All studies included in the pooled analysis performed HPV DNA testing on surgical tissue (FFPE and/or frozen) or biopsy specimens. Similarly the non-included studies performed HPV DNA testing on surgical or biopsy tissue specimens except for one study which utilized bronchial aspirates from lung cancer patients (64). Most of the studies in the pooled analysis as well as the non-included studies used PCR-based methods for HPV DNA detection, 93 and 80%, respectively. Although adenocarcinoma and SCC were the most common histological types included in the pooled dataset (56%, 1808 cases and 38%, 1220 cases, respectively) and in the non-included studies (33%, 1404 cases and 57%, 2379 cases, respectively), the pooled analysis consisted of a larger proportion of adenocarcinoma cases compared with the non-included studies. Similar proportions of other histological types were represented in both the pooled dataset and non-included studies (7%, 214 cases and 8%, 350 cases, respectively). Histology was not specified for 7 (0.2%) cases in the pooled dataset and for 68 (1.6%) cases in the non-included studies.

The predominant high-risk HPV types found in lung cancer patients were HPV 16 and HPV18 (Table I). Other high-risk HPV types (HPV31, 33, 35, 45, 51, 52, 68 and 82) were also detected. For all geographic regions (Asia, Europe as well as North America) and with the exception of South and Central America, large-to-moderate heterogeneity was observed between the studies with no evidence of publication bias (data not shown). In order to address the heterogeneity, HPV prevalence rates were calculated after adjusting for age, gender, smoking history and tumor stage; study was included as a random effect where appropriate (Table I). Significant differences in HPV16/18 prevalence were noted between geographic regions. HPV 16/18 was most prevalent in South and Central America (adjusted prevalence [AdjPr] = 21.90%, 95% CI = 19.61–24.20), followed by Asia (AdjPr = 4.60%, 95% CI = 3.48–5.73), North America (AdjPr = 3.78%, 95% CI = 3.35–4.22) and Europe (AdjPr = 3.03%, 95% CI = 2.76–3.30). For each geographic region, the prevalence of HPV16 was significantly higher than HPV18, with the exception of North America and Asia. South and Central America had the highest prevalence of HPV16 followed by Europe, North America and Asia, whereas HPV 18 was also highest in South and Central America, followed by North America and Asia. There was virtually no HPV 18 detected in European patients (AdjPr = 0.82%, 95% CI = 0.73–0.92).

Clinical and demographic characteristics of HPV16/18-positive lung cancer cases

The mean age of all lung cancer patients in the pooled dataset was 65.0 ± 10.1 (SD) years. The demographic and clinical characteristics for all cases are presented in Table II; comparisons were made between North American cases and the rest of the world. For the studies conducted in North America as well as the rest of the world, a higher proportion of the patients were male (53 and 72%, respectively), ever smokers (84 and 76%, respectively) and most of the lung cancers were not SCC (64 and 62%, respectively). North American patients were mostly White (89%) in contrast to the rest of the world where the majority were non-White (57%). Early-stage lung cancers were more represented in the North American studies (63%) in contrast to only 50% in studies from the rest of the world. For each geographic subgroup, there were no noted differences in the proportion of HPV16/18 patients according clinical and demographic characteristics with the exception of race. The majority of patients in North America were White and a higher proportion of HPV 16/18-positive lung cancers were also noted among White patients (72%). However, for the rest of the world, although a near equal proportion of White and non-White patients were included, the majority of HPV 16/18-positive lung cancers were observed in non-White patients (88%).

