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Expression of melanocyte-associated markers gp-100 and Melan-A/MART-1 in angiomyolipomas

An immunohistochemical and rt-PCR analysis

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Abstract Angiomyolipomas are tumours of uncertain histogenesis, most often occurring in association with the kidney. A characteristic finding is their reactivity with HMB-45, a monoclonal antibody to the melanocyte-associated antigen gp-100. We tested 18 angiomyolipomas for their reactivity with A103, a monoclonal antibody to Melan-A (MART-1), another melanocyte-associated marker, and compared it with HMB-45. All cases were positive with both antibodies, yet most cases showed a more homogeneous staining pattern with A103. Normal kidney was immunohistochemically negative for both antibodies. We also performed RT-PCR assays for gp-100 and Melan-A in 4 of the 18 angiomyolipoma samples and in three normal kidney samples. All 4 angiomyolipoma specimens revealed mRNA for both melanocyte differentiation markers. gp-100 mRNA was found in the samples of normal kidney, but Melan-A mRNA was not. Our study shows that angiomyolipomas express the melanocyte-associated antigens Melan-A and gp-100 at the protein and at the mRNA level, suggesting a true expression of these antigens rather than cross-reacting epitopes. Based on the mRNA expression pattern, immunohistochemical analysis is the preferred method for the detection of gp-100, while Melan-A can be used at the protein and mRNA levels. Our study demonstrates that A103 is a useful marker for the diagnosis of angiomyolipomas.

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Introduction

Angiomyolipomas are tumours of unknown histogenesis composed of variable amounts of fatty tissue, vascular structures and smooth muscle-like cells [1,38, 40, 42, 48]. They most commonly occur within or in close relation to the kidney, but can be found in other sites throughout the body [38, 40, 53]. Although they can be associated with the tuberous sclerosis complex [7, 8], the lesions often occur spontaneously [42, 44]. The histology of angiomyolipoma can vary; in typical cases there is a mixture of easily discernible vessels, mature lipocytes and areas of smooth muscle-type cells. In others, the tumour may be highly cellular, and pleomorphic, even carcinoma-like or sarcomatoid with little or no adipose tissue [24, 41, 43, 47, 48]. While the nature of the smooth muscle component has been confirmed by several reports at the ultrastructural and immunohistochemical levels [13, 18, 21, 24, 30], another characteristic property of angiomyolipomas is their reactivity with HMB-45 in a subpopulation of cells [4, 13, 18, 21, 31, 47, 51]. HMB-45 is a monoclonal antibody (mAb) commonly used for the diagnosis of malignant melanomas [27]. The antigen recognized by HMB-45 is a glycoprotein of 100 kDa which is hence called gp-100 [56]; this has recently been cloned [2, 3]. HMB-45 reactivity has been attributed to the melanosomes of melanoma cells [49, 52, 56] and can also be found at the ultrastructural level in the melanosome-like granular structures of angiomyolipoma [33]. Some studies, however, have questioned the melanosomal nature of the granules in angiomyolipoma [12, 58] or suspected cross-reacting epitopes [18].

Studies on melanoma antigens recognized by T cells led to the cloning of the *Melan-A* (*MART-1*) gene [22, 36]. Analysis of the mRNA expression indicated that *Melan-A* codes for an antigen expressed in cells and tumours of melanocytic lineage [22, 36, 55]. Owing to

their expression in cells of melanocyte differentiation, gp-100 and Melan-A are assigned to a group of antigens commonly referred to as melanocyte differentiation markers [22, 23]. Other terms frequently used are melanoma-specific, melanocyte-associated or melanocyte lineage antigens/markers [3, 14, 20, 31].

A monoclonal antibody, A103, to the Melan-A recombinant protein was recently developed by our group [20]. We showed that A103 detects the Melan-A antigen and that it can be used as a first line antibody for the diagnosis of melanomas and other melanocytic lesions [17, 32]. In melanomas, A103 showed a slightly greater sensitivity and a more homogeneous immunoreactivity pattern compared with HMB-45.

