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Investigation of Germline PTEN, p53, $p16^{INK4A}/p14^{ARF}$, and CDK4 Alterations in Familial Glioma

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Epidemiological studies suggest that some familial aggregations of glioma may be due to inherited predisposition. Many genes involved in familial cancers are frequently altered in the corresponding sporadic forms. We have investigated several genes known to be altered in sporadic gliomas for their potential contribution to familial glioma. Fifteen glioma patients with a family history of brain tumors were identified through the Mayo Clinic Department of Neurology (nine diffuse astrocytomas, two oligodendrogliomas, two mixed oligoastrocytomas, one pilocytic astrocytoma, and one pineal glioma). Eleven of the propositi had one or more first degree relative with a glioma. Lymphocyte DNA was derived from each of the patients and analyzed by polymerase chain reaction (PCR) and direct sequencing of the PTEN, p53, $p16^{INK4A}/p14^{ARF}$, and CDK4 genes. In addition, fluorescence in situ hybridization (FISH) was performed on EBV-transformed lymphocytes from each affected individual to detect germline copy number of the $p16^{INK4A}/p14^{ARF}$ tumor suppressor region. A p53 germline point mutation was identified in one family with some findings of Li-Fraumeni syndrome, and a hemizygous germline deletion of the $p16^{INK4A}/p14^{ARF}$ tumor suppressor region was demonstrated by FISH in a family with history of both astrocytoma and melanoma. Thus, whereas germ-line mutations of PTEN, p53, $p16^{INK4A}/p14^{ARF}$, and CDK4 are not common events in familial glioma, outside of familial cancer syndromes, point mutations of p53 and hemizygous deletions and other rearrangements of the $p16^{INK4A}/p14^{ARF}$ tumor suppressor region may account for a subset of familial glioma cases. Collectively, these data lend genetic support to the heritable nature of some cases of glioma. Am. J. Med. Genet. 92:136–141, 2000.

KEY WORDS: melanoma; tumor suppressor gene; familial glioma

INTRODUCTION

Gliomas are the most common primary central nervous system tumors of adults. These tumors may cluster in families, and an inherited susceptibility to glioma may be present in approximately 5–7% of these patients [Acqui et al., 1989; Cannon-Albright et al., 1994; Kleihues and Cavenee, 1997; Wrensch et al., 1997]. The molecular basis for the familial clustering of gliomas remains unknown, however, and our capacity to detect and appropriately monitor such susceptible individuals is limited.

In rare cases, gliomas may arise in kindreds with well-characterized cancer syndromes, including Li-Fraumeni syndrome, the neurofibromatoses, tuberous sclerosis, and Turcot syndrome [Kleihues and Cavenee, 1997]. Genes responsible for many of these inherited conditions have been identified and include p53, NF1, NF2, hMLH1, hPMS2, TSC1, and TSC2 [Hamilton et al., 1995; Malkin, 1994; Rouleau et al., 1993; The European Chromosome 16 Tuberous Sclerosis Consortium, 1993; van Slegtenhorst et al., 1997; Wallace et al., 1990]. Most patients with an apparent familial susceptibility to glioma, however, are not members of such families and thus are not accounted for by these alterations and syndromes.

Frequently, genes responsible for familial cancer syndromes also evidence alterations in corresponding sporadic tumors. Thus, characterization of the germline alterations found in familial cancers has proven useful in identifying somatic alterations in sporadic tumors and vice versa [Kamb et al., 1994; Liaw et al.,

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1997]. Several alterations important for the development of sporadic gliomas have been identified, including alterations of p53, PTEN, $p16^{INK4A}$/$p14^{ARF}$ (p16/p14), and CDK4. Further analyses have demonstrated the functional relevance of these genes. The p53, p16/p14, and CDK4 genes serve as critical cell cycle regulators, whereas PTEN is a dual lipid and protein phosphatase associated with cell growth and migration [Furnari et al., 1998; Kamb, 1995; Tamura et al., 1998]. To evaluate the potential contribution of the p53, PTEN, p16/p14, and CDK4 genes to familial glioma, we have analyzed these genes using direct sequencing and fluorescence in situ hybridization (FISH) in 15 glioma patients with a family history of brain tumors.

