Human Telomerase RNA Expression and MIB-1 (Ki-67) Proliferation Index Distinguish Hemangioblastomas From Metastatic Renal Cell Carcinomas.

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Human Telomerase RNA Expression and MIB-1 (Ki-67) Proliferation Index Distinguish Hemangioblastomas from Metastatic Renal Cell Carcinomas

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Abstract. Hemangioblastomas are low-grade, capillary rich neoplasms of the cerebellum and spinal cord that can occur sporadically or in the setting of Von Hippel-Lindau syndrome. The present study analyzed the utility of proliferation potential in differentiating hemangioblastoma from RCC metastatic to the central nervous system using a MIB-1 (Ki-67) labeling index and assessment of expression of the RNA component of telomerase. Immunohistochemical analysis for epithelial membrane antigen (EMA) and MIB-1 was performed on paraffin-embedded sections of 27 hemangioblastomas and 5 RCC metastatic to the central nervous system. All but one hemangioblastoma demonstrated low or negative MIB-1 immunoreactivity, while 4 of 5 RCC metastases had moderate or high labeling indices. Telomerase RNA expression was assessed in 10 hemangioblastomas and in all 5 metastatic RCC by in situ hybridization. All 10 hemangioblastomas demonstrated a lack of expression of telomerase RNA, while all 5 metastatic RCCs showed moderate to strong expression. Our results suggest that the MIB-1 labeling index is useful in differentiating hemangioblastoma from metastatic RCC and assessment of telomerase expression can also provide novel information on the difference in growth potential of these tumors.

Key Words: Hemangioblastoma; Metastatic renal cell carcinoma; MIB-1; Telomerase expression.

INTRODUCTION

Hemangioblastomas are low-grade, capillary rich neoplasms of the cerebellum, medulla, and spinal cord (1–4) containing variable amounts of lipidized stromal cells (5-7). These tumors represent one of the most common cerebellar neoplasms, present typically between the ages of 30 and 65, and can occur sporadically or in the setting of Von Hippel-Lindau syndrome (2, 4, 8, 9). The histopathologic differential diagnosis of hemangioblastoma includes metastatic clear cell carcinoma (10–13), which is especially relevant in light of the association between Von Hippel-Lindau syndrome and renal cell carcinoma (RCC) (14–16). The traditional pathological diagnostic workup includes an epithelial membrane antigen (EMA) immunohistochemical stain with demonstrable immunoreactivity in RCC, and nonreactivity in hemangioblastomas (10–13). However, there are cases in which the immunohistochemical profile is equivocal, which poses an acute diagnostic challenge.

The aim of the present study was to evaluate the utility of proliferation potential in differentiating hemangioblastomas and RCC metastatic to the central nervous system (CNS). Proliferation potential was assessed by immunohistochemical analysis using a monoclonal antibody to Ki-67 (MIB-1) and by in-situ hybridization for human telomerase RNA (hTR). Ki-67 is a nuclear-associated protein that is expressed in all phases of the cell cycle except G_0, and hence is a more sensitive marker for cell proliferation than traditional mitotic figure counts (17–22). The immunohistochemical analysis of Ki-67 (MIB-1) in paraffin sections is a recently developed technique (23–25), and currently there are limited studies available for standardization of Ki-67 (MIB-1) labeling in the area of CNS neoplasia (26–29). It is known that telomeres shorten progressively with each cell division in normal somatic cells (30–33). However, telomere length is stabilized in tumor cells (34–37). There is an emerging theory that re-expression of telomerase activity, an enzyme that maintains telomeres and prevents their shortening, may allow tumor cells to replicate indefinitely (34–37). Recently, telomerase activity has been assessed in a variety of neoplasms to investigate its prognostic significance (34, 38–44).

We hypothesize that hemangioblastomas are neoplasms of low proliferative capacity, while metastatic RCC are neoplasms of high proliferative capacity, and they can therefore be differentiated on this basis. This would allow for further classification of these neoplasms and aid in diagnostically challenging cases. We therefore undertook this study to evaluate the utility of proliferation potential in differentiating hemangioblastoma from RCC metastatic to the CNS.

