Rapid immunohistochemistry based on alternating current electric field for intraoperative diagnosis of brain tumors

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Abstract

Rapid immunohistochemistry (R-IHC) can contribute to the intraoperative diagnosis of central nervous system (CNS) tumors. We have recently developed a new IHC method based on an alternating current (AC) electric field to facilitate the antigen-antibody reaction. To ensure the requirement of R-IHC for intraoperative diagnosis, 183 cases of CNS tumors were reviewed regarding the accuracy rate of diagnosis without R-IHC. The diagnostic accuracy was 91.8% (168/183 cases) in which definitive diagnoses were not provided in 17 cases because of the failure of glioma grading and differential diagnosis of lymphoma and glioma. To establish the clinicopathological application, R-IHC for frozen specimens was compared with standard IHC for permanent specimens. 33 gliomas were analyzed, and the Ki-67/MIB-1 indices of frozen specimens by R-IHC were consistent with the grade and statistically correlated with those of permanent specimens. Thus, R-IHC provided supportive information to determine the grade of glioma. For discrimination between glioma and lymphoma, R-IHC was able to provide clear results of CD20 and Ki-67/MIB-1 in four frozen specimens of CNS lymphoma as well as standard IHC. We conclude that the R-IHC for frozen specimens can provide important information for intraoperative diagnosis of CNS tumors. (192 words)

Key words: Rapid immunohistochemistry (R-IHC), glioma, Central nervous system-lymhoma (CNS-lymphoma)

Introduction

The importance of intraoperative pathological diagnosis has increased in recent years [1]. Recently, in addition to the usefulness of CT and MRI images, additional multi-modal imaging techniques such as PET have improved the diagnostic accuracy. However, there are still limitations in obtaining an exact preoperative diagnosis [2] [3].

Pathological examination is performed based on the morphological findings such as cytological atypism, mitosis, vascular proliferation, and necrosis [4], in conjunction with the patient's clinical history, neuroradiologic images, and surgical findings. To date, the probability of intraoperative diagnosis has been reported as 66 to 95.6 % [5] [6].

In the last three decades, several methods for Rapid immunohistochemistry (R-IHC) have been proposed that use microwave [7], high quality reagents [8] [9] [10] [11] [12], intermittent microwave [13], and ultrasound [14]. In these studies, only a few papers were focused on the application of these techniques for central nervous system (CNS) tumors [10] [11]. Recently, we have developed a novel R-IHC method based on alternating current (AC) electric field which facilitates the antigen-antibody reaction, and reported its usefulness for detection of sentinel lymph nodes metastasis of lung cancer [15].

In this study, to examine the diagnostic advantage of our newly developed R-IHC methods for rapid diagnosis of CNS tumor, we compared the results of R-IHC on frozen specimens with that of ordinal IHC on permanent specimens of CNS tumor, and evaluated their diagnostic accuracy by combination of H&E staining and R-IHC in frozen sections.

Materials and methods

CNS tumor specimens and the criteria for diagnostic accuracy

To evaluate the intraoperative diagnostic accuracy of CNS tumors, we reviewed 183 cases of all CNS tumors that were diagnosed intraoperatively from January 2008 to May 2013 in Department of Cancer Pathology, Hokkaido University Graduate School of Medicine. The final diagnosis were as follows: 42.7 % (79/183 cases) of high grade glioma (HGG), 13.1% (24/183 cases) of low grade glioma (LGG), 18.6 % (34/ 183 cases) of metastatic carcinoma, 11.5% (21/ 183 cases) of meningioma, 4.3 % (8/ 183 cases) of CNS lymphoma, 3.3 % (6/ 183 cases) of schwannoma, 1.1 % (2/183 cases) of craniopharyngioma, and 4.9 % (9/ 183 cases) of non-neoplastic lesions. We classified our diagnoses into the following three degrees correlated to the accuracy of intraoperative diagnosis according to the modified criteria shown in [16]: (1) The intraoperative diagnosis was the same as the final diagnosis which means correct tumor lineage and grade; low or high (complete correlation); (2) the intraoperative diagnosis was not incorrect but was too broad to qualify as a complete correlation (partial correlation); and (3) the intraoperative diagnosis was incorrect and different from the final diagnosis (no correlation)

