Rapid immunocytochemistry based on alternating current electric field using squash smear preparation of central nervous system tumors

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Abstract

The role of intraoperative pathological diagnosis for central nervous system (CNS) tumors is crucial for neurosurgery when determining the surgical procedure. Especially, treatment of Carmustine (BCNU) wafers requires a conclusive diagnosis of high grade glioma (HGG) proven by intraoperative diagnosis. Recently, we demonstrated the usefulness of rapid immunohistochemistry (R-IHC) that facilitates antigen-antibody reaction under alternative current (AC) electric field in the intraoperative diagnosis of CNS tumors; however, a higher proportion of water and lipid in the brain parenchyma sometimes leads to freezing artifacts, resulting in poor quality of frozen sections. On the other hand, squash smear preparation of CNS tumors for cytology does not affect the frozen artifacts, and the importance of smear preparation is now being re-recognized as being better than that of the tissue sections. In this study, we established the rapid immunocytochemistry (R-ICC) protocol for squash smears of CNS tumors using AC electric field that takes only 22 min, and demonstrated its usefulness for semi-quantitative Ki-67/MIB-1 labeling index and CD 20 by R-ICC for intraoperative diagnosis. R-ICC by AC electric field may be a substantial tool for compensating R-IHC in inappropriate frozen sections and will be applied for broad antibodies in future.

(195 words)

Key words:

Central nervous system (CNS) tumor, intraoperative diagnosis, squash smear, immunocytochemistry (ICC), immunohistochemistry (IHC)

Introduction

Intraoperative pathological diagnosis of central nervous system (CNS) tumors is an essential tool for deciding the surgical procedure including details such as resection volume. Intraoperative implantation of Carmustine (BCNU) wafers (Gliadel ®) after maximal tumor resection has been reported as an effective and safe treatment for high grade glioma (HGG) [1]. As the implantation of BCNU wafers requires a conclusive diagnosis of HGG proven by intraoperative diagnosis, the rapid and accurate diagnosis is becoming increasingly important. Early studies on intraoperative diagnostic accuracy reported an exceptionally high figure of over 90% [2] [3]; however, low sensitivity in rates such as 50% was also reported by others about glioma grading [4]. The difficulties of intraoperative diagnosis have been reported in distinguishing low grade glioma (LGG) from gliosis, HGG from LGG, and primary CNS lymphoma (PCNSL) from HGG. Cellularity, nuclear atypia, mitosis and necrosis in tumors are evaluated, but pathologists sometimes face diagnostic difficulties because of the similarity of their morphology in frozen sections. The R-IHC method based on alternating current electric field was developed by Minamiya Y., et al in 2011 [5]. During the staining process,

incubation with primary and secondary antibody is performed under a combination of high-voltage (3.4 kV, offset 2.4 kV) and low-frequency (18 Hz) AC electric field for 5 min that facilitates the antigen-antibody reaction. As R-IHC takes only 30 min, it is able to provide immunohistochemical information in intraoperative pathological diagnosis. We have reported that Ki-67/MIB-1 and CD20 immunostaining in frozen sections by this new R-IHC method is valuable for intraoperative diagnosis for CNS tumors [6]. However, about 5% of cases have failed to good immunostaining results due to the low quality of frozen tissue because of artifacts in our previous experience. On the other hand, the squash smear tends to maintain the morphology of cells without frozen artifacts and is applicable for small biopsy specimens, bringing effective information to the diagnosis. In the current study, we dedicated ourselves to establishing a reliable protocol for rapid immunocytochemistry (R-ICC) of squash smears of CNS tumors using the new R-IHC method, and evaluated its usefulness for intraoperative diagnosis of CNS tumors.

