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# Mutation Screening of *Her-2*, *N-ras* and *Nf1* Genes in Brain Tumor Biopsies

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**Abstract.** *Background/Aim: A deeper understanding of the complex molecular pathology of brain malignancies is needed in order to develop more effective and targeted therapies of these highly lethal disorders. In an effort to further enlighten the molecular pathology of brain oncogenesis involving the her-2 (erbB-2/neu/ngl)/N-ras/nf1 pathway, we screened the genotypes of specimens from various types of brain tumors. Materials and Methods: The studied specimens included 35 biopsies of four general categories: 13 neuroglial tumors (4 astrocytomas, 2 oligodendrogliomas, 7 glioblastomas multiforme), 14 meningiomas, 3 other nervous system tumors (2 schwannomas, 1 craniopharyngioma) and 5 metastatic tumors (such as lung carcinomas and chronic myelocytic leukemia). Screening for most common mutations in oncogenes her-2, N-ras and tumor suppressor gene nf1 was conducted with molecular hybridization techniques (Southern blotting, dot blot and single-strand conformational polymorphism (SSCP) analysis, respectively), and was confirmed by DNA sequencing. Results: Gene amplification of her-2 was observed in only two cases (6%), namely in one glioblastoma and in one meningioma. Screening of 3 hot spot codons of the N-ras gene (12, 13 and 61) and subsequent DNA sequencing revealed mutations in 19 biopsies encompassing all categories (54%). Screening for mutations in exons of the nf1 gene by SSCP analysis detected a novel nonsense mutation in exon 31 in a unique case of a glioblastoma biopsy (3%) taken from a patient without neurofibromatosis type I. Conclusion: Activated N-ras appears to be a major oncogene in brain oncogenesis, exhibiting the most important role in the her-2/N-ras/nf1 pathway.*

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Despite advances in therapy, brain cancer remains a highly lethal malignant disease worldwide, with a median survival period of less than a year (1). Hence, further understanding of the complex molecular pathology of brain malignancies is needed in order to develop more effective and possibly targeted therapies.

According to the Knudson model, genesis and progression of tumors require the alterations of a number of somatic cell genes in a stepwise process. In the last decades, molecular genetic analysis revealed that oncogenesis results from accumulated mutations in two major classes of cell growth regulatory genes: the oncogenes and the tumor suppressor genes (1). Most of these genes belong to an intracellular signal transduction pathway or to cell cycle regulators.

The most well-known oncogene implicated in the development of brain tumors is *Neuroblastoma–rat sarcoma* (*N-ras*). It is located on chromosome 1p13.2 and encodes for a membrane bound GTPase switch protein, called p21Nras (2). Normal p21Nras is involved in the control of cell differentiation and growth, existing in the equilibrium between its inactive and active states (GDP-p21Nras *versus* GTP-p21Nras). Activating point-mutations in hot spot codons 12, 13 and 61 of *N-ras* gene result in protein products that inhibit GTP hydrolysis constitutively, leading to a prolonged growth-promoting event (2). There exists accumulating evidence for the involvement of *N-ras* in some types of brain tumors, such as glioblastomas, gliomas and neuroblastomas (2-4), in addition to other cancers such as leukemia, thyroid tumors, melanoma and colorectal carcinoma (5-8).

The rate of GTP-p21Nras hydrolysis is increased by GTP-activating proteins, the most important of which in cells of neuroectodermal origin is neurofibromin (nf1-GAP) that antagonizes the Ras function (9, 10). It is encoded by the tumor suppressor gene *nf1* (chromosome 17q11.2), mutations

in which cause neurofibromatosis type 1 (NF1), an autosomal dominant disorder characterized by predisposition to uncontrolled cell proliferation and tumors of the nervous system, including gliomas, neurofibrosarcomas and meningiomas (11-14). In the literature, somatic inactivating mutations or deletions in the *nf1* gene have been detected in about 23% of patients with glioblastomas (14, 15).