METHODS

Patients

Fifteen patients with glioma and a family history of brain tumors were selected for analysis (Table I). All patients provided informed consent, a detailed family history, and a blood sample. Of these patients, nine had diffuse astrocytic gliomas (three glioblastomas, four anaplastic astrocytomas, and two low grade astrocytomas), two had oligodendrogliomas, two had mixed oligoastrocytomas, one had a pilocytic astrocytoma, and one had a pineal glioma. Twelve patients had at least one first degree relative with a primary brain tumor, and the remaining three had at least one second degree relative with a primary brain tumor. Every effort has been made to designate the correct glioma subtype for these patients. Tumors for which specific subtype confirmation was not available have been designated as brain tumors that are not otherwise specified (Table I).

Mutational Analysis

Genomic DNA was prepared from EBV-transformed lymphocytes of 15 probands using a standard phenol/chloroform protocol. For each patient, mutational analysis was performed on all exons of the PTEN, p16, and p14 genes. In addition, mutational analysis was performed on exons 5-8 of the p53 gene and exon 2 of the CDK4 gene, regions corresponding to critical functional domains that have proven to harbor most sporadic and germline mutations [FitzGerald et al., 1996; Greenblatt et al., 1994; Kleihues and Cavenee, 1997; Louis, 1997; Soufir et al., 1998; Zariwala et al., 1994].

TABLE I. Summary of Brain Tumor Families*

Index patient			Other affected family members		
				Pathology	
Family no.	Age at diagnosis (yrs)	Pathology	Relation	Brain tumor	Other tumors
1	42	O	Father	GBM	
			Aunt	NOS	
			First cousin once removed	NOS	
2	64	GBM	Son	GBM	
			Son	GBM	
			Daughter	GBM	
			Father		Lung/gastric ca.
			Brother		Colon ca.
			Sister		Ovarian ca.
			Sister		Lung ca.
			Sister		Uterine ca.
3	40	AA	Father	GBM	
			Great aunt	NOS	
			First cousin once removed	NOS	
4	35	AA	Daughter	Ependymoma	
5	51	O	Mother	Astrocytoma	Breast ca.
6	55	GBM	Father	GBM	
7	64	PA, melanoma	Daughter	O	
8	44	AA	Father	GBM	
			First cousin once removed	NOS	
9	42	Astrocytoma	Sister	GBM	
			Nephew	NOS	
10	22	AA	Grandfather	GBM	
11	64	MOA	Brother	GBM	
			Nephew		Melanoma
			Grandson		Melanoma
12	66	GBM, melanoma	Brother	Astrocytoma	
			Sister	GBM	Melanoma
			Mother		Melanoma
			Nephew		Melanoma
13	41	MOA	Grandmother	NOS	
14	21	Pineal glioma	Grandmother	NOS	
			Great grandmother	NOS	
			Great aunt	NOS	
15	65	Astrocytoma	Son	NOS	

*AA: anaplastic astrocytoma; GBM: glioblastoma multiforme; O: oligodendroglioma; MOA: mixed oligoastrocytoma; PA: pilocytic astrocytoma; NOS: a brain tumor not otherwise specified; ca: carcinoma.

Exons were amplified by the polymerase chain reaction (PCR) using previously described primers and conditions [FitzGerald et al, 1996; Hussussian et al., 1994; Soufir et al., 1998; Steck et al., 1997; Zariwala et al., 1994]. After exonuclease I/shrimp alkaline phosphatase treatment, PCR products were manually sequenced using the ThermoSequenase kit (Amersham, Cleveland, OH).

