

D2-40 and calretinin—a tissue microarray analysis of 341 malignant mesotheliomas with emphasis on sarcomatoid differentiation

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Anti-calretinin antibodies are useful to differentiate adenocarcinomas from malignant mesotheliomas of the lung. Therefore, calretinin expression is rarely reported for sarcomatoid mesotheliomas. Anti-podoplanin antibodies (eg D2-40) react with lymphatic endothelia, Kaposi's sarcoma, lymphangioma and mesotheliomas. For the interpretation of spindle cell lesions of the pleura, knowledge of calretinin and D2-40 expression frequencies in sarcomatoid mesothelioma is desirable. To systematically investigate the sensitivity of calretinin and D2-40 antibodies in epithelioid and sarcomatoid areas of malignant mesotheliomas, a tissue microarray with 341 malignant mesotheliomas, including 112 epithelioid, 46 sarcomatoid and 183 biphasic tumors was constructed. Epithelioid and sarcomatoid differentiated tumor areas were clearly separated within the tissue microarray. Expression of calretinin and D2-40 was separately studied in epithelioid and sarcomatoid areas by immunohistochemistry. Calretinin expression was found in 91% of epithelioid and 57% of sarcomatoid tumor areas. D2-40 immunostaining was present in 66% of the epithelioid and 30% of the sarcomatoid tumor areas. A combination of calretinin and D2-40 increased the sensitivity in epithelioid tumor areas to 0.96 and in sarcomatoid tumor areas to 0.66. These data indicate that a combination of calretinin and D2-40 will improve diagnostic accuracy for spindle cell lesions of the pleura, whereas almost all epithelioid mesotheliomas are identified by calretinin alone.

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With its wide differential diagnosis and its therapeutic and prognostic implications, the accurate diagnosis of malignant mesothelioma is nowadays crucial. The most thoroughly investigated histologic mimic of malignant mesothelioma is adenocarcinoma of the lung. A variety of ancillary techniques including electron microscopy, histochemistry and immunohistochemistry assist in the differential diagnosis between malignant mesothelioma and adenocarcinoma. As surgical pathologists are confronted with an ever-decreasing size of biopsies, the necessity of a reliable marker is more and more apparent. Immunohistochemical panels are most promising and a wide range of sensitive and specific

markers for lung adenocarcinoma have been described.^{1–3}

Calretinin is a well-established immunohistochemical marker for malignant mesothelioma. It is a calcium-binding protein of the EF-hand family and has been shown to be of good discriminatory value in the distinction between epithelioid malignant mesothelioma and adenocarcinoma. Calretinin is expressed in both the cytoplasm and nucleus. According to the literature, its expression in malignant mesothelioma varies,^{3–20} but most authors report frequent expression in epithelioid malignant mesothelioma, with only few studies reporting expression below 90%. Interestingly, calretinin expression has been described in a wide variety of cells, including steroid-producing cells of the testis and ovary, adipocytes, eccrine-glands, keratinizing thymic epithelial cells and numerous tumors, for example Merkel cell carcinoma, Leydig cell tumors of the testis, esthesioneuroblastoma, adenomas of the adrenal gland, small cell carcinoma of the lung and adenomatoid tumor.^{5,21,22}

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In addition to calretinin, D2-40 has been recently recommended as a new marker for malignant mesothelioma.^{23,24} D2-40 is a novel monoclonal antibody that was reported to recognize a 40 kDa surface sialoglycoprotein expressed in germ cell neoplasia and fetal testicular gonocytes.²⁵ A recent investigation has demonstrated that this mucin-type glycoprotein is podoplanin.²⁶ Martín-Villar *et al*²⁸ and Ma *et al*²⁷ both described its human genome. D2-40 is selective for lymphatic endothelia and has been shown to react with Kaposi's sarcoma, lymphangioma and Dabska tumor.^{29,30} Membranous staining of D2-40 has been described in 51 of 53 (96%)²³ and 29 of 40 (72%) malignant mesotheliomas.²⁴

