Alterations of the INK4a/ARF Locus in Human Intracranial Germ Cell Tumors

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Abstract

Little is known about the molecular mechanisms responsible for the development of intracranial germ cell tumors (ICGTs). Recently, we demonstrated that the balance of the p53-mdm2 interactions is disrupted in ICGTs. The p14ARF product, a tumor suppressor gene located on the INK4a/ARF locus, acts as one of the major factors affecting p53-mdm2 interactions via its binding to mdm2 and the stimulation of mdm2 degradation. To evaluate whether genetic alterations of the INK4a/ARF locus occur in the genesis of ICGTs, we analyzed the INK4a/ARF genes in 21 ICGTs—10 pure germinomas and 11 nongerminomatous germ cell tumors. Fifteen (71%) of the 21 ICGTs displayed genetic alterations, including 14 homozygous deletions and 1 frameshift mutation. Furthermore, the frequency of the alterations was higher in pure germinomas (9 (90%) of the 10) than in nongerminomatous germ cell tumors [6 (55%) of the 11; P = 0.09]. These data suggested that INK4a/ARF gene abnormalities could play an important role in the genesis of ICGTs, especially in pure germinoma.

Introduction

ICGTs are rare neoplasms. The reported frequency of the tumor is 0.3–3.4% of primary intracranial tumors in Western countries and 2.1–12.7% in Japan (1–3), and their etiologies remain largely unknown. Studies on the histological nature of ICGTs revealed that ICGTs comprise five interrelated neoplasms: germinoma, teratoma, embryonal carcinoma, yolk sac tumor, and choriocarcinoma (2, 4). These five types can be divided into two groups, i.e., pure germinoma and nongerminomatous germ cell tumors (teratoma, embryonal carcinoma, yolk sac tumor, and choriocarcinoma). Prognosis varies between these groups and is highly dependent on the response to chemotherapy or radiotherapy (2, 5, 6). In general, pure germinoma has a better prognosis than nongerminomatous germ cell tumors.

We recently reported that a disruption in the balance of the p53-mdm2 interactions could play an important role in the tumorigenesis of these neoplasms. MDM2 gene amplification is found at a moderate frequency in ICGTs, whereas TP53 gene mutations are very rare. Furthermore, mdm2 protein is overexpressed at a very high frequency in ICGTs (7).

Increasing knowledge on the molecular genetic mechanisms underlying many types of tumors has demonstrated a high frequency of genetic alterations in a single locus, INK4a/ARF (8–10). The INK4a/ARF locus has two promoters and encodes two completely different proteins, p16INK4a and p14ARF (the mouse homologue is called p19ARF; Refs. 10–12). By acting as an inhibitor of cyclin-dependent kinases, the p16INK4a protein decreases the phosphorylation of retinoblastoma protein and results in cell cycle arrest at G1 (13–15). The other encoded protein, p14ARF, interacts with mdm2 and stimulates the degradation of mdm2 protein (16–19). The tumor-suppression function of p14ARF is dependent upon the presence of wild-type p53 (20), and ARF-deficient mice tend to develop carcinoma and tumors of the nervous systems (21).

Molecular genetic findings of the ICGTs, namely, the disruption of the balance of p53-mdm2 interactions, led us to speculate that alteration in the INK4a/ARF locus could be a major factor affecting p53-mdm2 interaction, and the tumorigenesis of ICGTs. To evaluate whether INK4a/ARF gene alterations play a role during ICGTs development, we examined a series of 21 ICGTs to identify mutations of both the p16INK4a and p14ARF genes.

Materials and Methods

Tissue Samples. Twenty-one intracranial germ cell tumor specimens (10 pure germinomas and 11 nongerminomatous germ cell tumors, including 4 mature teratomas, 3 immature teratomas, 2 choriocarcinomas, and 2 yolk sac tumors) were obtained at surgery. All of the cases were diagnosed and followed up at the Department of Neurosurgery, University Hospital, Kanazawa, Japan, between 1988 and 1998. The specimens were sent to the pathology laboratory for routine formalin fixation and paraffin embedding. All of the tumor specimens were examined microscopically, and only tumor tissue was dissected under the microscope before phenolic DNA extraction.

Homologous Deletion Analysis of the INK4a/ARF Genes. Homologous deletions of the INK4a/ARF genes were analyzed by a differential PCR method (22). Because the exon2 is a common open reading frame of both of these genes and the deletion breakpoint is located between exon3 and exon 1B (23), we amplified a 204-bp fragment of INK4a/ARF from exon2 (primer: ex2–1) with a 134-bp fragment of the APRF gene. Primer sequences are listed in Table 1. One of each pair was labeled with indodicarbocyanine (Cy5) fluorescent dye (Pharmacia Biotech, Uppsala) at the 5′ end. Differential PCR was performed in a final volume of 10 μl containing 2 ng of DNA, 50 mM KCl, 1.5 mM of MgCl2, 10 mM TRIS-HCL (pH 8.3), 200 μM each dNTP, 0.1% gelatin, 5 pmol of each primer set, 10% DMSO, and 0.25 units of Taq polymerase. Initial denaturation at 94°C for 3 min was followed by 28 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 55 s, and extension at 72°C for 55 s on a thermal cycler (thermal cycler 480, Perkin-Elmer, CA). A final extension step of 10 min at 72°C was used.