No statistically significant associations were observed between HPV16/18-positive lung tumors and gender, age, smoking history, stage and histology (Table III). However, HPV 16/18-positive lung cancer cases were less likely to be observed among White patients compared with non-White patients (adjusted odds ratio [Adj OR] = 0.33, 95% CI = 0.12–0.90). For larger studies, (≥100 cases, 4 studies with a minimum of 131 cases and a maximum of 399 cases), the association of HPV 16/18 with race persisted (White: Adj OR = 0.08, 96% CI = 0.02–0.33). For smaller studies (<100 cases, 16 studies with a minimum of 22 cases and a maximum of 99 cases), the HPV 16/18-positive

Table II. Comparisons of demographic characteristics of patients according to HPV16/18 status (published datasets only)

Characteristic	Asia, Europe and South/Central America				North America			
	Total (N = 2500)	HPV16/18 positive (N = 233, 9.3%)	HPV16/18 negative (N = 2267, 90.7%)	P value[a]	Total (N = 663)	HPV16/18 positive (N = 23, 3.5%)	HPV16/18 negative (N = 640, 96.5%)	P value[a]
Gender				0.157				0.632
Male	1749 (71.92)	154 (66.96)	1595 (72.43)		349 (52.64)	10 (43.48)	339 (52.97)	
Female	683 (28.08)	76 (33.04)	607 (27.57)		314 (47.36)	13 (56.52)	301 (47.03)	
Age (years)				0.529				0.557
≤66	1212 (51.33)	92 (42.4)	1120 (52.24)		315 (47.51)	10 (43.48)	305 (47.66)	
>66	1149 (48.67)	125 (57.6)	1024 (47.76)		348 (52.49)	13 (56.52)	335 (52.34)	
Race				0.059				0.102
White	1083 (43.32)	27 (11.59)	1056 (46.58)		239 (88.52)	13 (72.22)	226 (89.68)	
Others[b]	1417 (56.68)	206 (88.41)	1211 (53.42)		31 (11.48)	5 (27.78)	26 (10.32)	
Smoking history				0.762				0.256
Never smoked	602 (24.06)	67 (28.93)	534 (23.56)		104 (15.69)	3 (13.04)	101 (15.78)	
Ever smoked	1898 (75.94)	166 (71.07)	1733 (76.44)		559 (84.31)	20 (86.96)	539 (84.22)	
Histology				0.536				0.345
SCC	953 (38.12)	107 (45.92)	846 (37.32)		239 (36.43)	5 (22.73)	234 (36.91)	
Others[c]	1547 (61.88)	126 (54.08)	1421 (62.68)		417 (63.57)	17 (77.27)	400 (63.09)	
Stage				0.614				0.099
0/I[d]	1240 (49.60)	108 (46.52)	1132 (49.92)		422 (63.63)	15 (65.65)	407 (63.59)	
II	460 (18.41)	36 (15.41)	424 (18.72)		106 (16.01)	1 (5.59)	105 (16.39)	
III	479 (19.14)	43 (18.37)	436 (19.22)		109 (16.43)	3 (13.91)	106 (16.53)	
IV	321 (12.84)	46 (19.70)	275 (12.14)		26 (3.93)	4 (16.52)	22 (3.48)	

Data are presented as number of patients (%).
[a]P value was calculated by a logistic regression model with study as a random effect.
[b]Others include Black, Hispanic, Asian and race not specified.
[c]Others include adenocarcinoma and histology not specified.
[d]Includes four cases with stage 0 (*in situ*).

Table III. Association of demographic and clinical variables with HPV 16/18 (published datasets only in all regions)