No single antibody has demonstrated entire sensitivity or specificity for malignant mesothelioma,^{5,22,23,31–34} and an exact description of expression frequency in areas with sarcomatoid differentiation in sarcomatoid malignant mesothelioma or biphasic malignant mesothelioma has been largely neglected. The few publications that have evaluated calretinin expression in the three most frequent types of malignant mesothelioma separately present data that diverge from 18 to 100% positivity in sarcomatoid malignant mesothelioma, and 8 to 100% in biphasic malignant mesothelioma.^{5,7,15,18–20,33} The D2-40 staining frequency is also controversial in sarcomatoid and biphasic malignant mesothelioma. Whereas Chu *et al*²³ reported staining in all types of malignant mesothelioma, Ordóñez²⁴ did not detect any D2-40 staining in either sarcomatoid malignant mesothelioma or sarcomatoid areas of biphasic malignant mesothelioma.

With the availability of these novel mesothelioma markers, the differential diagnosis to adenocarcinoma has been facilitated in the last years. However, various spindle cell lesions of the pleura have broadened the differential diagnosis of sarcomatoid mesothelioma. These include especially synovial sarcomas of the pleura,^{35–39} but also solitary fibrous tumors, spindle cell carcinomas and high-grade sarcomas.

Consequently, we have constructed a tissue microarray with 341 malignant mesotheliomas and have clearly separated areas with epithelioid and sarcomatoid differentiation, in order to compare the value of D2-40 and calretinin immunostaining in distinct growth patterns of malignant mesothelioma. Tissue microarrays have proven highly representative in comparison with traditional evaluation through methods on large tissue samples.^{40–51}

Materials and methods

Tumors

All malignant mesotheliomas, diagnosed between 1975 and 2004, were retrieved from the archives of the Zurich Pneumoconiosis Research Group, Switzerland (Director M Rueeggger). The total of 341 cases

comprised 112 epithelioid, 46 sarcomatoid and 183 biphasic types. All but three cases are pleural malignant mesotheliomas, one case had both pleural and peritoneal involvement and two were peritoneal mesotheliomas. The diagnosis of malignant mesothelioma was based upon the characteristic clinicoradiographic and histopathological features. Occupational asbestos exposure was documented in the vast majority of cases. The tissue specimens were mainly derived from post-mortem examination (77% autopsy, 23% biopsy) and had uniformly been formalin-fixed and paraffin-embedded. They had all been originally examined and classified by one experienced lung pathologist (PV) and were reviewed (MH) to identify suitable areas for tissue microarray construction. Any discrepancy between the current assessment and original diagnosis was resolved by consensus upon simultaneous review (MH, PV and HM).

Tissue Microarray Construction

The construction of a set of three tissue microarrays was accomplished with a custom-made, semiautomatic tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA) as described before.^{40,46} The available tumors had been reinvestigated and grouped into three categories: epithelioid, sarcomatoid and biphasic malignant mesothelioma. Distinct areas with different morphological growth patterns had been marked. The most representative tumor blocks were then selected and four tissue cores, 0.6 mm in diameter, were taken from each case and transferred into the recipient paraffin block. Wherever applicable in biphasic mesotheliomas, areas were identified that clearly separated the two different growth patterns, epithelioid and sarcomatoid, and two cores were taken from each area, enabling us to compare directly the phenotypic diversity in one and the same tumor.