Materials and Methods

CNS tumor specimen preparation for R-ICC, R-IHC and conventional IHC

23 patients with CNS tumors who had undergone intraoperative diagnosis at Hokkaido University were enrolled in the study from May 2014 to March 2015. Intraoperatively, a small volume (1-2 mm^3) of tissue was squashed between two slides to prepare smears before embedding in white tissue-coat (U.I Kasei, Amagasaki, Japan) and immediately fixed with

95% ethanol for hematoxylin and eosin (H&E) stain and R-ICC. Smearing the specimens uniformly and thinly is very important for R-ICC to avoid cell clustering followed by non-specific staining. The rest of the samples were placed into the cryomold (Sakura Finetek Japan, Tokyo, Japan), and mounted with white tissue-coat, then frozen in liquid nitrogen, referred to as frozen sections. Frozen sections were sliced in thicknesses of 5μm and stained with 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 as permanent sections, were subjected to H&E stain and conventional IHC. This study was approved by the Medical Ethics Committee of Hokkaido University Graduate School of Medicine.

Establishment of R-ICC protocols

To establish the protocol for R-ICC in CNS tumor squash smears, we performed ICC for Ki67/MIB-1 in several HGG samples using five different protocols (Table 1): 1) conventional ICC method (protocol A), 2) R-ICC with antigen retrieval with incubation under AC electric field (protocol B), 3) R-ICC with antigen retrieval without incubation under AC electric field (protocol C), 4) R-ICC without antigen retrieval with incubation under AC electric field (protocol D), and 5) R-ICC without antigen retrieval without incubation under AC electric field (protocol E). Antigen retrieval was performed using Envision FLEX TRS

high pH at 97 ℃ for 20 min for conventional ICC and 90℃ for 5 min for R-ICC after fixation by 95% ethanol for 1 min. To examine the utility of AC electric field, the first antibody reaction was done under a combination of high-voltage (3.4 kV, offset 2.4 kV) and low frequency (18 Hz) AC electric field for 5 min using immunohistochemistry system, Histo-tek® R-IHC®(Sakura Finetek, Tokyo, Japan). The squash smears were washed once with wash buffer and incubated with EnVision FLEX/HRP (Dako, Glostrup, Denmark) for 5 min under high-voltage (3.4 kV, offset 2.4 kV) and low frequency (14 Hz) AC electric field. Reagents that reacted with peroxidase (PO) were visualized by diaminobenzidine (DAB) as a substrate at room temperature (RT) for 5 min, counterstained with hematoxylin, dehydrated, and mounted with cover slips. Ready to use monoclonal mouse anti-Ki-67/MIB-1 antibody: clone MIB-1 (Dako) and monoclonal mouse anti-CD20 antibody: clone L26 (Dako) were used in this study. Staining localizations such as nuclear in Ki-67/MIB-1 and membranous staining in CD20 were evaluated in cell non-clustering areas.

R-IHC for frozen sections and conventional IHC for permanent tissues

For R-IHC, frozen tissues were sectioned at 5μm thicknesses, placed on slide glasses, and fixed by Rapid Fix (Muto Pure Chemicals, Tokyo, Japan) for 1 min, and endogenous peroxidase (PO) was quenched by 3% H_2O_2 at RT for 1 min followed by immunostaining according to the previous paper [6]. Except for the duration of the preparation of frozen tissue

sections on the slide glass, it took approximately 25 min to accomplish the whole process from PO quenching to obtain H&E stained slide glasses for diagnosis. Conventional IHC was performed with automated immunostainer, Autostainer Link 48 (Dako), according to the standard protocol. Reacted antibodies were visualized by enzyme reaction with DAB as a substrate. IHC of Ki-67/MIB-1 labeling index and CD20 using FFPE sections used for permanent diagnosis.

Semi-quantification for Ki-67/MIB-1 labeling indices in R-ICC of squash smears and quantification for Ki-67/MIB-1 labeling indices in frozen and permanent sections

Ki-67/MIB-1 labeling indices in R-ICC of squash smears were evaluated semi-quantitatively based on the positive cell number: 0, no staining; 1+, < 5 %, 2+; 5 % ~ 15 %, 3+; 15 %<. Ki-67/MIB-1 labeling indices in frozen and permanent sections were evaluated by counting at least 200 cells in the maximum staining area.