Based on the initial observation that three angiomyolipomas were immunoreactive with A103 [32], the purpose of the present study was to analyse a larger number of these tumours for their expression of the melanocyte differentiation antigen Melan-A and to compare the immunoreactivity for A103 with that for HMB-45. Another aim of this study was to examine whether positive staining with A103 and HMB-45 is due to the actual expression of the *gp-100* and *Melan-A* gene products or cross-reactivity with related epitopes. This question was addressed by RT-PCR assays for both antigens, gp-100 and Melan-A. Since a recent investigation revealed low levels of gp-100 mRNA expression in various normal tissues, including kidney [14], specimens of normal kidney were included. Our immunohistochemical analysis shows that angiomyolipomas co-express both antigens Melan-A and gp-100, with a more homogeneous expression of Melan-A. The results of the RT-PCR assays parallel this immunohistochemical pattern and indicate that the immunoreactivity is based on the true expression of the epitopes and not due to cross-reacting epitopes. Normal kidney, which was immunohistochemically negative for A103 and HMB-45, revealed no Melan-A mRNA but low levels of gp-100 mRNA. Our study provides further evidence that angiomyolipomas carry features of melanocytic differentiation and indicates that Melan-A is a useful marker at the protein and mRNA levels for the diagnosis of angiomyolipoma.

Materials and methods

Eighteen cases of angiomyolipoma were retrieved from the archives of the Department of Pathology of MSKCC (Table 1). None of the patients carried a diagnosis of tuberous sclerosis. The slides were reevaluated and representative blocks were chosen for further analysis.

HE stains revealed the typical morphological spectrum of angiomyolipomas with varying proportions of vascular structures, adipose tissue and smooth muscle-type cells. While some cases revealed a fair amount of all three components, others showed predominance of one component. The latter consisted either primarily of fatty tissue with few vessels and scattered smooth muscle-type cells localized between the adipocytes or showed almost exclusively solid areas of smooth muscle-type cells with spindle and epitheloid appearance. Often, the smooth muscle type cells were arranged in cuffs around the vessels. Three cases (cases 7, 13, 18) showed atypical morphological features revealing areas or single cells with pleomorphic patterns. These consisted of foci with larger cells and giant cells with bizarre or multiple hyperchromatic nuclei. Because of these atypical features, 1 case (7) was initially diagnosed as a sarcoma.

Table 1 Localization, morphology, and immunohistochemical staining pattern of A103 and HMB-45 in 18 angiomyolipomas (staining of smooth muscle type cells: + <lt;25%; ++ 25–50%; +++ 50–75%; ++++75–100%; reactivity of only single cells indicated as "Focal"

Case	Site; size	A103	HMB-45
1	Kidney; 2 cm	+++	Focal
2	Kidney; 6 cm	+++	Focal
3	Kidney, 2.5 cm	++	Focal
4	Liver; 3 cm	++++	++++
5	Kidney, multiple; largest 5 cm	+++	Focal
6	Kidney; 7 cm	++	+
7	Kidney, 5.5 cm	++++	++
8	Kidney; multiple, largest 2 cm	++	++
9	Kidney, 10 cm	+++	Focal
10	Kidney, multiple, up to 4 cm	+++	+
11	Kidney, 16 cm	++++	+++
12	Kidney, 3.5 cm	+	+++
13	Kidney, 3 cm	++++	+
14	Kidney right	Focal	+
15	Kidney, 2 cm	+++	Focal
16	Kidney, 30 cm	++	Focal
17	Kidney, 3.5 cm	+	Focal
18	Kidney, 6 cm	+++	++++

For immunohistochemistry A103, an IgG1 murine mAb (already described [20]) was used at a concentration of 7.5 µg/ml. HMB-45 was purchased from DAKO (Carpinteria, Calif.) and applied at a dilution of 1:50. Incubations were done overnight at 4°C. A heat-induced antigen retrieval method (steam autoclave, 121°C, 10 min, 10 mM citrate buffer, pH 6.0) was used for both antibodies. Isotype controls were included for both antibodies. Endogenous peroxidase was suppressed by a 20 min incubation with 1% H_2O_2. Labelling of the secondary antibody was performed with an avidin-biotin complex system using a biotinylated horse anti-mouse-secondary (1:200; Vector, Burlingame, Calif.) for 30 min at 20°C and diaminobenzidine tetrahydrochloride (DAB, Biogenex, San Ramon, Calif.) as a chromogen.

