Cell Membrane Reactivity of MIB-1 Antibody to Ki67 in Human Tumors: Fact or Artifact?

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Abstract: Ki67 immunohistochemistry is a widely used marker of the tumor proliferative fraction. Apart from the nuclear staining of dividing cells, MIB-1 monoclonal antibody was also found to stain the cell membrane of some tumor types. Indeed, such membrane reactivity was proposed as a diagnostic feature of hyalinizing trabecular tumor (HTT) of the thyroid. To verify the diagnostic role of Ki67 membrane pattern, 6 HTTs, 8 pulmonary sclerosing hemangiomas (SH), and 6 other human tumors with MIB-1 cell membrane immunoreactivity were stained by immunoperoxidase with 5 different anti-Ki67 antibodies in different experimental conditions. We show here that the cell membrane reactivity reported in HTT is produced only by MIB-1 and not by other antibodies to Ki67 (including commercially available mouse and rabbit monoclonal antibodies). In addition, this peculiar pattern is obtained only if the reaction is performed at room temperature, because automated immunostainers which operate at 37°C do not produce any MIB-1 membrane localization. The same findings were obtained in the other 6 tumors. Conversely, sclerosing hemangioma of the lung did not produce any MIB-1 cell membrane reactivity in our hands. A cross-reactivity of the MIB-1 monoclonal antibody with an epitope expressed at the cell membrane level (rather than an artifact) seems the most likely explanation for this finding, because the immunoreactivity is generally intense and uniform in the membrane positive tumors. We conclude that when Ki67 immunohistochemistry is used for diagnostic purposes in a suspected HTT, only MIB-1 clone at room temperature should be employed.

Key Words: Ki67, MIB-1, immunohistochemistry, membrane, carcinoma, thyroid, hyalinizing trabecular tumor

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It is well known that the nuclear Ki67 antigen is expressed during all cell cycle phases, but G_0.[1] Ki67 immunostaining is therefore the most common histopathologic method to assess the tumor cell proliferation index (PI), in routine formalin-fixed paraffin-embedded samples.[2] In the literature, several studies have been reported on the prognostic role of the PI in neoplastic patients. The survival rate correlates with the percentage of Ki67 positive neoplastic cell of larynx, urogenital, digestive, and endocrine carcinomas.[3,4] In other neoplastic lesions (eg, neuroendocrine tumors), the PI is one of the parameters leading the therapeutic strategy.[5]

Ki67 immunostaining has also been proposed for diagnostic purposes, such as to differentiate benign (low PI) from malignant epithelial and mesenchymal lesions (high PI), to discriminate between typical and atypical bronchial carcinoids[6,7] and to take benign apart from malignant parathyroid and adrenocortical tumors.[8] Another useful immunohistochemical application of Ki67 is the localization and distribution of proliferating nuclei in pluristratified and pseudostratified epithelial lesions to grade the cervico-uterine and bladder urothelial lesions.[3,9,10] Ki67 is also useful in the differential diagnosis of follicular lymph node lesions, that is, follicular hyperplasia versus follicular lymphoma.[11]

The proliferative activity, as detected by Ki67 expression, is usually evaluated by means of the monoclonal antibody MIB-1, which recognizes a Ki67 epitope in archival, paraffin-embedded material. Recently, a peculiar cell membrane stain of Ki67 has been described in hyalinizing trabecular tumor (HTT) of the thyroid, suggesting that such membrane pattern was diagnostic in these lesions.[12–15] In sclerosing hemangioma (SH) of the lung (but not in a control group of conventional pulmonary carcinomas), a similar pattern was described in association with cytoplasmic staining.[16]

Recently, we happened to use Ki67 immunostaining to confirm the diagnosis of a case of HTT of the thyroid, but we had negative results. Therefore, an analysis of several commercially available anti-Ki67 antibodies in different technical conditions was designed with the aim of verifying if the cell membrane staining reported in HTT and, occasionally, in other human tumors was related to a specific Ki67 antibody clone and/or specific experimental conditions.

MATERIALS AND METHODS

A case of sarcomatous pleural mesothelioma with focal Ki67 cell membrane reactivity and 6 cases of HTT of the thyroid were retrieved from the pathology files of the University of Turin at San Giovanni Hospital. The clinicopathological and molecular data of HTT cases were reported elsewhere.[13,17] In addition, 4 cases of invasive breast carcinoma having a membrane reactivity were collected from the pathology files of the Treviso (3 cases) and Trento (1 case) Hospitals; 2 of them had been included in a study on rabbit monoclonal antibody applications in diagnostic immunohistochemistry.[18] Finally, 8 cases of SH of the lung and 1 case of renal oncocytoma were retrieved from the pathology files of the Arcispedale S. Maria Nuova, Reggio Emilia (3 SH), Ospedale S. Chiara, Trento (1 SH), and San Luigi Hospital in Orbassano, Turin (4 SH and 1 renal oncocytoma).

