Re-evaluation of MIB-1 immunostaining for diagnosing hyalinizing trabecular tumour of the thyroid: semi-automated techniques with manual antigen retrieval are more accurate than fully automated techniques

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Abstract. Hyalinizing trabecular tumour (HTT) immunohistochemically shows cell membranous immunoreactivity for MIB-1. This aberrant immunoreactivity is an important factor for the diagnosis of HTT. However, fully automated stainers frequently fail to confirm the immunoreactivity. The aim of this study is to investigate the cause of false negative cell membranous immunoreactivity for MIB-1 in HTT using fully automated stainers, to determine potential reasons for the problem, and to establish methods confirming cell membranous immunoreactivity for MIB-1 in HTT. Six participating institutions examined immunoreactivity for MIB-1 in 10 HTT cases using two approaches: fully automated and semi-automated methods. In the latter, antigen retrieval was carried out using manual methods adopted for routine assays at each institute. The autostainers used included the BOND-MAX, BOND-III, Benchmark XT, and Omnis systems. Using fully automated methods, institute E showed cell membranous MIB-1 positivity in all HTT cases. In contrast, at institute D, all HTT cases were negative. The positive rates of the remaining four institutes ranged from 10% to 20%. The incidence of positive cases using semi-automated methods was 100%, 90%, 90%, 30%, 80%, and 100% at institutes A, B, C, D, E, and F, respectively. We assert that antigen retrieval should be conducted manually for diagnosis of HTT; furthermore, definitively diagnosed HTT should be prepared as the external positive control.

Key words: Hyalinizing trabecular tumour, MIB-1, Immunohistochemical staining, Antigen retrieval
onstrated only in HTT; therefore, it is an important indicator for the diagnosis of HTT [3].

Methods and systems for immunohistochemistry (IHC) are continually being developed and improved. Today, many institutions use a fully automated stainer for IHC; these stainers simplify the complex IHC protocol and provide reliable results. In our experience, however, the fully automated stainers frequently fail to confirm cell membranous immunoreactivity for MIB-1 in cases with typical HTT. This same experience has been reported by Park et al. [4]. The aim of this study is to investigate the cause of false negative cell membranous immunoreactivity for MIB-1 in HTT using fully automated stainers, to determine potential reasons for the problem, and to establish methods confirming cell membranous immunoreactivity for MIB-1 in HTT.

Materials and Methods

We collected 10 cases with HTT for which tumours were resected and histologically diagnosed at Kuma Hospital between January 2014 and October 2016. The age ranged from 38 to 73 years (mean, 58.1 years) in nine women and one man. Tumour size varied from 12 to 44 mm. The diagnosis of HTT required a trabecular growth pattern, intertrabecular and intercellular basement membrane materials, intranuclear cytoplasmic inclusions, nuclear grooves, and yellow bodies. For immunostaining, 3-μm-thick formalin-fixed, paraffin-embedded tissues were used. Immunostaining was separately performed at six participating institutions, including A: Department of Clinical Laboratory, Kuma Hospital; B: Technical Development Department, Genostaff Co. Ltd.; C: Department of Diagnostic Pathology, Kobe University Graduate School of Medicine; D: Department of Diagnostic Pathology, Gunma University Graduate School of Medicine; E: Department of Diagnostic Pathology, Nara Medical University; and F: Department of Surgical Pathology, Sapporo Medical University School of Medicine. The primary antibodies for Ki-67 that each institute used in routine work are shown in Table 1. In all institutes, the clone of Ki-67 was MIB-1 and was supplied from DAKO (Glostrup, Denmark or Santa Clara, CA, USA).

The staining protocol is shown in Table 2. Institute A used the BOND-MAX system (Leica Biosystems, Wetzlar, Germany), Institute B used the BOND-III system (Leica Biosystems, Wetzlar, Germany), Institutes C and D used the Benchmark XT system (VENTANA, Roche Diagnostics, Basel, Switzerland), and Institutes E and F used the Omnis system (Dako, Agilent Pathology Solutions, CA, US), respectively. The participating institutes conducted immunostaining based on their routine protocols, according to the manufacturer’s recommendations (Study 1). In addition, semi-automated immunostaining was performed, with antigen retrieval carried out manually (Study 2). The retrieval methods used by each institute were A, D, and E, autoclave; C, pressure cooker; F, microwave oven; and B, pressure cooker in microwave. These methods were routinely employed at the respective institutes. Regarding MIB-1 immunoreactivity, nuclear positivity of lymphocytes and/or HTT tumour cells was evaluated as the internal positive control. The staining intensity for cell membranous immunoreactivity for HTT was divided into four groups: negative, weak, moderate, and strong. The number of positive tumour cells was divided into three levels: less than 30% of the tumour cells, more than 30% but less than 60% of the tumour cells, and more than 60% of the

Table 1  Primary antibodies used for immunohistochemical study at six institutes

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tumour cells. We assessed the statistical significance of the data using Fisher’s exact probability test. \( p \) values < 0.05 were considered statistically significant.

