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Mutation and deletion analysis of GFRα-1, encoding the co-receptor for the GDNF/RET complex, in human brain tumours

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Summary Glial cell line-derived neurotrophic factor (GDNF) plays a key role in the control of vertebrate neuron survival and differentiation in both the central and peripheral nervous systems. GDNF preferentially binds to GFRα-1 which then interacts with the receptor tyrosine kinase RET. We investigated a panel of 36 independent cases of mainly advanced sporadic brain tumours for the presence of mutations in *GDNF* and *GFRα-1*. No mutations were found in the coding region of *GDNF*. We identified six previously described *GFRα-1* polymorphisms, two of which lead to an amino acid change. In 15 of 36 brain tumours, all polymorphic variants appeared to be homozygous. Of these 15 tumours, one also had a rare, apparently homozygous, sequence variant at codon 361. Because of the rarity of the combination of homozygous sequence variants, analysis for hemizygous deletion was pursued in the 15 samples and loss of heterozygosity was found in 11 tumours. Our data suggest that intragenic point mutations of *GDNF* or *GFRα-1* are not a common aetiologic event in brain tumours. However, either deletion of *GFRα-1* and/or nearby genes may contribute to the pathogenesis of these tumours.

Keywords: *GDNF*; *GDNFRα*; *GFRα-1*; *RET*; brain tumours

Several receptor molecules, and their growth factor ligands, are expressed in the embryonic brain and are thought to play central roles in the development and differentiation of the nervous system (Weiner, 1995). In addition, altered function, or loss of function, of either receptor or ligand may lead to the development of malignancies of the nervous system (Weiner, 1995). Oncogenes (e.g. the gene encoding the epidermal growth factor receptor, *EGFR*) as well as tumour suppressor genes (e.g. *p53*, *PTEN*) are known to be involved in the development of brain tumours (Bogler et al, 1995; von Deimling et al, 1995; Wang et al, 1997; Duerr et al, 1998; Peters et al, 1998). Members of one broad class of growth factor receptors, the receptor tyrosine kinases (RTKs), have been shown to be involved in proliferation and differentiation during central nervous system (CNS) development (Weiner, 1995). The *RET* proto-oncogene encodes a RTK expressed in neural and neuroendocrine tissues (Takahashi and Cooper, 1987). To date, three related ligands for RET have been identified: glial cell line derived neurotrophic factor (GDNF), neurturin (NTN) and persephin (Durbec et al, 1996; Jing et al, 1996; Kotzbauer et al, 1996; Treanor et al, 1996; Trupp et al, 1996; Vega et al, 1996; Sanicola et al, 1997; Milbrandt et al, 1998). The receptor complex for GDNF, NTN and persephin comprises one of at least four membrane-bound adaptor molecules and RET (Jing et al, 1997; Thompson et al, 1998). GDNF preferentially binds GFRα-1 (*G*DNF *F*amily *R*eceptor alpha one, also known as GDNFR-α, RETL1 and TrnR1) with high affinity before this complex can interact with RET to effect downstream signalling (Jing et al, 1996; Treanor et al, 1996; Trupp et al, 1996; Vega et al, 1996; Davies et al, 1997; Sanicola et al, 1997). Similarly, NTN binds a membrane-bound adaptor GFRα-2 (related to GFRα-1 and also known as GDNFR-β, NTNR-α, RETL2 and TrnR2) with subsequent binding of RET (Baloh et al, 1997; Buj-Bello et al, 1997; Klein et al, 1997; Sanicola et al, 1997). GDNF can bind GFRα-2 as well, but with lower affinity, just as NTN can also bind GFRα-1 (Jing et al, 1997; Sanicola et al, 1997). A third co-receptor belonging to the same family, GFRα-3, has been identified, although formal binding studies have yet to be reported (Jing et al, 1997; Naveilhan et al, 1998; Worby et al, 1998). Recently, a fourth co-receptor, GFRα-4, was isolated that seems to be more closely related to GFRα-1 and GFRα-2 than to GFRα-3 (Thompson et al, 1998). Together with RET, GFRα-4 forms a functional receptor complex for persephin (Enokido et al, 1998).

