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Down-regulation of K-*ras* and H-*ras* in human brain gliomas

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ABSTRACT

Ras genes, a class of nucleotide-binding proteins that regulate normal and transformed cell growth, have been scarcely investigated in human brain tumours. We evaluated the mutational, mRNA and protein expression profile of the *ras* genes in 21 glioblastomas multiforme (grade IV), four fibrillary astrocytoma (grade II), four anaplastic astrocytoma (grade III) and 15 normal specimens. K-, H- and N-*ras* transcript levels were determined by real-time RT-PCR and mutational status by PCR-restriction fragment length polymorphism (RFLP) and direct sequencing. p21 protein was evaluated by Western blot analysis. Two K-*ras* mutations were found in codons 16 and 26 in one pathological and one normal sample, respectively. Glioblastoma multiforme cases exhibited significantly lower K- and H-*ras* mRNA levels compared to controls ($P < 10^{-4}$). K- and H-*ras* mRNA down-regulation was not associated with patient outcome or survival. K-*ras* was positively correlated with H-*ras* in glioblastomas ($P = 0.005$), but not in normal specimens. p21 protein was absent in all samples. Our findings provide evidence of K- and H-*ras* involvement in brain malignant transformation through transcriptional down-regulation, while N-*ras* seems to contribute less to brain carcinogenesis.

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1. Introduction

The human *ras* genes, K-, H- and N-*ras*, are members of a family of proto-oncogenes that are implicated in the development of tumours.[1] These genes encode for the plasma membrane p21 protein, which has guanosine triphosphatase activity. Wild-type *ras* genes have onco-suppressor properties, but can act as oncogenes and participate in the development and progression of human tumours upon their activation and aberrant expression of p21 protein.[2] Activation of *ras* oncogenes occurs by several routes, including point mutations, over-expression, gene amplification, and translocations, or by insertion of a promoter or enhancer element (Fig. 1). Codons 12, 13 and 61 are hot spots for mutations, resulting in a constitutive stimulation of cell growth, cell proliferation and malignant cell transformation. Mutations in normal *ras* alleles are not a prerequisite for rendering of oncogenic properties to the *ras* family genes, since it has been demonstrated that normal *ras* allele over-expression alone can trigger cell transformation.[3]

Activated *ras* oncogenes have been found in a significant proportion of all human cancers; however, their frequency varies considerably among different tumour types. K-*ras* mutations were found at a high frequency in astrocytoma,[4,5] colon,[6] intestinal[7] and lung[8] tumours, whereas low K-*ras* mutation incidence was observed in carcinomas of the uterine cervix,[9] breast[10] and kidney.[11] Spandidos and colleagues reported H-*ras* mRNA over-expression in breast cancer as compared to normal specimens,[12] while Whittaker and colleagues found N- and K-*ras* over-expression in malignant breast tumours compared to benign breast tumours.[13] p21 protein was found to be significantly elevated in gastric and

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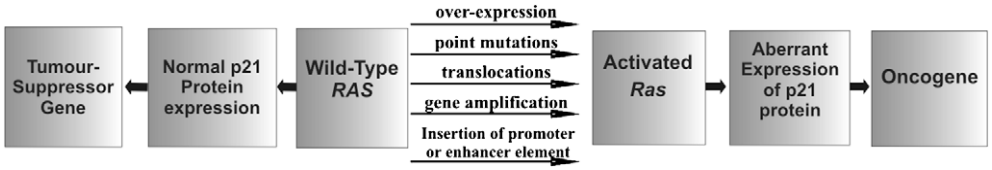

Fig. 1 – Dual role of *ras* genes. Wild-type *ras* can act as tumour suppressor genes, but when they are activated, they can act as oncogenes.

colon cancers,[14,15] while in lung cancer, ras p21 expression differed according to histological classification.[16]

The prognostic significance of *ras* activation has been demonstrated in a variety of human cancers. K-*ras* mutations have been associated with poor survival in colon and lung cancer patients.[17,18] Elevated K- and H-*ras* expression has been demonstrated to correlate with poor prognosis in colon neoplasms.[19] High ras p21 protein expression was correlated with disease recurrence in breast cancer.[20]

A limited number of studies, however, have examined the molecular alteration of the *ras* genes in gliomas. K-*ras* mutations have been reported in astrocytomas by two independent investigators,[21,22] whereas N-*ras* mutations were investigated only in neuroblastomas and medulloblastomas.[23,24] Decreased H-*ras* mRNA expression as well as over-expression of the ras p21 oncoprotein has been demonstrated in a small number of gliomas.[25,26] However, the combined mutational, mRNA and protein expression status of the three *ras* genes and its correlation with prognosis and survival have not been investigated in a large number of brain tumours.