Results

Establishment of R-ICC protocol

As shown in Figures 1a-e, fine staining was observed in R-ICC by protocol B (Fig. 1b) which is comparative with conventional ICC by protocol A (Fig. 1a) and conventional IHC in permanent section (Fig. 1f). Protocol A took 81 min, but protocol B was done within 22 min,

which is a much shorter time. There was no obvious positive staining by either protocol C, D, or E (Fig. 1c-e). From these results, we noticed that antigen retrieval and incubation under AC electric field were necessary for rapid and fine staining using squash smears, so we chose the protocol B as the optimal R-ICC method for further study.

Of the 23 enrolled cases, 15 cases were glioma, 4 cases were meningioma and 4 cases were PCNSL in the final diagnosis (Table 2). To evaluate the usefulness of intraoperative R-ICC in CNS tumors, the results were compared to those in both R-IHC in frozen sections and conventional IHC in permanent sections. For Ki-67/MIB-1 indices, almost all of the samples were compatible with those in both R-IHC and IHC in permanent sections. Lower proportion such as 1+ in squash smears reflected LGG or meningioma and higher proportion such as 3+ in smears reflected HGG or CNSL. Cases 1, 8 and 13 were not determined as HGG or LGG by H&E in frozen sections; however, the Ki-67/MIB-1 labeling index in squash smear was low enough to be compatible with LGG in Case 1 (Fig. 2a- e) and high enough to be compatible with HGG in Case 8 (Fig. 2f- j) . In Case 13, the Ki-67/MIB-1 labeling index in R-IHC was not evaluated due to its artifact, but a high Ki-67/MIB-1 labeling index was confirmed in R-ICC. In all cases of meningioma, the semi-quantitates of Ki-67/MIB-1 indices in squash smears were relatively low. Grade II atypical meningioma in Cases 18 and 19 showed a little higher Ki-67/MIB-1 indices in R-IHC (Fig. 2k- o) than those in grade I meningothelial meningioma (Cases 16 and 17). Although the tumors in Cases 19, 20, 21 and

21 were suspected to be HGG or PCNSL in H&E of frozen sections, high Ki-67/MIB-1 indices and membranous CD20 staining were observed in both R-ICC of squash smear and R-IHC in frozen sections that were compatible with permanent sections. These results provided supportive information for the intraoperative diagnosis of PCNSL (Fig. 2p- t). As shown in table 2, R-IHC result was not obtained in one of 23 cases (4%) due to sample processing failure.

Discussion

In this study, we have established a protocol for R-ICC based on AC electric field-facilitated antigen-antibody reaction which brought fine and rapid staining in 22 min. and demonstrated the utility in the intraoperative diagnosis of CNS tumors. Intraoperative diagnosis of CNS tumors is important for the determination of further surgical procedures and treatment plans including the intraoperative implantation of Carmustine (BCNU) wafers (Gliadel®). H&E stain of frozen tumor tissue sections constitutes the main diagnostic technique, but the diagnostic accuracy in the intraoperative diagnosis for CNS tumors using frozen sections is not always high, at rates of 50~96% [2] [3] [4]. The reason for the discrepant diagnosis is partly due to the high content of water as well as fat in fresh brain tumor, and the innately soft nature of brain tumor. The poor quality occurred in frozen sections, which led to diagnostic difficulties. On the other hand, the usefulness of imprint cytology of CNS tumors for intraoperative evaluation was described already in 1927 and

squash smears had also been reported as an important additional technique thereafter [7]. Recently, according to the progress in stereotactic biopsy, the technique was re-recognized as a useful tool for tiny biopsy specimens. [8] [9]

R-ICC by microwave-based method for intraoperative peritoneal washing cytology of pancreatic or biliary carcinoma was previously reported [10]. Recently, the usefulness of R-ICC of cytokeratin in touch print of sentinel lymph nodes for evaluating metastasis of breast cancer has been reported [11] [12]. With regard to CNS tumor, ICC of GFAP in squash smears was reported, but as the protocol takes 375 min for staining, it is not suitable for intraoperative diagnosis. Therefore, it is worthwhile to establish the R-ICC protocol using squash smear for intraoperative rapid diagnosis of CNS tumors [13].