The amount of cells stained was graded as 'focal' (staining of single cells, approximately not more than 5%), <25%, 25–50%, 50–75% and >75%. Since smooth muscle-type cells comprise the major component of angiomyolipoma immunoreactivity, the immunohistochemical evaluation was performed on those cells only. A malignant melanoma sample served as a control.

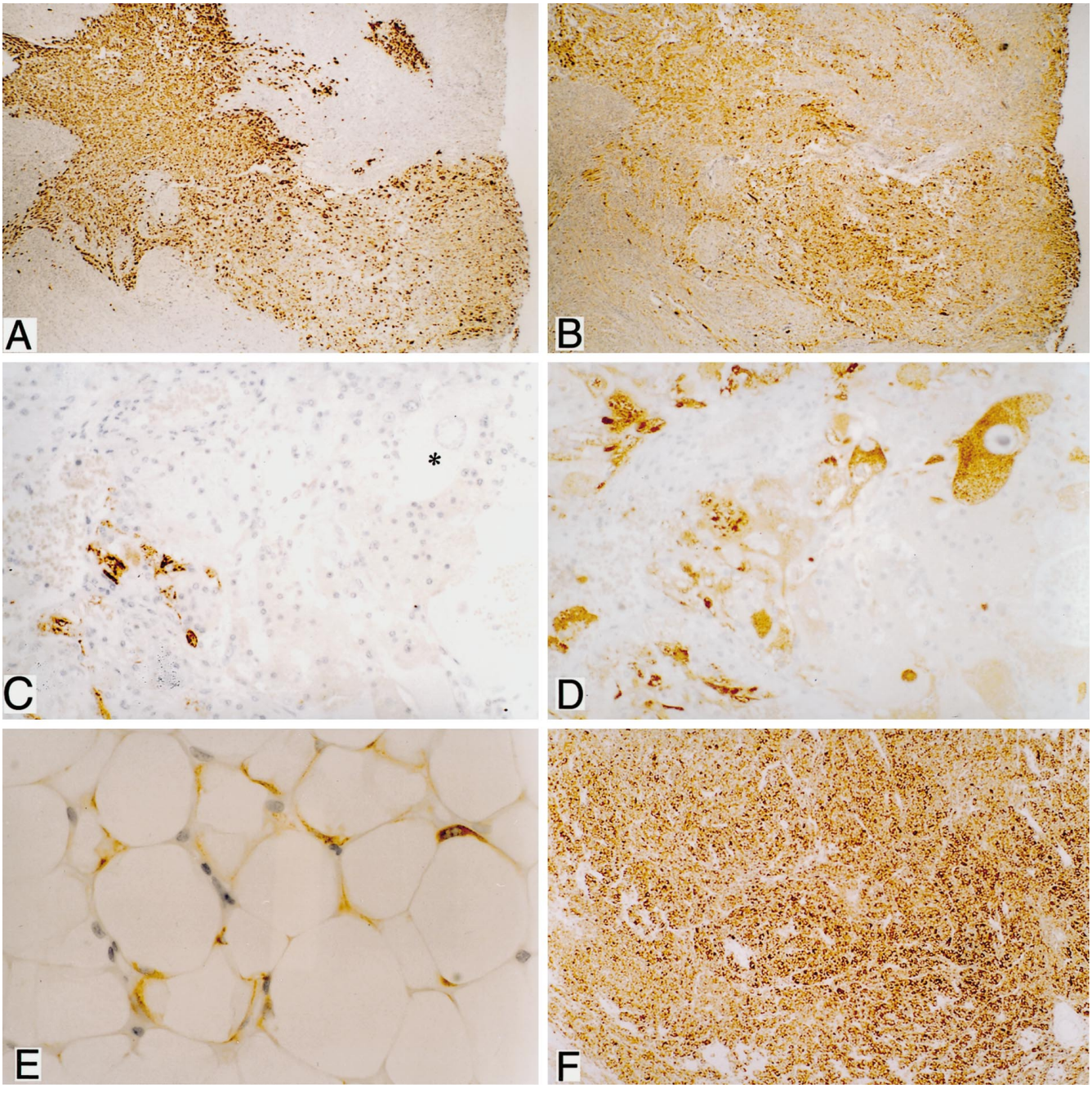
RT-PCR was done as previously described [20]. Briefly, total RNA was prepared from the available snap-frozen angiomyolipoma specimens of 4 tissues (cases 5, 9, 10, 18). In 2 of these cases, samples of uninvolved normal kidney tissue were available (case 10, 18). Another sample of normal kidney derived from a patient without primary disease of the kidney was included. Malignant melanoma was used as a positive control tissue. Oligonucleotide primers were synthesized based on published Melan-A [22] and gp-100 [3] sequences; both primers were synthesized commercially (Operon Technologies, Alameda, Calif.); gp-100: (S): 5'-AGTTCTAGGGGGCCCAGTGTCT-3', (AS): 5'-GGGCCAGGCTCCAGGTAAGTAT-3'; Melan-A: (S): 5'-CTGACCCTACAAGATGC-

