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Intracranial Germ Cell Tumors: Detection of p53 Gene Mutations by Single-strand Conformation Polymorphism Analysis

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Using polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis, p53 gene mutation was examined in 12 intracranial germ cell tumors (5 yolk sac carcinomas and 7 germinomas), many of which were derived from young patients in the first to the second decade. A total of 10 mutations were detected in 4 of the 12 cases and, in 3 of them, the mutations were multiple or tandem. Among the 10 mutations, 7 were missense, 1 was splicing and 2 were silent. The 7 missense mutations were located at previously proposed hot spot codons or in their vicinity or, when outside the hot spots, at a codon encoding an amino acid conserved in most vertebrates. These findings suggested that all 7 missense mutations may actually give rise to functional alteration of the p53 protein. The splicing mutation was considered to be a germ-line mutation, though its biological effect was equivocal, since the neoplastic tissue contained an additional mutation. The pattern of the mutations was predominancy of G:C-A:T transition with frequent involvement of the CpG site. These mutations were more frequently detected in yolk sac carcinomas (60%; 3/5 cases) than in germinomas (14%; 1/7 cases), suggesting that the contribution of the p53 mutation to carcinogenesis differed with the histological type of the intracranial germ cell tumor.

Key words: Germ cell tumor — Brain tumor — Teratoma — p53 gene mutation — Tumor suppressor gene

Germ cell tumors are considered to be derived from primordial germ cells and are classified into germinoma (seminoma and dysgerminoma), embryonal carcinoma, yolk sac carcinoma, choriocarcinoma and immature and mature teratomas.[1] These tumors occur not only in the gonadal organs, but also in extragonadal sites, usually in the midline structures of the body.[1] In the brain, the tumors develop in the pineal region and also in the suprasellar region, mostly in young patients in the second decade.[1] Though the incidence of the intracranial germ cell tumors is high (around 10–15%) among brain tumors in young patients, the genetic alteration involved in these tumors remains largely unknown.

Recently, mutations of the p53 gene have been detected in various human tumors.[2] The p53 gene is a tumor suppressor gene located on the short arm of chromosome 17 consisting of 11 exons and 10 introns and encoding 393 amino acids of p53 protein.[3–8] In a wide variety of human tumors, p53 mutations have been detected mainly in exons 5 through 8 which included phylogenetically highly conserved domains 2–5.[2,9] Regarding brain tumors, p53 gene mutation has been examined in astrocytic gliomas[10–19] and some other tumors.[14,20–22] However, to our knowledge, no analysis of p53 gene mutation in intracranial germ cell tumors has been reported.

In this study, we examined a total of 12 intracranial germ cell tumors for p53 gene mutation by means of single strand conformation polymorphism (SSCP) analysis.

MATERIALS AND METHODS

Tumor specimens Twelve autopsied intracranial germ cell tumors including 5 yolk sac carcinomas and 7 germinomas were examined (Table I). One yolk sac carcinoma contained admixed immature teratoma components. Many of these tumors were derived from young patients in the first to the second decade. Normal brain and/or spleen tissues of the same patients were also examined. All specimens were formalin-fixed, paraffin-embedded and preserved at room temperature for more than 20 years.

DNA extraction Genomic DNA was extracted from formalin-fixed, paraffin-embedded sections according to the method of Goelz *et al.*[23]

PCR-SSCP analysis Oligonucleotides (20-mer) were chemically synthesized with a DNA synthesizer (ABI, Foster, CA) and used as primers in the polymerase chain reaction (PCR). The primers which were designed to

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Table I. Summary of p53 Gene Mutations in Intracranial Germ Cell Tumors