CNS tumor specimens for R-IHC

We have performed R-IHC using 15 cases of glioma and four cases of CNS lymphoma from the specimens for intraoperative diagnosis at Hokkaido University that were unintentionally selected. Case 3 to 15 in glioma and case 37 in CNS lymphoma were

performed R-IHC at the time of intraoperative diagnosis and Case 1, 2, 34, 35 and 36 were performed R-IHC in their recut frozen section that were kept in deep freezer after intraoperative diagnosis (Table 1a). 18 cases (Case 16 to 33) of glioma at the Department of Neurosurgery, Akita University Hospital were consecutively used for this study from September 2011 through May 2013 to compare the institutional differences (Table 1b). This study was approved by the Medical Ethics Committee of Hokkaido University Graduate School of Medicine and Akita University Hospital.

Preparation of frozen and FFPE tissues

Surgically resected specimens for intraoperative diagnosis were placed into the plastic cassette, and mounted with OCT compound medium (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), then frozen by liquid nitrogen, referred to frozen sections were performed H&E stain and R-IHC. Additionally resected tumors were fixed with 10% neutralized buffered formalin and embedded with paraffin. The Formalin-fixed paraffin-embedded **(**FFPE) tissues, referred to permanent sections were performed H&E stain and ordinal IHC.

R-IHC for frozen tissues

R-IHC was performed using a newly developed machine as described[15]. Briefly, frozen tissues were sectioned at 5 μm thicknesses, placed on slide glasses, and fixed by acetone at 4°C for 30 sec., and endogenous peroxidase (PO) was quenched by 3% H_2O_2 at RT for 1 min. Subsequently, the sections were incubated with primary antibody under a combination of

high-voltage (3.4 kV, offset 2.4 kV) and low-frequency (18 Hz) with altered current (AC) electric field for 5 min. The sections were washed 3 times with PBS with 0.05% Tween20 and incubated with EnVision TM+ System/HRP Mouse/ Rabbit (Dako, Glostrup, Denmark) for 5 min under a high-voltage (3.4 kV, offset 2.4 kV) and low-frequency (14 Hz) AC electric field. Reagents that reacted with PO were visualized by diaminobenzidine (DAB) as a substrate at RT for 2 min., counterstained with hematoxylin, dehydrated, and mounted with coverslips. Except for the duration of the preparation of frozen tissue sections on the slide glass, approximately 16 min. were enough to accomplish whole process from PO quenching to obtain IHC slide glass for diagnosis. The machine was used during incubation period with primary or secondary antibodies to facilitate the reaction. R-IHC for Ki-67/MIB-1, CD3, and CD20 were performed using frozen sections that used for intraoperative diagnosis.

Standard IHC for permanent tissues

Standard IHC was performed as described elsewhere [15]. Briefly, sectioned specimens were incubated with primary antibody at RT for 60 min. and washed with PBS with 0.05% Tween20 for 5 min. 3 times, and then incubated with Envision at RT for 30 min. Reacted antibodies were visualized by enzyme reaction with DAB as a substrate. Standard IHC for Ki-67/MIB-1, CD3, and CD20 were performed using FFPE sections that used for permanent diagnosis.

Antibodies

The following antibodies were used as the primary antibody with the appropriate dilution shown in parentheses: monoclonal mouse anti-Ki-67/MIB-1 antibody (monoclonal, clone MIB-1; Dako, 1:100), anti-CD20 antibody (monoclonal, clone L26; Dako, 1:400), and anti-CD3 antibody (polyclonal, rabbit; Dako, 1:200).

Statistical analysis

The correlation between frozen and permanent sections of Ki-67/MIB-1 indices were evaluated by Pearson's correlation coefficient. A value of $P < 0.05$ was considered as significant.