Variable	HPV16/18 positive					
	All studies ($N = 3163$)		Small studies[a] ($N = 918$)		Large studies[b] ($N = 2245$)	
	OR[c] (95% CI)	*P* value	OR[c] (95% CI)	*P* value	OR[c] (95% CI)	*P* value
Gender		0.141		0.049		0.824
Male	1.0 (ref.)		1.0 (ref.)		1.0 (ref.)	
Female	1.27 (0.92, 1.75)		1.61 (1.00, 2.59)		1.05 (0.68, 1.62)	
Age (years)		0.420		0.029		0.361
≤66	1.0 (ref.)		1.0 (ref.)		1.0 (ref.)	
>66	1.13 (0.84, 1.54)		1.65 (1.05, 2.59)		0.82 (0.54, 1.25)	
Race		0.029		0.708		< 0.001
Others[d]	1.0 (ref.)		1.0 (ref.)		1.0 (ref.)	
White	0.33 (0.12, 0.90)		0.80 (0.25, 2.59)		0.08 (0.02, 0.33)	
Smoking history		0.930		0.859		0.974
Never smoked	1.0 (ref.)		1.0 (ref.)		1.0 (ref.)	
Ever smoked	1.02 (0.66, 1.57)		1.06 (0.58, 1.91)		1.01 (0.59, 1.74)	
Histology		0.812		0.576		0.827
Others[e]	1.0 (ref.)		1.0 (ref.)		1.0 (ref.)	
SCC	1.05 (0.71, 1.54)		1.16 (0.69, 1.93)		0.94 (0.53, 1.66)	
Tumor stage		0.561		0.044		0.338
I/II	1.0 (ref.)		1.0 (ref.)		1.0 (ref.)	
III	0.87 (0.56, 1.37)		0.94 (0.53, 1.66)		0.78 (0.36, 1.68)	
IV	1.10 (0.66, 1.85)		2.55 (1.08, 6.04)		0.68 (0.36, 1.27)	

Ref.: reference level.
[a]Small studies include studies with 22 ≤ number of cases ≤ 99.
[b]Large studies include studies with 131 ≤ number of cases ≤ 399.
[c]Study was included as a random effect.
[d]Others include Asian, Black, Hispanic and race not specified.
[e]Others include adenocarcinoma and histology not specified.

patients were more likely to be female (Adj OR = 1.61, 95% CI = 1.00–2.59), older than 66 years (Adj OR = 1.65, 95% CI = 1.05–2.59) and diagnosed with stage IV tumors than stage I/II (Adj OR = 2.55, 95% CI = 1.08–6.04). There were no differences in the association of HPV with stage IV tumors when we compared studies that reported verification of cancer diagnosis to those that did not report verification of cancer diagnosis in their publication. Similarly, for studies that reported efforts to avoid contamination, there was no difference in the association of HPV16/18 with stage IV tumors compared with those studies that did not include this information in their publication (data not shown). When North American studies were evaluated separately, there was no association of HPV 16/18 with gender, age, smoking history or histology and a marginal association with race was observed, which was significant for larger studies only (data not shown).

HPV prevalence in normal versus lung cancer tissues

There were 16 studies that evaluated HPV prevalence in lung cancer tissues (1208 cases) in addition to normal lung tissues (732 controls). Normal lung tissues were either from lung cancer patients (i.e. adjacent normal or non-adjacent normal, $n = 255$) or from normal lung tissues from non-cancer patients ($n = 477$). Lung cancer tissues were almost four times more likely to be positive for any HPV DNA compared with normal (OR = 3.86, 95% CI = 2.87–5.19; study was included as a random effect).

Evidence of HPV16/18 DNA integration in lung cancer

Because integration of high-risk HPV DNA correlates with the overexpression of HPV oncogenes, we investigated the physical status of HPV16 in a subset of cases. In our pooled international dataset, physical status data were available for three studies conducted in Asia, South/Central America and North America (32,48,49) ($n = 28$, HPV16-positive patients). For the HPV16-positive lung tumors, 75% (21/28) carried integrated HPV16 genomes, 3.6% (1/28) carried episomal HPV16 genomes and 21.4% (6/28) carried both integrated and episomal forms of HPV16 DNA. There was no significant difference in the distribution of HPV physical status according to each study ($P = 0.436$).

An equal number of male and female patients were HPV16 positive, but the majority of female patients, i.e. 92.9% (13/14), carried integrated HPV 16 DNA compared with 57% (8/14) of male patients (Table IV). None of the female patients had episomal HPV genomes in their tumors and only 7.1% (1/14) had both integrated and episomal forms. On the other hand, 7.1% (1/14) of male patients had episomal HPV genomes, and 35.7% (5/14) had both integrated and episomal HPV genomes in their tumors.