Immunohistochemistry

Sections (4.5 μ m) of tissue microarray blocks were transferred to an adhesive-coated slide system (Instrumedics, Hackensack, NJ, USA) supporting the cohesion of 0.6 mm array elements on glass. De-paraffined sections were manually stained after heat-induced epitope retrieval (3 min, 110°C, citrate buffer, pH 6.0) using a standard multilink detection kit (Medi-Stain HRP DAB, mediteAG, Nunningen, CH, Switzerland), including endogenous peroxidase block, block of nonspecific binding, horse radish peroxidase, and diaminobenzidine as chromogen. The primary antibodies were a polyclonal rabbit antiserum for calretinin (Cell Marque, Hot Springs, AR, USA), prediluted by the manufacturer, and a monoclonal mouse antibody for podoplanin (clone D2-40) (DakoCytomation, Baar, CH, Switzerland) at 1:50 dilution. They were used for overnight incuba-

tion at 4°C. For both antibodies adequate positive controls were used, vermiform appendix for calretinin and uterus for D2-40, according to the manufacturer's recommendations.

Interpretation of Results

The sections were semiquantitatively assessed by one observer (MH) and assistance was received for ambiguous cases (HM, PV). The growth pattern was confirmed on every individual spot. If both growth patterns were present on one spot of a biphasic malignant mesothelioma we rated them independently of each other, if the number of cells attributed to each type allowed us to do so. In a first step, staining intensity of positive tumor cells was recorded as follows: weak (1+), moderate (2+) or strong (3+). Strong staining intensity was defined as staining comparable to control tissue. Weak staining was regarded as any faint staining in tumor cells.

In a second step, the percentage of positive tumor cells was calculated and a three-tiered score (negative, weak and strong staining) was generated for statistical analysis. All tumors without staining or staining in up to 10% of tumor cells were regarded as *negative*. Tumors with 1+ staining intensity in more than 10% or 2+ intensity in 10–30% of cells were considered *weakly positive*. Spots with 2+ intensity in 30% tumor cells and more or 3+ intensity in more than 10% of cells were considered *strongly positive*. The relatively high cutoff value of 10% was set to rule out false positive results.

Fluorescence *In Situ* Hybridization

For FISH, a Dual Color Break Apart Rearrangement Probe for the detection of SYT (18q 11.2) gene translocation (LSI[®] SYT (18q 11.2), Abbott/Vysis Molecular Diagnostics, IL, USA) was used according to the manufacturer's instructions with minor modifications. These included tissue preincubation with 30 mg pepsin (Sigma) at 37°C for 25 min. FISH signals of at least 40 nuclei were evaluated for each spot (four spots per case), using a fluorescence microscope (Olympus BX61) with filters DAPI, Spectrum Orange and Spectrum Green. The images were acquired with a CCD camera and analyzed with AnalySIS software (Soft Imaging System, Munster, Germany). If at least two spots of each case contained not less than 25% of split red and green signals, the tumor was regarded as being positive for the SYT translocation.⁵² The signal was considered as split if the red and green signals were smallest separated by more than twice the distance occupied by a several signal.

Statistics

All cases were morphologically and/or immunologically defined mesotheliomas. Therefore, the

sensitivity for D2-40 and calretinin could be calculated with the formula: Sensitivity = true positives / (true positives + false negatives).

Results

Tumors

The tissue microarray comprised a total of 1372 tissue cylinders. For calretinin, 816 epithelioid and 369 sarcomatoid spots, and 139 with both growth patterns could be evaluated. For D2-40, there were 818 epithelioid and 373 sarcomatoid spots, and 140 with both growth patterns. Forty-eight spots for calretinin, and 41 spots for D2-40 were not interpretable because of necrosis or lack of sufficient cells on the spot. The average of evaluable cells per spot was 340 tumor cells (minimum 80 tumor cells). Two cases were lost entirely on both slides because none of the four spots was interpretable. After exclusion of these tumors, 341 malignant mesotheliomas remained for statistical analysis.

Calretinin

There was both, cytoplasmic and nuclear staining for calretinin (Figure 1a). Two hundred fifty-two (91%) epithelioid malignant mesotheliomas or biphasic malignant mesotheliomas with an epithelioid component were calretinin positive. Twenty-two (8%) cases showed weak and 230 (83%) strong positivity. In contrast, only 95 (57%) sarcomatoid malignant mesotheliomas or biphasic malignant mesotheliomas with a sarcomatoid component exhibited calretinin positivity. Thirty-four (20%) showed weak and 61 (37%) strong calretinin positivity (Table 1).