The aim of this study was to investigate the role of the *ras* genes in human brain tumours of various grades and specifically astrocytomas of grades II, III and IV. Therefore, we evaluated the mRNA expression of the K-, H- and N-*ras* genes, as well as the expression of ras p21 protein, in human brain astrocytomas and in normal brain tissue. Furthermore, we examined the presence of activating mutations of the three *ras* genes, and correlated our results with the clinicopathological characteristics and survival data of the patients.

2. Materials and methods

2.1. Tissue specimens

Samples were surgically obtained from 21 glioblastoma multiforme (GBM) tumours, eight astrocytomas and 15 normal brain tissues (12 from glioblastoma multiforme patients, 2 from fibrillary astrocytoma patients and 1 from an anaplastic astrocytoma patient) (Table 1) from patients who had undergone therapeutic surgical tumour removal without prior radio- or chemotherapeutic treatment, at the Department of Neurosurgery of the University Hospital of Crete between 2005 and 2007. The normal tissue specimens were cerebral tissue debris that was produced during tumour resection in patients, who had undergone surgery for deep sighted tumours. The mean age (±SEM) of the patients at the time of surgery was 64.1 (±2), with a range of 35–78 years.

Sample sections were histologically evaluated by board-certified neuropathologists and were categorised according to the World Health Organisation (WHO) classification (Table 1). The purity of the tumour samples was estimated using H&E staining to ensure that each sample had >80% neoplastic cells. Tissue specimens were obtained at the time of the surgery, and they were snap frozen and stored at –80 °C until RNA extraction.

Table 1 – Clinical and histological characteristics of the patients.

Characteristic	Number of patients
Total number of patients	29
Age	
Mean ± SEM	64.1 ± 2
Range	35–78
Histological type	
Glioblastomas	21
Astrocytomas	8
Histological grade	
Grade II	4
Grade III	4
Grade IV	21

The study was approved by the Ethics Committee of the University of Crete, and all patients gave written informed consent.

2.2. RNA and protein extraction and reverse transcription (RT-PCR)

Total RNA and protein were extracted from each tissue sample by the use of the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) with the aid of a power homogeniser according to the manufacturers' instructions. RNA concentration and purity were evaluated by a spectrophotometer. Protein concentration was determined using the Bradford assay. Aliquots of RNA and protein were stored at –80 and –20 °C, respectively, until use.

cDNA from each sample was derived by reverse transcription of 2 μg of total RNA using the AffinityScript™ Multi Temperature cDNA synthesis kit (Stratagene, La Jolla, CA, USA). Random hexamers were used as amplification primers. To remove the RNA template, cDNA was incubated with *Escherichia coli* RNase H, and was stored at –20 °C until use.

2.3. Real-time PCR

Transcript levels of K-, H- and N-*ras* were determined using the Mx3000P Real-Time PCR system (Stratagene) and SYBR® Green I Master Mix (Stratagene) according to the

Table 2 – Primer sequences used for quantitative real-time RT-PCR.

Oncogene	Primer pair sequence (5′-3′)	Product size (bp)
K-*ras*	For: GGGGAGGGCTTTCTTTGTGTA Rev: GTCCTGAGCCTGTTTTGTGTC	174
H-*ras*	For: GGGGCAGTCGCGCCTGTGAA Rev: CCGGCGCCCACCACCACCAG	110
N-*ras*	For: CTTCCTCTGTGTATTTGCCATCA Rev: GCACCATAGGTACATCATCCGA	107
GAPDH	For: GGAAGGTGAAGGTCGGAGTCA Rev: GTCATTGATGGCAACAATATCCACT	101

