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EXPRESS COMMUNICATION

**Christiane B. Knobbe · Julia Reifenberger**
**Guido Reifenberger**

# Mutation analysis of the Ras pathway genes *NRAS*, *HRAS*, *KRAS* and *BRAF* in glioblastomas

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**Abstract** Aberrant activation of Ras signaling is a common finding in human glioblastomas. To determine the contribution of Ras gene mutations to this aberration, we screened 94 glioblastomas for mutations in the three Ras family genes *NRAS*, *KRAS* and *HRAS*. All tumors were additionally analyzed for mutations in *BRAF*, which encodes a Ras-regulated serine/threonine kinase with oncogenic properties. Mutation analysis of the entire coding regions of *NRAS* and *KRAS*, as well as the known mutation hot-spot sites in *HRAS*, identified somatic point mutations in two glioblastomas, both affecting codon 12 of *NRAS* (c.35G > A, p.G12D). Three additional tumors carried *BRAF* mutations altering the known hot-spot codon 599 (c.1796T > A, p.V599E). None of these five glioblastomas showed amplification of the *EGFR* or *PDGFRA* genes, while three of the tumors, including two with *NRAS* and one with *BRAF* mutation, demonstrated *PTEN* missense mutations or loss of *PTEN* mRNA expression. Taken together, our data suggest activating mutations in *NRAS* or *BRAF* as a molecular alteration that contributes to aberrant Ras signaling in a small fraction of glioblastomas.

**Keywords** *NRAS* · *BRAF* · Glioblastoma · Mutation

## Introduction

Glioblastomas are the most frequent and most malignant primary brain tumors. Molecular studies have implicated alterations in different pathways in the pathogenesis of glioblastomas [21]. For example, the vast majority of glioblastomas carry genetic changes resulting in a loss of p53- and pRb1-dependent control mechanisms, such as mutation of *TP53*, homozygous deletion of *CDKN2A* and $p14^{ARF}$, as well as amplification of *CDK4*, *MDM2* or *MDM4* [21]. In addition, glioblastomas frequently carry genetic changes leading to aberrant signaling via the Ras and Akt pathways, including *EGFR* and *PDGFRA* amplification, *PTEN* mutation, and *CTMP* hypermethylation [14, 15]. At the protein level, activation of these pathways has been corroborated by the finding of high levels of Ras-GTP and frequent phosphorylation of Akt in glioblastomas [10, 11, 15]. The importance of aberrant Ras signaling for glial tumorigenesis is also supported by experimental data obtained in transgenic mice showing that expression of oncogenic Ha-Ras in astrocytes results in the development of astrocytic gliomas [8]. In addition, combined activation of Ras and Akt in murine neural progenitor cells has been reported to induce highly malignant gliomas histologically resembling glioblastomas [11].

The Ras proteins are highly homologous small G-proteins with GTPase activity that mediate the cellular response to external growth stimuli by signaling via different effector cascades [1, 18]. A major mechanism of Ras-induced oncogenic transformation is related to an enhanced mitogen-activated kinase (MAPK) pathway signaling caused by Ras-dependent activation of Raf serine/threonine-specific kinases. In addition, signal transduction via other pathways, such as the phosphatidylinositol 3-kinase (Pi3k) and the Ral guanine nucleotide exchange factors (Ral-GEFs) cascades, may be important for Ras-induced transformation [1, 18].

Oncogenic point mutations in the three human Ras genes (*NRAS*, *KRAS*, *HRAS*) have been detected in a wide variety of human cancers, with carcinomas of the pancreas, colon, thyroid, and lung showing the highest incidence [2]. More recently, activating mutations in the Ras effector protein Braf were identified as frequent genetic aberrations in malignant melanomas [7, 22] and

C. B. Knobbe · G. Reifenberger (✉)
Department of Neuropathology, Heinrich-Heine-University, Moorenstrasse 5, 40225 Düsseldorf, Germany
E-mail: reifenberger@med.uni-duesseldorf.de
Tel.: +49-211-8118660
Fax: +49-211-8117804

J. Reifenberger
Department of Dermatology, Heinrich-Heine-University, Moorenstrasse 5, 40225 Düsseldorf, Germany

in different types of carcinomas [6, 13, 20]. Here we report on the systematic analysis of 94 human glioblastomas for mutations in the *NRAS*, *KRAS*, *HRAS* and *BRAF* genes. All tumors were additionally investigated for *NRAS*, *KRAS* and *HRAS* expression at the mRNA level. Our findings indicate that *NRAS* and *BRAF* are aberrantly activated by point mutations in a small subset of glioblastomas.

## Materials and methods

### Tumor samples

We investigated 94 primary glioblastomas, including 74 classic glioblastomas, 6 giant cell glioblastomas and 14 gliosarcomas. From one patient, the primary tumor and a recurrent tumor were investigated. The tumors were from 43 female and 50 male patients (mean age at operation 55.3 years; range 10–83 years). All tumors were selected from the archives at the Department of Neuropathology, Heinrich-Heine-University, Düsseldorf, Germany. Peripheral blood samples for extraction of leukocyte DNA were available from 53 patients.