We established the R-ICC protocol in squash smears by comparing five different protocols as shown in Table 1. Antigen retrieval is a necessary step for R-ICC, although it is not needed for R-IHC protocol in frozen sections using the same machine [6]. It is most important to make proper squash smears that are thin and homogenously spreading before R-ICC procedure. R-ICC by AC electric field method facilitates shorter staining time and as fine quality as that by the conventional method. We also confirmed that ready to use primary antibodies can be used in this protocol. To examine the usefulness of R-ICC using smear samples by the established protocol, we stained Ki-67/MIB-1 and/or CD20 in 23 CNS tumors. Although three cases of glioma were difficult to diagnose whether they were low or high

grade glioma in H&E in frozen sections and squash smears, Ki-67/MIB-1 by R-ICC clearly distinguished them. In four cases of tumor that were not determined as HGG or PCNSL by H&E in frozen sections and squash smears, R-ICC showed clear membranous CD20 staining. As the Ki-67/MIB-1 indices do not always give a clear distinction between grades II and III, the low or high Ki-67/MIB-1 indices provide supplementary information. In addition to Ki-67/MIB-1 and CD20, we have performed both R-ICC and R-IHC for GFAP, olig2, ATRX, p53, pHH3 and cytokeratin in several cases and obtained good staining results (data not shown). Although mutant IDH1 seems to be difficult for staining by R-IHC, R-ICC might be suitable to such a specific antibody.

In conclusion, we established a protocol for R-ICC of CNS tumor squash smears using a new method which used AC electric field to facilitate antigen-antibody reaction that was accomplished for 22 min and demonstrated the usefulness of R-ICC in squash smears for intraoperative rapid diagnosis in CNS tumors. R-ICC by AC electric field may be a substantial tool for compensating R-IHC in inappropriate frozen sections and will be applied to broader antibodies for not only CNS tumors but also other cancers in future.

Appendix: **R-IHC Study Group**; Shinya Tanaka, Mishie Tanino, Tomoko Takenami, Jun Moriya, (Hokkaido University), Akagami, Masami, Satoru Kamata, Eichi Suzuki, Yoichi Kagaya, Ryuta Nakamura (Akita Industrial Technology Center), Yoshihiro Minamiya, Toshio Sasajima, Akiteru Goto,

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Figure Legends

Fig. 1. Immunocytochemical staining of Ki-67/MIB-1 in squash smears and immunohistochemical staining of Ki-67/MIB-1 in permanent section of glioblastoma. (a) Protocol A, conventional ICC; (b) protocol B, R-ICC with antigen retrieval and Histotek® R-IHC®; (c) protocol C, R-ICC with antigen retrieval and without Histotek® R-IHC®; (d)

protocol D, R-ICC without antigen retrieval and with Histotek® R-IHC®; (e) protocol E, R-ICC without antigen retrieval and without Histotek® R-IHC®; (f) conventional IHC in permanent section. Bars indicate 100 μ m. Inserts showed higher magnification.

Fig. 2. H&E staining in frozen sections (a, f, k, and p), H&E staining in squash smears (b, g, l and o), R-ICC of Ki-67/MIB-1 (c, h, and m) and CD20 (r) in squash smears, R-IHC of Ki-67/MIB-1 (d, i, and n) and CD20 (s) in frozen sections. Conventional IHC of Ki-67/MIB-1(e, j, and o) and CD20 (t) in permanent sections. Insert in Fig. 2r shows membranous staining of CD20 by R-ICC in high magnification. Case 1 is astroblastoma. Case 8 is glioblastoma. Case 18 is atypical meningioma. Case 21 is PCNSL. Bars indicate 50μm.

Fig. 1.