Case No.	Age/Sex[a]	Site of tumor[b]	Histology[c]	p53 mutations					Radio-therapy[f]
				Exon	Codon	Nucleotide[d] d.s. (c)	Amino acid	Normal tissue[e] d.s.	
1	8/M	p	ysc(+it)	—	—	—	—		+
2	15/M	p	ysc	8	273	CGT→TGT (2/2)	Arg→Cys	—	+
3	10/M	p	ysc	—	—	—	—		+
5	19/M	p	ysc	5	156	CGC→AGC (3/4)	Arg→Ser	—	+
					176	TGC→TTC (3/4)	Cys→Phe	—	
					177	CCC→CCT (3/4)	Pro→Pro	—	
				8	273	CGT→CAT (1/2)	Arg→His	—	
6	8/M	p	ysc	intron 5		agG→aaG (2/4)	—	+	+
				8	273	CGT→CAT (1/2)	Arg→His	—	
4	40/M	p	g	5	140	ACC→ATC (3/5)	Thr→Ile	—	—
					176	TGC→TTC (3/5)	Cys→Phe	—	
					177	CCC→CCT (3/5)	Pro→Pro	—	
7	13/F	ss	g	—	—	—	—		—
8	18/M	p	g	—	—	—	—		+
9	29/M	p	g	—	—	—	—		—
10	19/M	p	g	—	—	—	—		—
11	13/M	p	g	—	—	—	—		—
12	23/M	p	g	—	—	—	—		—

a) Age, years; M, male; F, female.
b) p, pineal region; ss, suprasellar region.
c) ysc, yolk sac carcinoma; g, germinoma; it, immature teratoma.
d) Mutations from left to right nucleotides at underlined positions. Mutations were detected by direct sequencing (d.s.) of PCR products and confirmed by a second PCR and subsequent nucleotide analysis of cloned PCR products (c). Parentheses: number of clones with mutation/number of clones examined in analysis of plasmid clones of the second PCR products (c).
e) Results of direct sequencing (d.s.). +, the same mutation as in neoplastic tissue; −, absence of the same mutation.
f) +, Patients received irradiation therapy prior to death; −, no irradiation therapy.

amplify individual exons 5–8 together with parts of their adjacent introns were previously described by Hsu *et al.*[24] The primers for amplification of exon 4 were those described by Mashiyama *et al.*[14] At the 5′-end of these primers, either a *Hin*dIII or an *Eco*RI site was introduced according to Fults *et al.*[12] The PCR was conducted essentially according to Orita *et al.*[25] Briefly, the PCR mixture (20 μl) containing 2 μg of genomic DNA, 100 ng each of labeled primers, 3.125 nmol each of dNTP, 1 U of Taq polymerase (Takara, Kyoto), 10 m*M* Tris-HCl (pH 8.3) and other reagents as described[13] was subjected to amplification for 40 cycles. The temperature profile for denaturation, annealing and extension was 93°C for 1 min, 50°C for 2.5 min and 72°C for 2.5 min, respectively, except for the last cycle, in which the extension time was 15 min. For SSCP analysis, the PCR products were adequately diluted with a diluent solution, denatured at 95°C for 5 min, electrophoresed in a 6% non-denatured polyacrylamide gel at room temperature and exposed to X-ray film as described previously.[25]

Nucleotide analysis PCR products were prepared by the same method as described, except for the use of unlabeled primers instead of labeled ones. The direct sequencing was performed by the dideoxy termination method[26] using a sequencing kit, fmol DNA Sequencing System (Promega, Madison, WI). Briefly, an extension/termination reaction mixture, 6 μl in volume, containing 25 ng of the PCR products, 25 ng of one of the two primers described, 1.25 U of Taq polymerase, 46 kBq of [α-^{32}P]dATP and dNTP/ddNTP mix was subjected to 30 cycles of PCR amplification using the temperature profile recommended by the manufacturer. The ^{32}P-incorporating PCR products were electrophoresed in a sequencing gel and autoradiographed. All mutations detected by direct sequencing were confirmed by a second PCR on fresh genomic DNA samples and subsequent nucleotide analyses of cloned PCR products. Briefly, PCR products were purified by 6% polyacrylamide gel electrophoresis followed by electro-elution. The purified DNAs were digested with *Hin*dIII and *Eco*RI and ligated directionally into the cloning sites of pUC 118 plasmid vector. The recombinant plasmids were transfected into competent JM 109 *Escherichia coli* cells, propagated, purified and sequenced on both strands by

the dideoxy method. Sequencing was performed on 2–5 plasmid clones derived from different bacterial colonies.

RESULTS

Upon PCR-SSCP analysis, the DNA of normal tissues was used as a negative control. The samples that showed electrophoretic mobility shifts compared with the control DNA were considered to contain a mutant p53 gene. Aberrant bands were seen in 4 of 12 germ cell tumors, in exon 5 (case 4), in exon 8 (case 2), in exons 5 and 8 (case 5) and in exons 6 and 8 (case 6), in 1 case each. Normal bands were not distinct or were invisible in these cases. Fig. 1 shows representative aberrant migrations in SSCP analyses.