FISH Analysis

The Expand Long Template PCR System (Boehringer-Mannheim, Indianapolis, IN) was used to amplify an 8 kb DNA fragment, mapped to the approximately 15 kb interval between exon 1α of p16 and exon 1β of p14, according to supplied protocol. PCR primer sequences were: 5′-CAAGAGATGAGATGCACGCTGC-3′ and 5′-ACCATAAAGAGACAGAAAGA-3′. The copy number of this probe was enumerated in patient samples using FISH as previously described [Smith et al., 2000]. Briefly, EBV-transformed lymphocytes from each patient were dropped onto glass slides, air dried, microwave treated in citrate buffer (pH 6.0) for 10 min, digested in pepsin solution (4 mg/mL in 0.9% NaCl, pH 1.5) for 15 min at 37°C, rinsed in 2× SSC at room temperature for 5 min, and air dried. Each sample was hybridized concomitantly with two probes, the p16/p14 probe labeled with digoxigenin and a chromosome 9 centromere probe labeled with SpectrumGreen (Vysis, Downers Grove, IL). Probes and target DNA were denatured simultaneously in an 80°C oven for 5 min, followed by overnight incubation at 37°C. Slides were then washed in 1.5 M urea/0.1× SSC at 45°C for 10 min (×3) and in 2× SSC at room temperature for 2 min. After washing, the digoxigenin-labeled probes were detected using a rhodamine detection kit (Oncor, Gaithersburg, MD). Nuclei were counterstained with 4,6-diamidino-2-phenylindole and the antifade compound *P*-phenylenediamine. A Zeiss Axioplan microscope equipped with a triple-pass filter (DAPI/Green/Orange; Vysis) was used to assess the number of FISH signals for each probe. Approximately 100 non-overlapping nuclei were enumerated per hybridization for all cases except Case 12, for which 300 non-overlapping nuclei were enumerated.

RESULTS

Mutational Analysis

We used direct sequencing to screen 15 families in which two or more members had brain tumors, most often diffuse gliomas (Table I). All 15 index cases had a glioma, and 11 of these had at least one first degree relative with a pathologic diagnosis of glioma. Two of these families had multiple members afflicted by malignant glioma, malignant melanoma, or both. The clinical findings in one of these families has been previously reported [Kaufman et al., 1993]. The index cases from each of the 15 families were screened for germ-line alterations in the PTEN, p16/p14, CDK4, and p53 genes.

The index case of Family 2 (Table I) exhibited a G-to-A transition at codon 248 of the p53 gene (Arg248Gln), a previously reported hot spot for point mutations [Kleihues and Cavenee, 1997]. No other germline alterations were identified in this patient. No germline mutations of the PTEN, p16/p14, CDK4, or p53 genes were identified by sequencing in any of the other patients.

FISH Analysis of the p16/p14 Region

An 8 kb PCR product from the p16/p14 tumor suppressor region was labeled and used for FISH. Analysis of EBV-transformed lymphocytes from each patient demonstrated one case (Table I, Family 12) with a germline hemizygous deletion of this region. Figure 1 shows a representative FISH image and probe enumeration for this patient. Notably, this patient has multiple relatives with malignant glioma, malignant melanoma, or both. Normal copy number of this probe was enumerated in each of the remaining probands.

DISCUSSION

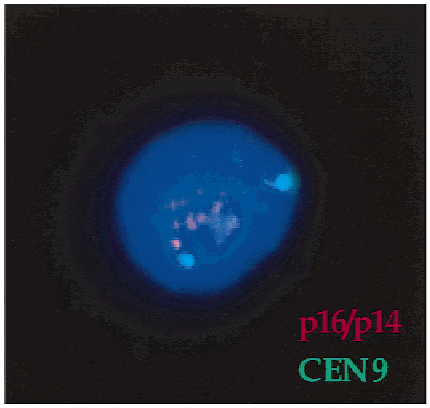
Multiple tumor suppressor genes demonstrate frequent alteration in sporadic gliomas. To investigate the potential contribution of a subset of these genes to familial glioma, we have examined p53, $p16^{INK4A}$/$p14^{ARF}$, CDK4, and PTEN for germline alteration in 15 glioma patients with a family history of brain tumors. The APC and Rb genes were not examined, given no history of familial polyposis or retinoblastoma in this set of patients.

