Immunohistochemical analysis of p53 and p21^{WAF1/Cip1} expression in primary intracranial germ cell tumors

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Primary intracranial germ cell tumors (GCTs) comprise 3.1% of all brain tumors and 13.6% of those in patients younger than 15 years of age in Japan. They are classified into five basic histological types: germinoma, teratoma, choriocarcinoma, yolk sac tumor, and embryonal carcinoma; or into mixed tumor types when they consist of two or more components. Radiation therapy with or without chemotherapy has proven effective in the treatment of germinoma, whereas there is a poor prognosis for choriocarcinoma, yolk sac tumor, embryonal carcinoma, and mixed tumors having components of the group of malignant intracranial GCTs. The underlying mechanisms for such different responses to radio- and chemotherapies of intracranial GCTs remain unknown.

In this study, the authors analyzed the expression of p53 and p21^{WAF1/Cip1} proteins by immunohistochemical analysis in 35 intracranial GCTs. Expression of p53 protein was observed in 33 (94%) of 35 intracranial GCTs. Expression of p21^{WAF1/Cip1} was detected in seven (20%) of 35 intracranial GCTs. None of the 15 germinomas was immunoreactive for p21^{WAF1/Cip1} protein, whereas in a group of malignant intracranial GCTs, four (80%) of five cases showed immunoreactivity for p21^{WAF1/Cip1} protein. Analysis of the data suggests that overexpression of p21^{WAF1/Cip1} in intracranial GCTs may correlate with decreased sensitivity to radio- and chemotherapy and suggest a poor prognosis.

Key Words * germ cell tumor * p53 * p21^{WAF1/Cip1} * immunohistochemistry

The primary intracranial germ cell tumors (GCTs) are classified into the five basic histological types of germinoma, teratoma, choriocarcinoma, yolk sac tumor, and embryonal carcinoma, or they are classified into mixed tumor types when they consist of two or more components.[18] In Western countries, GCTs constitute only 0.3 to 0.5% of all primary brain tumors and approximately 3% of those in children and adolescents,[33] whereas in Japan GCTs account for 3.1% of all primary brain tumors and 13.6% of those in children less than 15 years of age.[16] Surgical extirpation followed by radiation therapy with or without chemotherapy has been proven effective in the treatment of germinoma, which has a 5-year survival rate of 95.4%.[24] On the other hand, choriocarcinoma, yolk sac tumor, embryonal carcinoma, and mixed tumors composed mainly of these tumor types would be categorized into a malignant group of GCTs having a 5-year survival rate of only 17.4%.[24] Despite intensive treatments including whole-brain radiation with combination chemotherapy, in more than 80% of the patients with the malignant group of GCTs the tumors recurred and about half of the recurrences were distant metastases. Each type of GCT represents the neoplastic correlate of a cell type from a specific stage of embryonic development: choriocarcinoma corresponds to the trophoblast, yolk sac tumor

to yolk sac endoderm, embryonal carcinoma to the pluripotent stem cell of the embryo proper, teratoma to the embryonic differentiated cell, and germinoma corresponds to the primordial germ cell.[35] Tumor cells resembling the cells from an earlier stage of histogenesis (choriocarcinoma, yolk sac tumor, and embryonal carcinoma) are more malignant and refractory to treatments compared to the more differentiated cells such as teratoma and germinoma cells.[24]

The **p53** tumor-suppressor gene is involved in the apoptotic response to radiation therapy and to many chemotherapeutic drugs.[22] Gonadal GCT cells overexpress wild-type p53 protein,[9,13,29,32,36] and GCT cells undergo p53-mediated apoptosis in response to DNA damage.[23] The **p21^{WAF1/Cip1}** is a downstream effector in the p53 pathway of growth control in mammalian cells.[5] In response to DNA damage, p53 expression promotes the transcription of p21^{WAF1/Cip1} resulting in growth arrest through inhibition of cyclin-dependent kinases, which are required for G1 to S transition.[14,26,44] When overexpressed in tumor cells, p21^{WAF1/Cip1} inhibits cell growth.[5,45] On the other hand, p21^{WAF1/Cip1} can also be induced by p53-independent pathways[25,28,39] and protects normal cells as well as cancer cells from apoptosis.[11,12,19,30,31,34,42,43] Cells that are resistant to apoptosis will be less responsive to treatment. In some cancers, including acute myelogenous leukemia, head-and-neck cancer, and breast cancer, elevated levels of p21^{WAF1/Cip1} expression were shown to be associated with increased resistance to chemotherapy and shorter relapse-free survival rates.[1,7,46]