Stimulation of the GTP-activating proteins, and therefore of the GTP-Ras pathway, is also effected by activated growth factor receptors with tyrosine kinase activity, such as human epidermal growth factors 1 (her-1/EGFR) and 2 (her-2/erbB-2/neu/ngl). While alterations in the *her-1* gene have been frequently reported in brain tumors (16), less clear remains the role of *her-2* gene in brain tumorigenesis (locus 17q21.2), although it was first characterized in rat neuro/glioblastoma cell lines (14). There exist only few reports that overexpressed or activated protein her-2 may be involved in the development of brain tumors (14, 17).

In an effort to contribute to the understanding of the molecular pathology of brain oncogenesis and the involvement of the her-2/N-ras/nf1 pathway, we studied 35 biopsies of various types of brain neoplasias for the most common mutations in these three tumor-related genes.

## Materials and Methods

Brain biopsies were collected from 35 patients without NF1 who underwent craniotomy for brain tumor surgery at the Evangelismos Hospital, Athens, Greece. The patients included 18 males and 17 females and their ages at the first diagnosis ranged from 26 to 77 years (median 63 years). Six of them (17%), aged from 58 to 77 years (median 67 years), had a first-degree relative affected with a specific form of cancer other than a brain tumor. The specimens included biopsies of four general categories of pathological diagnosis: 13 neuroglial tumors (4 astrocytomas, 2 oligodendrogliomas, 7 glioblastomas multiforme), 14 meningiomas, 3 other nervous system tumors (2 schwannomas, 1 cranio-pharyngioma) and 5 metastatic tumors (4 lung adenocarcinomas and 1 chronic myelogenous leukemia). The metastatic tumors were analyzed as controls. Each biopsy sample was given a code number.

The mutation screening was conducted blindly. Total DNA was isolated from brain biopsies with the use of a NaCl extraction method. DNA samples from all biopsies were screened for most common mutations in the 3 studied genes. Mutation screening in 3 hot spot codons of the *N-ras* gene (numbers 12, 13 and 61) was performed with a Bio-Dot apparatus (Bio-Rad, Irvine, CA, USA) using hybridization of dot blotted amplified DNA sequences of *N-ras* gene with 7-8 biotin-labeled oligonucleotide probes (19mers; Oncogene Inc., New York, NY, USA) per codon according to standard procedure (18), *i.e.* mutation detecting oligonucleotides in addition to a w.t. detection one (Table I). The results of dot blotting were confirmed by direct DNA sequencing. The screening for the most common mutation of the *erbB-2* gene (its amplification in multiple copies) was performed by hybridization of *BamHI* Southern blots with a biotin-labeled 1.6 kb cDNA probe. The screening for mutations in all exons of the *nf1* gene was performed with SSCP analysis of PCR products in glycerol containing 80% native polyacrylamide gels. The candidate mutations were analysed by direct DNA sequencing.

Table I. *Screened mutations at 3 hot spot codons of N-ras gene. Mutant codons are named after the amino acid they encode for.*

| | Codon 12 $T_a$ | Codon 13 $T_a$ | Codon 61 $T_a$ |
|---|---|---|---|
| wt | Gly (GGT) (64˚C) | Gly (GGT) (62/64˚C) | Gln (CAA) (58/60˚C) |
| mut | Ala (62/64˚C) | Ala (62˚C) | Arg (58˚C) |
| | Asp (62˚C) | Asp (60˚C) | Gly (60˚C) |
| | Arg (62˚C) | Arg (60˚C) | Leu (56˚C) |
| | Cys (62˚C) | Cys (60˚C) | Lys (56˚C) |
| | Ser (62˚C) | Ser (60˚C) | His (CAC) (60˚C) |
| | Val (62˚C) | Val (60˚C) | His (CAT) (58˚C) |
| | | | Pro (60˚C) |

Ta: Dot blot hybridization temperature of the respective oligonucleotide probes; wt, wild-type codon; Mut, mutant codon.

## Results

The results of the blind mutation screening of the three studied genes in the 35 biopsies of brain tumors are presented in Table II. At least one mutation was detected in 20 biopsies (57%). There was no statistically significant difference in male *versus* female patients for whom mutations were found ($\chi^2$=2.32, $p$>0.05).