manufacturers' instructions. All primers were designed to span at least one intron in order to avoid amplification of contaminating genomic DNA. Primers sequences and optimal annealing temperatures are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control to normalise K-, H- and N-*ras* mRNA expression levels. For each target gene, 1 μl of cDNA was amplified in a total volume of 20 μl containing 2X Brilliant SYBR® Green QPCR Master Mix supplemented with 300 nM of each primer set. A representative pool of all samples was diluted in a series of seven 2X dilutions and was used to construct a standard curve for the quantification process. Melting curves for each amplicon were generated to evaluate the specificity of the products. Data were collected and analysed using Mx3000P Real-Time PCR software version 2.00, Build 215, Schema 60 (Stratagene). For all the samples tested, we initially calculated the normalised expression of the target versus the housekeeping gene for each pathological or normal sample using the following formula: Normalised Sample or Control = $(1 + E_{Ras})^{-\Delta Ct}Ras/(1 + E_{GAPDH})^{-\Delta Ct}GAPDH$. Afterwards, we divided the normalised expression of each tumour sample with the mean normalised value of the controls (expression ratios). A twofold increased or decreased expression was considered significant over-expression or down-regulation, respectively. The expression ratios provided by the division of the normalised expression of each tumour sample to that of its adjacent normal tissue, i.e. [(gene/*GAPDH*) tumour/(gene/*GAPDH*) normal] were used to provide a distinct molecular portrait of each tumour in our brain tissue-pair analysis. Again, a twofold increased or decreased expression was considered to reflect over-expression or down-regulation, respectively.

2.4. Western blot analysis

Proteins extracts (20 μg) were electrophoresed through a 10% polyacrylamide gel, transferred onto nitrocellulose membranes and incubated with an anti-ras antibody (Cat. No. 05-516, Upstate Biotechnology, USA) at a dilution of 1:1000, and with anti-actin antibody (MAB1501, Chemicon Int., CA). Antibody binding was revealed by a peroxidase-labelled secondary antibody. Bands were visualised using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA), according to the manufacturers' protocol. The analysis was performed twice for each sample (normal or tumour) using a pre-neoplastic 208F rat fibroblast cell line bearing a G12V H-*ras* mutation as a control for ras p21 expression, and normal endometrial tissue protein extracts as a control of endogenous ras p21 expression. The positive controls (fibroblasts/endometrium) were run concomitantly with the test samples.

2.5. DNA extraction and PCR amplification

Genomic DNA was extracted using proteinase K followed by phenol extraction and ethanol precipitation according to the standard procedures. PCR reactions were performed using Go Taq Flexi DNA Polymerase (Promega, USA). The set of primers used in PCR and amplification conditions was as previously described.[27]

2.6. DNA sequencing and restriction fragment length polymorphisms

Codon 12 was analysed by RFLP,[27] while codons that are included in our amplified sequence (hot spot codons 12 and

Table 3 – K-*ras* and H-*ras* mRNA expression in pathological and normal specimens.

Gene	Fibrillary astrocytomas (Grade II) (N=4)	Anaplastic astrocytomas (Grade III) (N=4)	Glioblastoma Multiforme (Grade IV) (N=21)	Normal (N=15)	*P* value*
K-*ras*	0.66±0.16	0.49±0.18	0.59± 0.09	1.34±0.11	$<10^{-4}$
P value**	0.029	0.004		$<10^{-4}$	
H-*ras*	1.24± 0.38	0.92±0.10	0.77±0.17	2.16±0.52	0.002
P value**	NS	NS		$<10^{-4}$	

Data are presented as the mean +/– SEM (standard error of the mean).
*Kruskal-Wallis test. P<0.05 is considered statistically significant.
**Bonferroni's correction and Mann-Whitney test. $P < 0.05$ is considered statistically significant. NS, not significant.

13, as well codons 14–47) were analysed by direct sequencing. PCR products were purified with the Wizard SV Gel and PCR clean-up system (Promega, USA) and were amplified with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) in a 10-μl volume containing purified PCR product using the previously described primers (Table 2). Sequencing and results analysis was performed onto the ABI PRISM 3100 Genetic Analyser (Applied Biosystems) using the Sequence Analysis 3.7 software (Applied Biosystems, USA).

2.7. *Statistical analysis*

The Kolmogorov–Smirnov test was used to determine whether the expression data obtained follow a normal distribution pattern. The mRNA expression of K-, H- and N-*ras* was compared between the groups of normal and pathological samples, as well as between the groups of different histological features using non-parametric procedures (the Kruskal-Wallis and Mann-Whitney tests). The Spearman rank correlation (non-parametric test) was used to evaluate the significance of the *ras* gene mRNA correlation pair-wise. Finally, the Chi-square (χ^2) test was applied to evaluate the significant statistical differences in K-, H- and N-*ras* mRNA expression status (over-expression or down-regulation). Survival analysis was performed according to the Kaplan-Meier method and was analysed using the log-rank test. Survival was assessed as corrected survival from the date of surgery to the date of death or to the closing date of the study: 23 months survival time.