The direct sequencing revealed a total of 10 mutations in PCR products derived from the 4 cases which showed mobility shifts in SSCP (Table I). The mutations were located at codon 273 in 1 case (case 2), at codons 140, 176 and 177 in 1 case (case 4), at codons 156, 176, 177 and 273 in 1 case (case 5) and at codon 273 and the splice acceptor signal in intron 5 in 1 case (case 6). At the nucleotide positions of mutations, only aberrant ladders were distinct and normal ladders were always faint to invisible, suggesting loss of the normal allele in these cases (Fig. 2). Nucleotide analyses of the cloned PCR products confirmed all 10 mutations, and 3 mutations in 1 case (case 4) and 3 of the 4 mutations in another 1 case (case 5) were shown to be tandem in the same p53 gene alleles (Table I, Fig. 3).

Among the 10 mutations detected, 7 were missense mutations leading to amino acid substitutions, 1 was a splicing mutation and 2 were silent. Of the 7 missense mutations, 6 were located in the conserved domains 2 (codon 140 in case 4), 3 (codon 176 in cases 5 and 4) and 5 (codon 273 in cases 2, 5 and 6) of the p53 gene. While 5 (codons 176 and 273) of them were located on previously proposed hot spot codons (codons 175, 196, 213, 248, 273 and 282)[2] or in their vicinity, 1 (codon 140) was located outside the hot spots, but at a codon encoding an amino acid conserved in most vertebrates. The remaining 1 missense mutation (codon 156 in case 5) was located outside the conserved domains, but involved a hot spot region (codons 156–166 outside the domains) proposed by Frankel *et al.*[11]

On examination of the normal tissues of the same patients, no missense or silent mutations were detected (Table I), indicating that the 7 missense and 2 silent mutations observed in the neoplastic tissues were somatic, but not of polymorphic or germ-line nature. On the other hand, the splicing mutation in 1 case (case 6) was considered to be a germ-line mutation. The normal tissue showed aberrant and presumed normal bands in SSCP analysis of exon 6 and both aberrant and normal ladders at the splicing signal in intron 5 in the direct sequencing (Fig. 4). In the neoplastic tissue, the aberrant band and ladder were more distinct than the normal ones in the same examinations (Fig. 4). In addition, the neoplastic tissue revealed another mutation (codon 273) (Table I) and the splicing mutation seemed to coexist with this additional mutation in the same allele, since

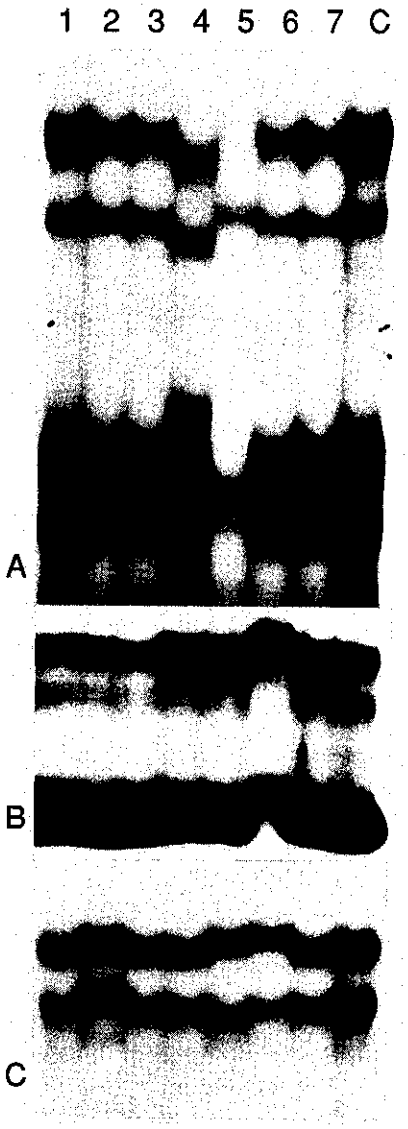

Fig. 1. SSCP analysis of p53 gene in intracranial germ cell tumors. Electrophoretic patterns of representative germ cell tumors in analyses of exons 5 (A), 6 (B) and 8 (C). 1–7, cases 1–7; C, control. A. Aberrant migration patterns are seen in cases 4 and 5. B. Aberrant migration pattern in case 6. C. Aberrant migration patterns in cases 2, 5 and 6. In A–C, normal bands are not distinct or are invisible when aberrant bands are seen.