CAAGAG-3', (AS): 5'-ATCATGCATTGCAACATTTATTGATG-GAG-3'. The RNA was reverse transcribed into cDNA and PCR-amplified with AmpliTaqGold (Perkin Elmer, Norwalk, Conn.) for 30 cycles in a thermal cycler (Perkin Elmer) at an annealing temperature of 60°C. RT-PCR products were visualized with ethidium bromide.

Results

An overview of the immunohistochemical assays performed is given in Table 1. All 18 angiomyolipomas showed immunoreactivity with both antibodies. HMB-45 revealed a more restricted staining pattern than A103 (Fig. 1a–d). With HMB-45, a reactivity of less than 25% of the area was observed in 12 tissues, 8 of which showed staining restricted to single cells, which was indicated as focal. The reactivity was found mainly in spindle and epitheloid smooth muscle-type cells and in pleomorphic cells. However, not all of the latter were immunoreactive. Staining of single lipocytes was observed occasionally.

Fig. 1 Immunohistochemical staining of angiomyolipomas with HMB-45 (**A, C**) and A103 (**B, D, E, F**). **A, B** intense HMB-45 staining in circumscribed area and A103 staining both in HMB-45-positive area and in the surrounding tissue in case 13. ×15 **C, D** several pleomorphic cells positive for A103 but restricted reactivity with HMB-45 (*asterisk* denotes unstained cell corresponding to intensely stained cell) in case 7. ×150 **E** A103 staining in lipocytes in case 16. ×300 **F** A103 control, malignant melanoma. ×75