D2-40

Podoplanin expression was seen in 180 (66%) epithelioid malignant mesotheliomas or epithelioid components of biphasic malignant mesotheliomas (6% weak and 60% strong positivity). Fifty (30%) sarcomatoid malignant mesotheliomas or sarcomatoid components of biphasic malignant mesotheliomas were D2-40 positive (6% weak and 24% strong positivity). D2-40 positive tumor cells showed membranous and/or cytoplasmic staining. Immunostaining was frequently patchy. Epithelioid areas showed predominantly membranous D2-40 staining whereas sarcomatoid areas exhibited cytoplasmic staining (Figure 1b and c; Tables 2 and 3). An interesting observation was a dot-like D2-40 staining in a few cells, which was detected in five cases (Figure 1d).

Sensitivity of Calretinin and D2-40

There was no significant difference, neither for calretinin nor for D2-40 staining, between tissue

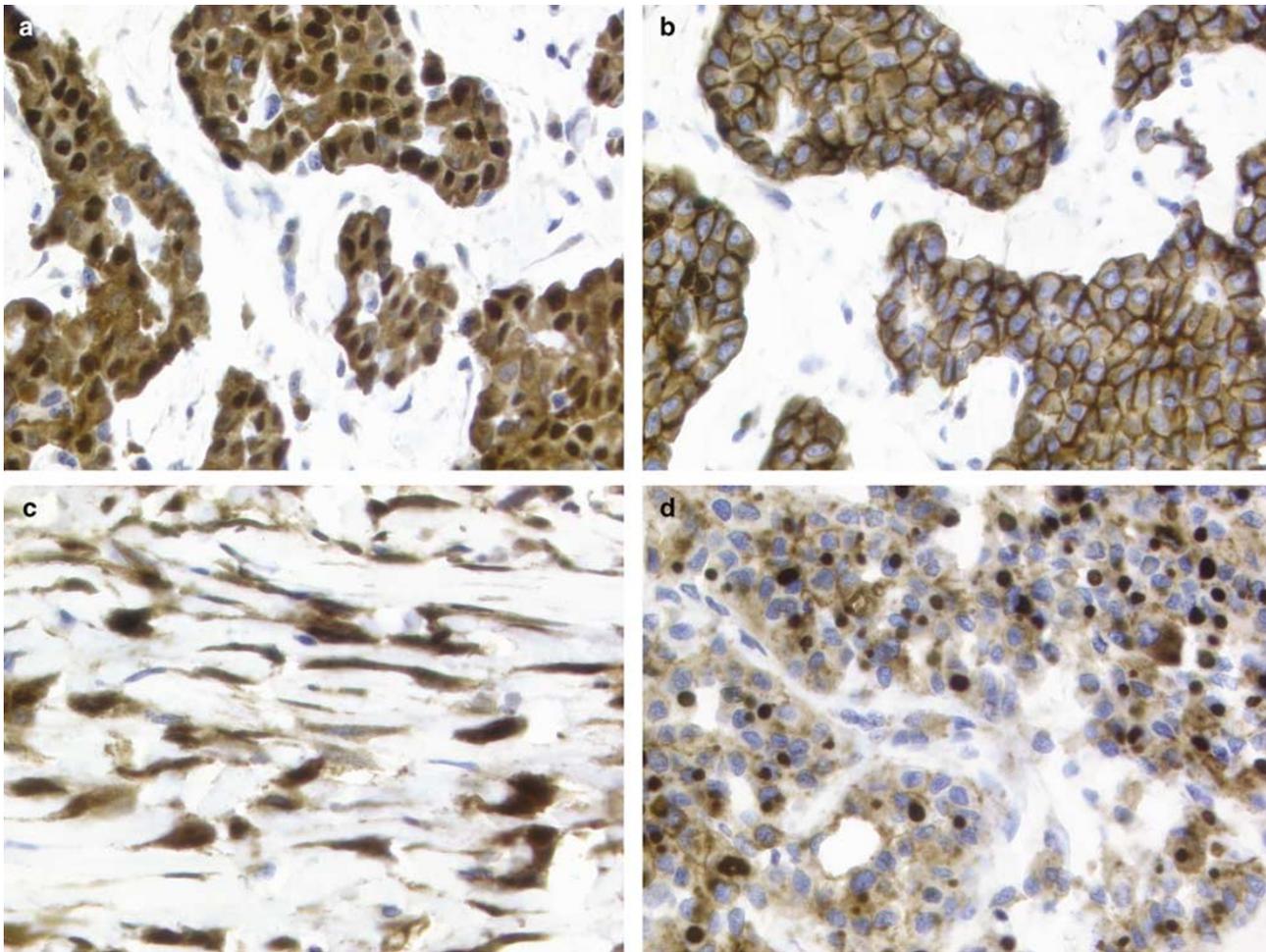


Figure 1 (a) Strong cytoplasmic and nuclear staining of calretinin in an epithelioid area. (b and c) Membranous and cytoplasmic staining pattern of D2-40 in an area of epithelioid or sarcomatoid differentiation, respectively. (d) Dot-like staining of D2-40 in an epithelioid case.

Table 1 Calretinin expression in epithelioid and sarcomatoid areas of malignant mesothelioma (MM)

Score	Calretinin expression			
	Epithelioid MM	Sarcomatoid MM	Biphasic MM, epithelioid areas	Biphasic MM, sarcomatoid areas
	n (%)	n (%)	n (%)	n (%)
0	8 (7)	21 (46)	16 (10)	51 (42)
1	7 (6)	11 (24)	15 (9)	23 (19)
2	97 (87)	14 (30)	133 (81)	47 (39)

Table 2 D2-40 expression in epithelioid and sarcomatoid areas of malignant mesothelioma (MM)