In this study, we investigated the expression of p53 and p21^{WAF1/Cip1} proteins by immunohistochemical analysis in 35 primary intracranial GCTs. Our aim was to compare the expression of those two proteins to the clinicopathological characteristics of the tumors including histological classification and treatment response.

CLINICAL MATERIAL AND METHODS

Thirty-five primary intracranial GCTs from 34 patients were obtained at surgery. Both primary and recurrent tumors were available from one patient with an immature teratoma. Eleven cases of germinoma, four cases of germinoma with syncytiotrophoblastic giant cells (STGCs), nine cases of immature teratoma, two mature teratomas, four mixed tumors of germinoma and immature teratoma components, two choriocarcinomas, one yolk sac tumor, one embryonal carcinoma, and one mixed tumor of embryonal carcinoma and germinoma components were included. All the histological sections were reevaluated by one of the authors (M.M.), and the classification was confirmed by immunohistochemical staining for tumor markers including human chorionic gonadotropin-beta, alpha-fetoprotein, and placental alkaline phosphatase when necessary. All patients underwent radiotherapy after surgery except for one with a mature teratoma that was completely resected. Except for the cases treated before 1981, all patients received adjuvant chemotherapy including cisplatin or carboplatin before or after radiation therapy.

Immunohistochemical Analysis

Surgically resected tissues were fixed in 10% formalin and embedded in paraffin. Sections (4 µm thick) were deparaffinized, rehydrated, and incubated in hydrogen peroxide to block endogenous peroxidase activity in a standard manner. An anti-p53 monoclonal antibody, DO-7 (DAKO A/S, Copenhagen, Denmark), was diluted (1:50) and applied to sections with a microwave treatment for antigen retrieval. The sections were incubated for 1 hour at room temperature. An anti-p21^{WAF1/Cip1} monoclonal antibody, EA10 (Oncogene Research Products, Cambridge, MA), was diluted (1:10) and applied to sections with a heat treatment using a pressure cooker, and they were incubated for 18 hours at 4°C. A standard avidin-biotin complex (ABC) method was used according to the manufacturer's recommendations (Vectastain, Vector Laboratory, Inc., Burlingame, CA) and diaminobenzidine tetrahydrochloride was used to visualize the immunoreactivities. Slides were lightly counterstained with hematoxylin. For negative control, phosphate-buffered saline was used in place of

primary antibodies. Staining for p21^{WAF1/Cip1} was nuclear and intense in vascular smooth-muscle cells when present, which served as positive internal controls. Immunoreactivities were scored as absent, focal, or diffuse for both p53 and p21^{WAF1/Cip1} staining.

RESULTS

Analyses of Germinoma

Fifteen cases of germinoma, including eleven pure and four germinomas with STGCs, were analyzed. Diffuse nuclear staining for p53 protein was observed in each of the 15 germinomas analyzed. Positive staining was confined to tumor cells and was not present in infiltrating lymphocytes. Although the distribution of p53 immunoreactive cells was diffuse, some tumor cells showed prominent immunoreactivity (Fig. 1). There was no difference in immunoreactivity between pure germinomas and those with STGCs. All 15 cases of germinoma were negative for p21^{WAF1/Cip1} protein expression.

Fig. 1. Case 8. Photomicrograph showing expression of p53 protein in a germinoma. ABC, original magnification X 400.

Analyses of Immature and Mature Teratoma

We analyzed 11 cases of teratoma: nine immature and two mature. Positive immunoreactivity for p53 protein was observed in nine of the 11 tumors: seven of nine immature teratomas and both of the mature teratomas. Immunoreactivity for p53 was localized to tumor cells displaying differentiation into epithelia, whereas other tumor cells were negative for p53 expression (Fig. 2).

