMark XT, Tucson, AZ, USA). The primary antibodies, their dilutions, and sources are listed in Table 1. Appropriate positive and negative controls were added on each automated immunohistochemistry run to confirm the sensitivity and specificity of each antibody. The iVIEW DAB Detection Kit (Ventana) was used as a revelation system. Immunoreactivity was scored as negative (no immunostaining) or positive. The percentage of immunostained cells was recorded as follows: 1+ (1%-25%), 2+ (26%-50%), 3+ (51%-75%) 4+ (76%-100%).

## Results

### *Primary carcinomas of the lung*

The immunohistochemical results in the different types of primary lung carcinomas are shown in Table 2.

**Table 1 - Antibodies used for immunohistochemical analysis**

| Molecule/antibody | Clone | Antibody dilution | Source |
|---|---|---|---|
| Thrombomodulin | 1009 | 1:30 | Dako* |
| Calretinin | Polyclonal | Ready to use | Ventana§ |
| Cytokeratin 5/6 | D5/16B4 | Ready to use | Ventana |
| D2-40 | D2-40 | Ready to use | Ventana |
| WT-1 | 6F-H2 | Ready to use | Ventana |

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**Table 2 - Immunohistochemical results in 172 primary lung tumors**

| Markers | Positive reactions | | Grading of reactivity | | | | |
|---|---|---|---|---|---|---|---|
| | n. | % | 0 | 1+ | 2+ | 3+ | 4+ |
| Squamous cell carcinoma (55 cases) | | | | | | | |
| Thrombomodulin | 39 | 71 | 16 | 8 | 10 | 8 | 13 |
| Calretinin | 11 | 20 | 44 | 8 | 2 | 1 | 0 |
| CK 5/6 | 52 | 94.5 | 3 | 6 | 4 | 10 | 32 |
| D2-40 | 23 | 42 | 32 | 4 | 4 | 6 | 9 |
| WT-1 | 1 | 2 | 54 | 1 | 0 | 0 | 0 |
| Small cell lung carcinoma (18 cases) | | | | | | | |
| Thrombomodulin | 2 | 11 | 16 | 2 | 0 | 0 | 0 |
| Calretinin | 8 | 44 | 10 | 3 | 2 | 0 | 3 |
| CK 5/6 | 0 | 0 | 18 | 0 | 0 | 0 | 0 |
| D2-40 | 0 | 0 | 18 | 0 | 0 | 0 | 0 |
| WT-1 | 0 | 0 | 18 | 0 | 0 | 0 | 0 |
| Adenocarcinoma (71 cases) | | | | | | | |
| Thrombomodulin | 3 | 4 | 68 | 3 | 0 | 0 | 0 |
| Calretinin | 3 | 4 | 68 | 3 | 0 | 0 | 0 |
| CK 5/6 | 3 | 4 | 68 | 3 | 0 | 0 | 0 |
| D2-40 | 2 | 3 | 69 | 2 | 0 | 0 | 0 |
| WT-1 | 0 | 0 | 71 | 0 | 0 | 0 | 0 |
| Large cell carcinoma (4 cases) | | | | | | | |
| Thrombomodulin | 2 | 50 | 2 | 1 | 0 | 0 | 1 |
| Calretinin | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| CK 5/6 | 2 | 50 | 2 | 0 | 0 | 0 | 2 |
| D2-40 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| WT-1 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| Large cell neuroendocrine carcinoma (4 cases) | | | | | | | |
| Thrombomodulin | 1 | 25 | 3 | 1 | 0 | 0 | 0 |
| Calretinin | 1 | 25 | 3 | 1 | 0 | 0 | 0 |
| CK 5/6 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| D2-40 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| WT-1 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| Sarcomatoid carcinoma (10 cases) | | | | | | | |
| Thrombomodulin | 1 | 10 | 9 | 0 | 0 | 1 | 0 |
| Calretinin | 1 | 10 | 10 | 0 | 0 | 0 | 1 |
| CK 5/6 | 3 | 30 | 7 | 0 | 0 | 1 | 2 |
| D2-40 | 0 | 0 | 10 | 0 | 0 | 0 | 0 |
| WT-1 | 0 | 0 | 10 | 0 | 0 | 0 | 0 |
| Carcinoid tumor (10 cases) | | | | | | | |
| Thrombomodulin | 0 | 0 | 10 | 0 | 0 | 0 | 0 |
| Calretinin | 0 | 0 | 10 | 0 | 0 | 0 | 0 |
| CK 5/6 | 0 | 0 | 10 | 0 | 0 | 0 | 0 |
| D2-40 | 0 | 0 | 10 | 0 | 0 | 0 | 0 |
| WT-1 | 0 | 0 | 10 | 0 | 0 | 0 | 0 |