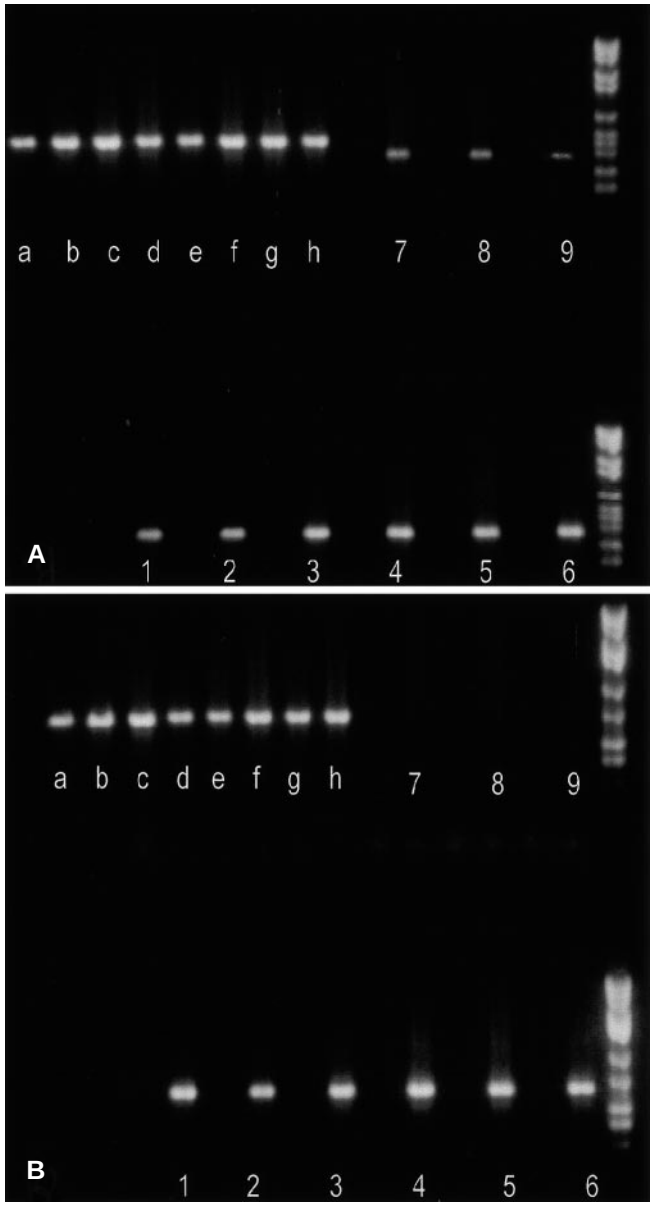


Fig. 2 RT-PCR analysis of **A** gp-100 and **B** Melan-A, ethidium bromide. *Lane 1* malignant melanoma control, *lanes 2–6* angiomyolipoma, *lanes 7–9* normal/uninvolved kidney, *lane 2* case 9, *lane 3, 9* case 10, *lane 4* case 5, *lanes 5, 6, 7* case 18 (two angiomyolipoma samples were included from case 18), *lane 8* normal kidney from unrelated case; *lane a–h* G3PDh control

A103 revealed more widespread staining than HMB-45 in 13 cases (Fig. 1). This difference was striking in 5 cases (1, 2, 5, 9, 15), which revealed only focal reactivity of HMB-45 whereas larger areas consisting of more than 50% of the cells were immunoreactive with A103. In 8 other tissues the difference in immunostaining was less pronounced: 3 of these (cases 3, 17, 16) showed focal HMB-45 reactivity contrasting with more widespread A103 staining. This difference was also apparent in the case with sarcomatoid features (case 7), which showed immunoreactivity with A103 in many cells while HMB-45 was restricted to a smaller subpopulation of cells (Fig. 1c, d). A103 showed pronounced immunoreactivity with lipocyte-type cells in several cases, with a narrow rim of immunoreactivity that could not be attributed to cells between the fat cells (Fig. 1e).

In contrast to these 13 cases, 2 tissues (cases 4, 8) revealed a similar staining pattern for both antibodies, and in 3 cases (11, 14, 18) more cells were immunoreactive with HMB-45 than with A103. However, the difference in immunoreactivity was not as pronounced as in the cases with a predominance of A103. In most cases, the immunoreactivity was overlapping: that is to say the staining patterns consisted of similar cell populations with additional cells that were immunoreactive for one antibody. Only occasionally did the staining patterns consist of a different cell population for each antibody. The inner cells of the cuffs surrounding the vessels remained negative with both antibodies. No staining with either antibody was present in adjacent normal tissue areas.

RT-PCR revealed a product for both genes in all 4 angiomyolipomas available for evaluation (Fig. 2). Though there was a difference in the immunohistochemical reactivity, this difference was not reflected in the RT-PCR result. The two samples from the uninvolved areas of the kidney in angiomyolipoma cases and the sample from the patient with no primary kidney disease showed faint bands indicating a product for gp-100 (Fig. 2a), while no product could be detected for Melan-A (Fig. 2b).