Mice lacking RET or GDNF have been shown to have defects in the enteric nervous system and components of the peripheral nervous system (Schuchardt et al, 1994; Moore et al, 1996; Pichel et al, 1996; Sanchez et al, 1996). GDNF, like NTN, was initially isolated due to its ability to sustain the survival of embryonic dopaminergic neurons in vitro (Lin et al, 1993). In vivo studies subsequently demonstrated that GDNF was a target-derived trophic factor for dopaminergic neurons (Stromberg et al, 1993; Hudson et al, 1995; Tomac et al, 1995). In several animal models of Parkinson's

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disease, a disease in which dopaminergic neurons degenerate, treatment with GDNF has a protective effect (Lindsay, 1995; Moore et al, 1996). GDNF is, therefore, thought to play a key role in the control of vertebrate neuron survival and differentiation in both the central and peripheral nervous systems (Pichel et al, 1996).

Taking these data together, *GDNF* and *GFRα-1* appear to represent good targets for mutations which may play a pathogenetic role in the development of brain tumours. Additionally, the localization of *GFRα-1* to 10q26 (Gorodinsky et al, 1997; Eng et al, 1998), a region known to be somatically deleted at high frequency in malignant human brain tumours (Fults and Pedone, 1993), further supports its candidacy as a brain tumour gene.

MATERIALS AND METHODS

Patient samples

A panel of 36 sporadic brain tumours of various histologies and WHO grades (Table 1), from 36 unrelated German patients, was analysed. Tumour DNA was extracted from fresh frozen tissue and corresponding germline DNA from peripheral blood leucocytes, using standard protocols (Sambrook et al, 1989).

Mutation analyses

Polymerase chain reaction (PCR) conditions and primers to amplify *GDNF* have been described (Dahia et al, 1997; Marsh et al, 1997). PCR of *GFRα-1* was carried out using 1 μM each of forward and reverse primer pairs (see below) in 1 × PCR buffer (Perkin-Elmer Corp.), 200 μM dNTP, 2.5 U *Taq* polymerase (Perkin-Elmer Corp.), TaqStart™ Antibodies (Clontech, San Francisco, CA, USA), and 100–200 ng of DNA template in a final volume of 50 μl. Reactions were subjected to 40 cycles of 94°C for 1 min, 58–62°C for 1 min and 72°C for 1 min followed by 10 min at 72°C. The PCR amplicons were then fractionated on 1% low melting point agarose (Bio-Rad Lab., Hercules, CA, USA) and visualized with UV transillumination after ethidium bromide staining. Before sequencing, these products were column purified (Wizard PCR Prep, Promega, Madison, WI, USA). Semi-automated sequencing was performed using either the forward or reverse primer and the ABI dye terminator cycle sequencing ready reaction kit as previously described (Liaw et al, 1997).

GFRa-1 primers

Primers used to amplify *GFRα-1* for sequencing are:

Exon 1: RA-1F (5′-GTCGGACCTGAACCCCTAAAA-3′) (f)
RA-1R (5′-CCAAAAAGAAACTTCTTCCTTCC-3′)(r)
Exon 2: RAF22 (5′-GCAGACTTGCTCCTGTCGGC-3′) (f)
RAF7 (5′-CGCACGCTAAGGCAGTGCGT-3′) (f)
HRAR2N (5′-GGCTCTGGTACATGCTCCAGT-3′) (r)
HRAR2 (5′-CTGGTACATGCTCCAGTA-3′) (r)
Exon 3: RA-3F (5′-CAGCAAAAACCTGCTTGAAATA-3′) (f)
RAI-3F (5′-CAGCAAAAACCTGCTTGAAA-3′) (f)
RA-3R (5′-TGCCTCTTCATTATCATCATCCT-3′) (r)
RAI-3R (5′-TTCAAGCACACAAAGGCATC-3′) (r)
Exon 4: RAI-4F (5′-TGTGACCATGCCTGTCTTTC-3′) (f)
RAI-4R (5′-TCATTAATCACCAGCTGCCA-3′) (r)
Exon 5: RAI-5F (5′-CCCCACCCTTTTTCCTATTG-3′) (f)
RAI-5R (5′-CAGGCATGTCCTCAAGGATT-3′) (r)