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*Thrombomodulin.* Squamous cell carcinomas showed the highest percentage of thrombomodulin-positive cases and the highest score of reactivity. Thirty-nine (71%) of 55 cases were found to be positive. The highest percentage of positive cells (3+ and 4+) was found in well- to moderately differentiated tumors. All keratinizing tumors showed strong and diffuse staining for the marker (Figure 1A). Three (4%) of 71 adenocarcinoma cases were thrombomodulin positive, and immunostaining was extremely focal (1+) with fewer than 5% positive cells. Small cell carcinomas showed thrombomodulin immunoreactivity in 2 (11%) of 18 cases; both cases showed less than 5% (1+) positive tumor cells. Thrombomodulin immunoreactivity was found in 2 (50%) of 4 large cell carcinomas; one case was focally positive, the other case showed diffuse and strong (4+) immunostaining. Focal positivity was also found in 1 (25%) of 4 neuroendocrine large cell carcinomas. One (10%) of 10 sarcomatoid carcinomas showed moderately diffuse (3+) thrombomodulin immunoreactivity. The tumor was subtyped as pleomorphic carcinoma consisting of a squamous cell carcinoma component associated with more than 10% of malignant giant cells; both components were thrombomodulin immunoreactive (Figure 1B). No carcinoid tumor was found to be positive. Regardless of tumor histology, the staining pattern of thrombomodulin was always typically membranous.

*Calretinin.* Squamous cell carcinomas showed calretinin positivity in 11 (20%) of 55 cases (Figure 1C). The highest percentage of positive tumor cells was found in 3 well-differentiated keratinizing tumors (2+ in 2 cases and 3+ in one case), and the moderately and poorly differentiated calretinin-positive squamous cell carcinomas were all scored 1+. Three (4%) of 71 primary adenocarcinomas of the lung showed calretinin expression; all cases showed less than 5% (1+) positive cells. The highest frequency of positivity for calretinin and the highest percentage of calretinin-positive cells were found in small cell carcinomas (Figure 1D). Globally, 8 (44%) of 18 cases were calretinin positive. Three cases showed diffuse (4+) immunostaining. One of these cases was a combined small cell carcinoma (small cell carcinoma associated with an adenocarcinoma component) in which calretinin was expressed only by the neuroendocrine cells. Two cases were scored 2+ and 3 cases showed only focal (1+) immunoreactivity. Focal (<5%) immunoreactivity was found in 1 (25%) of 4 large cell neuroendocrine carcinomas. Strong and diffuse calretinin immunostaining was found in one sarcomatoid carcinoma. Carcinoid tumors were all found to be negative for this marker. All positive cases showed the typical nuclear and cytoplasmic pattern of staining.

*CK 5/6.* All well-differentiated, keratinizing squamous cell carcinomas were found to be diffusely and strongly (4+) CK 5/6-positive. Moderately differentiated tumors and non-keratinizing tumors were more commonly scored 3+ and 2+. Cases with the lowest scores of immunoreactivity and the 3 (5%) negative cases were all poorly differentiated tumors. Within adenocarcinomas, 3 (4%) of 71 were immunoreactive for CK 5/6. All positive cases (mixed adenocarcinomas, predominantly acinar type) showed less than 5% of immunostained tumor cells. Diffuse CK 5/6 immunostaining was found in 2 (50%) of 4 large cell carcinomas. Three (30%) sarcomatoid tumors were found to have diffuse or moderately diffuse (4+ and 3+) CK 5/6 immunostaining. Two positive tumors were classified as pleomorphic carcinomas composed of squamous cell carcinoma associated with a giant cell component and squamous cell carcinoma associated with a spindle cell component. The third positive case was subtyped as spindle cell carcinoma. Positive immunoreactions were found within the squamous cell component, the giant cell and the spindle cell component. No CK 5/6 immunostaining was found in small cell carcinomas or in carcinoid tumors.