A representative formalin-fixed, paraffin-embedded tissue block was selected for each case and 5-μm-thick serial sections were dewaxed, rehydrated, and processed for immunoperoxidase using different Ki67 antibody clones and different technical conditions, as summarized in Table 1. For all Ki67 antibody clones tested, antigen retrieval was based on three 5-minute passages in microwave oven at 750 W in 10 mM citrate buffer solution at pH 6. Streptavidin-biotin-peroxidase complex (LSAB DakoCytomation, Glostrup, Denmark) and biotin-free methods (EnVision, DakoCytomation) were used to detect the immune reaction. Diaminobenzidine and hydrogen peroxide were applied as chromogen and substrate, respectively. Both manual and automated immunostains (DakoCytomation and Ventana, Tucson, Arizona) were performed for each antibody, at room temperature or at 37°C, respectively.

RESULTS

Automated immunostains and manual methods showed basically the same pattern at comparable experimental conditions. No differences were observed using the 2 different detection systems (either biotin-based or biotin-free). In all selected lesions, except for SH cases, tumor cells showed membrane and (occasionally) cytoplasmic staining only when MIB-1 clone (either from DakoCytomation or from Immunotech) was used at room temperature (Fig. 1). No MIB-1 membrane reactivity was found when immunostaining was performed at 37°C with the same clone. No Ki67 membrane localization was observed when any other clone (from both mouse and rabbit sources; Table 1) was employed, irrespective of the incubation temperature of the antibody. In these same cases, MIB-1 nuclear staining was detected in proliferating tumor cells, as expected, and in few reactive lymphocyte nuclei, irrespective of the reaction temperature.

DISCUSSION

Ki67 is a nuclear protein expressed during the entire cell cycle activity, being switched off during the G_0 phase.[1] MIB-1 is a related monoclonal antibody that works in paraffin-embedded tissue and recognizes a fixation-resistant epitope of this antigen.[2,19,20] Neither the exact epitope sequence recognized by the different Ki67 antibodies is known nor the commercial data sheets of the reagents provide any additional information. Despite this lack of information, the various reagents are routinely used as if they were identical, although differences in LI may be obtained using different reagents.[21,22]

The Ki67 cell membrane staining has been sometimes interpreted in the literature as an artifact. However, the possibility of a real cross-reaction by a similar epitope should rather be considered, on the basis of the intense and sometimes crisp membrane staining, which is different from nonspecific background signal.

In this study, we show that only the MIB-1 clone was able to stain the cell membrane in HTT of the thyroid and in the other tumors here collected (cases of breast carcinoma, renal oncocytoma, and mesothelioma). By contrast, we were not able to reproduce the same "artifact" described by Hattori[16] in SH, where, at least in our cases, only an MIB-1 nuclear pattern was observed, despite the same strong antigen retrieval conditions described by the author[16] were applied. The other Ki67 antibodies were invariably negative at the cell membrane level (although positive in the proliferating nuclei, as expected). This indicates that the different anti-Ki67 clones either identify different Ki67 epitopes or have a different grade of avidity. This latter possibility is unlikely because MIB-1 clones did not produce membrane staining when the temperature of the immunohistochemical reaction was increased at 37°C, although it is well known that temperature increases the level of antibody avidity.[23] Rather, the presence of a partial antigen homology, which may be possibly related to a peculiar neoplastic phenotype, is probably a better explanation, supported by the fact that MIB-1 stained tumor cell membranes are restricted to different types of neoplastic lesions, including HTT cases and, occasionally, sarcomatous mesothelioma, invasive breast carcinoma, and renal oncocytoma. On the other hand, we think that fixation and other tissue processing did not contribute to produce this phenomenon, because similar immunostaining patterns have been observed in cases that have been processed in 3 different laboratories, with similar but not identical protocols of fixation and embedding.

A final issue deserves a comment: when the MIB-1 incubation was performed at 37°C, such as in the Ventana automated immunostainer, no staining was observed at the cell membrane level, but only in proliferating nuclei, as expected. This apparently paradoxical phenomenon may be explained by a temperature-induced modification of the antigen spatial conformation, that is, tertiary or quaternary molecular arrangement. Despite the increased avidity and affinity of the antibody binding induced by higher temperature, the minimal conformational change of the antigen may prevent the antibody recognition of a partially similar antigen.