Fig. 2. Case 20. Photomicrograph showing expression of p53 protein confined to tumor cells displaying epithelial differentiation in an immature teratoma. ABC, original magnification X 400.

Among the nine teratomas immunoreactive for p53 protein, three of the tumors (two immature and one mature) showed positive immunoreactivity for p21^{WAF1/Cip1}. Both of the p53-negative teratomas were also negative for p21^{WAF1/Cip1} immunoreactivity. In total, three of 11 teratomas were positive for p21^{WAF1/Cip1} expression. Immunoreactivity for p21^{WAF1/Cip1} protein was strong in cells displaying epithelial differentiation, similar to that for the p53 protein, although some tumor cells in the mesenchymal component were also positive for p21^{WAF1/Cip1} expression (Fig. 3). A primary and recurrent tumor from a patient with an immature teratoma were included in this series of samples. Six months after surgery for the primary tumor followed by chemotherapy, the tumor recurred. The histological phenotypes were indistinguishable between the primary and recurrent tumors. Although both tumors were immunoreactive for p53 protein, immunoreactive cells for p21^{WAF1/Cip1} were observed in the recurrent tumor but not in the primary tumor.

Fig. 3. Case 20. Photomicrograph showing expression of p21^{WAF1/Cip1} protein in an immature teratoma (a recurrent tumor of Case 20). Immunoreactivity for p21^{WAF1/Cip1} protein was strong in cells displaying epithelial differentiation, similar to that for p53, although some tumor cells in

the mesenchymal component were also positive for p21^{WAF1/Cip1} expression. ABC, original magnification X 400.

Analyses of Mixed Tumor of Germinoma and Teratoma Components

Four tumors of mixed histology consisting of germinoma and teratoma components were analyzed. In each of the four cases, tumor cells in the germinomatous component were immunoreactive for p53, and the distribution and intensity of staining were similar to those in pure germinomas. In teratomatous components, two of the four mixed tumors showed immunoreactivity for p53 protein localized to tumor cells that displayed epithelial differentiation, similar to that seen in the pure teratomas. Immunoreactivity for p21^{WAF1/Cip1} protein was not detected in any components of the four mixed tumors analyzed.

Group of Malignant GCTs

Five cases of GCTs of the malignant group were investigated. Two choriocarcinomas, one yolk sac tumor, one recurrent tumor of embryonal carcinoma treated with radio- and chemotherapy, and one mixed tumor of embryonal carcinoma and germinoma components were included. All five of the malignant GCTs showed immunoreactivity for the p53 protein, and p21^{WAF1/Cip1} protein expression was positive in four of the five tumors. Both of the choriocarcinomas showed localized but strong immunoreactivity for both p53 and p21^{WAF1/Cip1} proteins as shown in Figs. 4 upper and lower, respectively. The yolk sac tumor showed diffuse immunoreactivity for the p53 protein and localized immunoreactivity for p21^{WAF1/Cip1} protein. The embryonal carcinoma component of the mixed tumor showed localized immunoreactivity for both p53 and p21^{WAF1/Cip1} proteins, whereas the germinomatous component was positive for p53 and negative for p21^{WAF1/Cip1} expression, similar to the pure germinomas. A recurrent embryonal carcinoma displayed localized immunoreactivity for p53 but not for p21^{WAF1/Cip1} protein.

Fig. 4. Case 31. Upper: Photomicrograph showing expression of p53 in a choriocarcinoma. ABC, original magnification X 400. Lower: Photomicrograph showing expression of p21^{WAF1/Cip1} in a choriocarcinoma. ABC, original magnification X 400.

Correlation Between Expression of p53 and p21^{WAF1/Cip1} Proteins and Treatment Results

Results of immunoreactivity for p53 and p21^{WAF1/Cip1} proteins and clinical treatment results of all 34 patients (35 tumors) are summarized in Table 1.