*D2-40.* Immunostaining with the monoclonal antibody D2-40 was found to be positive in 23 (42%) of 55 squamous cell carcinomas (Figure 1E). Immunoreactivity was graded 4+ in 9 cases, 3+ in 6 cases, 2+ in 4 cases and 1+ in 4 cases. Most positive cases were well- and moderately differentiated tumors. Only 2 poorly differentiated squamous cell carcinomas were D2-40-positive, both showing diffuse and strong immunoreactivity in 80% and 100% of tumor cells, respectively. The immunostaining was more evident in the outer cells of the neoplastic nests with a membranous pattern of staining along the entire surface of the cells. Two (3%) of 71 adenocarcinoma cases were focally (1+) D2-40-positive; both positive adenocarcinomas were mixed subtype with a predominantly acinar pattern of growth. No positivity at all was found in any of the other cases included in the study. Only membranous staining was considered as a positive immunoreaction.

*Wilms' tumor 1 protein.* WT-1 protein was negative in all our study cases, except for 1 case of moderately differentiated squamous cell carcinoma in which few tumor nests showed nuclear WT-1 positivity (Figure 1F).

### *Pleural epithelioid mesothelioma*

The immunohistochemical results of the mesothelioma markers in epithelioid mesotheliomas are shown in Table 3. Each mesothelioma marker was found to be positive in a high percentage of epithelioid mesotheliomas. Thrombomodulin was found to be positive in 58 (77%) mesothelioma cases. Grading of reactivity showed heterogeneous results, as shown in Table 3. The lowest percentage of positive cells was observed in the less differentiated tumors with a solid pattern of growth. Immunoreactivity was often limited to the superficial

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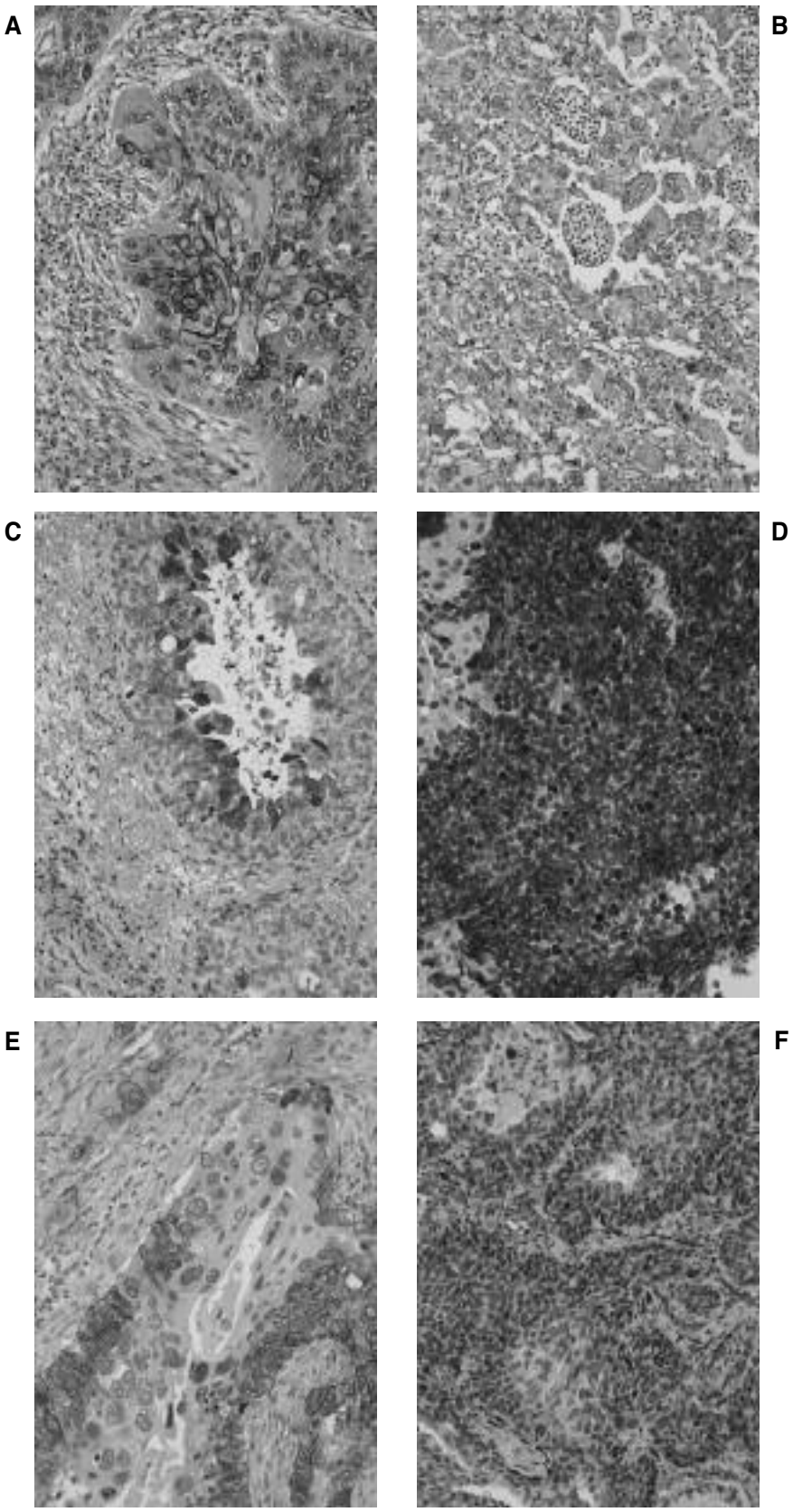