Discussion

Though often referred to as hamartomas [7, 53] rather than neoplastic tumours, recent findings indicate that sporadic and tuberous sclerosis-associated angiomyolipomas are clonal lesions [28, 35, 45] and that the genetic alterations in sporadic cases can resemble those of the tuberous sclerosis-associated cases [29, 41]. Nevertheless, their histogenesis remains controversial. Various studies have produced ultrastructural and immunohistochemical evidence for the smooth muscle character of a fraction of the proliferating cells [1, 4, 15, 18, 24, 30, 31, 40, 43], but the precise nature of angiomyolipoma is still unclear.

Recently the concept of a peculiar cell type, the perivascular epitheloid cell (‘PEC’), common to angiomyolipoma and related lesions was introduced to explain their morphological and immunohistochemical properties and similarities [11, 12, 46]. An interesting finding is the immunoreactivity of these tumours with HMB-45 [1, 4, 15, 18, 21, 24, 47, 51], a mAb widely used for the diagnosis of malignant melanoma. Originally raised against a human melanoma cell line [27], HMB-45 has been shown to detect gp-100 [2, 3], an antigen expressed in melanocytes and their respective tumours [2, 6, 56]. HMB-45 was shown to localize to melanosomes in melanoma cells [49, 52] and to label granular structures resembling melanosomes within angiomyolipomas [33]. However, some doubt that these structures are indeed melanosomes [18, 47, 57, 58]. Further confusion was caused by several studies reporting HMB-45 immunoreactivity in a wide

variety of normal and neoplastic nonmelanocytic tissues [9, 26, 39, 54, 59, 60]. Though it was later shown that these additional nonspecific staining properties were caused by contamination of a commercially available ascites fluid preparation [5, 10, 33], they are still causing concern about the specificity of HMB-45, as they are discussed as possible artifacts in more recent reports on angiomyolipomas [4, 18]. Nevertheless, HMB-45 has become an immunohistochemical marker for the diagnosis of angiomyolipoma.

In our angiomyolipoma study, the HMB-45 distribution pattern is consistent with that previously reported by others, being present in all cases and ranging from mostly focal to intense labelling of quite large tumour areas in some cases [4, 18, 24, 47, 51]. Also, the HMB-45 staining was primarily seen in the smooth muscle type cells. No staining was observed in adjacent or in additional specimens of normal kidney tissue.

Melan-A [22] – also named MART-1 [36] – was identified by analysing the T cell response to autologous melanoma cell lines. Since Melan-A mRNA expression is restricted to cells and tumours of melanocytic lineage [14, 22, 36], it is, like gp-100, regarded as a marker of melanocyte differentiation. We previously generated 'A103', a mAb to the Melan-A recombinant protein [20], and showed that it can be used for the diagnosis of melanocytic lesions in a standard diagnostic setting [16, 17, 32]. In the present study, A103 showed immunoreactivity in all angiomyolipoma cases. Interestingly, in most cases the staining was more homogeneous than with HMB-45; that is wider areas of the tumour were immunoreactive with A103. Only 3 cases revealed a more homogeneous staining with HMB-45. These results parallel our report on the distribution pattern of A103 in metastatic malignant melanomas in which we showed that A103 had a more homogeneous staining pattern than HMB-45 [32]. The similarities and differences in staining patterns might reflect closely linked antigens present in certain stages of cell development.

Though the sequence suggests a transmembrane protein [22], the biochemical properties, cellular function, and subcellular localization of the Melan-A protein are not known, but it is believed to be localized in the melanosomes [23]. A103 reactivity is present in most melanocytes in normal skin [16, 32]. However, gp-100 – as detected by HMB-45 – is known to be present only in early stages of the melanosome formation (stages I–III), while late stage melanosomes lack gp-100 [49, 52]. This is also reflected in the HMB-45 reactivity in normal skin, which is present in only a fraction of melanocytes with activated melanogenesis [5, 27].