Probability values (P values) < 0.05 were considered statistically significant. Statistical calculations were performed using SPSS 11.5 software (SPSS, Chicago, IL, USA).

3. Results

In the present study, we examined the presence of mutations in codon 12 of the K-, H- and N-*ras* genes by PCR-RFLP and direct sequencing, and we evaluated the mRNA expression profile of these *ras* family genes in 21 glioblastomas, four anaplastic astrocytomas (grade III), four fibrillary astrocytomas (grade II) and 15 normal brain tissues specimens by Real-time PCR. Ras p21 protein was assessed by Western blot analysis in tissue protein extracts of both tumour and normal specimens.

3.1. *Mutation analysis*

PCR-RFLP and sequencing analysis did not detect the GGT → GTT (Gly12Val) mutation in codon 12 of the three *ras* genes, but did reveal a loss of A in codon 16 of K-*ras* in a pathological sample, leading to the change of the amino acid Lys to Arg (Fig. 2A). A C insertion in K-*ras* codon 26 resulting in the change of the amino acid Asn to Gln in the produced protein was found in a normal specimen (Fig. 2B). No H- and N-*ras* codon 12 mutations were found.

3.2. *Transcript levels of K-, H- and N-ras*

Normal brain specimens were found to have significantly elevated K- and H-*ras* mRNA levels (normalised to GAPDH) compared to glioblastomas ($P < 10^{-4}$ for both; Mann-Whitney test) (Fig. 3). Furthermore, K-*ras* transcript levels were found to be lower in fibrillary as well as in anaplastic astrocytomas compared to normal specimens ($P = 0.009$ and $P = 0.006$, Mann-Whitney test; $P = 0.029$ and $P = 0.004$, respectively, after the Bonferroni correction for small number of samples) (Fig. 3A, Table 3). N-*ras* normalised transcript levels were similar in glioblastomas, astrocytomas and in the normal specimens.

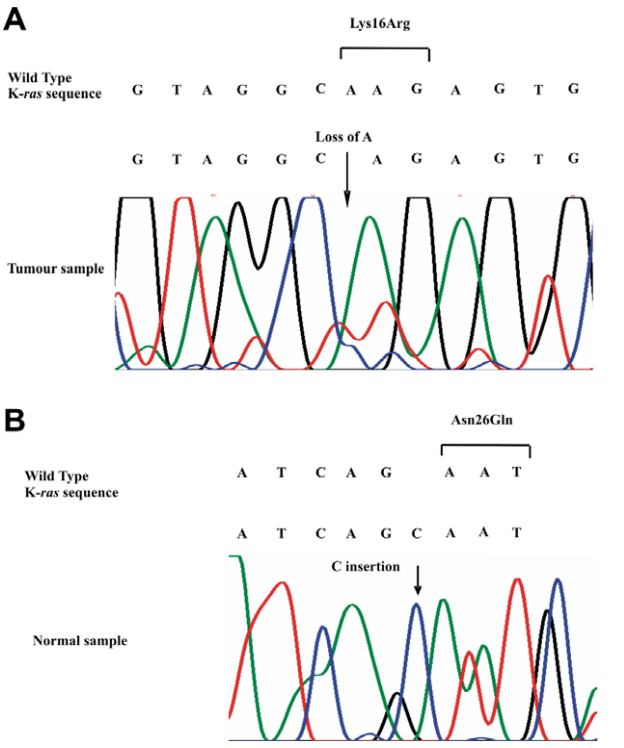

Fig. 2 – (A) Sequencing chromatogram showing the loss of A in codon 16 of a glioblastoma multiforme sample (AAG → AGA; Lys16Arg) and (B) Sequencing chromatogram showing the C insertion in codon 26 of a normal brain sample (AAT → CAA; Asn26Gln).