We recognize that there is heterogeneity of tumor types within this collection of patients, however, this heterogeneity is representative of families with glioma that are encountered clinically. In addition, five of the glioma patients studied have a first degree relative with a different subtype of glioma, suggesting that initial predisposing genetic alterations may be shared by multiple glioma subtypes and further suggest that additional inherited or acquired alterations may account for intra-familial glioma subtype heterogeneity.

p53

The p53 tumor suppressor gene is the most frequently mutated gene in human cancer [Varmus and Weinberg, 1993] and serves multiple functions, including regulation of cell cycle and apoptosis. Sporadic gliomas frequently exhibit alterations of p53 [Chung et al., 1991; Rasheed et al, 1994; van Meyel et al., 1994]. For example, alterations of p53 have been identified in approximately 30% of both low and high grade astrocytomas, suggesting that abrogation of p53 function is an early and critical event in astrocytoma progression [Louis, 1997].

Because most sporadic and germline p53 mutations occur within the evolutionarily-conserved regions of exons 5–8 [Greenblatt, et al., 1994; Kleihues et al., 1997b; Louis, 1997], we, as have other investigators [Zariwala et al., 1994; Li et al., 1995], focused our screening efforts on these exons. Of the 15 patients, only the individual representing Family 2 (Table I) exhibited a germline point mutation in p53. Careful examination of the corresponding family history showed multiple first degree relatives with a variety of cancers, many of them occurring before the age of 45. The family history



CEN9 copy number	p16/p14 copy number				
	0	1	2	3	Total
0	0.0	07	0.3	00	10
1	03	10	07	00	20
2	03	940	2.3	00	966
3	0.0	04	00	00	04
Total	0.6	961	33	0.0	1000

Fig. 1. Probe enumeration and a representative FISH image are shown for familial glioma index Case 12. FISH was performed on EBV-transformed lymphocytes. The green signal maps to the centromere of chromosome 9 (CEN 9), and the red signal maps to the p16/p14 tumor suppressor gene cluster. Thus, the nuclei in the representative image exhibit two copies of centromere 9 and a single copy of the p16/p14 region, indicating germline hemizygous deletion of the latter. Probe enumerations are converted to percentages and represent a total of 300 nuclei. For example, 94 percent of the nuclei exhibited two CEN 9 signals and one p16/p14 signal. Probe enumeration for the remaining 6 percent of nuclei was likely distorted by background hybridization or overlapping nuclei.

and the germline mutation of p53 are suggestive of Li-Fraumeni syndrome, an autosomal dominant trait of multiple neoplasms, including brain tumors, in children and young adults [Kleihues et al., 1997b]. This case, however, does not represent a classical example of Li-Fraumeni syndrome, because it does not meet all of the criteria [Garber et al., 1991; Hisada et al., 1998; Li et al., 1988]; specifically, no family members developed a sarcoma.

$p16^{INK4A}/p14^{ARF}$

The p16 and p14 tumor suppressor genes share two of their three exons and both serve as critical cell cycle regulators, controlling the transition from the G_1 phase to the S phase. The p16 gene was identified based on familial melanoma linkage analyses and homozygous deletions in melanoma cell lines [Kamb et al., 1994] and was subsequently shown to exhibit germline point mutations or microdeletions in a significant proportion of familial melanoma cases [FitzGerald et al., 1996; Platz et al., 1997]. In addition, p16 alterations have been demonstrated in several other sporadic cancers, including most high grade astrocytomas in which inactivation is usually achieved through homozygous deletion [Perry et al., 1997; Ueki et al., 1996].

Using an 8 kb FISH probe mapped to the p16/p14 region, we identified one case (Table I, Family 12) with a germline hemizygous deletion. This family has been studied previously and has been suggested to harbor a deletion in this region using quantitative PCR and linkage [Bahuau et al., 1998]. Thus, the present analysis has confirmed the presence of this hemizygous deletion using FISH. Further efforts to map the extent of this deletion are currently underway. We observed no other alterations of p16/p14.