Survival analysis

A subset of patients had available follow-up data for four published studies (11,31,55,56) ($n = 451$). For all patients combined, the 2 year and 5 year overall survival rates for HPV16/18-negative patients were 60.9 and 38.5%, respectively, whereas the 2 year and 5 year overall survival rates for HPV16/18-positive patients were 71.4 and 64.3%, respectively. The Kaplan–Meier curves according to HPV16/18 status (data not shown) were not statistically significantly different (log-rank P value = 0.263).

Discussion

To date, >100 studies (including case reports) have analyzed HPV DNA in lung cancers (7). Although the majority of these studies have reported the prevalence of HPV16 and/or 18 DNA and a few publications have shown evidence of HPV oncogene (E6 and E7) expression (12,57), definite evidence of a causal relationship is still missing. Previously published meta-analyses of HPV in lung cancer show that HPV16 and HPV18 are the two most common genotypes detected in lung tumors worldwide (5–7); however, the characteristics of patients with these tumors were not determined. This study is the first international pooled analysis of individual data received from various research groups and was focused to define the demographic, behavioral and clinical characteristics of lung cancer cases with HPV16 and/or 18 DNA. We have also for the first time reported and compared published data from five North American studies with other parts of the world. Similar to previous reports, as expected, in our study (5–7), HPV16 and/or 18 were the most prevalent high-risk genotypes

Table IV. HPV 16 physical status in primary lung carcinomas

Case	Age	Sex	Histology	Physical status
1	69	Female	Adenocarcinoma	Mixed
2	84	Male	Adenocarcinoma	Integrated
3	67	Male	Adenocarcinoma	Integrated
4	74	Male	SCC	Mixed
5	62	Female	Adenocarcinoma	Integrated
6	58	Male	SCC	Integrated
7	66	Female	Adenocarcinoma	Integrated
8	75	Male	SCC	Integrated
9	70	Female	Adenocarcinoma	Integrated
10	72	Female	Adenocarcinoma	Integrated
11	80	Male	SCC	Mixed
12	72	Female	Adenocarcinoma	Integrated
13	55	Female	Adenocarcinoma	Integrated
14	79	Male	SCC	Integrated
15	59	Female	Adenocarcinoma	Integrated
16	74	Female	SCC	Integrated
17	71	Female	Adenocarcinoma	Integrated
18	70	Male	SCC	Mixed
19	74	Male	SCC	Integrated
20	69	Male	Adenocarcinoma	Integrated
21	78	Male	SCC	Mixed
22	68	Male	SCC	Episomal
23	59	Female	Adenocarcinoma	Integrated
24	63	Male	Adenocarcinoma	Integrated
25	69	Female	Adenocarcinoma	Integrated
26	39	Female	SCC	Integrated
27	66	Male	SCC	Mixed
28	62	Female	Adenocarcinoma	Integrated
SiHa				Integrated
Caski				Mixed

Mixed: integrated and episomal genomes.

detected in lung cancers, but still counting for a low fraction of lung cancers and with heterogeneity among studies.

It is plausible that differences in the geographic location, study size, demographic and clinical makeup of each study could contribute to heterogeneity in HPV prevalence between studies. Furthermore, the majority of studies included in this pooled analysis were small (<100 cases) and may be less representative samples, thus contributing to heterogeneity in HPV prevalence. Therefore, for each geographic region, the prevalence of HPV 16/18 was adjusted for each study as well as potential confounders yet variations in HPV16/18 prevalence remained. The highest prevalence of HPV16/18-positive lung cancers was observed in South and Central American, whereas North American and Asian studies had similar prevalence and European studies had significantly lower prevalence of HPV 16/18. Similar observations were made for HPV 16 and HPV 18 independently. However, within each geographic region, distinct differences in the prevalence of HPV 16 and HPV18 genotypes were noted with the exception of Asia and North America. In North America, although the prevalence of each genotype was low, the prevalence of HPV 18 was slightly higher than that of HPV 16. In contrast, for Asia, Europe and South and Central America, if any HPV DNA detected in lung cancer, HPV 16 appeared to be more prominent than HPV18. In Europe, the prevalence of HPV18 was near zero. Factors that may be responsible for variability in HPV prevalence could be sexual behaviors of participants, genetics and possible environmental contamination as argued in the study by Koshiol *et al.* (54).