### DNA and RNA extraction

Extraction of high molecular weight DNA and RNA from frozen tumor samples was carried out by ultracentrifugation as reported elsewhere [25]. Extraction of DNA from peripheral blood leukocytes was performed according to a standard protocol [23].

### cDNA synthesis

From each tumor, 3 μg total RNA were reverse-transcribed into cDNA in a volume of 50 μl using random hexanucleotide primers and Superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany).

### Single-strand conformation polymorphism/heteroduplex analysis and DNA sequencing

The entire coding regions of *NRAS* and *KRAS* were amplified from cDNA templates, each in three overlapping fragments. In case of *HRAS*, we amplified the first exon from genomic DNA. This exon encodes the part of the protein that contains the three mutation hot spot codons 12, 13 and 61, respectively. For mutation analysis of *BRAF*, exons 11 and 15 were amplified from genomic DNA using primers binding to intronic flanking sequences. Exon 11 encodes a protein segment including the G loop ATP-binding region at amino acid residues 463–468. Exon 15 encodes the mutation hot spot region involving residues 594–599 within the Braf kinase domain. The respective primer sequences were reported elsewhere [22]. PCR products were heat-denatured and then subjected to electrophoresis on non-denaturing polyacrylamide gels using at least two different running conditions, with variations in polyacrylamide concentration and/or temperature (room temperature versus 4°C). Single-strand conformation polymorphism (SSCP)/heteroduplex band patterns were visualized by silver staining. PCR products showing aberrant band patterns were sequenced in both directions using cycle-sequencing (BigDye cycle sequencing kit, Applied Biosystems, Foster City, CA) and an ABI PRISM 377 semi-automated DNA sequencer (Applied Biosystems). Somatic origin of mutations was confirmed by sequencing the respective constitutive (leukocyte) DNA, when available.

### *Hpa*II restriction enzyme digestion

*Hpa*II restriction enzyme digestion was performed as an additional method to detect mutations affecting the *HRAS* hot spot codon 12. Exon 1 of *HRAS* was amplified, and PCR products were digested with *Hpa*II for 12 h at 37°C, then separated on agarose gels and visualized under ultraviolet light after ethidium bromide staining.

### Expression analyses at the mRNA level

Duplex reverse transcription-PCR analyses were carried out to determine the expression of *HRAS*, *NRAS* and *KRAS* transcripts. The mRNA expression levels of these genes were normalized to the mRNA expression level of the housekeeping gene γ-actin (*ACTG1*). Different non-neoplastic brain samples from patients operated on for chronic epilepsy (temporal lobe, white and gray matter), as well as samples from two autopsy cases were used as non-neoplastic reference tissues.

## Results and discussion

We performed a systematic mutation analysis of the *NRAS*, *HRAS*, *KRAS* and *BRAF* genes in 94 human glioblastomas. Mutational analysis of *NRAS* identified one glioblastoma (GB105D) and one gliosarcoma (GS22D) that both harbored a point mutation resulting in the substitution of glycine to aspartic acid at codon 12 (c.35G > A, p.G12D) (Fig. 1a, b). *NRAS* codon 12 is a known mutation hot spot that is commonly altered in various human cancers. According to the online catalogue of somatic mutations in cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/), the p.G12D mutation is the second most common *NRAS* missense mutation in human cancers.

None of the 94 investigated glioblastomas demonstrated evidence for mutations in *KRAS* and *HRAS* by SCCP/heteroduplex analysis. In addition, agarose gel