MATERIALS AND METHODS

Specimens

Twenty-seven patients with hemangioblastomas undergoing resection at Parkland Memorial Hospital or Zale Lipshy University Hospital at the University of Texas Southwestern Medical Center (Dallas, Texas) between 1991 and 1996 were included in this retrospective study. All patients underwent

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TABLE 1
Summary of Immunohistochemistry and Telomerase (hTR) Expression

Immunohistochemical stain in situ hybridization	Hemangioblastoma (n = 27)	Metastatic RCC (n = 5)
EMA	2(+) 25(−)	5(+)
MIB-1 (Ki-67)	5 rare + cells 21 low (<7%) 1 low/medium (12%)	1 low (4%) 2 medium (15%, 20%) 2 high (35%, 70%)
hTR	10(−)*	2(2+) 3(3+)

* 10 cases randomly selected.

curative surgery with a gross total resection. There were 13 (48%) female and 14 (52%) male patients, ranging in age from 17 to 73 years (mean, 42). Nineteen (70%) tumors were located in the cerebellum/posterior fossa and 8 (30%) tumors were within the spinal cord. In addition, 5 patients with suspected RCC metastatic to the CNS undergoing biopsy were included. All 5 patients were male, ranging in age from 51 to 68 years (mean, 60).

Immunohistochemical Analysis

All immunostaining was performed in a standard fashion at room temperature on a BioTek Solutions TechMate 1000 automated immunostainer (Ventana BioTek Systems, Tucson, AZ) with appropriate positive and negative controls run concurrently. Briefly, paraffin sections were cut at 3 μm, mounted on positively charged glass slides (POP100 capillary gap slides, Ventana BioTek Systems), air dried overnight, and were then deparaffinized. After incubation with blocking serum, sections were incubated with primary antibody (MIB-1, monoclonal, BioGenex, San Ramon, CA , 1:10 dilution, 2 hour primary incubation; EMA, mouse monoclonal, Dako, Carpenteria, Calif, 1:300 dilution) followed by a biotinylated polyvalent secondary antibody solution. Sections were then incubated with horseradish peroxidase–conjugated avidin-biotin complex (HRP/ABC) followed by diaminobenzidine (DAB) and H_2O_2, and then counterstained with hematoxylin.

Quantification of Staining Results (Immunohistochemistry)

Quantification of the proliferation marker MIB-1 was performed by identifying those areas demonstrating maximum reactivity for MIB-1. All nuclei with staining were rated as positive, and proliferative activity was quantified by counting 1000 cells at 400× magnification to arrive at a MIB-1 labeling index percentage. Epithelial membrane antigen immunohistochemical stains were interpreted as diffusely positive, occasional positive cells, or negative.

Telomerase (hTR) Expression Analysis

Slide preparation: Paraffin sections were cut at 3 μm, mounted on positively charged glass slides, and air dried overnight. The sections were deparaffinized, rehydrated in phosphate buffered saline (PBS), and treated with proteinase K (20 μg/ml) in 50 mM HCl (*p*H7.5), 5 mM EDTA for 20 min at room temperature. After rinsing for 5 min in PBS, sections were postfixed in 4% paraformaldehyde/PBS, rinsed in water, and acetylated in freshly prepared 0.25% acetic anhydride/0.1M triethanolamine (45). The slides were then dehydrated in gradually increasing concentrations of ethanol prior to hybridization.

Probe Preparation

The plasmid pGEM-5Z (Promega Corp., Madison, Wis) containing an hTR complementary 559 nucleotide DNA (Geron Corp., Menlo Park, Calif) was used as a template to generate sense and anti-sense probes (46).

Hybridization and Washing Procedures

Sections were hybridized overnight (45), and following stringent washing, the slides were dehydrated and dipped in Kodak NTB-2 nuclear track emulsion and exposed for 3 weeks in photoresistant boxes with a desiccant at 4°C. The microautoradiographs were developed in Kodak Dektol developer (3.5 min), washed in water (20×s), fixed in Kodak fixer (7 min), rinsed in water, and counterstained with hematoxylin-eosin (45). To confirm the presence of intact RNA, replicate slides from each sample utilized for hTR expression were also tested for expression of a "housekeeping" gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The Zba/HindIII fragment from the GAPDH cDNA in pBR322 obtained from the American Type Culture Collection in Rockville, Md, was subcloned into pBluescript. Intense GAPDH hybridization signals were present in all sections studied.