In addition to geographic location, the recent meta-analysis by Syrjanen *et al.* (7) reported that the heterogeneity between studies might be related to differences in histologic type. In this study, there were no differences in the proportion of histologic types in North America compared with the rest of the world and we observed no association of HPV 16/18 status with histology. Although South and Central American studies had the highest HPV prevalence, there were only two studies from this region; therefore, this finding will need to be confirmed by additional studies. Second to South and Central America, we observed that Asia had the highest prevalence of HPV 16 and 18. A significant association of HPV 16/18 DNA was also observed with race, where non-White lung cancer patients were more likely to have HPV-positive tumors compared with White lung cancer patients. There may be a number of reasons for this observed difference in HPV prevalence some of which might include differences in sexual practices or differences in susceptibility. Further investigation of the potential reasons for the racial difference in the association of HPV16/18 is needed.

Park *et al.* (65) evaluated HPV status in 112 non-small-cell lung cancer patients from Korea and reported that HPV16 was more common in younger lung cancer patients and HPV18 was more common in patients diagnosed with advanced stage. Although we categorized HPV16 and HPV18-positive tumors into a single group, we found no associations with age or stage, neither for gender, smoking history or histology. However, for small studies only, HPV16/18 was associated with old age (>66 years), female gender and advanced stage. It is possible that patients with metastatic disease (by definition, stage IV) could include metastases from other or mixed tumors, such as head and neck or cervical cancers (59), and the resulting misclassification could account for some of these findings. Therefore, the association with older females with stage IV tumors warrants further investigation in a single, larger and more representative study.

Although HPV-positive oropharyngeal cancers have been found to be associated with non-smokers, these tumors are also associated with patients having lower number of pack-years of cigarettes compared with heavy smokers (66). *In vitro* studies also show that tobacco smoke carcinogens have been shown to increase HPV16 and 18 viral synthesis as well as interact with HPV16 E6/E7 oncoproteins to increase lung cell proliferation (67). Furthermore, smoking suppresses the host innate immunity including functional and structural changes in the respiratory ciliary epithelium, lung surfactant protein and immune cells in the lung (68), thus may facilitate HPV infection and persistence in the lung in a subset of tumors. Mechanistically, it is not yet clear how HPV might promote lung carcinogenesis; however, the cooperation between HPV and tobacco smoke carcinogens for lung carcinogenesis is plausible. In this study, the association of HPV with smoking status was not conclusive. Therefore, further studies are needed to investigate the relationship between HPV and smoking status among patients with HPV-positive lung tumors.

Although our study cannot conclusively confirm the carcinogenetic role of HPV in lung cancer, we have shown that lung cancer tissues were almost 4-fold more likely to be HPV-positive compared with normal lung tissues. Secondly, our preliminary investigation of HPV16/18 physical status among a subset of tumors shows that the majority of the female tumors carried integrated HPV DNA while the physical status of HPV16/18 in male tumors was inconsistent. Given the predominance of integrated HPV genomes in female lung cancer patients, it is possible that HPV may play a role in lung cancer development but is unlikely to contribute to a large proportion of lung cancer cases. Although the presence of integrated DNA would suggest that the DNA detected was not simply due to contamination, it is important to note that there are no published studies comparing E2/E6-based integration with direct integration detection methods, which are more reliable. Thus, the results must be interpreted with caution. Our findings suggest an association of HPV DNA with a small fraction of lung tumors, with large geographic variations, but further comprehensive analysis is needed to assess whether this association reflects a causal relationship. Such detailed analysis should include not only HPV DNA testing but evaluation of all criteria that were postulated to prove a causal involvement of HPV in carcinogenesis, such as p16 expression and HPV E6/E7 oncogene expression (2,69), and measures to exclude pulmonary metastases (59).