K-, H- and N-*ras* transcript levels in each pathological sample were, respectively, divided by the mean value of K-, H- and N-*ras* expression in the normal specimen group. A twofold increased or decreased expression ratio was considered to reflect over-expression or down-regulation. K- and H-*ras* down-regulation was, respectively, observed in 57% and 81% of the glioblastomas, while normal expression was observed in 43% and 19% of the corresponding cases. K- and H-*ras* down-regulation occurred more often than normal expression in glioblastomas ($P = 0.048$, odds ratio (OR) = 0.57, confidence interval (CI) = 0.31–1.03; $P < 10^{-7}$, OR = 0.06, CI = 0.03–0.12, respectively). N-*ras* down-regulation was more frequent in glioblastomas (62%) compared to up-regulation (9.5%) or normal expression (28.5%) ($P < 10^{-7}$, OR = 4.50, CI = 2.36–8.64; $P < 10^{-6}$, OR = 0.25, CI = 0.13–0.47).

In fibrillary astrocytomas, K-*ras* mRNA down-regulation was the most frequent event (75%) ($P < 10^{-7}$, OR = 9.00, CI = 4.53–18.4), whereas H- and N-*ras* mRNA was either down regulated (50%) or normally expressed (50%). In anaplastic astrocytomas, transcriptional down-regulation of all *ras*

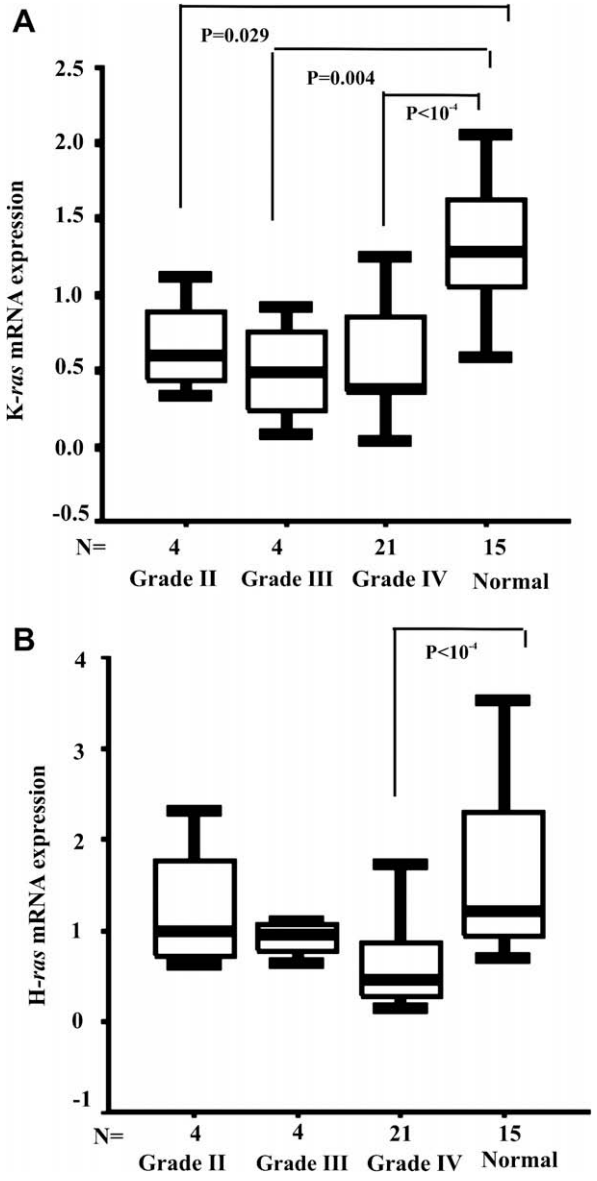

Fig. 3 – (A) K-*ras* mRNA levels in glioblastoma multiforme (grade IV), anaplastic astrocytomas (grade III), fibrillary astrocytomas (grade II) and normal tissues. Glioblastoma multiforme, as well as fibrillary and anaplastic astrocytomas, demonstrated lower K-*ras* mRNA levels than normal brain specimens ($P < 10^{-4}$, Mann-Whitney test; $P = 0.009$ and $P = 0.006$, respectively, Bonferroni's correction). (B) H-*ras* mRNA levels in glioblastoma multiforme (grade IV), anaplastic astrocytomas (grade III), fibrillary astrocytomas (grade II) and normal tissues.

genes was the most common event, occurring in 100% of the cases for K-*ras* and in 75% of the cases for H- and N-*ras* ($P < 10^{-7}$, OR = 9.00, CI = 4.53–18.4) (Table 4).