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TABLE 1. Type of Ki67 Antibody and Technical Procedures Employed

Primary Ki67 Clone	Source /Dilution	Detection System (DakoCytomation)	Immunostaining Procedure	Temperature Incubation	Nuclear Staining	Membrane Staining
MIB-1	DakoCytomation, Glostrup, Denmark 1/300	LSAB	Manual	Room	Yes	Yes
			Automated Dako	Room	Yes	Yes
			Automated Ventana	37°C	Yes	No
		En Vision	Manual	Room	Yes	Yes
			Automated Dako	Room	Yes	Yes
			Automated Ventana	37°C	Yes	NO
MIB-1	Immunotech, Marseilles France 1/300	LSAB	Manual	Room	Yes	Yes
			Automated Dako	Room	Yes	Yes
			Automated Ventana	37°C	Yes	NO
		En Vision	Manual	Room	Yes	Yes
			Automated Dako	Room	Yes	Yes
			Automated Ventana	37°C	Yes	NO
7B11	Zymed, San Francisco, 1/200	LSAB	Manual	Room	Yes	NO
			Automated Dako	Room	Yes	NO
			Automated Ventana	37°C	Yes	NO
		En Vision	Manual	Room	Yes	NO
			Automated Dako	Room	Yes	NO
			Automated Ventana	37°C	Yes	NO
KIS5	DakoCytomation 1/300	LSAB	Manual	Room	Yes	NO
			Automated Dako	Room	Yes	NO
			Automated Ventana	37°C	Yes	NO
		En Vision	Manual	Room	Yes	NO
			Automated Dako	Room	Yes	NO
			Automated Ventana	37°C	Yes	NO
KI88	Biogenex, San Ramon (kit dilution)	LSAB	Manual	Room	Yes	NO
			Automated Dako	Room	Yes	NO
			Automated Ventana	37°C	Yes	NO
		En Vision	Manual	Room	Yes	NO
			Automated Dako	Room	Yes	NO
			Automated Ventana	37°C	Yes	NO
SP6 (rabbit)	Lab Vision, Fremont, 1/400	LSAB	Manual	Room	Yes	NO
			Automated Dako	Room	Yes	NO
			Automated Ventana	37°C	Yes	NO
		En Vision	Manual	Room	Yes	NO
			Automated Dako	Room	Yes	NO
			Automated Ventana	37°C	Yes	NO

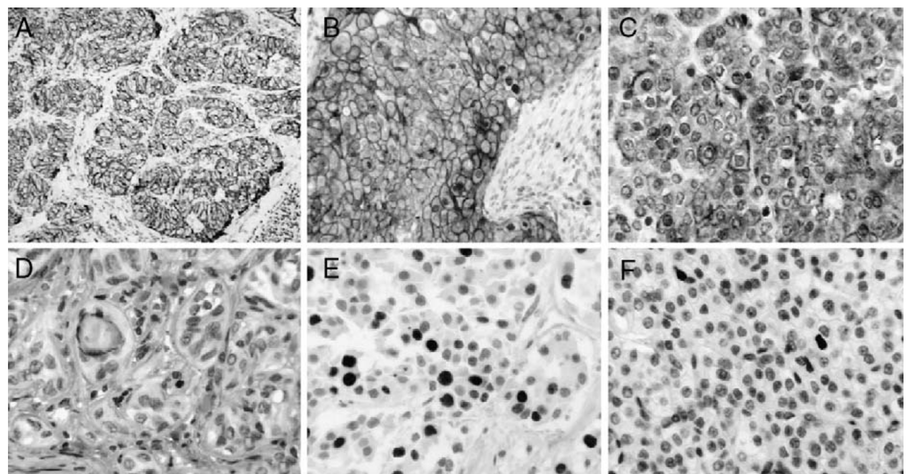

FIGURE 1. MIB-1 cell membrane reactivity in hyalinizing trabecular tumor of the thyroid (A) (250 ×), invasive breast carcinoma (B) (250 ×), and renal oncocytoma (C) (400 ×) using an immunoperoxidase procedure at room temperature and a biotin-free detection system. The same cases were negative at the membrane level when MIB-1 was employed in the thyroid tumor at 37°C in an automated stainer (D) (400 ×), when the SP6 rabbit monoclonal antibody to Ki67 was employed in the breast carcinoma (E) (250 ×), and when the 7B11 clone was used in the renal oncocytoma (F) (400 ×).

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In conclusion, we have shown that the reported Ki67 cell membrane reactivity in some human tumors occurs only when using the MIB-1 monoclonal antibody at room temperature. Therefore, from a practical standpoint, when Ki67 immunohistochemistry is used for diagnostic purposes in the suspicion of HTT, only MIB-1 clone at room temperature should be employed. Conversely, apart from HTT, the role of MIB-1 clone as a diagnostic test should be discouraged until its better definition.

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