The survival data in this pooled analysis only included 14 HPV16/18-positive cases and 5 events, and there were no significant differences between the Kaplan–Meier curves. However, based on the 2 and 5 year survival rates between HPV16/18-positive and HPV16/18-negative patients, there was some suggestion that HPV16/18-positive lung cancer patients had improved survival. This trend toward improved survival is consistent with HPV-related

carcinogenesis in non-cervical sites, such as the head and neck and penile cancer, where HPV-positive cancer patients have an improved overall and disease-free survival compared with HPV-negative patients (70–72). This observation needs to be verified in a much larger number of cases/events and adjustment for known prognostic factors including a history of HPV-related disease as well as treatment for previous HPV-related disease.

The availability of individual data from each study included in the analysis reduces the potential errors associated with aggregate data. However, a number of limitations are inherent in this study design as well. One of these limitations is the lack of a uniform gold standard in the HPV DNA detection methods leading to variability in HPV prevalence. Only 27 of the 81 eligible studies participate in this analysis. We have compared the characteristics of the cases in the pooled dataset and with that of the non-included studies and remain confident that both subsets are similar in characteristics. Nevertheless, possible selection bias cannot completely be ruled out. Although the overall sample size was large, some of the subgroup analyses included small numbers. Although this study has good representation of populations from the Americas, Europe as well as Asia, China, Korea and Taiwan were not as well represented. In addition, the North American dataset was limited, in that, the majority of cases were of European descent; a larger US study with broad ethnic diversity is needed to allow for further investigation related to HPV and race. Although the present analysis provides evidence in favor of an association of HPV DNA with a small subset of lung cancer, these findings need to be validated and further comprehensive analysis is needed to examine a potential causal relationship. A detailed analysis of a larger and more representative case series, including the testing of all criteria that were postulated to prove a causal involvement of HPV in carcinogenesis and excluding pulmonary metastases, needs to be performed. Most importantly, the preliminary evidence on the potential prognostic role of HPV in lung cancer needs confirmation because of its potential clinical implications, e.g. in therapy of lung cancer.

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ORIGINAL ARTICLE

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 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 90.7 % (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.

Keywords Rapid immunohistochemistry (R-IHC) · Glioma · Central nervous system-lymphoma (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 multimodal 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,

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and surgical findings. To date, the probability of intraoperative diagnosis has been reported as 66–95.6 % [5, 6].

In the last three decades, several methods for rapid immunohistochemistry (R-IHC) have been proposed that use microwave [7], high-quality reagents [8–12], 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 was 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. Cases 3–15 in glioma and Case 37 in CNS lymphoma were performed R-IHC at the time of intraoperative diagnosis and Cases 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 16–33) 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 s., 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) 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

Table 1 Summary of intraoperative and final findings

Case	Age/sex	Frozen HE	MIB-1 (R-IHC)	Frozen HE + R-IHC	Permanent MIB-1	Final diagnosis
a. Hokkaido University						
1[a]	48/F	II	3.5	II	3	II
2[a]	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
b. Akita University						
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	II or 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

[a] Recut section from reserved frozen blocks

facilitate the reaction. R-IHC for Ki-67/MIB-1, CD3, and CD20 was performed using frozen sections 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 was performed using FFPE sections 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