Table 1 WHO histologic classification of the 36 brain tumours

	WHO grade	*n*
Glioblastoma multiforme	IV	18
Glioblastoma multiforme (recurrence)	IV	3
Oligoastrocytoma anaplastic	III	1
Astrocytoma anaplastic (recurrence)	III	1
Ependymoma		1
Ependymoma anaplastic (recurrence)	III	1
Malignant peripheral nerve sheath tumour (MPNST)		1
Meningioma atypical		2
Meningioma anaplastic	III	1
Medulloblastoma	IV	2
Medulloblastoma (recurrence)	IV	2
Haemangiopericytoma		1
Primitive neuroectodermal tumour (PNET, recurrence)	IV	2

Exon 6: GRI-1128-1F (5′-CTCAAGATAAATTGCCGA-GAAAAT-3′) (f)
RAI-6F (5′-GGCCATGGAAAAGTATCATCA-3′) (f)
GRI-1261-1R (5′-TACAGGCACAAGGTACAA-GAGGTA-3′) (r)
RAI-6R (5′-CTGGAGCTCGGAGAAGAAAA-3′) (r)
Exon 7: RAI-7F (5′-CGTTTGCTGCTTGACTTTGA-3′) (f)
RAI-7R (5′-GGAATCTGGACGCAGTTCTC-3′) (r)
Exon 8: RAI-8F (5′-TTTTTCTTGTCCCTCTCCAG-3′) (f)
RAR13 (5′-TCTATAAATGCACGAAGCCT-3′) (r)
Exon 9: GRI-1549-IF (5′-GCAGTGATGATAATGAAAC-CATTC-3′) (f)
GRA20R (5′-TTTTTCATGTCCATATTG-TATTTTT-3′) (r)

Deletion analysis

DNA samples derived from brain tumours which were apparently homozygous for all *GFRα-1* polymorphisms underwent further analysis to determine if this represented hemizygous whole gene deletion. Corresponding germline DNA was examined for the presence of heterozygosity at the intragenic polymorphic sites that were apparently homozygous either by direct sequencing or differential restriction enzyme digestion. If the germline DNA was heterozygous for any one sequence variant, that tumour was defined as having loss of heterozygosity (LOH) of that marker, representing hemizygous gene deletion. If all intragenic polymorphisms were also homozygously present in the germline DNA, the result was not informative. In those cases, we performed a semi-quantitative duplex PCR using the tumour-derived DNA and primers for exon 2 of *GFRα-1* (RAF7, HRAR2) and those for the housekeeping gene beta-glucuronidase (GUSB) (Ivanchuk et al, 1997). The relative amount of the *GFRα-1* fragments versus that of *GUSB* were determined by visual inspection and densitometric scanning using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

RESULTS AND DISCUSSION

We analysed *GDNF* and *GFRα-1* for DNA sequence variants in a panel of 36 mainly high-grade human brain tumours by direct sequencing. No mutations were found in the coding region of *GDNF*. Analysing *GFRα-1*, we detected six distinct single nucleotide polymorphisms (Table 2) that have been previously

Table 2 *GFRα-1* germline frequencies of sequence variants in patients with brain tumours, HSCR disease and control DNA (Myers *et al*., 1999)

Amino acid change	Nucleotide		Frequency		
			Brain tumours	HSCR	Control
N/A	–106	G	0.93	0.96	ND
		A	0.07	0.04	ND
N/A	–78	T	0.67	0.69	0.68
		C	0.33	0.31	0.32
Y85N	253	T	0.96	0.98	0.96
		A	0.04	0.02	0.04
N179N	537	T	0.54	0.60	0.59
		C	0.46	0.40	0.41
N/A	IVS5+21	G	0.85	0.92	0.82
		A	0.15	0.08	0.18
T361A	1081	A	0.91	0.93	0.88
		G	0.09	0.07	0.12
N/A	IVS8+28	T	0.97	ND	ND
		A	0.03	ND	ND