The incidence of K-*ras* down-regulation was significantly higher in grade III (100%) than in grade II gliomas (75%), followed by grade IV cases (57%). H-*ras* mRNA down-regulation was most frequently observed in grade IV (81%) followed by grade III (75%) and grade II (50%) gliomas. H-*ras* down-regulation was more frequent in grades IV and III compared to grade II gliomas, respectively ($P < 10^{-6}$, OR = 4.26, CI = 2.16–8.47; $P < 10^{-3}$, OR = 3.00, CI = 1.55–5.72). N-*ras* down-regulation was more frequent in grade III (75%) compared to grade IV (62%) ($P = 0.047$, OR = 1.94, CI = 0.96–3.53) followed by grade II gliomas (50%).

K-, H- and N-*ras* mRNA levels were not found to be associated with any of the clinicopathological features of the grade IV specimens examined, such as tumour location or patient age. Due to the limited number of grade II and III samples, the association between mRNA levels and clinicopathological features of the patients was not evaluated.

3.3. *Prognostic value of K-, H- and N-ras mRNA expression*

K-, H- and N-*ras* transcript levels were not found to correlate with the survival of patients with glioblastoma multiforme tumours. Specifically, the Kaplan-Meier analysis did not reveal any prognostic value for the observed down-regulation of K-, H- and N-*ras* mRNA levels compared to glioblastoma multiforme patients with normal expression levels of the same genes (Fig. 4).

3.4. *mRNA co-expression analysis*

The Spearman correlations for evaluation of K-, H- and N-*ras* co-expression patterns in the groups of malignant and normal brain tissue specimens are shown in Table 5.

No correlations were observed in the group of normal specimens, whereas K-*ras* was found to be positively correlated with H-*ras* ($P < 10^{-5}$, the Spearman correlation) in glioblastoma multiforme specimens ($P = 0.005$, the Spearman correlation).

3.5. *Brain tissue-pair analysis*

In the present study, normal brain tissue specimens were available from 12 patients with glioblastoma multiforme. It was, therefore, considered appropriate to compare the mRNA levels of each pathological sample with those of its corresponding normal specimen. The ratio of the transcript levels of each gene to *GAPDH* in the tumour sample was calculated to that of its adjacent normal tissue, i.e. [(gene/*GAPDH*) tumour/(gene/*GAPDH*) normal]. This ratio was used to provide a distinct molecular portrait of each tumour, which was subsequently compared with clinicopathological features. A two-fold increased or decreased expression ratio was considered to reflect over-expression or down-regulation, respectively. The purpose of the present analysis was to investigate whether the results of tissue-pair analysis and of the 'non-pair analysis' of the specimens are in agreement. Transcript levels of K-*ras* were found to be under-expressed in 75% of glioblastoma multiforme pairs, while normal K-*ras* expression was observed in 25% of the cases, indicating that down-regulation occurs significantly more often than normal regulation ($P < 10^{-7}$, OR = 0.11, CI = 0.06–0.22). H-*ras* mRNA levels were down regulated in 92% and over expressed in 8% of the cases ($P < 10^{-7}$, OR = 0.01, CI = 0.00–0.02, χ^2 test). N-*ras* exhibited mRNA over-expression in 42% of tissue pairs, and under-expression and normal expression in 33% and 25% of the cases, respectively. The incidence of N-*ras*

Table 4 – Relative mRNA expression of K-, H- and N-*ras* in brain tumours.

A. Glioblastomas (n=21)

Gene:	*K-ras*			*H-ras*			*N-ras*		
	↑ (%)	- (%)	↓ (%)	↑ (%)	- (%)	↓ (%)	↑ (%)	- (%)	↓ (%)
Cancer	-	43	57	-	19	81	9.5	28.5	62
P value*		0.048			$P<10^{-7}$		$P<10^{-7}$		$P<10^{-6}$

↑, over-expression, - , normal expression, ↓, reduced expression, *x^2 test, (df=2)

B. Fibrillary astrocytomas (n=4)

Gene:	*K-ras*			*H-ras*			*N-ras*		
	↑(%)	- (%)	↓(%)	↑(%)	- (%)	↓(%)	↑(%)	- (%)	↓(%)
Cancer	-	25	75	-	50	50	-	50	50
P value*		$P<10^{-7}$			NS			NS	