Figure 1 - Immunoreactivity of thrombomodulin, calretinin, cytokeratin 5/6, D2-40 and WT-1 in different types of primary carcinomas of the lung. Strong membrane immunostaining for thrombomodulin in a squamous cell carcinoma of the lung (A). Diffuse and strong thrombomodulin immunostaining in the giant cell component of a pleomorphic carcinoma of the lung (B). Nuclear and cytoplasmic calretinin immunostaining in a moderately differentiated squamous cell carcinoma of the lung (C). Small cell carcinoma of the lung with diffuse nuclear and cytoplasmic immunostaining for calretinin (D). D2-40-positive well-differentiated squamous cell carcinoma; the pattern of staining is membranous along the entire surface of the cells and it is clearly more evident in the outer cells of the neoplastic nests (E). Focal nuclear WT-1 immunostaining in a poorly differentiated squamous cell carcinoma (F).

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**Table 3 - Immunohistochemical results in 75 epithelioid mesotheliomas**

| Marker | Positive reactions | | Grading of reactivity | | | | |
|---|---|---|---|---|---|---|---|
| | No. | % | 0 | 1+ | 2+ | 3+ | 4+ |
| Thrombomodulin | 58 | 77 | 17 | 21 | 21 | 11 | 5 |
| Calretinin | 75 | 100 | 0 | 0 | 0 | 20 | 55 |
| CK 5/6 | 70 | 93 | 5 | 7 | 9 | 11 | 43 |
| D2-40 | 73 | 97 | 2 | 4 | 0 | 19 | 50 |
| WT-1 | 67 | 89 | 8 | 1 | 7 | 30 | 29 |

cells, being negative in the deeper portions of the tumor. The staining pattern was always typically membranous.

Calretinin expression was shown by all mesothelioma cases. The staining was nuclear and cytoplasmic, strong and diffuse (3+ or 4+) in all cases.

Cytoplasmic cytokerkatin 5/6 immunostaining was shown in 70 (93%) mesotheliomas. Fifty-nine cases were moderately or diffusely immunoreactive (3+ and 4+), whereas in 16 cases the grading of reactivity was focal (1+) or mild (2+).

Seventy-three (97%) mesotheliomas stained with the monoclonal antibody D2-40. Immunoreactivity was strong and diffuse (4+) in 50 cases and moderately diffuse (3+) in 19 cases; 4 cases showed focal staining (1+). In most cases, the staining occurred along the apical surface of the cells in a continuous pattern. In solid areas, the staining was milder and sometime discontinuous.