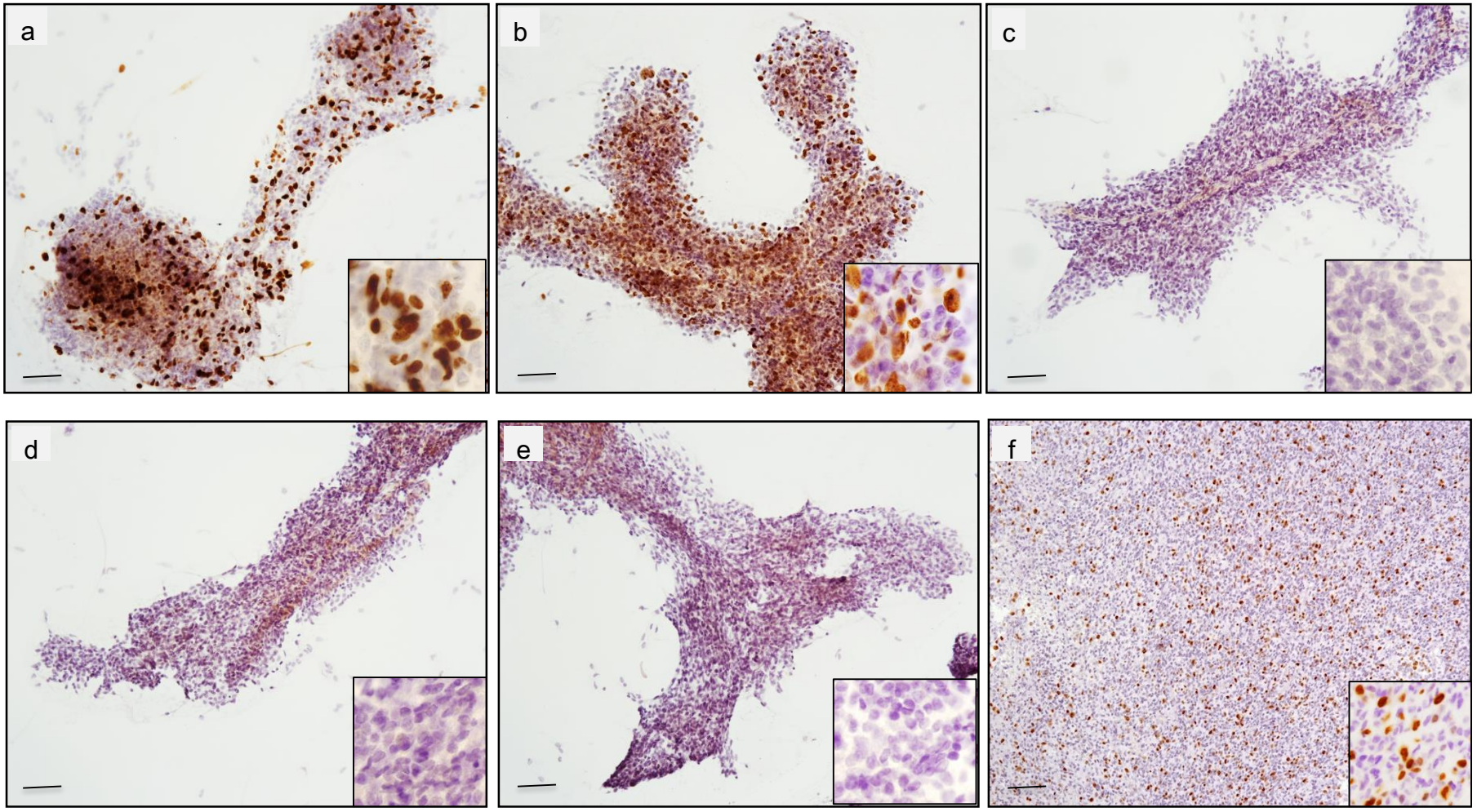

Fig. 2.