TABLE 1
EXPRESSION OF P53 AND P21^{WAF1/CIP1} PROTEINS AND CLINICAL RESPONSE TO TREATMENT IN 34 PATIENTS WITH INTRACRANIAL GCTs*

Case No.	Age (yrs), Sex	Tumor Histology	Tumor Location	Immunohistochemistry		Outcome†
				p53	p21	
1	13, M	germinoma	pineal	++	-	CR for 18 yrs‡
2	12, M	germinoma	pineal	++	-	CR for 13 yrs‡
3	27, M	germinoma	pineal	++	-	CR for 9 yrs
4	14, M	germinoma	pineal	++	-	CR for 7 yrs‡
5	16, M	germinoma	pineal	++	-	CR for 6 yrs
6	12, M	germinoma	pineal	++	-	CR for 6 yrs
7	19, M	germinoma	pineal	++	-	CR for 4 yrs
8	17, M	germinoma	pineal	++	-	CR for 9 yrs
9	12, M	germinoma	pineal	++	-	CR for 3 yrs‡
10	9, F	germinoma	neurohypophyseal	++	-	CR for 3 yrs
11	23, M	germinoma	pineal & neurohypophyseal	++	-	NA
12	9, M	germinoma w/ STGCs	neurohypophyseal	++	-	CR for 2 yrs
13	10, M	germinoma w/ STGCs	basal ganglia	++	-	CR for 2 yrs
14	14, M	germinoma w/ STGCs	pineal	++	-	dissem 2 yrs posttreatment
15	24, M	germinoma w/ STGCs	neurohypophyseal	++	-	recur 16 mos posttreatment
16	16, M	immature teratoma	pineal	+	-	CR for 7 yrs
17	9, M	immature teratoma	intraventricular	-	-	CR for 7 yrs
18	8, M	immature teratoma	pineal	+	-	CR for 5 yrs
19	14, M	immature teratoma	pineal	+	-	CR for 4 yrs
20	12, M	immature teratoma	pineal	+	-	recur 6 mos posttreatment§
		immature teratoma	pineal	+	+	CR for 3 yrs
21	9, F	immature teratoma	neurohypophyseal	-	-	NA
22	24, M	immature teratoma	neurohypophyseal	+	+	NA
23	3 mos, F	immature teratoma	cerebellar	+	-	NA
24	5, M	mature teratoma	pineal	+	+	CR for 17 yrs‡
25	3, M	mature teratoma	pineal	+	-	CR for 4 yrs**
26	12, M	germinoma	pineal	++	-	CR for 6 yrs
		w/immature teratoma		-	-	
27	9, M	germinoma	pineal	++	-	CR for 5 yrs
		w/immature teratoma		-	-	
28	20, M	germinoma	pineal	++	-	CR for 3 yrs
		w/immature teratoma		+	-	
29	18, M	germinoma	pineal	++	-	NA
		w/immature teratoma		+	-	
30	11, M	choriocarcinoma	pineal	+	+	refractory & progressive
31	20, M	choriocarcinoma	neurohypophyseal	+	+	NA
32	12, M	yolk sac tumor	pineal	++	+	dissem 8 mos posttreatment
33	24, M	embryonal carcinoma††	pineal	+	-	refractory & progressive
34	23, M	embryonal carcinoma	pineal	+	+	CR for 2 yrs
		w/germinoma		++	-	

* CR = complete remission; dissem = dissemination of tumor; NA = not applicable (follow-up period shorter than 1 year); recur = recurrence of tumor; - = absent; + = focal; ++ = diffuse.

† Radio- and chemotherapy administered after surgery except in cases indicated.

‡ Treated only by surgery and radiation therapy.

§ Recurrent tumor appeared during scheduled courses of chemotherapy after surgery.

|| Recurrent tumor 6 months after surgery and chemotherapy.

** No radio- or chemotherapy administered after surgery.

†† Recurrent tumor 9 years after surgery, radio-, and chemotherapy.