WT-1 immunoreactivity was seen in 67 (89%) mesotheliomas. Most cases showed strong, diffuse or moderately diffuse nuclear staining. Negative cases showed a solid pattern or a poorly differentiated, pleomorphic histology.

## Discussion

A large number of immunohistochemical markers for the positive diagnosis of epithelioid mesothelioma has proven to be valuable[1-4,6]. Specificity and sensitivity of mesothelioma markers have been mainly and extensively studied with regard to the differential diagnosis between epithelioid mesothelioma and lung adenocarcinoma[1-4,6]. Yet, mesothelioma is notorious for phenotypic versatility, both from case to case and even within the same tumor. The various histologic patterns and cytomorphologic features of the tumor underlie its capacity to mimic many other neoplasms involving the pleura or adjacent tissues. Moreover, besides pulmonary adenocarcinoma, lung carcinomas in general are inherently heterogeneous tumors that show a wide range of cytohistological aspects within each major histotype. Additionally, morphology and immunohistochemical results may both be confusing when dealing with biopsy specimens, due to limited tissue samples, artefacts and non-specific changes.

Thrombomodulin is a 75-kDa transmembrane glycoprotein that exerts anticoagulant activity[7]. It is expressed in a variety of tumors other than those of vascular origin, such as mesothelioma, trophoblastic tumors, squamous and transitional cell carcinomas[8,9]. Since the first studies on the value of this marker in differentiating epithelioid mesothelioma from lung adenocarcinoma, results on its sensitivity and specificity have been controversial[8]. The reported percentage values of positive mesotheliomas are highly variable, as highly variable as the percentage of thrombomodulin expression in lung adenocarcinomas[4,8]. Such discrepancies have been widely analyzed and attributed to a variety of possible factors[8]. Nonetheless, thrombomodulin was the first positive mesothelioma marker that could be used on formalin-fixed, paraffin-embedded tissue[10]. In our experience[11,12], including the present study, about two-thirds of mesotheliomas expressed thrombomodulin, but the grading of reactivity was heterogenous, with many cases showing only focal staining. As regards the expression of thrombomodulin in lung carcinomas other than adenocarcinomas, data from the literature are reported in Table 4. Most investigations considering large series of squamous cell carcinomas reported diffuse expression of the marker[8,13,14]. In our investigation, a large number (71%) of squamous cell carcinomas was found to be thrombomodulin positive. As previously reported, strong and diffuse immunostaining characterized the most well-differentiated keratinizing squamous cell carcinomas[13]. Within the remaining histotypes, other than adenocarcinoma and squamous cell carcinoma, data from the literature are very few (Table 4). Concerning neuroendocrine tumors, results are extremely variable due to differences in the number of cases examined in the different studies[8,14,15]. The largest number of cases was analyzed by Miettinen *et al.*[14] The authors found thrombomodulin-positive immunostaining in 27% of small cell carcinomas and in 18% of large cell neuroendocrine carcinomas. In our study, thrombomodulin immunoreactivity was focally observed in 2 (11%) of 18 cases of small cell carcinoma and in 1 of 4 cases of large cell neuroendocrine carcinomas. Carcinoid tumors were all found to be thrombomodulin negative in the investigation by Ordóñez[8]. The same results were obtained in the present study. Within undifferentiated large cell carcinomas, no immunoreactivity was found in the study by Ordóñez[8], whereas Miettinen *et al.*[14] found 29 (25%) thrombomodulin-positive large cell carcinomas out of 117 cases. In our limited series of cases, we found 2 (50%) thrombomodulin-positive large cell carcinomas, one focally positive and the other one diffusely positive. Discordant results on thrombomodulin expression in sarcomatoid carcinomas of the lung have been reported[8,14-18]. In fact, posi-