Results

Limitation of the accuracy of intraoperative diagnosis of CNS tumors without R-IHC

The overall diagnostic accuracy (the complete correlation) was 91.8% (168/183 cases), and this was as high as the accuracy described in previous studies. The accuracy for each diagnosis was as follows; glioma 91% (94/103), metastatic carcinoma 100% (34/34), meningioma 90% (19/21), CNS lymphoma 50 % (4/8), shcwannoma 66% (4/6), and craniopharyngioma 100% (2/2). The partial correlation was 8.2% (15/ 183 cases) and no correlation was 1.1% (2/ 183 cases). In these two no-correlation cases, we could not determine the glioma grading even as low or high grade, and failed to make a differential diagnosis of glioma from lymphoma.

Application of intraoperative R-IHC for Ki-67/MIB-1 to diagnosis of glioma.

In case of the discrimination of low and high grade glioma, Ki-67/MIB-1 index may become supportive information, and R-IHC can be completed within 16 min. and provide clear staining of Ki-67/MIB-1. Both in Case 6 and Case 13, H&E staining for frozen specimens showed increased cell numbers with nuclear atypia suggesting diagnosis as grade II or III glioma (Fig. 1a and 1c). By using R-IHC, Ki-67/MIB-1 index in frozen specimens were 6.7% and 23 %, respectively (Fig. 1b and 1d); thus, we considered Case 6 as being grade II and Case 13 as Grade III. These decisions were consistent with the final diagnosis using FFPE tissues with Ki-67/MIB-1 index in ordinal IHC (Fig. 1e, 1f, 1g, 1h and Table 1a).

Application of intraoperative R-IHC for lymphocyte surface antigen for diagnosis of CNS lymphoma

For the intraoperative diagnosis, discrimination between glioma and lymphoma is occasionally difficult without any support of R-IHC. Thus, we examined the application of lymphocyte surface antigen to R-IHC methods, and among the four cases of CNS lymphoma analyzed, CD20 and Ki-67/MIB-1 indices were successfully stained in all frozen samples by using R-IHC. Ki-67/MIB-1 indices were as high as those found in permanent sections (Fig. 2 and Table 2).

Comparison of the results of Ki-67/MIB-1 in glioma between R-IHC for frozen tissues and standard IHC for FFPE tissues

Clinicopathological application of the new R-IHC machine to CNS tumor diagnosis

was evaluated. The diagnosis of H&E stain with or without Ki-67/MIB-1 index in frozen sections and final diagnosis with immunohistochemstry in permanent sections of this study were shown in Table 1a and 1b. As measurement of Ki-67/MIB-1 index is critical for glioma grading, we compared the results of immunostaining of Ki-67/MIB-1 between R-IHC for frozen tissues and standard IHC for FFPE tissues in two independent facilities, Hokkaido University and Akita University.

Correlation of Ki-67/MIB-1 indices with glioma grading were observed with Grade II as 2.4±1.2(SD)%, Grade III as 11.6±9.9(SD)%, and Grade IV as 19.1±5.9(SD)% in Hokkaido University and Grade I as 0.5%, Grade II as 0.8±1.1(SD)%, Grade III as 13.0±8.2(SD)%, and Grade IV as 29.6±16.3(SD)% in Akita University (Fig. 3a and 3b). In addition, the Ki-67/MIB-1 indices based on R-IHC for frozen sections significantly correlated with those of permanent sections in both Hokkaido University. ($P<0.01$, $r=0.87$) and Akita University ($P<0.01$, $r=0.93$) (Fig. 3c and 3d).

Discussion

We have established a new machine for R-IHC based on a novel principle of AC-facilitated antigen-antibody reaction, by which the turn-around time to obtain the results for surgeons in case of intraoperative diagnosis can be almost within 30 min. It is obvious that this new R-IHC method can be applied for various specimens of the intraoperative diagnosis, but it should also possess advantages in the field of CNS tumor diagnosis. To