↑, over-expression, - , normal expression, ↓, reduced expression, *x^2 test, (df=2)

C. Anaplastic astrocytomas (n=4)

Gene:	*K-ras*			*H-ras*			*N-ras*		
	↑(%)	- (%)	↓(%)	↑(%)	- (%)	↓(%)	↑(%)	- (%)	↓(%)
Cancer	-	-	100	-	25	75	-	25	75
P value*		-			$P<10^{-7}$			$P<10^{-7}$	

↑, over-expression, - , normal expression, ↓, reduced expression, *x^2 test, (df=2)

D. Glioblastoma tissue-pairs (n=12)

Gene:	*K-ras*			*H-ras*			*N-ras*		
	↑(%)	- (%)	↓(%)	↑(%)	- (%)	↓(%)	↑ (%)	- (%)	↓(%)
Cancer	-	25	75	8	-	92	42	25	33
P value*		$P<10^{-7}$			$P<10^{-7}$		P=0,01		

↑, over-expression, - , normal expression, ↓, reduced expression, *x^2 test, (df=2)

over-expression was significantly higher than that of under-expression and normal expression ($P < 10^{-4}$, OR = 0.3, CI = 0.15–0.59; $P = 0.01$, OR = 2.17, CI = 1.14–4.15, respectively) (Table 4D).

Pair-wise mRNA co-expression analysis demonstrated no significant correlation between the three *ras* genes in glioblastoma multiforme tissue pairs. No association was found between grade IV samples and the clinicopathological features of the patients, such as tumour location or patient age. Due to the limited number of grade II and III samples, the association between mRNA levels and clinicopathological features of the patients was not evaluated.

3.6. *Western blot analysis*

Western blot analysis did not result in detectable levels of ras protein in our samples, while beta-actin, which was used as an internal control, was constantly expressed in the normal and tumour specimens (Fig. 5).

4. Discussion

In the present study, we evaluated the expression of *ras* gene mRNA and ras p21 protein in glioblastoma multiforme and anaplastic and fibrillary astrocytoma brain tumours, as well as in normal specimens. The mRNA expression levels obtained were associated with the clinicopathological features of the patients. We also examined the presence of codon 12 K-, H- and N-*ras* mutations in these samples.

Reports of only one K-*ras* codon 13 mutation in 21 philocytic astrocytomas (grade I), and one codon 12 mutation in a group of 25 grade I and II astrocytomas indicate that K-*ras* mutations are a rare event.[5,22] Our evaluation is, to the best of our knowledge, the first to investigate the incidence of