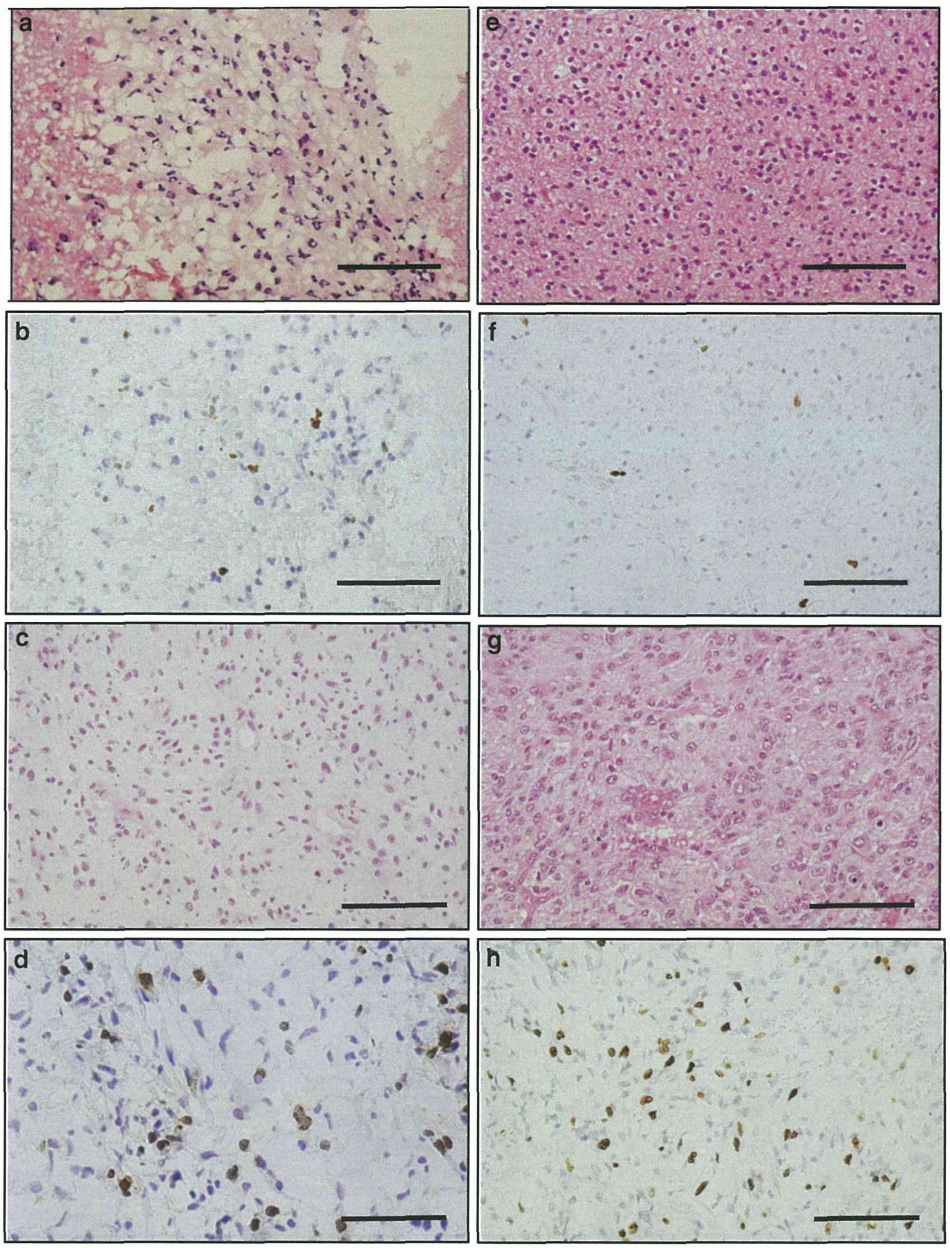

Fig. 1 Histological findings in Case 6 (**a** H&E staining in frozen specimen, **b** Ki-67/MIB-1 staining by R-IHC in frozen specimen, **c** H&E staining in FFPE specimen, **d** Ki-67/MIB-1 staining by standard IHC in FFPE specimens). Histological findings in Case 13 (**e** H&E staining in frozen specimen, **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

(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 was 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 90.7 % (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),