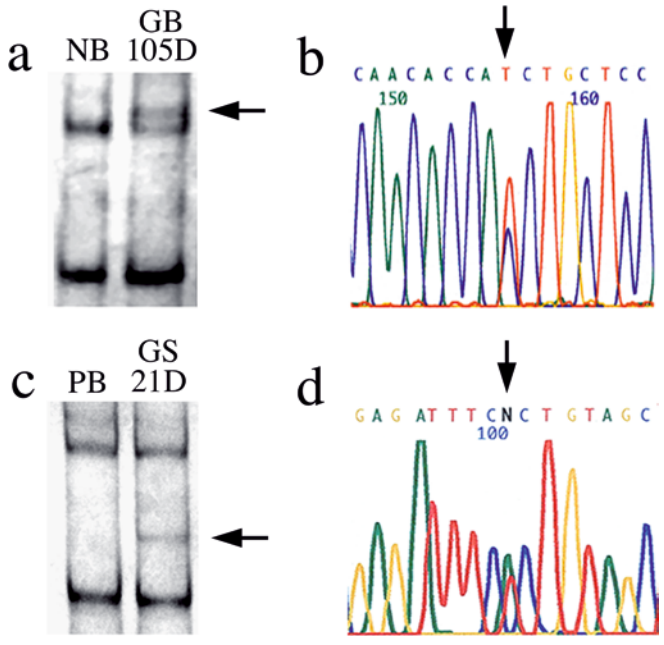


**Fig. 1** **a** SSCP/heteroduplex analysis showing an aberrant band pattern of the PCR product amplified from exon 1 of *NRAS* in tumor GB105D (*arrow*) (*NB* non-neoplastic brain). **b** DNA sequencing of the same fragment revealed a point mutation at nucleotide 35 (c.35G > A, p.G12D). The *arrow* indicates the mutation site (shown is the sequence of the non-coding strand). **c** SSCP/heteroduplex analysis demonstrating an aberrant band pattern for *BRAF* exon 15 in tumor GS21D (*arrow*) [*PB* peripheral blood (leukocyte) DNA]. **d** DNA sequencing identified a point mutation (*arrow*) affecting codon 599 (c.1796T > A, p.V599E). The figure shows the sequence of the non-coding strand

electrophoresis of *Hpa*II restriction digestion products derived from *HRAS* exon 1 did not reveal any alterations. Our findings are in line with previous data from a smaller series of glioblastomas, which did not detect any Ras gene mutations [9]. Similarly, an investigation of 9 radiation-induced astrocytomas found no *KRAS* mutations [3]. Other authors screened 46 pediatric brain tumors for Ras gene mutations and detected activating *KRAS* mutations in three astrocytomas, while *NRAS* and *KRAS* mutations were absent [17].

To investigate our glioblastoma series for potential overexpression of the Ras family genes at the mRNA level, we performed duplex reverse transcription-PCR analyses. These experiments revealed mRNA expression ratios that were approximately equal to non-neoplastic brain tissue in most cases, with only individual tumors showing slightly elevated levels up to 2.5-fold relative to the reference tissue. None of the glioblastomas showed a marked overexpression of *NRAS*, *HRAS* or *KRAS* transcripts that would be indicative of gene amplification. In line with these data, other authors did not detect any amplification of *NRAS* or *HRAS* in astrocytic gliomas [4, 16].

Despite the rare or absent mutation of *NRAS*, *HRAS* and *KRAS* in glioblastomas, aberrant activation of Ras signaling is common in these tumors. Guha et al. [10] showed that the majority of glioblastomas display elevated levels of Ras-GTP, the active form of Ras proteins. Data from transgenic mice indicate that activation of Ras, either alone or in combination with other oncoproteins, can cause the development of astrocytic gliomas [8, 11]. In addition, inhibition of Ras signaling has been shown to inhibit glioma growth and specific Ras inhibitors are already being tested in clinical trials on glioma patients [19].

The serine/threonine-specific kinase Braf is an important Ras effector protein that links Ras to the MAPK pathway. The *BRAF* gene has been shown to carry activating point mutations in various tumors, most notably in malignant melanomas, papillary thyroid carcinomas and colorectal carcinomas [6, 7, 13, 22]. Approximately 90% of the *BRAF* mutations affected the catalytic domain of the protein, with the p.V599E mutation being by far the most commonly detected change [6, 7, 13, 22]. Here, we report on the first mutational analysis of *BRAF* in human gliomas. We investigated 94 glioblastomas for mutations in exons 11 and 15 of *BRAF* and identified two glioblastomas (GB3D, GB47D) and one gliosarcoma (GS21D) that carried p.V599E mutations (Fig. 1c, d). None of these tumors showed *NRAS* mutations, which would be in line with findings in other tumors indicating that *NRAS* and *BRAF* mutations are mutually exclusive [7, 20, 22, 24]. Comparison of our mutation data with previously published results obtained in the same tumors [14] revealed that none of the *NRAS* or *BRAF* mutant glioblastomas had *EGFR* or *PDGFRA* gene amplification. However, one glioblastoma with a *BRAF* mutation (GB47D) and the *NRAS* mutant gliosarcoma (GS22D) carried the same *PTEN* missense mutation (c.395G > A, p.G132D). In addition, the *NRAS* mutant glioblastoma (GB105D) exhibited markedly reduced *PTEN* mRNA expression levels in the absence of any mutation or homozygous gene deletion [14].

Taken together, our data indicate that mutations in *NRAS* or *BRAF* likely contribute to the aberrant activation of Ras signaling in approximately 5% of glioblastomas. In most glioblastomas, however, Ras activation must be due to other alterations, such as amplification and overexpression of growth factor receptor genes or aberrations in yet other Ras pathway genes. Interestingly, recent studies have implicated the Ras-related genes *RASSF1A* [12], *RSU1* [5], and *p190RhoGAP* [26] in glioma pathogenesis, indicating that the molecular mechanisms contributing to altered Ras signaling in gliomas are complex and need to be explored further.

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