Mutation Analysis of the INK4a/ARF Genes. For all of the cases without INK4a/ARF gene homozygous deletion, all of the exons of each gene were analyzed by fluorescence-based SSCP and direct sequencing (24). PCR was performed in a final volume of 10 μl containing 2ng DNA, 50 mM KCl, 1.5 mM MgCl2, 10 mM TRIS-HCL (pH 8.3), 200 μM each dNTP, 0.1% gelatin, 5 pmol of each primer set, 10% DMSO, and 0.25 units of Taq polymerase. Initial denaturation at 94°C for 3 min was followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 55 s, and extension at 72°C for 55 s on a thermal cycler (thermal cycler 480, Perkin-Elmer, CA). A final extension step of 10 min at 72°C was used.

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1 The abbreviations used are: ICGT, intracranial germ cell tumor; APRF, adenosine phosphoribosyltransferase; SSCP, single-strand conformational polymorphism; TGT, testicular germ cell tumor.

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Once separated, the products were analyzed by an automated DNA sequencer (Pharmacia Biotech Model ALFred) running with a fragment analysis program (Pharmacia biotech AlleleLinks version 1.00).

The materials with variant SSCP were realigned and sequenced bidirectionally. Sequence analysis was carried out with a semiautomated sequencer (Model 373, Applied Biosystems, CA). The primers for SSCP analysis and direct sequencing are listed in Table 1.

**Statistical Analysis.** Two-tailed Fisher’s exact test was carried out to compare the frequency of INK4a/ARF alterations in pure germinomas and nongerminomatous germ cell tumors.

**Results**

To determine the variation in the ratio of the PCR products of the INK4a/ARF genes to those of the APRT gene in normal DNA, we studied 60 genomic DNA samples from peripheral lymphocytes. The mean ratio and SD of the coamplified genes to those of the APRT gene after the p53 degradation via the stabilized mdm2 could abrogate the normal balance of each molecule in the ARF-mdm2-p53 pathway. This could break down the G1-S phase checkpoint and subsequently lead to cell proliferation. Thus, the high frequency of ARF gene alterations in ICGTs could confirm the mechanism of ICGTs tumorigenesis suggested above. Furthermore, another important pathogenesis of ICGTs in addition to INK4a/ARF alterations might be the ARF alterations.

**Discussion**

Little is known about the molecular mechanisms occurring in ICGTs. Although TP53 gene mutations are rare, some subsets of ICGTs carry the MDM2 gene amplification (7). This suggests that the disruption of the balance of p53-mdm2 interaction might play an important role in the tumorigenesis of ICGTs.

In this series, 9 (90%) of 10 pure germinomas and 6 (55%) of 11 nongerminomatous germ cell tumors exhibited INK4a/ARF gene alterations. The alterations in the ARF gene after the p53 degradation via the stabilized mdm2 could abrogate the normal balance of each molecule in the ARF-mdm2-p53 pathway. This could break down the G1-S phase checkpoint and subsequently lead to cell proliferation. Thus, the high frequency of ARF gene alterations in ICGTs could confirm the mechanism of ICGTs tumorigenesis suggested above. Furthermore, another important pathogenesis of ICGTs in addition to the ARF alterations might be the INK4a alterations.

The higher frequency of INK4a/ARF alterations in pure germinomas (90%) compared with that in nongerminomatous germ cell tumors (55%) may help explain why pure germinomas are more sensitive to chemotherapy and/or radiotherapy than nongerminomatous germ cell tumors. In an earlier report (25), INK4a gene transfection and forced expression of p16INK4a in the p16INK4a-deficient cell line were found to increase the radiosensitivity of the cells. This is opposed to our findings, which suggested that pure germinoma, the more radiosensitive neoplasm, carried INK4a gene alterations. On the other hand, the abrogation of p53 function was found to increase the response to radiation in some types of neoplasms (26). This was consistent with our findings, which showed that the ARF alterations and subsequent abrogations of p53 function were more frequent in
pure germinomas. In ICGTs, disorder in ARF-p53 pathway may overcome the INK4a-Rb pathway abrogation at the point of radiosensitivity.

ICGTs and TGTs may share similar cellular origins. Both types of tumors have the same molecular genetic abnormalities in TP53 gene and MDM2 genes (7, 27, 28). However, deletions or mutations in INK4a/ARF genes are rare in TGTs (29, 30). In contrast, in our study, a high frequency of INK4a/ARF gene deletions was demonstrated in ICGTs. In ARF-deficient mice, tumors of the nervous system are likely to emerge (21). The difference in the status of the ARF gene between ICGTs and TGTs may affect the site of germ cell tumor development.

The silencing of the INK4a gene by methylation at the promoter site has been reported in a variety of tumors (31). Recently, it has also been shown that the ARF gene is silenced by methylation at the ARF-specific promoter site in some types of cell lines, although not in natural tumors (32, 33). In our series of 21 ICGTs, cases without deletions or mutations in INK4a/ARF may carry a promoter site methylation. In the future, in addition to the analysis for genetic alterations, additional examinations for gene modifications of this type will be required to evaluate the biological features of p16INK4a and p14ARF in, and their influences on, ICGTs.

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