ensure the requirement of R-IHC for the intraoperative diagnosis of CNS tumors, we have surveyed our 183 cases and found that the accuracy of the intraoperative diagnostic without IHC was 91.8 %, as same as in previous reports. Among them, the most difficult diagnosis was glioma grading, especially discrimination between grade II and grade III. In this study, we clarified that R-IHC for Ki-67/MIB-1 index can provide important information for glioma grading in two independent institutes. Ki-67/MIB-1 indices of R-IHC in frozen sections were statistically correlated to those of conventional IHC in permanent sections. In addition, discrimination of CNS lymphoma from glioma was occasionally difficult, and this new R-IHC method clearly demonstrated the positivity of the lymphocyte surface antigen CD20. Although R-IHC obviously provide further information about the tumor, the number of analyzed cases in this study was not enough to provide clear cut-off value for Ki-67/MIB-1 index according to the WHO grading. Furthermore, we should notice the limitation of intraoperative diagnosis using R-IHC mainly due to sampling divergency including tumor heterogeneiety in glioma, poor demarcation of CNS lymphoma, and differential location of sampling. Indeed, Ki-67/MIB-1 index by R-IHC in case 21 were not matched with that by standard IHC by the tumor heterogeneity.

Similar to our present study, several groups have reported the limitations of intraoperative diagnosis of CNS tumors such as glioma grading and determination of astrocytic versus oligodendroglial origin in addition to the differential diagnosis of CNS

lymphoma, spindle cell lesions, reactive lesions as gliosis, poorly differentiated metastatic carcinoma, and primitive neuroectodermal tumor (PNET) [5] [11]. In this study, as we focused on the utility for diagnosis of glioma and CNS lymphoma by using limited number of antibodies as Ki-67/MIB-1, CD3 and CD20, we could not distinguish between astrocytic and oligodendroglial tumor. The combination of several markers will provide more precise diagnosis in the future. At least, to distinguish gliosis from low grade glioma, Ki-67/MIB-1 by R-IHC is useful as shown in case 4. In the near future, application of anti-IDH1-R132H antibody on this R-IHC method is expected to the discrimination of glioma and gliosis.

To date, several methods for R-IHC were proposed including microwave, specific reagent, and a combination of microwave and ultrasound, but such specific methods have not been widely accepted in the field of clinicopathological diagnosis for several reasons, such as the requirement of higher concentrated primary antibody or non-specific reaction due to the possible increase of temperature of the specimens by microwave. By our new method, fine staining can be obtained by using standard or sometimes lower concentrations of primary antibodies compared to standard IHC methods (data not shown). As compared to another R-IHC methods for Ki-67/MIB-1, our method would be more beneficial from the standpoint of antibody concentration (Table 3). In addition, we have measured the temperature of the specimens within AC stimulation and found a constant temperature (data not shown).

In conclusion, the new R- IHC method using AC field provides reliable results of IHC

for CNS tumor diagnosis on frozen sections, and will contribute to an appropriate intraoperative rapid diagnosis.

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Figure legends

Fig. 1. Histological findings in Case 6. (a. H&E staining in frozen specimen; b. Ki-67/MIB-1 staining by R-IHC in frozen specimen; c. H&E staining in FFPE specimen; d. Ki-67/MIB-1 staining by standard IHC in FFPE specimes) . Histological findings in Case 13. (e. H&E staining in frozen specimen; f. Ki-67/MIB-1 staining by R-IHC in frozen specimen; g. H&E staining in FFPE specimen; h. Ki-67/MIB-1 staining by standard IHC in FFPE specimen) . Scale bars, 20 μm.

Fig. 2. Histological findings of frozen specimen in Case 37 (a. H&E staining; b. CD20 staining by R-IHC; c.CD3 staining by R-IHC; d. Ki-67/MIB-1 staining by R-IHC), and FFPE specimens for final diagnosis (e. H&E staining ; f. CD20 staining by standard IHC; g.CD3 staining by standard IHC ; h. Ki-67/MIB-1 staining by standard IHC). Scale bars, 20 μm.

Fig. 3. Ki-67/MIB-1 indices in frozen and FFPE specimens in glioma. a. Ki-67/MIB-1 indices by R-IHC in 15 frozen specimens of grade II to IV glioma at Hokkaido University. b. Ki-67/MIB-1 indices by R-IHC in 18 frozen specimens of grade I to IV glioma at Akita University Hospital. c. Correlation between Ki-67/MIB-1 indices by R-IHC using frozen specimens and those by standard IHC using FFPE specimens at Hokkaido University d. Correlation between Ki-67/MIB-1 indices by R-IHC using frozen specimens and those by standard IHC using FFPE specimens at Akita University Hospital.