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**Table 4 - Positive mesothelioma markers reported in lung carcinomas other than adenocarcinoma**

| Marker | Histotype | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SCC | | SCLC | | LCC | | LCNEC | | CT | | SC | |
| | Any positivity of total cases (%) | Ref. | Any positivity of total cases (%) | Ref. | Any positivity of total cases (%) | Ref. | Any positivity of total cases (%) | Ref. | Any positivity of total cases (%) | Ref. | Any positivity of total cases (%) | Ref. |
| Trombomodulin | 94/188 (50) | 8,13-15 | 11/51 (22) | 8,14,15 | 29/117 (25) | 8,14 | 6/33 (18) | 14 | 0/16 (0) | 8 | 19/45 (42) | 8,14-18 |
| Calretinin | 71/242 (29) | 14,15,19-23 | 27/60 (45) | 14,15,20 | 50/157 (32) | 14,20,21 | 15/33 (45) | 14 | 0/2 (0) | 19 | 36/60 (60) | 14,16-18, 24,25 |
| Cytokeratin 5/6 | 116/124 (94) | 14 | 11/41 (27) | 14,15,27, 31 | 70/125 (56) | 14,31 | 6/42 (14) | 14,27 | ND | | 5/40 (12) | 14-17 |
| D2-40 | 73/148 (49) | 23,34,39,40 | 0/53 (0) | 39,40 | ND | | 1/11 (9) | 40 | ND | | 26/51 (51) | 18,25,41 |
| WT-1 | 0/58 (0) | 23,44-46 | 1/24 (4) | 45,47 | 0/1 (0) | 45 | ND | | 0/1 (0) | 45 | 7/35 (20) | 18,44,45 |

SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma; LCC, large cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; CT, carcinoid tumour; SC, sarcomatoid carcinoma; ND, no data.

tive values in the different studies ranged from 0% to 43.5%, but again, the number of cases studied was extremely variable. In the present study, thrombomodulin positivity was found in 1 (10%) sarcomatoid carcinoma (giant cell carcinoma) with moderately diffuse immunostaining.

In summary, the use of this marker in differentiating mesothelioma from lung adenocarcinoma has certainly declined since the identification of more sensitive positive mesothelioma markers. Moreover, such common thrombomodulin positivity in squamous cell carcinoma and, less frequently, in other histotypes further limits the value of this marker in the positive diagnosis of mesothelioma.

Calretinin is a 29-kDA protein that is a member of the EF-hand family of calcium-binding proteins. First described in the central and peripheral neural tissues, calretinin expression has also been reported in a variety of epithelial and non-epithelial human tissues under normal and neoplastic conditions[19,20]. Since the first study[19] on the use of the marker in the differential diagnosis between mesothelioma and lung adenocarcinoma, calretinin has almost always been considered the most useful marker for the positive diagnosis of mesothelioma[4,21]. In the present study, calretinin was diffusely and strongly positive in all mesothelioma cases, thus confirming the results of previous investigations. Moreover, only 3 (4%) of 71 lung adenocarcinomas showed focal calretinin immunostaining, in accordance with the well-known high specificity of this marker in the differential diagnosis between the latter tumor and epithelioid mesothelioma. Relatively few studies have been published on calretinin immunoreactivity in squamous cell carcinoma of the lung[14,15,19-23]. The data from previous studies are shown in Table 4. Grading of reactivity has almost always been reported as focal or moderate. A correlation between grade of differentiation and calretinin expression was reported in the investigation by Miettinen *et al.*[14]; the authors found a higher grading of reactivity in nonkeratinizing tumors. In the current study, 11 (20%) of 55 squamous cell carcinomas were calretinin positive. Grading of reactivity was focal in most cases. In contrast with previous results, we found higher reactivity score values in well-differentiated keratinizing tumors. Calretinin expression in neuroendocrine carcinomas has been evaluated in few reports[14,15,19,20]. Concerning small cell lung carcinoma, most studies found high percentages of calretinin-positive cases with values ranging from 41% to 49%[14,20]. Large cell neuroendocrine carcinomas have been evaluated only in one previous study[14], showing 45% calretinin-positive cases. We found calretinin positivity in 50% of small cell carcinomas and in 25% of large cell neuroendocrine carcinomas. In contrast to high-grade neuroendocrine carcinomas, no calretinin immunoreactivity was found in our series of carcinoid tumors or in the previous study by Doglioni *et al.*[19] With regard to large cell carcinoma, the largest series was evaluated by Miettinen *et al.*[14], who reported 38% calretinin-positive cases. In the current study, no calretinin immunoreactivity was observed. Finally, reported results on the expression of calretinin in sarcomatoid carcinomas have been controversial[14,16-18,24-26]. Positive calretinin immunostaining has been reported in both the spindle cell and the giant cell component of sarcomatoid carcinomas. The data from the literature (Table 4) indicate about 60% positivity, although some results are questionable since cytoplasmic staining alone was considered to be positive[26]. Our investigation showed one giant cell carcinoma with diffuse and strong calretinin staining.