*N-ras* mutations were detected in 19 biopsies (54%), encompassing all four general categories. More specifically, they were detected in 9/13 neuroglial tumors (69%), in 6/14 meningiomas (43%), in 1/3 other nervous system tumors (33%) and in 3/5 metastatic tumors (60%). Interestingly, more than one *N-ras* mutations were detected in 9 of the 19 biopsies (47%): two mutant codons in 7/19 (37%) and three mutant codons in 2/19 (10%). Codon 12 mutations were found in 8 biopsies (of all categories), codon 13 mutations in 7 biopsies (4 of them were gliomas) and codon 61 mutations in 14 biopsies (7 of them gliomas and the rest of other categories).

The incidence of the most common screened mutations in the other two studied genes was much lower. Amplified gene copies of *her-2* were observed in only two cases (6%). They were detected in 1 of 13 neuroglial tumors (8%) and in 1 of 14 meningiomas (7%).

In a unique case (3%), a novel mutation was detected in exon 31 of *nf1* gene in a glioblastoma biopsy. In codon 1948, a C5842T transition resulted in a change of a Gln codon (CAA) to a stop codon (TAA).

## Discussion

Several sequential genetic alterations appear to be required to direct cells toward malignancy, namely the activation of growth promoting oncogenes and the inactivation of growth inhibiting tumor suppressor genes. The major causal events of oncogene activation include either hot spot point

Table II. *Mutations in her-2, N-ras and nf1 genes detected in brain tumors and reported by codon and encoded amino acid or stop. Fam: family history of cancer; amplif: gene amplification, mut: mutation.*

| Type of tumor (N) | Sample No (Gender/Age/Fam) | *Her-2* amplif | *N-ras* mut | *nf1* mut |
|---|---|---|---|---|
| 1.Neuroglial tumors (13) | | | | |
| Astrocytomas (4) | 5 (M/62/-) | - | - | - |
| | 20 (F/46/-) | - | 13Ser/61His | - |
| | 35 (M/76/Yes) | - | 13Ser | - |
| | 37 (F/63/-) | - | 61His | - |
| Oligodendrogliomas (2) | 22 (F/77/Yes) | - | 13Ala,13Asp/61Lys | - |
| | 31 (M/44/-) | - | 61His | - |
| Glioblastoma multiforme (7) | 9 (F/65/-) | - | - | - |
| | 12 (M/67/-) | - | - | - |
| | 14 (M/67/Yes) | - | 13Ser/61Leu | Gln1948Stop |
| | 18 (M/67/-) | - | - | - |
| | 24 (M/60/Yes) | - | 12Ser/61His | - |
| | 34 (F/58/-) | - | 12Ser | - |
| | 38 (M/67/-) | + | 61Pro | - |
| 2. Mesenchymatic tumors (14) | 7 (F/68/-) | - | - | - |
| | 10 (M/63/-) | - | 12Ser | - |
| | 11 (M/66/-) | - | 61His | - |
| | 15 (M/44/-) | - | 61His | - |
| | 16 (M/26/-) | - | 12Cys/61Arg | - |
| | 21 (F/62/Yes) | - | - | - |
| | 26 (M/60/-) | - | - | - |
| | 28 (F/60/-) | - | - | - |
| | 29 (F/72/-) | - | 12Ala/13Ala | - |
| | 30 (F/68/-) | - | 61His | - |
| | 32 (F/65/-) | - | - | - |
| | 33 (F/65/-) | - | - | - |
| | 36 (F/68/-) | - | - | - |
| | 42 (F/58/Yes) | + | - | - |
| 3. Other nervous system tumors (3) | | | | |
| Craniopharyngioma (1) | 6 (M/52/-) | - | 12Ser/13Ser/61His | - |
| Schwannomas (2) | 1 (F/52/-) | - | - | - |
| | 27 (F/62/-) | - | - | - |
| 4. Metasyatic tumors (5) | 2 (F/48/-) | - | 12Ser/61Lys | - |
| | 3 (M/67/-) | - | - | - |
| | 8 (M/54/-) | - | - | - |
| | 19 (M/47/-) | - | 12Ser | - |
| | 25 (M/71/-) | - | 13Ala/61Leu | - |

mutations (such as in the case of *N-ras*) or gene amplification (such as in the case of *her-2*), while inactivation of tumor suppressor genes occurs mainly as a result of loss-of-function mutations (such as in *nf1* gene). Cumulative evidence suggests that genetic alterations affecting components of the cell growth regulating signal transduction pathway her-2/Ras/RTK/P13K sum up to 88% in brain tumors such as malignant gliomas (19).