N/A = not applicable, ND = not done.

found in the normal population (Myers et al, 1998). Two of these were in the 5′ untranslated region 106 bp (–106G>A) and 78 bp (–78T>C) upstream of the translational start site. One silent base-pair substitution at codon 179 (c.537T>C) and a non-coding sequence polymorphism within intron 5 (IVS5+21G>A) were also observed. The remaining two polymorphisms were in exon 2 and exon 7 and resulted in amino acid substitutions (Y85N, 253T>A; T361A, 1081A>G). For codon 85, both the amino acids tyrosine and asparagine are neutral and polar. However, tyrosine is an aromatic, polar residue while the polymorphic asparagine is small and non-aromatic. Similarly, the substitution of threonine (neutral and polar) with alanine (neutral and hydrophobic) at codon 361 could also change the structure of GFRα-1. In those cases harbouring rare and/or apparently homozygous polymorphisms that lead to amino acid substitutions, it is conceivable that the stearic structure, and presumably, function, of GFRα-1 could be subtly altered such that the other co-receptors of RET (e.g. GFRα-2 or GFRα-3) may bind preferentially to GDNF and RET, thus leading to altered activation and/or specificity. We, therefore, examined the frequency of each of the *GFRα-1* single nucleotide polymorphisms in this series of patients with brain tumours and found them to be no different from those in normal controls or in non-cancer patients (Myers et al, 1999).

In addition to the six previously described single nucleotide polymorphisms, we also detected a homozygous non-coding sequence variant within intron 8, 28 basepairs downstream of the exon 8–intron 8 boundary (IVS8+28T>A) in the brain tumours of two unrelated patients (one glioblastoma multiforme, one anaplastic oligoastrocytoma). Interestingly, these two samples, together with 13 others, appeared to be homozygous at all six polymorphic sites. Further, the glioblastoma multiforme with the IVS8 sequence variant was apparently homozygous for the rare polymorphism at codon 361 (Table 2). Subsequent analyses of these 15 samples revealed somatic hemizygous deletion of *GFRα-1* in 11 of these 15 tumours (73% of 15; 31% of total), including both tumours with the intronic sequence variant.

In the present study, we did not find any obvious disease-associated somatic *GDNF* or *GFRα-1* intragenic mutations in DNA from human brain tumours from 36 individuals. We found seven single nucleotide polymorphisms within the genomic sequence of *GFRα-1* in this series, six of which have been previously noted (Myers et al, 1999). Of interest, we identified a novel intronic sequence variant in IVS8 in two patients. Our results indicate that intragenic mutations of *GDNF* and *GFRα-1* are not common aetiologic events in brain tumorigenesis. However, we did find that 31% of the 36 brain tumours had hemizygous *GFRα-1* deletion. This datum may support either of two postulates. First, it might well be possible that hemizygous deletion of *GFRα-1* is aetiologic in the pathogenesis of brain tumours. Second, the deletion of *GFRα-1* might be merely coincidental, an innocent bystander when large segments of chromosome 10q become deleted (Dalrymple et al, 1995; Simon et al, 1995; Albarosa et al, 1996). Putative tumour suppressor genes like *PTEN* or *DMBT* have been mapped in this region (Li et al, 1997; Mollenhauer et al, 1997; Steck et al, 1997; Duerr et al, 1999).

While it is obvious that 'high penetrance' mutations of *GDNF* and *GFRα-1* are not associated with brain tumorigenesis, it is becoming more and more evident that development of a cancer can result from an interplay of either a few 'high penetrance' mutations in key genes or from several, or many, sequence variants of unknown significance (Storey et al, 1998). In this respect, variant–variant interactions and/or variant–environment interactions may all be involved in predisposing to many common tumours. It is, therefore, intriguing that a few of these sequence variants in *GFRα-1* involve amino acids (Y85N, T361A) that are highly conserved among species (rat, chicken and human) [Genbank accession #U90541, #U59486, #U97144]. Further informatics-based and functional studies need to be performed to investigate whether these 'polymorphic' amino acid changes and seemingly neutral sequence variants have any impact on the function of this receptor.

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