In summary, calretinin seems to be more frequently expressed in lung carcinomas other than adenocarcinoma. In particular, neuroendocrine carcinomas and sarcomatoid carcinomas may exhibit high percentages of positive tumor cells, whereas squamous cell carcinomas are more often focally immunoreactive.

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CK 5/6 are high-molecular-weight basic cytokeratins. They are normally expressed in basal cells of complex epithelia such as respiratory and squamous epithelia as well as in the basal-myoepithelial cell layer of the prostate, breast and salivary glands[27]. This keratin is typically present in normal mesothelial cells and in mesotheliomas[27,28]. Significant differences in the cytokeratin expression pattern between lung adenocarcinoma and mesothelioma have been highlighted[28,29]. Since the introduction of the commercially available monoclonal antibody D5/16B4 anti-CK 5/6, most investigations have indicated high sensitivity and specificity of this marker[30,31]. Less specificity of CK 5/6 has been documented when analyzing adenocarcinomas of various tissue origins other than the lung[31]. In accordance with previous investigations, we found a high percentage (97%) of CK 5/6-positive mesotheliomas. In our experience, diffuse immunostaining is observed mostly in well-differentiated tubular or papillary epithelioid mesotheliomas, whereas less differentiated tumors often show only mild and focal CK 5/6 immunostaining. Few positive CK 5/6 tumor cells were found in 3 adenocarcinoma cases. CK 5/6 is typically expressed in most squamous cell carcinomas. Only few studies have investigated the expression of CK 5/6 in lung carcinomas other than adenocarcinoma and squamous cell carcinoma. CK 5/6 is almost always found to be negative in high-grade neuroendocrine tumors[14,15,27,31]. In our series, all high-grade neuroendocrine tumors and all carcinoid tumors were CK 5/6 negative. The data reported on CK 5/6 immunoreactivity in undifferentiated large cell carcinomas are few and controversial[14,31]. In our study, 2 (50%) cases showed strong and diffuse CK 5/6 immunostaining, but the number of cases is too small to make any considerations. Positivity for CK 5/6 in sarcomatoid carcinomas has been reported in the squamous cell component of pleomorphic carcinomas, in spindle cell carcinomas and in giant cell carcinomas[14-17]. In the present study, we observed 3 (30%) CK 5/6-positive sarcomatoid carcinomas (1 spindle cell carcinoma and 2 pleomorphic carcinomas).

In summary, CK 5/6 is typically expressed in mesothelioma and lung squamous cell carcinoma. These cytokeratins may also be expressed in large cell carcinomas and in sarcomatoid carcinomas of the lung.

D2-40 is a monoclonal antibody directed against human podoplanin, a 38-kD transmembrane mucin-type glycoprotein[32]. Until recently, the clinical use of D2-40 included the demonstration of lymphatic invasion by primary tumors and its use as a marker of tumors with lymphatic differentiation[33]. Subsequently, data from the literature have demonstrated the expression of D2-40 a variety of different neoplasms, including mesothelioma[33-35]. Most studies have indicated the utility of D2-40 differentiating epithelioid mesothelioma from lung adenocarcinoma[4,34-39]. Our results are in accordance with those of the literature: 93% of the mesothelioma cases were D2-40-positive, whereas adenocarcinomas showed immunoreactivity in 3% of the cases. Data from the literature on D2-40 immunoreactivity in squamous cell carcinomas show positive immunostaining in about 50% of the evaluated cases[23,34,39,40]. In the present study, D2-40 was found to be positive in 23 (42%) of 55 squamous cell carcinomas. Different results from previous investigations were observed concerning the grading of reactivity, since we found 65% of squamous cell carcinomas with high percentages of D2-40-positive cells. In agreement with Ordóñez[23], immunostaining was stronger in the peripheral cells of the tumor nests and the staining pattern was typically membranous. Moreover, differences in D2-40 expression were found in relation to the grade of differentiation. In fact, all but 2 D2-40-positive cases were classified as well- or moderately differentiated tumors. We found no comments on this issue in previous investigations. With regard to neuroendocrine tumors, data from the literature are very few[39,40] and demonstrate no immunoreactivity in small cell lung carcinomas or, except for one case, in large cell neuroendocrine carcinomas[40]. So far, there are no published data on the expression of D2-40 in carcinoid tumors. Small cell carcinomas, large cell neuroendocrine carcinomas and carcinoid tumors included in the present study were all found to be D2-40-negative. The expression of D2-40 in sarcomatoid carcinomas of the lung has been reported in recent studies[18,25,41]. Immunostaining has been described mainly as focal and/or weak. In the present study, no D2-40-positive immunostaining was observed in sarcomatoid carcinomas.