Quantification of Staining Results (hTR Expression)

Telomerase expression was interpreted as positive or negative on each case. The intensity of expression in the positive cases was then assessed as mild (1+), moderate (2+), or strong (3+) based on the density of silver grains overlying tumor nuclei (Fig. 1). A sense probe slide served as the control for comparison with the anti-sense probe slide for each individual case. Ten hemangioblastomas and all 5 metastatic RCC were studied.

RESULTS

EMA Immunohistochemistry (Table 1)

Of 27 cases of hemangioblastoma, 25 were negative for EMA and the remaining 2 cases showed faint staining. In all cases there was no evidence of a primary RCC

after thorough clinical evaluation. All 5 cases of RCC metastatic to the CNS were immunoreactive for EMA.

MIB-1 Labeling (Table 1)

Five of the hemangioblastoma cases showed a <1% MIB-1 labeling index. Twenty-one hemangioblastomas showed a low labeling index with a range of 1%–7% (Fig. 2), a mean of 2.5%, and a median of 3%, all based on counts of 1000 cells. A single hemangioblastoma showed a low to moderate labeling index of 12%. The overall mean for hemangioblastoma cases was 2.8%.

The metastatic RCC cases overall demonstrated much higher labeling indices. Two cases showed a high MIB-1 labeling index (Fig. 2) with a range of 35%–70%, and a mean of 52.5%. Two cases showed a moderate labeling index with a range of 15%–20%, and a mean of 17.5%. A single metastatic RCC case showed a low labeling index of 4%. The overall mean for the metastatic RCC cases was 28.8%.

We compared the MIB-1 labeling indices in hemangioblastoma vs metastatic RCC cases using a 2 sample t-Test. Although there was some overlap between the 2 groups (Fig. 3), the average percentage of labeled nuclei in the group of metastases was significantly greater than the average percentage in the hemangioblastoma group (28.8% ± 25.6% [mean ± standard deviation] in metastatic RCC, 2.8% ± 2.4% in hemangioblastoma, $p < 2.5 \times 10^{-6}$) (Table 2).

Telomerase (hTR) Expression (Table 1)

Ten of 27 hemangioblastomas were randomly selected for assessment of hTR expression. All 10 cases showed no detectable expression and were read as negative (Fig. 2). Two of the metastatic RCC cases demonstrated moderate (2+) expression and 3 cases demonstrated strong (3+) expression (Fig. 2).

In comparing the telomerase expression in hemangioblastomas vs metastatic RCC, it was not possible to perform a statistical analysis, since all cases in the hemangioblastoma group had no detectable hTR expression. The mean degree of expression in the metastatic group was 2.6 ± 0.6 in the 5 cases studied (Table 2).

DISCUSSION

The primary consideration in the histopathologic differential diagnosis of CNS hemangioblastoma is metastatic RCC (10–13). Although the hematoxylin-eosin histologic appearance is extremely similar in these 2 entities, in most cases a definitive diagnosis can be made utilizing both clinical and radiographic information in addition to immunohistochemical analysis, namely EMA (10–13). Whereas the histogenesis of the stromal clear cells in hemangioblastomas remains disputed (5–7,11–12), reports have demonstrated a lack of immunoreactivity for the epithelial marker EMA (10–13), while there is often immunoreactivity for EMA in the clear cells of RCC (10–13). However, there are cases in which the staining profile is equivocal and the diagnosis is not straightforward.

In our series, there were 2 hemangioblastomas that demonstrated EMA immunoreactivity. These cases illustrate the occasional problem with what is to date the most reliable method of differentiating these 2 histologically similar lesions. Postulating that hemangioblastomas are neoplasms of low proliferative capacity while metastatic RCC are of high proliferative capacity and can therefore be differentiated on this basis, we assessed proliferation potential in these neoplasms using MIB-1 immunostaining and hTR expression.

Immunohistochemical analysis of MIB-1 activity is a rapid technique that is a useful indicator of growth potential in a variety of neoplastic conditions including RCC (47–49). We demonstrated a significant difference in labeling indices between hemangioblastomas and metastatic RCC. When we compared the MIB-1 labeling index in the 2 groups, we found that 26 of 27 hemangioblastoma cases had low labeling indices, with only a single hemangioblastoma case showing a low to moderate labeling index (12%). In metastatic RCC cases, the labeling indices varied from low to high, showing some overlap with the hemangioblastoma group, although 4 of 5 cases had labeling indices above 15%, whereas there were no cases of hemangioblastoma with labeling indices above 12%. These results provide evidence that in the majority of cases, the MIB-1 index can contribute additional useful information that will aid in distinguishing between hemangioblastoma and metastatic RCC.