**Results**

Table 3 shows the results of MIB-1 cell membranous immunoreactivity for 10 HTTs. All preparations that were stained at each institute showed nuclear positivity of the tumour cells and/or lymphocytes as positive internal controls.

Study 1: Concerning cell membranous reactivity of HTTs for MIB-1 using fully automated stainers, the incidence of positive cases varied considerably among the participating institutions. Institute E showed positivity in all HTT cases. On the other hand, at institute D, all HTT cases were negative. The positive rates for the remaining four institutes ranged from 10% to 20%. Institutes A and B found immunoreactivity in two cases (cases 6 and 10) and one case (case 6), respectively, but the staining was weak. At institute C, two cases (cases 2 and 3) were positive, but at institute D, these same cases were negative. Institute E found moderate to strong positivity in all cases. At institute F, less than 30% of the tumour cells in cases 5 and 8 showed positive staining.

Study 2: The incidence of positive cases using semi-automated immunostaining was 100%, 90%, 90%, 30%, 80%, and 100% at institutes A, B, C, D, E, and F, respectively (Fig. 1). The incidence at institutes A, B, C, and F was significantly higher (A, B, and F; \( p < 0.001 \), C; \( p < 0.01 \)), and the staining was more intense than those in study 1, except for that at institute E. At institute E, the immunoreactivity of study 1 tended to be stronger than that of study 2. The incidence of positive cases was also significantly different (\( p < 0.01 \)) between institutes C and D, although they employed the same type of instrument. In contrast, there was no significant difference in the incidence of positive cases between institutes A and B and between institutes E and F.

**Discussion**

In 1995, Hirokawa et al. first reported that HTT displayed intense cell membranous immunopositivity for MIB-1, which is a clone of Ki-67 [3]. The aberrant staining pattern was not identified in other thyroid tumours [5-7], and other Ki-67 clones, such as 7B11, KISS, KI88, and SP6, did not show this phenomenon [3, 8]. Therefore, immunostaining for MIB-1 has been generally used in diagnosing HTT. Of note, the aberrant staining pattern is also observed in breast carcinoma [9], salivary gland pleomorphic adenoma [10], and pulmonary sclerosing hemangiomas [11], but no diagnostic significance has been demonstrated. Leonardo et al. described that cross-reactivity of MIB-1 monoclonal antibody with a cell membrane epitope seems the most likely explanation for the findings [8].

Concerning immunohistochemical studies, a variety of pitfalls and troubles can cause false positive or false negative results. False negative results may be caused by under-fixation or over-fixation, degradation of antibody, inadequate retrieval, few epitopes, tumour heterogeneity, technical error, and misinterpretation [12-14]. In our study, as all preparations stained at all institutes used
confirmed positive internal controls, we believe that immunostaining for MIB-1 was properly carried out. Nevertheless, cell membranous staining for HTT varied among the institutes and stainers. These findings indicate that, regarding reactivity for MIB-1, nuclear antigenicity differs from cell membranous reactivity in HTT. The unreliable and unpredictable results are likely due to the cross-reactivity.

In our study, the positive incidence of cell membranous reactivity of HTTs for MIB-1 using fully automated stainers was up to 20% at five of six institutes. In contrast, when retrieval was carried out manually using autoclave, pressure cooker, or microwave, the incidence was 80% or more at five of six institutes. These findings indicate that retrieval by fully automated stainers is insufficient for cell membranous staining of MIB-1 for HTTs. A similar phenomenon was reported by Park et al. [4]. In their study, all of eight HTT cases exhibited immunoreactivity for MIB-1 using the BOND X system, but the positive rate decreased to 62.5% (five cases) using the BOND MAX system. The BOND X system is a semi-automated stainer in which antigen retrieval is conducted manually, whereas the BOND MAX system is a fully automated stainer. Based on these findings, in
conjunction with those of the current report, we believe that the retrieval method seems to be the key to appropriate staining. Interestingly, in institute E, which used the Omnis system for fully automated staining, cell membranous positivity for MIB-1 was found in all HTT cases. The Omnis system uses a water bath (97.0°C) for antigen retrieval; in contrast, the BOND MAX and Benchmark XT systems use a hot plate (100°C). This difference in antigen retrieval may influence the results.

In conclusion, we demonstrated that cell membranous staining of HTT for MIB-1 using fully automated stainers may be negative, even though the nuclei of lymphocytes and/or HTT tumour cells, used as the internal control, were positive. Medical technologists and pathologists should be aware of this pitfall. Moreover, definitively diagnosed HTT should be prepared as the external positive control for diagnosis of HTT. We assert that manual antigen retrieval should be used to confirm cell membranous immunoreactivity for MIB-1 in HTT. In order to calculate the Ki-67 labelling index of HTT, we recommend the use of Ki-67 except for MIB-1, or the use of fully automated strainers. It is the reason that the cell membranous staining of MIB-1 masks the nuclear staining.

Disclosures

The authors declare no conflicts of interest.

References