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Table 1. Summary of intraoperative and final findings

a. Hokkaido University

Case	Age/Sex	Frozen HE	MIB-1 (R-IHC)	Frozen HE +R-IHC	Permanent MIB-1	Final diagnosis
1*	48/F	II	3.5	II	3	II
2*	37/M	II	2.7	II	4.5	II
3	62/M	II	3.2	II	4	II
4	37/F	II or gliosis	2.1	II	2.6	II
5	16/M	II	0.5	II	0.1	II
6	37/M	II or III	6.7	II	5	II
7	63/F	III	5	III	6	III
8	55/F	IV	15.1	IV	15	IV
9	83/M	IV	16.7	IV	30	IV
10	60/F	IV	15.6	IV	39.7	IV
11	59/M	III or IV	20.4	III or IV	25	IV
12	39/M	IV	12	IV	25	IV
13	30/F	II or III	23	III	15	III
14	81/F	IV	28	IV	50	IV
15	65/M	IV	25.8	IV	50	IV

*: recut section from reserved frozen blocks

b. Akita University

Case	Age/Sex	Frozen HE	MIB-1 (R-IHC)	Frozen HE +R-IHC	Permanent MIB-1	Final diagnosis
16	4/F	I	0.5	I	2	I
17	32/F	II	2	I	8	II
18	43/F	II	0.2	I	1.8	II
19	49/M	II or III	0.1	II	2.7	II
20	29/M	IIor III	15	III	17.9	III
21	32/F	II	4	II	12.7	III
22	33/M	II or III	20	III	25.6	III
23	46/F	III or IV	15	III or IV	23	IV
24	50/F	IV	25	IV	36.7	IV
25	60/M	IV	30	IV	28.9	IV
26	63/M	IV	12	IV	14.3	IV
27	65/M	IV	39.5	IV	77.4	IV
28	69/F	IV	26.7	IV	40.4	IV
29	71/F	IV	25	IV	40	IV
30	72/M	IV	30	IV	26.7	IV
31	74/F	III or IV	46	IV	81	IV
32	76/F	IV	10	IV	11.7	IV
33	87/M	IV	66	IV	71	IV

Table 2. Summary of intraoperative and final findings

Case	Age/Sex	Frozen HE	CD20 (R-IHC)	MIB-1 (R-IHC)	Frozen HE +R-IHC	Permanent MIB-1	Final Dx.
34	68/F	CNSL	diffuse	90%	CNSL	100%	CNSL
35*	86/F	CNSL	diffuse	90%	CNSL	90 %	CNSL
36*	49/F	CNSL	diffuse	50%	CNSL	60%	CNSL
37*	62/F	HGG or CNSL	diffuse	90%	CNSL	90 %	CNSL

*: recut section from reserved frozen blocks

Table 3. Representative rapid-IHC methods

Author	Year	Method	Ki-67 Ab clone	Dilution	Total time(min)	Reference
Ichihara T, et al.	1989	Microwave	N.D.	N.D.	13	〔7〕
Richiter T, et al.	1999	EPOS	N.D.	N.D.	12	〔8〕
Kammerer U, et. al.	2001	Modified EnVision	KISS	1:10	12	〔9〕
Haapasalo J, et al.	2005	Ultrarapid-ki67 kit	N.D.	N.D.	14	〔10〕
Monig SP, et. al.	2006	En Vision, Histofine	N.D.	N.D.	10~13	〔12〕
Hatta H, et. al.	2006	Intermittent microwave	N.D.	N.D.	15	〔13〕
Hatta H, et al.	2010	Ultrasound	MIB-1	1:40	10	〔14〕
Uzuka T, et al.	2011	Vectastain kit	MIB-1	1:200	70	〔11〕
Toda Y, et al	2011	AC field	N.D.	N.D.	21	〔15〕
Tanino M, et al.	2014	AC field	MIB-1	1:100	16	This study