All hemangioblastoma cases on which telomerase RNA expression was assessed were negative in comparison with metastatic RCC, in which hTR expression was detected in all cases. Specifically, all cases of RCC metastatic to the CNS demonstrated moderate to strong expression (2+ to 3+). This is in sharp contrast to the hemangioblastoma cases, in which there was no detectable hTR expression. Although not currently a rapid technique (3 weeks) (45), in situ telomerase assessment might provide vital information when a diagnosis remains less than definitive. The paraffin in situ technique (45) for assessing hTR expression has recently been shown to correlate with the telomeric repeat amplification protocol (TRAP) assay on frozen tissue (50–51). The in situ technique allows determination of hTR expression while obviating the need for frozen tissue, which may not be available on all cases (45). This technique also allows identification of the specific cell populations expressing telomerase, which is not possible with the TRAP assay. The development of a paraffin immunohistochemical antibody to telomerase is the next logical step in the progression of this area of study. This would allow for a more useful

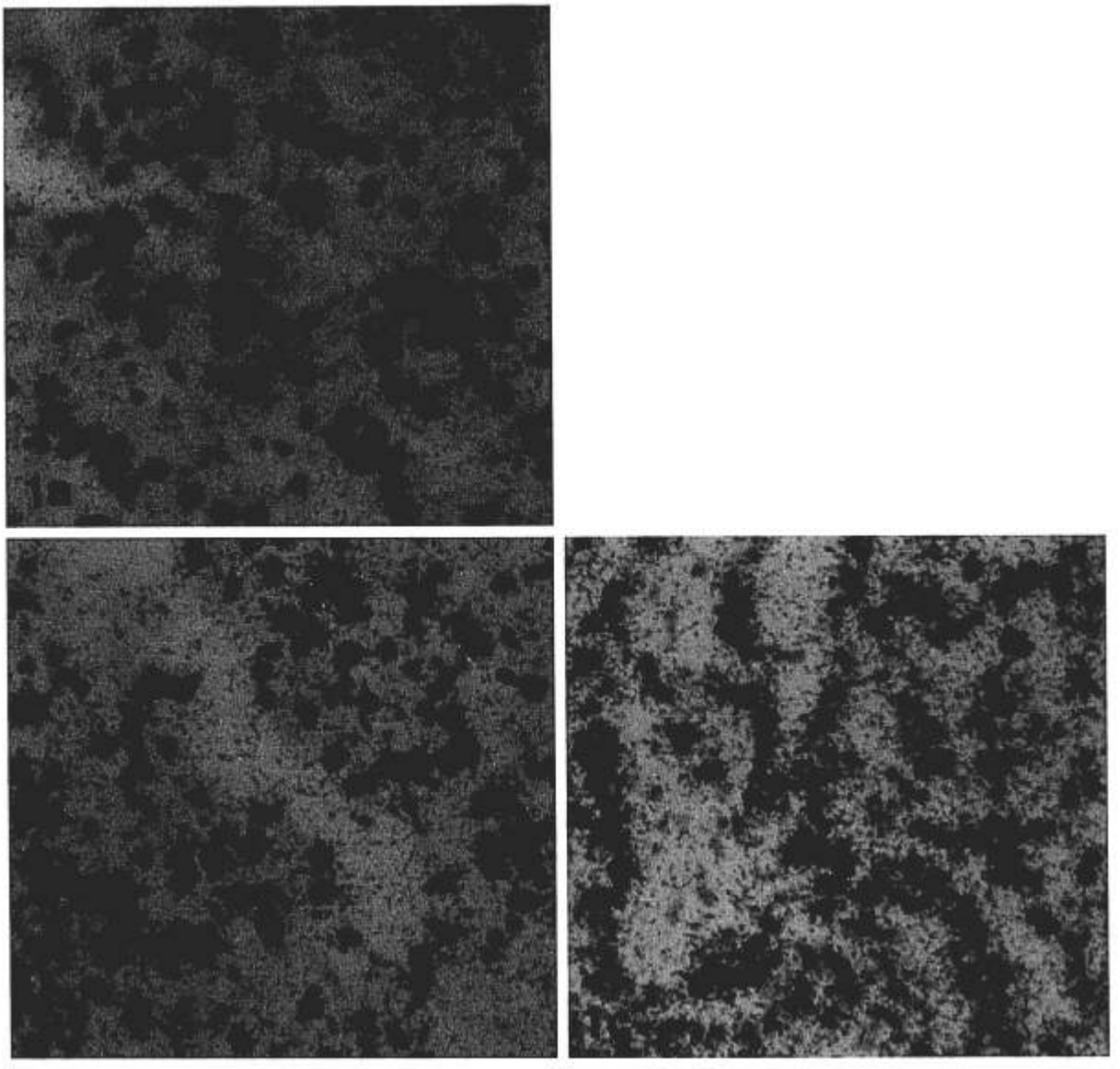

Fig. 1. Telomerase (hTR) microautoradiographs of varying degrees of expression. Formalin-fixed, paraffin-embedded sections (renal cell carcinoma) hybridized with hTR probe, developed, and counterstained with hematoxylin-eosin. a: mild, 1+ expression. b: moderate, 2+ expression. c: strong, 3+ expression. Original magnification: 1a, 400×; 1b, 400×; 1c, 400×.

and rapid method for the determination of tumor proliferative potential, similar in value to the Ki-67 antigen.

Although both MIB-1 labeling and hTR expression were able to distinguish between hemangioblastomas and metastatic RCC, hTR expression was clearly more definitive. These results suggest that although MIB-1 labeling is a very useful method for assessing tumor cell proliferation, in situ determination of hTR expression may be

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Fig. 2. Comparison of hemangioblastoma and renal cell carcinoma (RCC) metastatic to the central nervous system. a: Hemangioblastoma. b: Metastatic RCC. c: Hemangioblastoma immunostained with MIB-1 antibody showing a low labeling index (1%) (HRP-DAB/ABC with light hematoxylin counterstain). d: Metastatic RCC immunostained with MIB-1 antibody showing a moderate to high labeling index (40%) (HRP-DAB/ABC with light hematoxylin counterstain). e: Hemangioblastoma assessed for degree of hTR expression showing no detectable expression (hematoxylin-eosin counterstain). f: Metastatic RCC assessed for degree of hTR expression showing strong (3+) expression (hematoxylin-eosin counterstain). Original magnification: 2a, 250× (inset 600×); 2b, 250× (inset 600×); 2c, 400×; 2d, 400×; 2e, 400×; 2f, 400×.