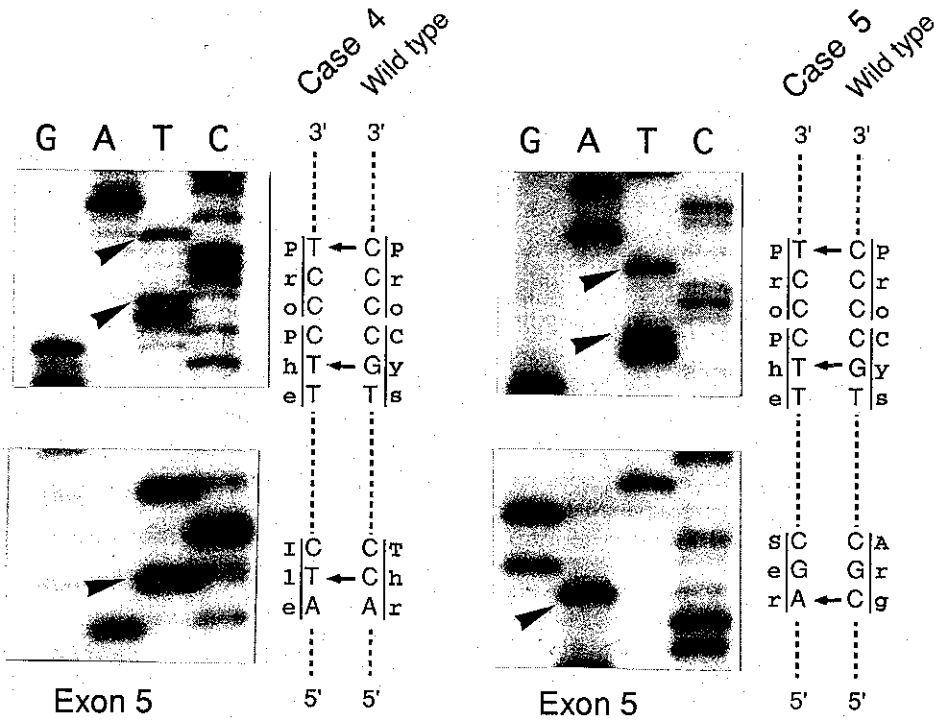

Fig. 2. Direct sequencing analysis of p53 gene in intracranial germ cell tumors. Tandem 3 mutations (arrowheads) at codons 140 (lower), 176 (middle) and 177 (upper) in case 4 (germinoma) and those at codons 156 (lower), 176 (middle) and 177 (upper) in case 5 (yolk sac carcinoma) are shown. Note faint to invisible normal ladders at the nucleotide positions of mutations.

lack of the normal allele was suggested in the neoplastic tissue from the SSCP and the direct sequencing analyses, as described.

The pattern of the mutations was predominantly G:C-A:T transition with frequent involvement of the CpG site. Of the 9 somatic mutations (7 missense and 2 silent mutations), 6 (67%) were G:C-A:T transitions and 3 (33%) were G:C-T:A transversions. Three of the 6 G:C-A:T transitions were located at the CpG site of the hot spot codon 273.

It seemed of interest that these mutations were seen more frequently in yolk sac carcinomas than in germinomas. Yolk sac carcinomas revealed the mutations in 3 (60%) of the 5 cases, while germinomas exhibited mutation in only 1 (14%) of the 7 cases (Table I).

DISCUSSION

In this study, we carried out PCR-SSCP analysis on DNA samples extracted from paraffin-embedded tissue sections of 12 intracranial germ cell tumors, many of which were derived from young patients of the first to the second decade. We found p53 gene mutations in 4 of the 12 germ cell tumors; in 3 of the 5 yolk sac carcinomas and 1 of the 7 germinomas. The immature teratoma components which involved about 40% of the neoplastic field in 1 of the 5 yolk sac carcinoma cases were considered to be negative for p53 mutation, since DNA extracted from the entire neoplastic field of this case showed no mutation. Thus, although its incidence differed with the histological type, p53 gene mutation was shown to be related to the genesis of intracranial germ cell tumors.