Another intriguing observation in our study is the presence of immunoreactivity in single lipocytes with both A103 and to a lesser extent with HMB-45. No immunoreactivity has been reported with either antibody in normal fatty tissue [5, 32]. While some studies have confirmed HMB-45 reactivity in adipose tissue of angiomyolipoma [1, 58], others disclaimed the existence of lipocyte staining [4, 31, 47]. In the present study the vast majority of stained cells are of the smooth muscle type, but the focal adipocyte immunoreactivity supports the notion that all cellular components might derive from the same cell type [28, 35, 45]. This observation is supported by an earlier ultrastructural study, which found transitional forms of cells linking the smooth muscle-type cell to the lipocytes [30].

Besides the controversies surrounding the nature of the structures detected by HMB-45 in angiomyolipoma, there is evidence that A103 is not an entirely Melan-A-specific antibody. In a previous study, A103 was immunoreactive with some normal tissues and neoplastic lesions containing steroid-hormone-producing cells, such as adrenal cortex and adrenocortical tumours [17]. These tissues were negative for Melan-A mRNA. In order to analyse whether the immunohistochemical reactivity of A103 and HMB-45 in angiomyolipoma is a specific reaction, we tested for the presence of both gp-100 and Melan-A at the mRNA level by RT-PCR. All 4 available samples were positive for mRNA of gp-100 and of Melan-A, supporting the notion that both HMB-45 and A103 react with the specific gene product of gp-100 and Melan-A in immunohistochemical assays.

Earlier analysis of Melan-A revealed mRNA expression strictly confined to tissues and cells of melanocytic lineage [14, 22, 36]. Previous studies employing northern blot assays claimed a similar distribution for gp-100 mRNA [37]. A recent investigation using the RT-PCR technique, however, disclosed low levels of gp-100 mRNA in various tissues of non-melanocytic lineage while the protein expression of gp-100 remained purely melanocyte-associated [14]. Consequently we included samples of normal kidney in our study. These were derived from uninvolved kidney parenchyma of 2 patients with angiomyolipoma and from a patient without primary disease of the kidney. While RT-PCR of *Melan-A* revealed no gene product, RT-PCR of *gp-100* resulted in bands in all three samples. As the assays were not performed in a quantitative setting, no conclusion about the actual mRNA levels can be made. In additional assays (data not included) we confirmed the presence of gp-100 mRNA in various normal organs, such as heart, liver, small intestine, and lung. Since in our study normal kidney parenchyma was immunohistochemically negative for A103 and HMB-45, the mRNA expression paralleled the protein expression only for Melan-A. The significance of the gp-100 mRNA expression in kidney and other non-melanocyte-associated tissue deserves further evaluation. Current knowledge based on the extensive analysis of the gp-100 antigen by immunohistochemistry [5], immunoblotting assays [14] and our study, however, suggest that no detectable levels of gp-100 protein are expressed. Low-level mRNA expression of tissue-specific genes in various tissues without detectable protein expression has been reported for several other genes [19].

In addition to our immunohistochemical and molecular findings, the histochemical demonstration of cytoplasmatic dopa-oxigenase in angiomyolipoma is a further indication of melanosomal differentiation [19].

Also, an association of gene loci allegedly linked to tuberous sclerosis and loci assigned to enzymes of melanogenesis has been postulated by some [25, 50] but disputed by others [28, 29, 34].

Our data provide additional evidence to support the notion that angiomyolipomas carry features of melanocytic differentiation. Our study shows that aside from gp-100 antigen, Melan-A antigen is expressed in a significant proportion of cells. While Melan-A mRNA is confined to lesional tissue in angiomyolipoma, gp-100 mRNA can also be found in samples of normal kidney. Owing to this expression pattern, the RT-PCR evaluation of gp-100 in angiomyolipomas (and other lesions) should be conducted with great caution. Furthermore, our study indicates that aside from HMB-45, anti-Melan-A immunomarkers such as A103 and the detection of Melan-A mRNA can be used for the diagnosis of angiomyolipoma.

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