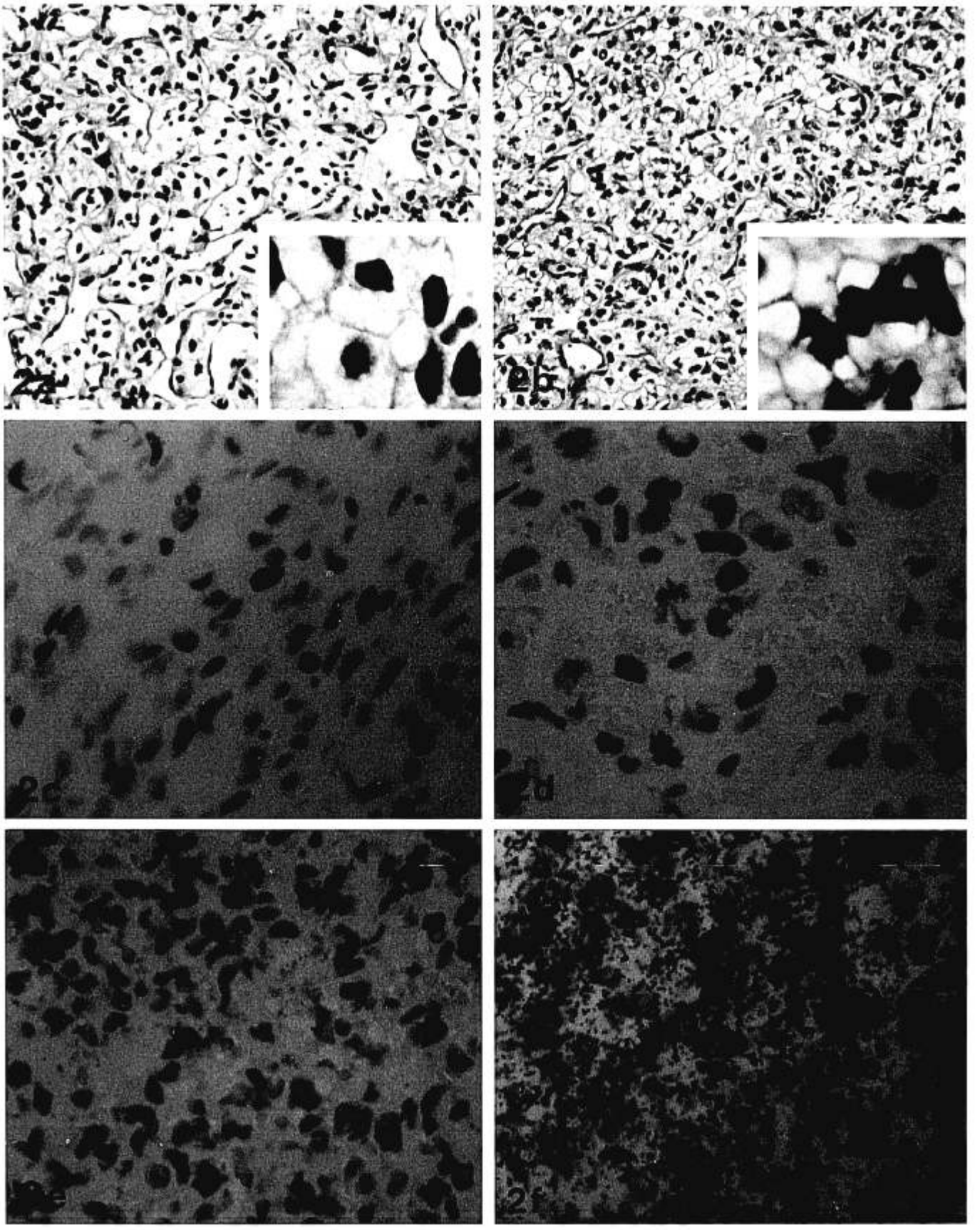

TABLE 2
Comparison of MIB-1 Labeling Indices and Telomerase (hTR) Expression in Hemangioblastomas and Metastatic Renal Cell Carcinomas

Proliferation marker	Hemangioblastoma	Metastatic RCC	p Value (two sample t-test)
MIB-1 labeling index	2.8 ± 2.4% (n = 27)	28.8 ± 25.6% (n = 5)	$p < 2.5 \times 10^{-6}$
hTR expression	0.0 (n = 10)	2.6 ± 0.6 (n = 5)	N/A

[Mean ± Standard Deviation]

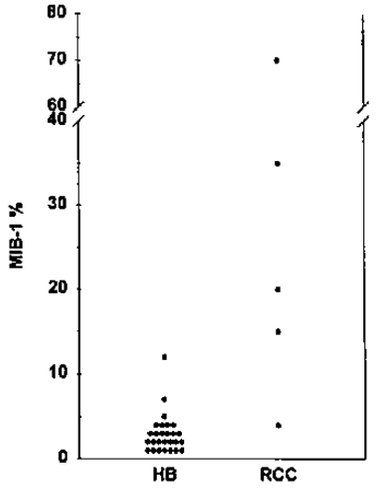

Fig. 3. Comparison of MIB-1 labeling indices in hemangioblastoma (HB) and renal cell carcinoma metastatic to the central nervous system (RCC).

a more sensitive technique, allowing more accurate detection of proliferation potential in light of a low MIB-1 index.

In conclusion, our results demonstrate that the MIB-1 (Ki-67) labeling index is useful in differentiating hemangioblastoma from metastatic RCC, two entities that bear striking hematoxylin-eosin histologic resemblance. In addition, assessment of hTR expression may also provide novel information on the difference in growth potential of these tumors, allowing further delineation between hemangioblastomas and RCC. Additional pursuit in the area of proliferation markers including larger series and variety of tumor types will aid in establishing standards for comparison and in developing information on biologic behavior as it relates to prognosis and clinical outcome.

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