Score	D2-40 expression			
	Epithelioid MM	Sarcomatoid MM	Biphasic MM, epithelioid areas	Biphasic MM, sarcomatoid areas
	n (%)	n (%)	n (%)	n (%)
0	28 (25)	35 (76)	66 (41)	81 (68)
1	4 (4)	1 (2)	12 (7)	9 (7)
2	80 (71)	10 (22)	84 (52)	30 (25)

from pure epithelioid and sarcomatoid malignant mesothelioma compared to epithelioid and sarcomatoid areas of biphasic malignant mesothelioma. Thus, sensitivity was calculated separately in epithelioid and sarcomatoid differentiated areas, independent of its original type. Calretinin reached a sensitivity of 0.91 for epithelioid and 0.57 for sarcomatoid areas. The sensitivity of D2-40 was 0.66 and 0.3, respectively (Table 4). A combination of

D2-40 and calretinin increased the sensitivity to detect sarcomatoid malignant mesothelioma to 0.66 and epithelioid malignant mesothelioma to 0.96. The percentage of additional malignant mesotheliomas detected using a panel of calretinin and D2-40 was 8.5% for sarcomatoid malignant mesothelioma and 4.5% for epithelioid malignant mesothelioma (Figure 2). There was one desmoplastic variant of sarcomatoid mesothelioma, which was negative for

Table 3 Staining pattern of D2-40 in epithelioid and sarcomatoid areas of malignant mesothelioma (MM)

Staining pattern	Epithelioid n (%)	Sarcomatoid n (%)
Membranous	448 (77)	5 (4)
Both	54 (9)	3 (2)
Cytoplasmic	80 (14)	120 (94)

Table 4 Sensitivity of D2-40 and calretinin in epithelioid and sarcomatoid areas of malignant mesothelioma (MM)

Marker	Epithelioid MM areas	Sarcomatoid MM areas
Calretinin	0.91	0.57
D2-40	0.66	0.3
Calretinin and D2-40	0.96	0.66

Sensitivity = true positives/(true positives+false negatives).