Among the 14 patients with germinoma with a follow-up period of longer than 1 year, two germinomas with STGCs recurred within 2 years. There were no differences in immunoreactivity for p53 and p21^{WAF1/Cip1} proteins in cases with relapse compared with those without relapse, as the expression pattern of both proteins was similar in all the germinomatous cases.

Eight of the patients with teratomas and three of the patients with mixed tumors of germinoma and teratoma components have been followed for more than 1 year after treatment. Among these 11 tumors, a single immature teratoma recurred in one patient 6 months after the initial surgery and chemotherapy. In the primary tumor, p53-immunoreactivity was present but localized, whereas p21^{WAF1/Cip1} immunoreactivity was absent. In the recurrent tumor from this patient, however, expression of p21^{WAF1/Cip1} protein was detected. This patient has been tumor-free for more than 3 years since receiving radio- and chemotherapy for the recurrent tumor. All of the other six patients with teratoma and three patients with mixed tumors of germinoma and teratoma components survived more than 3 years without tumor recurrence. Among these nine cases, p53 expression was not detected in one immature teratoma and two teratomatous components of mixed tumor; however, it was present in five teratomas, two teratomatous components of the mixed tumors, and all of the germinomatous components of the mixed tumors. In one case of mature teratoma, p21^{WAF1/Cip1} staining was positive. Therefore, we could not detect any significant correlation between treatment results and immunoreactivity for p53 or p21^{WAF1/Cip1} proteins in the teratomatous cases analyzed.

Among the five GCTs of the malignant group that were analyzed, four cases have been followed for more than 1 year. A patient with a mixed tumor of embryonal carcinoma and germinoma components has been without recurrence for more than 2 years posttreatment, whereas one choriocarcinoma and one recurrent embryonal carcinoma were refractory to treatment, and a yolk sac tumor recurred with spinal dissemination 8 months after initial treatment. Immunoreactivity for p53 protein was present in all four of these cases, whereas p21^{WAF1/Cip1} protein was present in three. The germinomatous component in the mixed tumor of embryonal carcinoma and germinoma was negative for p21^{WAF1/Cip1}, whereas the embryonal carcinoma component was positive for p21^{WAF1/Cip1} expression. This patient was the only one in this group to survive more than 2 years. One choriocarcinoma and one yolk sac tumor were immunoreactive for p21^{WAF1/Cip1} protein while a recurrent embryonal carcinoma was negative for p21^{WAF1/Cip1} expression, and all were refractory to treatment.

DISCUSSION

In this study, 33 (94%) of 35 intracranial GCTs were positive for p53 expression, consistent with previous studies on gonadal GCTs.[2,13,21,32,36,40] Although p53 overexpression in tumors is commonly associated with mutation of the **p53** gene, **p53** mutations have not been found in GCTs overexpressing p53 protein[9,13,29,32,36] except for a few intracranial tumors.[8] On the other hand, expression of p21^{WAF1/Cip1} protein, a downstream effector of the p53-mediated growth control in mammalian cells, was not detected in testicular GCTs.[13] Overexpression of wild-type p53 in tumor cells generally triggers cell cycle arrest or apoptosis.[20] Absence of p21^{WAF1/Cip1} expression in gonadal GCTs was suggested as one of the mechanisms by which tumor cells survive the growth-inhibitory pressure induced by accumulated wild-type p53 protein.[13,23] In contrast to the previous study[13] of testicular GCTs, we observed p21^{WAF1/Cip1} expression in seven (20%) of 35 intracranial GCTs: three (27%) of 11 teratomas and four (80%) of five GCTs of the malignant group. Frequent expression of p21^{WAF1/Cip1} was observed in various cancers including breast,[1] colon,[41] stomach,[10] pancreas,[4] and glial tumors.[17] In some cancers, including acute myelogenous leukemia, head-and-neck cancer, and breast cancer, elevated levels of p21^{WAF1/Cip1} expression were associated with increased resistance to chemotherapy as well as with short relapse-free survival.[1,7,46] In cancers of the pancreas, stomach, and uterus, p21^{WAF1/Cip1} expression was reported to indicate a better prognosis.[3,10,15] We did not observe a significant correlation between immunoreactivity for p21^{WAF1/Cip1} protein and treatment results in this study. However, our findings that none of the germinoma cases expressed p21^{WAF1/Cip1} protein and four of five GCTs of the malignant group expressed p21^{WAF1/Cip1} protein suggest that expression of p21^{WAF1/Cip1} in intracranial GCTs may be