N.D.; not determined

Fig. 1

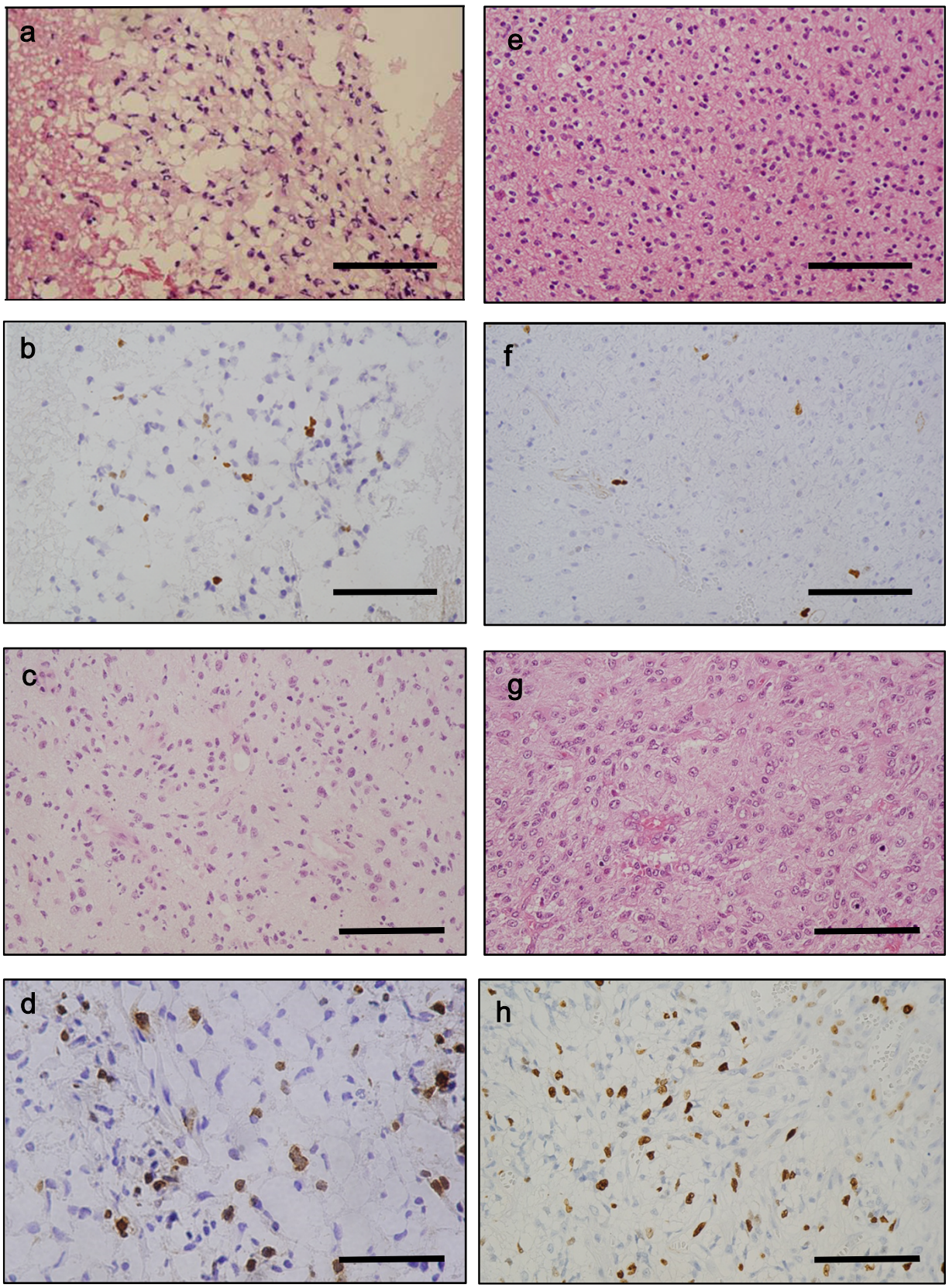


Fig. 2

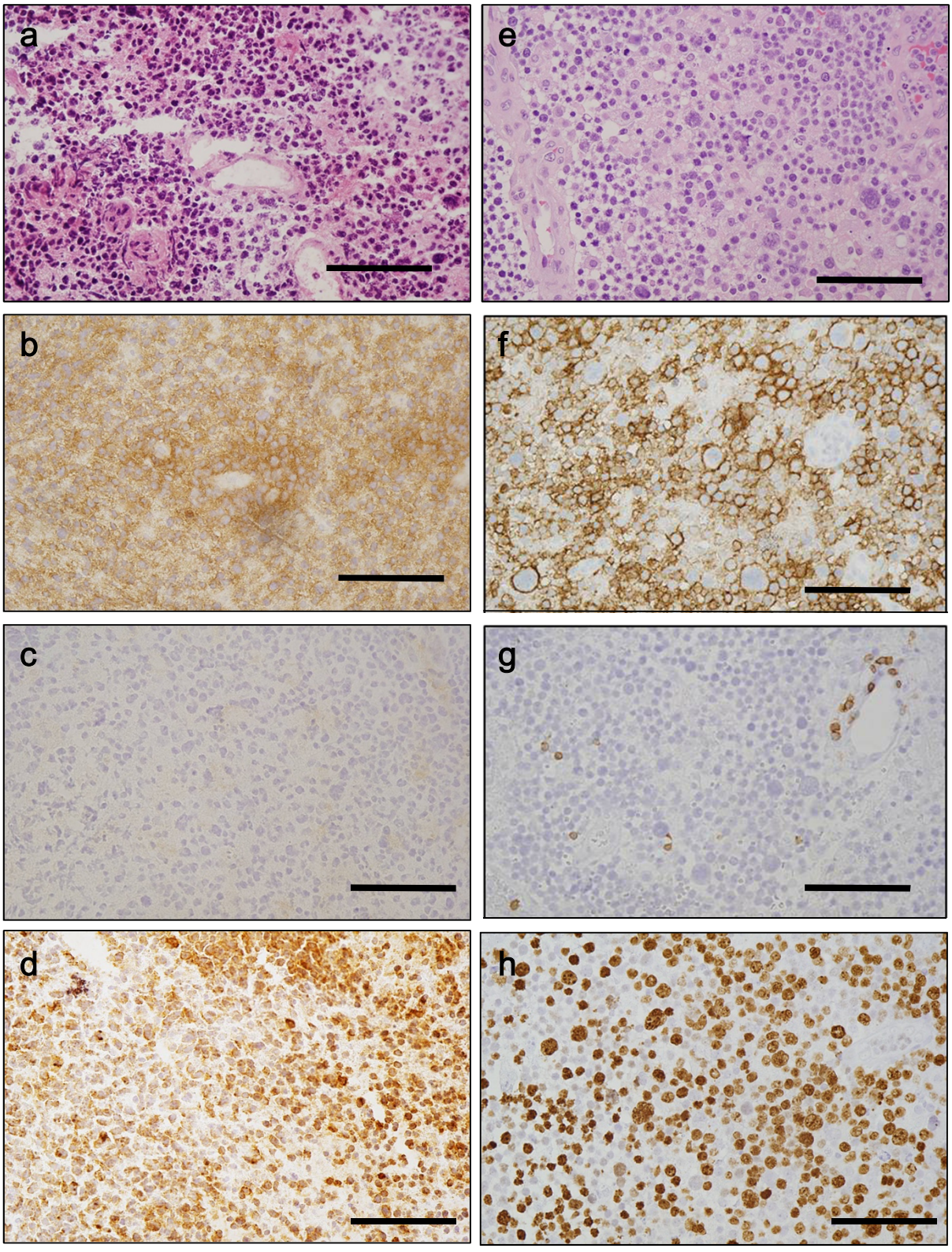
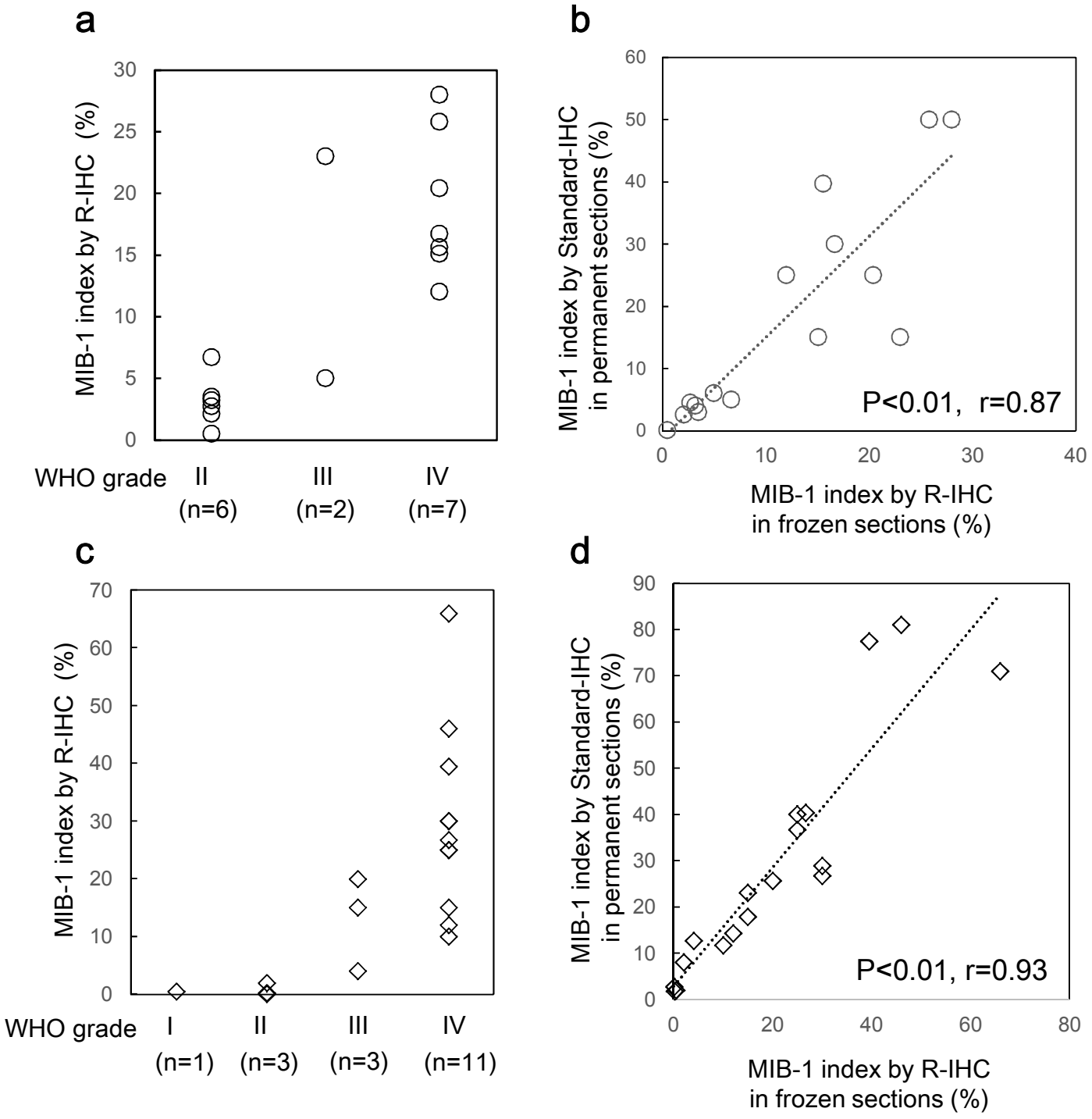


Fig. 3


a
MIB-1 index by R-IHC (%)
WHO grade
II (n=6)
III (n=2)
IV (n=7)
b
MIB-1 index by Standard-IHC in permanent sections (%)
MIB-1 index by R-IHC in frozen sections (%)
P<0.01, r=0.87
c
MIB-1 index by R-IHC (%)
WHO grade
I (n=1)
II (n=3)
III (n=3)
IV (n=11)
d
MIB-1 index by Standard-IHC in permanent sections (%)
MIB-1 index by R-IHC in frozen sections (%)
P<0.01, r=0.93