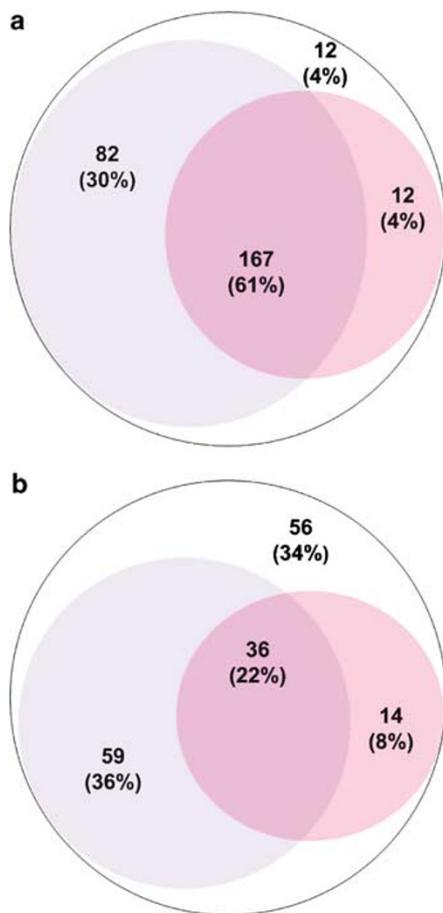


Figure 2 Calretinin (blue) and D2-40 (red) positivity in epithelioid (a) and sarcomatoid (b) tumor areas of malignant mesothelioma. (a) Thirty percent of epithelioid areas were positive only for calretinin, 4% only for D2-40 and 61% for both. No expression for both markers in 4% of epithelioid areas (blank). (b) Thirty-six percent of sarcomatoid areas were positive only for calretinin, 8% only for D2-40 and 22% for both. No expression for both markers in 34% of sarcomatoid areas.

calretinin and D2-40. There were no tumors with heterologous differentiation.

Discussion

In this study, we have used the tissue microarray technology to compare two mesothelioma antibodies, calretinin and D2-40. The use of tissue microarrays allowed to compare the sensitivity of both antibodies within epithelioid and sarcomatoid components of malignant mesothelioma. We have shown that calretinin is of superior value in detecting both epithelioid and sarcomatoid components of malignant mesothelioma.

Numerous investigations have meanwhile provided strong evidence on the representativeness of tissue microarray data for biomarker analysis. Tissue microarray studies have almost always reproduced the expected biological relevance of a given biomarker, for example, the clinical significance of Ki-67 labeling index in bladder cancer, the prognostic role of steroid hormone receptor expression and Her2 neu amplification/overexpression in breast cancer as well as many other established or new associations between molecular markers and tumor phenotype or clinical outcome.^{41,45,48} Also in our study, there was a high concordance of tissue microarray results and data obtained on large tissue specimens. Previous studies have found calretinin positivity in 50–100% of epithelioid malignant mesothelioma, with only very few studies showing expression frequencies below 90% on large tissue sections. In our large tissue microarray analysis, we observed calretinin expression in 91% of epithelioid areas of malignant mesothelioma. A previous tissue microarray analysis reported calretinin positivity in 85.7%.²² Therefore, the present study is another example for the representativeness of tissue microarrays.

In contrast to the traditional large section method, tissue microarrays are an ideal tool to compare different antibodies. Large section studies generate a huge workload and the number of tumors always remains low if multiple parameters are analyzed. Recent large section studies examining diagnostic antibody panels in malignant mesotheliomas only included 173, 111 or 40 malignant mesotheliomas.^{24,53,54} It is evident that the use of larger mesothelioma numbers has a higher statistical value for the evaluation of diagnostic markers. Since other proposed diagnostic markers can easily be included in one study, the use of tissue microarrays allows to rapidly determine optimal antibody panels for diagnostic purposes and to accelerate characterization of novel markers.