In summary, D2-40 is a highly sensitive and specific mesothelial marker when differentiating epithelioid mesothelioma from lung adenocarcinoma, large cell carcinomas and neuroendocrine tumors. D2-40 positivity has been reported in about 40% to 50% of squamous cell carcinomas. In our opinion, such a common D2-40 positivity limits the value of this marker in differentiating squamous cell carcinoma from epithelioid mesothelioma, especially when dealing with small biopsy fragments.

The WT-1 tumor suppressor gene, located on the short arm of chromosome 11 (11p13), encodes a protein with the structural features of a DNA-binding transcription factor[42,43]. Currently, its main practical use as a diagnostic marker concerns Wilms' tumor, epithelioid mesothelioma, ovarian serous cancers, serous carcinoma of the peritoneum and rhabdomyosarcomas. As for other positive mesothelioma markers, the value of WT-1 has been investigated mainly in differentiating epithelioid mesothelioma from lung adenocarcinomas[4,44]. Depending on the type of antibody used, rabbit polyclonal antibody against WT-1 or monoclonal antibody clone 6F-H2, nuclear positivity for the marker in epithelioid mesothelioma has been reported to range from 43% to 96%[44]. In accordance with most studies that used the

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monoclonal antibody 6F-H2, we found WT-1 immunoreactivity in a high percentage (89%) of epithelioid mesotheliomas; negative cases were those with less differentiated histology. As reported by most investigations, despite less sensitivity of WT-1 in comparison with calretinin and D2-40, we found very high specificity (100%) of this marker in differentiating epithelioid mesothelioma from lung adenocarcinoma. Very little information is available on WT-1 immunoreactivity in lung tumors other than adenocarcinoma (Table 4)[23,44-47]. All the data from the literature show very high specificity of this marker. More recently, the investigation by Takeshima *et al.*[18] reported WT-1 immunoreaction in 7 (36.8%) of 19 lung sarcomatoid carcinomas. However, most cases in the study showed cytoplasmic staining, which, in our opinion, should be considered as negative immunostaining. To the best of our knowledge, our study reports the largest series published to date on the expression of WT-1 in primary lung carcinomas. We found focal, nuclear WT-1 immunoreactivity only in one case, a squamous cell carcinoma.

In summary, in our experience and consistent with most investigators, WT-1 seems to be slightly less sensitive than other available positive mesothelioma markers, but very specific in differentiating epithelioid mesothelioma from primary carcinomas of the lung.

In conclusion, in accordance with previous published data, the present study showed thrombomodulin, calretinin, CK 5/6, D2-40 and WT-1 to be useful in the identification of most mesothelioma cases. These markers may react with different types of primary pulmonary carcinomas with a variable frequency. Thrombomodulin, CK 5/6 and D2-40 may be expressed especially in squamous cell carcinomas, whereas calretinin may be positive in high-grade neuroendocrine carcinomas. WT-1 seems to be the most specific positive mesothelioma marker when differentiating epithelioid mesothelioma from primary lung carcinomas. The pattern of immunostaining is important for certain markers such as calretinin and WT-1: the former requires both nuclear and cytoplasmic staining, the latter should show only nuclear staining. These data should be taken into consideration when dealing with biopsy specimens and poorly differentiated tumors. Thus, especially in cases of small pathological samples, accurate morphological examination and appropriate clinical, radiological and surgical findings are mandatory to select the most sensitive and specific positive and negative mesothelial markers.

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