Notably, Family 12 has multiple members with malignant astrocytoma, malignant melanoma, or both malignancies. Although germline alterations of p16 are frequently seen in familial melanoma, they have not been previously associated with familial glioma [Gao et al., 1997]. Furthermore, because an increased incidence of glioma has not been reported among the plethora of melanoma families with germline mutations of p16, it is unlikely that germline alterations specifically targeting this gene are responsible for the phenotype of Family 12. Interestingly, approximately 10% of mice specifically deficient for $p19^{ARF}$, a mouse homologue of $p14^{ARF}$, but retaining intact p16 activity develop tumors of the nervous system, predominantly gliomas [Kamijo et al., 1999]. Collectively, these data suggest that the germline deletion exhibited in case 12 may target both p16 and p14, because the FISH probe maps to the region between exon 1α and exon 1β. Thus, p16 and p14 may contribute to the phenotype of this family, the former increasing susceptibility to melanoma and the latter increasing susceptibility to glioma. Alternatively, the deletion may specifically target p14 that may increase susceptibility to both of the malignancies observed in Family 12.

CDK4

The CDK4 gene promotes cell cycle progression by phosphorylating the retinoblastoma protein [Kamb, 1995]. Germline point mutations of CDK4, presumed to be activating mutations that drive the cell cycle, have been identified in a limited number of familial melanoma cases [FitzGerald et al., 1996; Soufir et al., 1998; Zuo et al., 1996], whereas amplification of CDK4, that also drives the cell cycle, is observed in a variety of sporadic cancers, including high grade astrocytomas [He et al., 1995]. Notably, CDK4 kinase activity is inhibited when bound to p16 [Kamb, 1995], consistent with the observation that p16 and CDK4 alterations seem to have an inverse correlation [FitzGerald et al., 1996; He et al., 1995; Soufir et al., 1998; Zuo et al., 1996]. Our CDK4 screening efforts focused on exon 2, because all germline mutations of the CDK4 gene identified to date have been confined to this exon [FitzGerald et al., 1996; Soufir et al., 1998; Zuo et al., 1996]. Because no alterations of CDK4 were identified in the

15 familial glioma probands analyzed, it is unlikely that germline point mutations of this gene are a common etiology of familial glioma.

PTEN

Recently, the new tumor suppressor gene, PTEN, that encodes a dual lipid-protein tyrosine phosphatase, was identified [Li et al., 1997; Steck et al., 1997] and shown to be frequently inactivated in high grade gliomas through mutation or homozygous deletion [Liu et al., 1997]. Moreover, germline mutations of the PTEN gene have been shown in patients with Cowden disease, that is characterized by multiple hamartomas in a variety of organs [Liaw et al., 1997]. Germline PTEN mutations have also been identified in Bannayan-Riley-Ruvalcaba syndrome and juvenile polyposis coli, that share juvenile intestinal polyps as a common feature [Marsh et al., 1997; Olschwang et al., 1998]. Consistent with a report by Laugé et al. [1999], however, we did not identify germline alterations of PTEN in any probands from families with gliomas, suggesting that PTEN is not frequently involved in familial glioma.

Our results suggest that germline mutations of the p53, p16/p14, CDK4, and PTEN genes are not frequently associated with familial glioma. A p53 point mutation was identified in one of the index cases from 15 families with multiple brain tumors. Although this family exhibited a broad range of cancers, there were no reports of sarcoma, and thus this case does not represent classical Li-Fraumeni syndrome. Another family with multiple glioma demonstrated a germline deletion in the p16/p14 region. Interestingly, this family was multiply afflicted with both glioma and melanoma, suggesting complex involvement of this tumor suppressor region in the development of both malignancies. Collectively, these data lend genetic support to the hereditary nature of some glioma cases.

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