The availability of mesothelioma-specific antibodies facilitated the differential diagnosis between adenocarcinoma and epithelioid malignant mesothelioma. However, the diagnostic spectrum of spindle cell lesions of the pleura has also been

expanded by other entities, recently. It includes pleural synovial sarcomas, pulmonary sarcomatoid carcinomas, malignant solitary fibrous tumor, malignant pleural smooth muscle tumor and extraskeletal osteosarcoma.⁵⁵ Therefore, exact knowledge of the sensitivity of malignant mesothelioma markers is of increasing importance. To obtain a comprehensive overview of two different diagnostic antibodies in different tumor components, we have selected tissue cores from both epithelial and sarcomatoid areas. By this approach, we could immediately compare the sensitivity of both antibodies in different histologic types of malignant mesothelioma.

Noteworthy, there are only limited data on calretinin expression in sarcomatoid differentiated areas of malignant mesothelioma in the literature and the results diverge from 18 to 100% positivity in sarcomatoid malignant mesothelioma. We found calretinin staining in 57% and D2-40 staining in only 30% of areas with sarcomatoid differentiation. These results are consistent with data by Chu *et al*²³ but contrast with data by Ordóñez,²⁴ who was not able to detect D2-40 immunoreactivity in sarcomatoid malignant mesothelioma. Importantly, the sensitivity of D2-40 was lower than that of calretinin in both, epithelioid and sarcomatoid areas. We expected a higher sensitivity for D2-40 than calretinin in sarcomatoid malignant mesothelioma, because D2-40 is also positive in other spindle cell lesions, for example Kaposi's sarcoma, and lymphangioma, whereas calretinin is expressed in many epithelioid tumors of non-mesothelial origin, for example, Merkel cell carcinoma, Leydig cell tumors of the testis, esthesioneuroblastoma, adenomas of the adrenal gland and small cell carcinoma of the lung.^{5,21,22} According to our data, calretinin is a more robust marker for malignant mesothelioma than D2-40, although D2-40 probably does increase the specificity of an immunohistochemical panel considerably, because calretinin staining by itself can be seen in a variety of tumors. A panel including calretinin and D2-40 would increase the sensitivity especially for the detection of sarcomatoid malignant mesothelioma.

In order to rule out the presence of synovial sarcomas in our mesothelioma specific tissue microarray, we performed a fluorescence *in situ* hybridization for detection of the translocation t(X,18). Synovial sarcomas have previously been found to be reactive for D2-40²⁴ and calretinin.^{15,56–58} Unfortunately, we were able to obtain sufficient FISH signals in only 18% of the tumors. This low number might be due to different fixation procedures used in different institutions, but the presence of synovial sarcomas could be ruled out at least in these cases (Figure 3).

In summary, our results demonstrate that calretinin is of superior diagnostic value than D2-40. A diagnostic antibody panel including calretinin and D2-40 significantly improves the detection rate of sarcomatoid malignant mesothelioma.

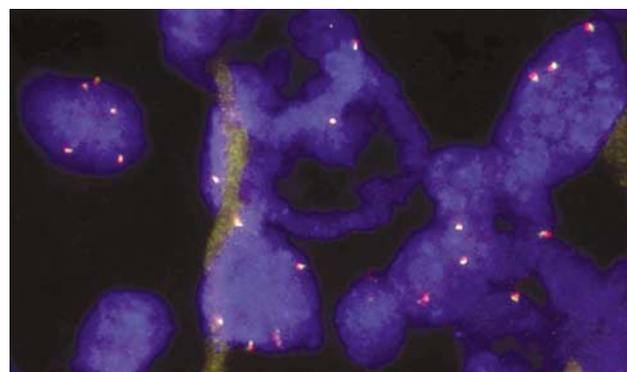


Figure 3 FISH with a break apart probe for the rearrangement of the SYT gene shows only fused signals in the nucleus indicating the absence of a t(X,18) translocation.

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