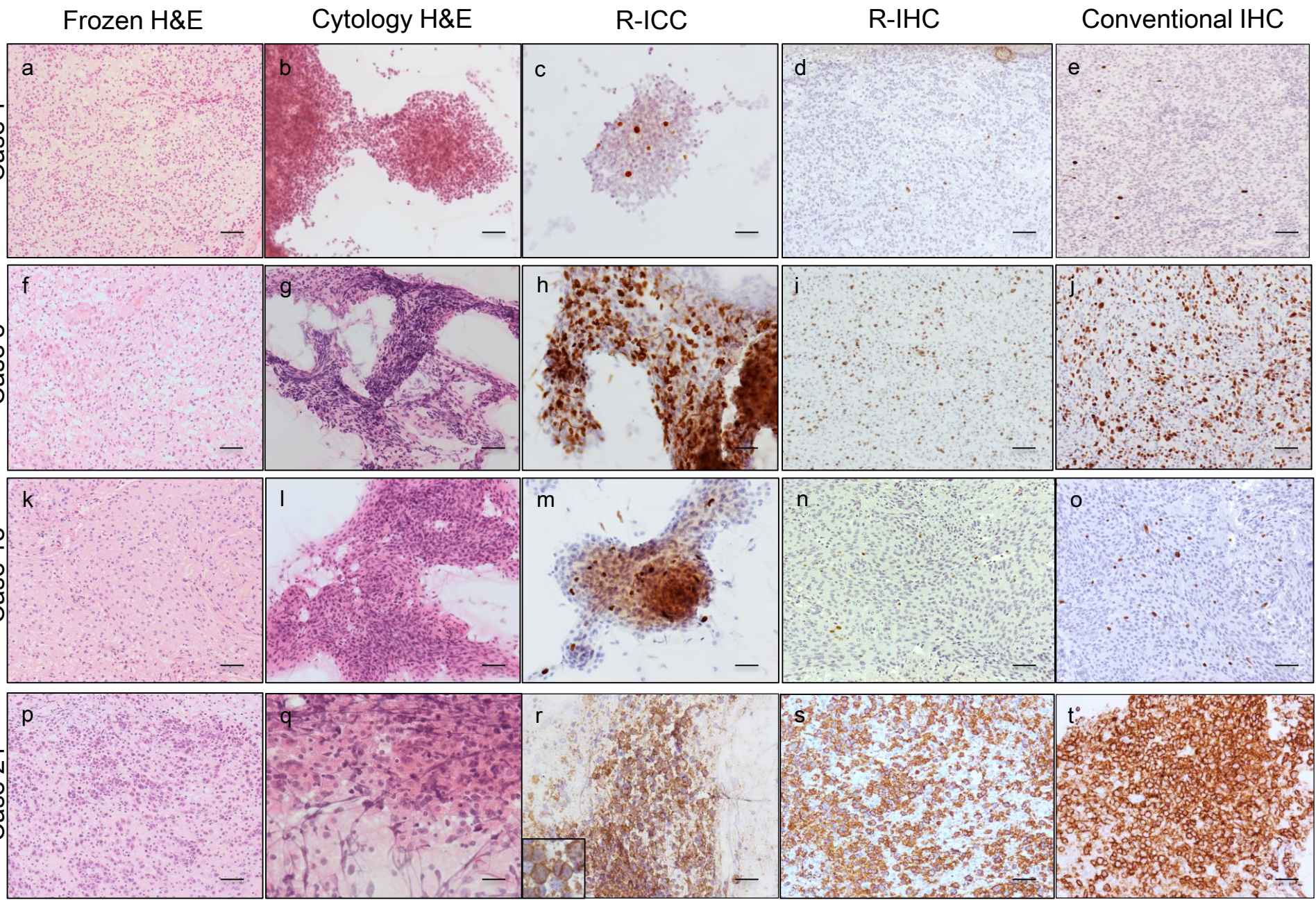
Frozen H&E
Cytology H&E
R-ICC
R-IHC
Conventional IHC
Case 1
Case 8
Case 18
Case 21
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Table 1. Protocols for conventional and rapid immunocytochemistry