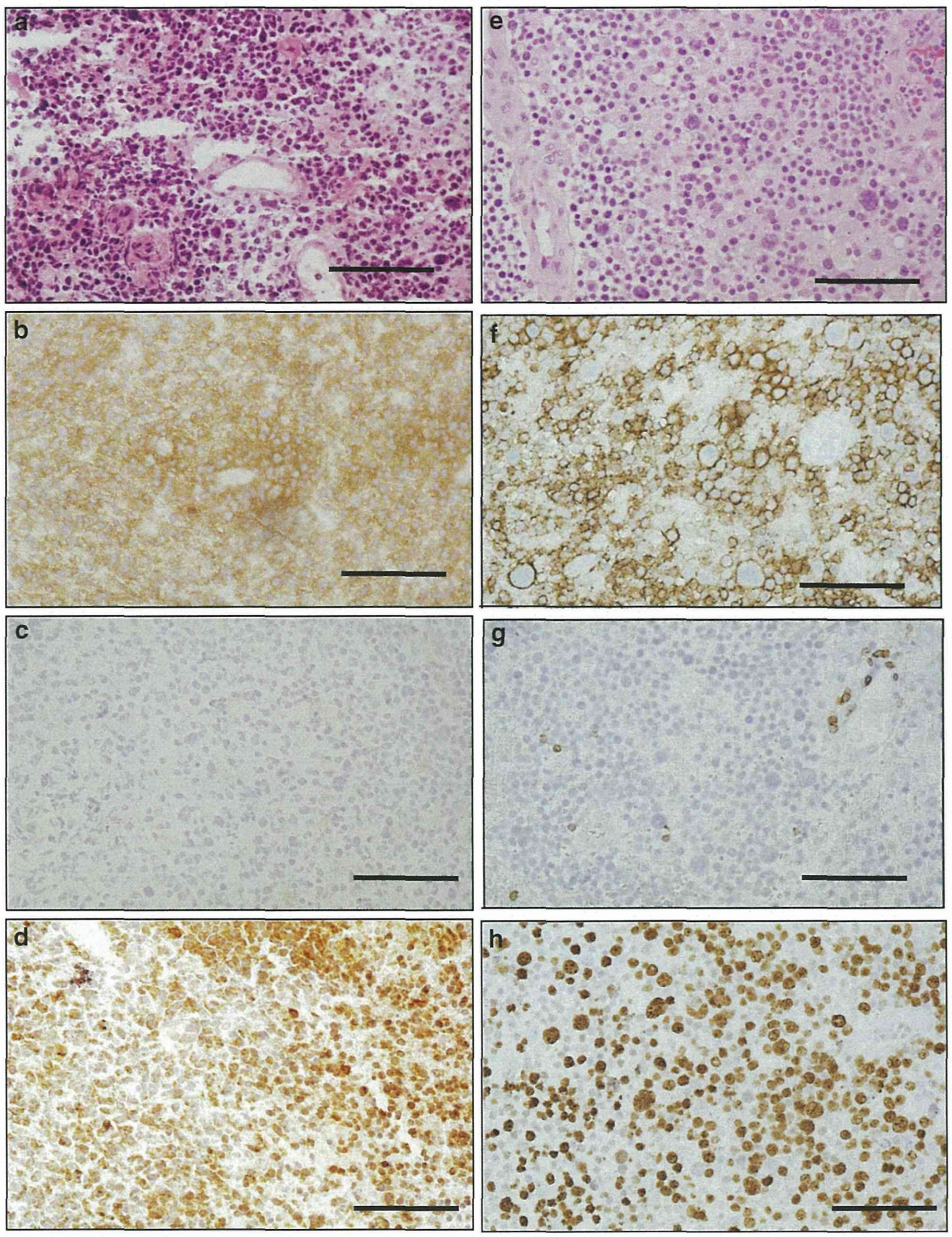

Fig. 2 Histological findings of frozen specimen in Case 37 (**a** H&E staining, **b** CD20 staining by R-IHC, **c** CD3 staining by R-IHC, **d** Ki-67/MIB-1 staining by R-IHC), and FFPE specimens for final diagnosis (**e** H&E staining, **f** CD20 staining by standard IHC, **g** CD3 staining by standard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

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