correlated with decreased sensitivity to radio- or chemotherapy and poor prognosis. Clinical analyses for evaluating possible prognostic factors in GCTs may be biased by several factors, including the extent of surgical extirpation, the effect of previous radio- or chemotherapy, and histological heterogeneity of GCTs. For example, an embryonal carcinoma was negative for p21^{WAF1/Cip1} protein expression but refractory to treatment. However, this tumor had recurred after radio- and chemotherapy, and radiation may have downregulated the expression of p21^{WAF1/Cip1}. [27] On the other hand, a mixed tumor of embryonal carcinoma and germinoma components responded well to therapies, and the tumor has not recurred in the 2 years posttreatment. The section was composed of approximately 50% embryonal carcinoma and 50% germinoma, and p21^{WAF1/Cip1} expression was positive only in the embryonal carcinoma component. The germinomatous component must have responded well to treatment; however, a longer follow-up period is needed. Further studies in a larger patient population are necessary to elucidate whether immunoreactivity for p21^{WAF1/Cip1} protein could be a prognostic factor in intracranial GCTs.

In teratomas, immunoreactivity for p53 and p21^{WAF1/Cip1} proteins was mostly confined to tumor cells displaying epithelial differentiation. Overexpression of p53 protein in embryonic pulmonary and intestinal epithelia was observed. [37] Upregulation of p53 expression in early stages of mouse embryo development corresponds to each type of malignant GCT: upregulation of p53 expression in chorionic villi in the placenta corresponds to overexpression of p53 protein in choriocarcinoma; upregulation of p53 expression in yolk sac endoderm corresponds to overexpression of p53 in yolk sac tumor; and upregulation of p53 expression in all tissues of the embryo during early morphogenetic stages corresponds to overexpression of p53 in embryonal carcinoma. As embryonic development enters a phase of organogenesis and histogenesis, the p53 expression pattern becomes heterogeneous and restricted to certain specific tissues, such as lung, intestine, brain, liver, salivary gland, and kidney, which corresponds well to the restricted expression of p53 in teratoma cells displaying epithelial differentiation. Overexpression of p53 protein in intracranial GCTs can result from a developmental mistiming event, in which genes that are normally expressed in the embryonic period become abnormally upregulated in tumors. In addition, the testis is one of the few adult tissues in which positive staining for p53 has been observed, [38] which again corresponds to the expression of p53 in germinomas.

We found frequent p21^{WAF1/Cip1} expression in the most malignant types of GCTs, such as choriocarcinoma, yolk sac tumor, and embryonal carcinoma, and in a few cells displaying epithelial differentiation of teratomas. Upregulation of p21^{WAF1/Cip1} expression is linked to terminal differentiation of muscle, nasal, pulmonary, and intestinal epithelia, as well as other cell types. [6,28] Contrary to p53 expression, p21^{WAF1/Cip1} expression was not detected in the early embryonic stage of mouse development, before day 10 postfertilization, except for postmitotic muscle cells. [28] Therefore, p21^{WAF1/Cip1} expression in teratoma cells displaying epithelial differentiation may reflect their differentiation status, whereas that in GCTs of the malignant group may be a tumor-specific event.

Several basic issues remain to be solved. First, is the **p53** gene wild-type in intracranial GCTs? If the gene is wild-type, similar to that in gonadal GCTs, then how does wild-type p53 protein accumulate and how is p21^{WAF1/Cip1} expression downregulated in germinomas? Second, what is the biological significance of p21^{WAF1/Cip1} protein overexpression in the malignant group of intracranial GCTs? Further unraveling of the biological significance of p53 and p21^{WAF1/Cip1} expression in intracranial GCTs may lead to more appropriate and successful treatment modalities for tumor types that are now considered to be resistant to treatment.

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