	Conventional ICC	R-ICC			
Protocol	A	B	C	D	E
95％Ethanol fixation	RT 1min	RT 1min	RT 1min	RT 1min	RT 1min
Washing	once	10 sec	10 sec	10 sec	10 sec
Hydrogen peroxide	5 min	10 sec	10 sec	10 sec	10 sec
Washing	once	10 sec	10 sec	10 sec	10 sec
Antigen retrieval	97 ℃ 20 min	90 ℃ 5 min	90℃ 5 min	–	–
Washing	once	10 sec	10 sec	10 sec	10 sec
Primary antibody	20 min	AC 5 min	RT 5min	AC 5min	RT 5min
Washing	once	10 sec	10 sec	10 sec	10 sec
FLEX HRP	20 min	AC 5 min	RT 5 min	AC 5 min	RT 5 min
Washing	5 min once	10 sec	10 sec	10 sec	10 sec
3,3`diaminobenzidine	10 min	5 min	5 min	5 min	5 min
Time required	81 min	22 min	22 min	17 min	17 min

ICC, immunocytochemistry; IHC, immunohistochemistry; RT, room temperature;
AC, Alternative current electric field.

Table 2. Summary of intraoperative and final diagnosis of 23 CNS tumors

Case	Gender/age	Frozen H&E Diagnosis/WHO grade	R-ICC Ki-67/ MIB-1	R-IHC Ki-67/ MIB-1	R-ICC CD20	R-IHC CD20	IHC Ki-67/ MIB-1	IHC CD20	Final diagnosis
1	F/18	LGG or HGG/ Ⅱ or Ⅲ	1+	3%	-	-	5%	-	Astroblastoma
2	F/50	LGG/ Ⅱ	1+	1%	-	-	4%	-	Oligoastorocytoma
3	F/44	LGG/ Ⅱ	1+	1%	-	-	4%	-	Oligoastorocytoma
4	M/53	HGG/ Ⅲ	3+	13%	-	-	15%	-	Anaplastic Astrocytoma
5	F/64	HGG/ Ⅳ	3+	17%	-	-	40%	-	Glioblastoma
6	M/43	HGG/ Ⅳ	3+	23%	-	-	40%	-	Glioblastoma
7	M/66	HGG/ Ⅳ	2+	10%	-	-	20%	-	Glioblastoma
8	F/64	LGG or HGG/ Ⅱ or Ⅲ	3+	16%	-	-	20%	-	Glioblastoma
9	F/61	HGG /Ⅳ	3+	13%	-	-	15%	-	Glioblastoma
10	F/35	HGG/ Ⅳ	3+	32%	-	-	25%	-	Glioblastoma
11	M/57	HGG/ Ⅳ	3+	48%	-	-	50%	-	Glioblastoma
12	M/55	HGG/ Ⅳ	3+	35%	-	-	30%	-	Glioblastoma
13	M/78	LGG or HGG/ Ⅱ or Ⅲ	3+	NA	-	-	40%	-	Glioblastoma
14	M/71	HGG/ Ⅳ	3+	44%	-	-	40%	-	Glioblastoma
15	F/66	HGG/ Ⅳ	3+	27%	-	-	30%	-	Glioblastoma
16	M/61	Meningioma/ Ⅰ	1+	1%	-	-	3%	-	Meningioma
17	F/74	Meningioma/ Ⅰ	1+	2%	-	-	3%	-	Meningioma
18	F/52	Meningioma/ Ⅰ	1+	4%	-	-	5%	-	Atypical meningioma
19	F/74	Meningioma/ Ⅰ or Ⅱ	1+	5%	-	-	8%	-	Atypical meningioma
20	M/73	PCNSL or HGG	3+	71%	Diffuse	Diffuse	79%	Diffuse	PCNSL
21	M/70	PCNSL or HGG	3+	79%	Diffuse	Diffuse	80%	Diffuse	PCNSL
22	M/74	PCNSL or HGG	3+	72%	Diffuse	Diffuse	94%	Diffuse	PCNSL
23	M/76	PCNSL or HGG	3+	75%	Diffuse	Diffuse	95%	Diffuse	PCNSL

LGG, low grade glioma; HGG, high grade glioma; PCNSL, primary central nervous system lymphoma; NA, not available.