The mutations were multiple in 3 of these 4 cases and, in 2 of them (cases 4 and 5), the multiple mutations were localized in one p53 gene allele by sequence analysis of cloned PCR products. Among a total of 10 mutations detected, 7 were missense, 1 was splicing and 2 were silent. Regarding the missense mutations, all 7 were located at previously proposed hot spot codons[2,11] (codon 156 in 1 and codon 273 in 3 mutations) or in their vicinity (codon 176 in 2 mutations) or, when outside the hot spots, at a codon encoding an amino acid conserved in most vertebrates (codon 140 in 1 mutation). These findings suggested that all 7 missense mutations distributed in the 4 cases may actually give rise to functional alteration of the p53 protein.

As suggested by the analysis of the normal tissues of the same patients, all 7 missense and 2 silent mutations

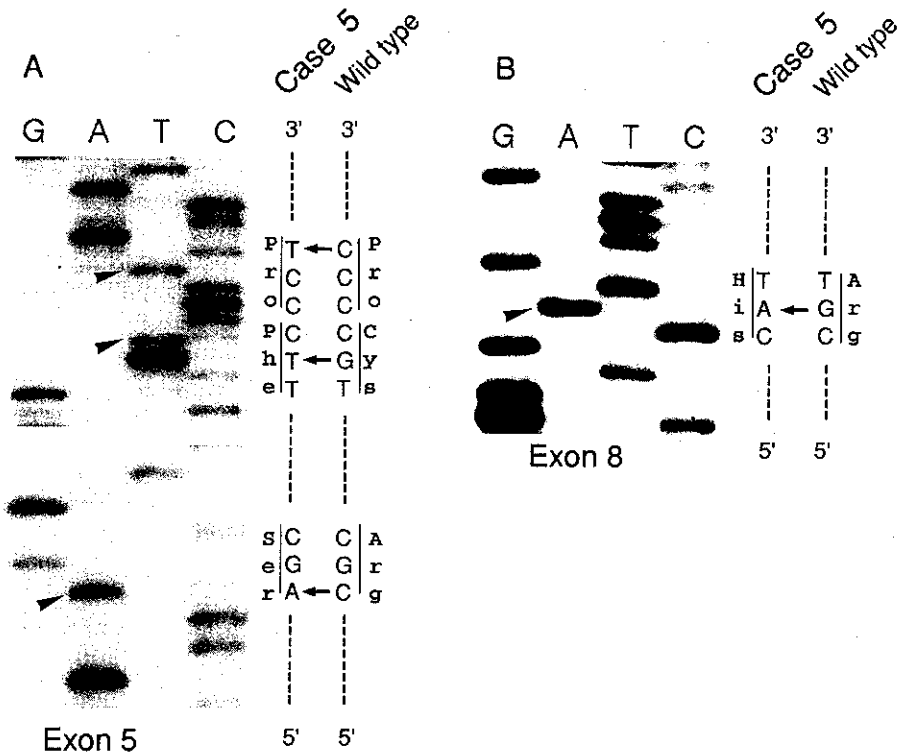

Fig. 3. Sequencing analysis of cloned PCR products of p53 gene in a yolk sac carcinoma (case 5). A. Tandem 3 mutations (arrowheads) at codons 156 (lower), 176 (middle) and 177 (upper) in exon 5 are shown. B. A mutation (arrowhead) at codon 273 in exon 8 is shown.