In an effort to elucidate the oncogenic role of key components of the initial part of the her-2/Ras/RTK/P13K pathway, namely the her-2/N-ras/nf1, we screened various brain tumor types for genetic mutations on these genes. Our data support the notion that activated *N-ras* is a major oncogene in central nervous system malignancies playing the most important role in activation of the her-2/N-ras/nf1 pathway. *N-ras* gene codon 61 in particular appears to be an important mutation hot spot in both neuroglial tumors and meningiomas (mutations found in 7/9 and 4/6, respectively). The important role of *N-ras* in brain oncogenesis has been previously reported in neuroblastoma and glioblastoma multiforme (20). In addition, *N-ras* mutations were detected in more than half of the metastatic brain tumors in this study:

two lung adenocarcinomas and one chronic myeloid leukaemia. Activated N-ras has been previously reported to be associated with both types of cancers (6, 21), however it is unclear whether the somatic mutation events occurred prior or after metastasis.

Interestingly, more than one mutations in the *N-ras* gene were found in 8 of 19 biopsies: 2 mutations in different codons in 7 samples and 3 mutations in 1 biopsy (13Ala, 13Asp/61Lys). It is unclear whether these mutations represent genetic events within the same or different cell populations of the studied tumors. Similar findings have been occasionally reported in the literature (22, 23). It is possible that genomic instability in a parent cell population, induced by mutations in genes important for cell cycle regulation and DNA repair (for example p53 gene ), may lead to multiple gene alterations in the daughter cell populations (1).

Hereditary predisposition to oncogenesis may not be excluded in 5 patients with positive family history, although their ages were quite advanced (58-77 years). Four patients with N-ras mutations in their gliomas had a first degree relative with tumors previously associated with N-ras activation: cutaneous melanoma, endometrial carcinoma, colorectal carcinoma and lung carcinoma (7, 8, 21, 24). One of the above mentioned patients with glioblastoma multiforme had an additional mutation in nf1 gene. That was probably a somatic one, since the corresponding patient had a mother with colorectal carcinoma, a tumor not previously been reported to be associated with nf1 genetic alteration. The fifth patient with positive family history had no mutation in the N-ras gene but instead amplified gene *her-2* copies in her meningioma and his father with salivary gland adenocarcinoma, a tumor which amplification of *her-2* was previously reported (25).

Our findings indicate that activated N-ras is a major oncogene in brain tumors, while the roles of *her-2* and *nf1* genes seem to be less important, although their protein products closely interact with the N-ras protein in the same cellular pathway. In accordance to our observations, low incidence of overexpressed her-2 protein and amplification of its gene have been previously observed in gliomas, while moderate incidence has been reported in meningiomas (14, 26). The low incidence of nf1 mutations in brain tumor biopsies is in accordance to previous reports in gliomas, meningiomas and primitive neuroectodermal tumors of non-NF1 patients (2, 4), but at the same time in contrast to a large scale multidimensional study which has reported nf1 somatic inactivation mutations or deletions at a moderate rate (14).

Somatic mutations in oncogenes such as N-ras are the most common activating lesions found in human cancers. These mutations are frequently associated with poor response to standard cancer therapies. Despite the genetic complexity and the pathologic heterogeneity of brain tumors, major components of her-2/Ras/RTK/P13K pathway may be appropriately targeted by molecular and immunological therapies. In this context, the frequently observed hyperactivation of N-ras could represent an opportunity to develop new targeted therapies in order to increase treatment effectiveness and decrease the toxicity at the same time (27-29). Combination therapies aiming at multiple molecular targets of the activated signaling pathway including her-1, N-ras and P13K might be more effective than those of single target.

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