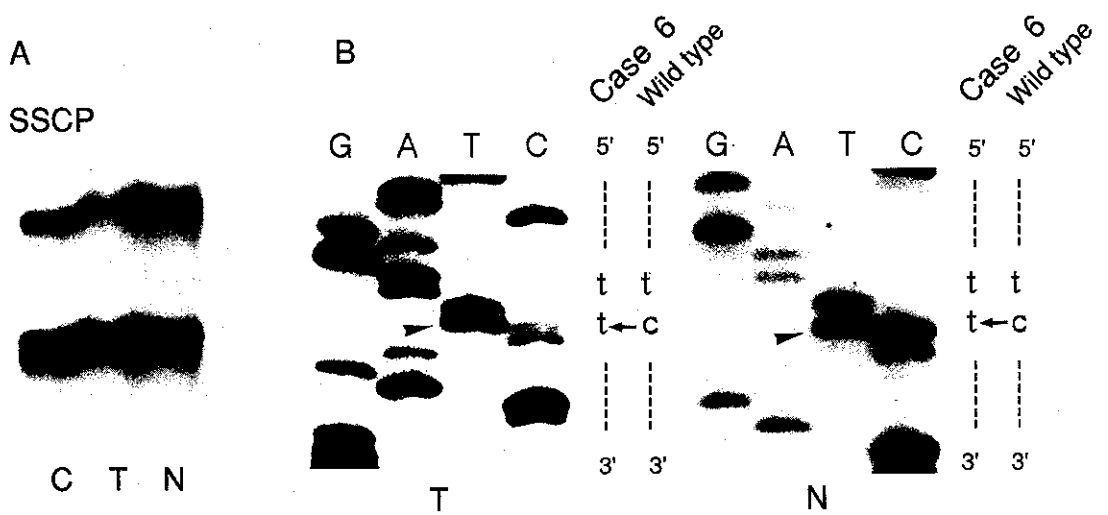

Fig. 4. SSCP (A) and direct sequencing (B) analyses of p53 gene in a yolk sac carcinoma (case 6). A. SSCP analysis of exon 6. Compared with wild-type bands in control DNA (C), aberrant migrations are seen in both the normal (N) and the tumor (T) tissues. The normal band is presumed to be present in the normal tissue, while it is not distinct in the tumor tissue. B. Direct sequencing shows a mutation at the splice acceptor site (arrowheads) (change from tc to tt in antisense strand corresponding to change from ag to aa in sense strand) in intron 5 in both the normal (N) and the tumor (T) tissues. Note that a normal ladder at the position of mutation is distinct in the normal tissue and weak in the tumor tissue.

were considered to be somatic, but not of polymorphic or germ-line nature. On the other hand, the splicing mutation was detected in both the neoplastic and the normal tissues of the same patient (case 6). Considering that splicing mutations usually result in abnormal splicing and the same splicing mutation has been detected in some sporadic tumors,[13, 27] this mutation was considered to be a germ-line mutation, but not of polymorphic nature. However, the neoplastic tissue in this case revealed an additional mutation at codon 273 which is known to lead to functional alteration of p53 protein,[28] making the biological effect of the splicing mutation equivocal. If abnormal splicing actually occurred, the amino acid sequence downstream to exon 5 might be greatly changed, irrespective of the presence of mutation at codon 273. However, if abnormal splicing occurred only occasionally despite the splicing mutation, the mutation at codon 273 might be crucial in functional alteration of p53 protein.

As described, the incidence of p53 gene mutation differed with the histological type of the tumor. The p53 mutations were more frequently detected in yolk sac carcinomas than in germinomas. The reason for this difference between different histological types belonging to the same tumor group is unknown. Regarding germ cell tumors in the gonadal organs, only a few studies[29–31] have analyzed the p53 gene mutations. Kihana *et al.*[30] examined 2 dysgerminomas (equivalent to germinomas) and 2 immature teratomas of ovarian origin and detected multiple mutations in 1 dysgerminoma case. Peng *et al.*[31] examined samples in which the neoplastic field involved more than 20% of the tissue and detected no mutation in a total of 22 germ cell tumors of the testis including 10 seminomas (equivalent to germinomas), 1 yolk sac carcinoma (mixed type) and some other types. Similar negative findings in testicular germ cell tumors were also reported by Heimdal *et al.*[29] Taking the findings in these studies together, it appears that p53 mutation is relatively rare in gonadal germ cell tumors. The negative finding in seminomas coincided with the low frequency of p53 mutation in our germinomas. However, the previous studies examined only 1 yolk sac carcinoma (mixed type), which is insufficient for comparison with our intracranial yolk sac carcinomas.

Clinically, all 5 patients with yolk sac carcinoma in this study received radiotherapy prior to death, but only 1 germinoma patient did (Table I). Thus, the high incidence of p53 mutation in yolk sac carcinomas might have occurred as a result of the treatment, and this would suggest that caution is necessary in the treatment of germ cell tumors. However, no relationship between incidence of p53 mutation and history of radiotherapy has been observed in various tumors, including brain tumors.[11, 19] Therefore, it is more likely that the contribution of the p53 mutation to carcinogenesis differs with the histological type of the intracranial germ cell tumors, although further studies using tumor specimens from untreated patients are required.

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