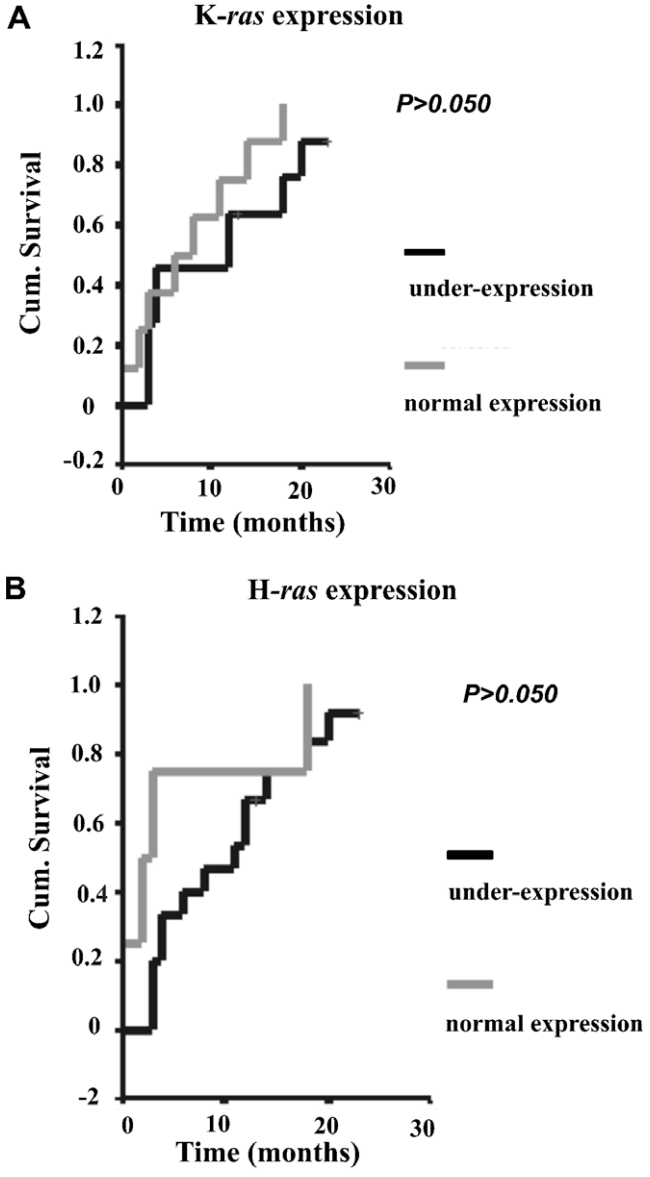

Fig. 4 – The Kaplan-Meier analysis survival plots of the glioblastoma multiforme patients (N = 21) demonstrating the effect of (A) K-*ras* and (B) H-*ras* mRNA down-regulation compared to normal expression status in the tumour tissues examined (P > 0.050) at the end-point, defined as the time period of survival in months.

Table 5 – The Spearman correlation rho and P values.

		K-*ras*	H-*ras*	N-*ras*
A. Normal specimens				
K-*ras*	Spearman's rho	1.000		
	Sig. 2-tailed			
H-*ras*	Spearman's rho	0.191	1.000	
	Sig. 2-tailed	0.494		
N-*ras*	Spearman's rho	0.166	0.131	1.000
	Sig. 2-tailed	0.553	0.643	
B. Glioblastomas				
K-*ras*	Spearman's rho	1.000		
	Sig. 2-tailed			
H-*ras*	Spearman's rho	0.587	1.000	
	Sig. 2-tailed	**0.005**		
N-*ras*	Spearman's rho	0.174	0.326	1.000
	Sig. 2-tailed	0.451	0.149	
C. Glioblastoma tissue pairs				
K-*ras*	Spearman's rho	1.000		
	Sig. 2-tailed			
H-*ras*	Spearman's rho	0.093	1.000	
	Sig. 2-tailed	0.742		
N-*ras*	Spearman's rho	–0.096	–0.1	1.000
	Sig. 2-tailed	0.732	0.723	

K-, H- and N-*ras* mutations in glioblastoma multiforme and in grade II and III astrocytic tumours. Two different non-activating K-*ras* mutations were found in one glioblastoma multiforme sample and in a normal specimen derived from a patient with glioblastoma, supporting the previous findings that *ras* mutations are a rare event in brain carcinogenesis. Sequencing analysis demonstrated the absence of H- and N-*ras* mutations in all samples, suggesting that H- and N-*ras* mutations are not a feature of gliomas.

Regarding mRNA expression profiles of the three *ras* genes being analysed, this is the first time that brain tumour samples were compared to normal tissue specimens from the same patient in order to investigate the molecular portrait of each tumour. In the previous studies, transcript levels in tumours were compared to the mean mRNA levels of normal brain specimens derived from a different set of patients. In the present study, the expression analysis and co-expression pattern were evaluated in both ways to investigate whether the results of tissue-pair analysis and of 'non-pair analysis' are in accordance in terms of molecular pattern.

According to the non-pair analysis, K- and H-*ras* transcript levels were significantly lower in glioblastoma multiforme than in normal brain specimens. Our findings are in accordance with a previous report[25] of decreased H-*ras* mRNA levels in a small number of glioblastomas (N = 4) compared to normal brain tissues. Additionally, grade II and III astrocytomas exhibited substantially lower K-*ras* mRNA levels than

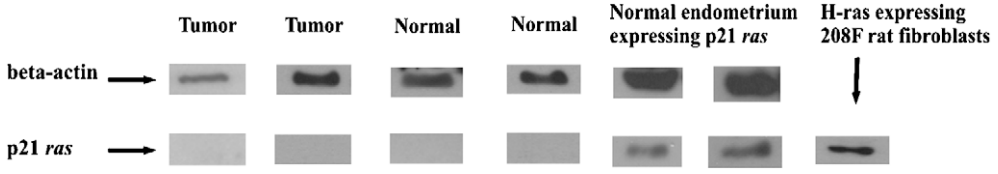

Fig. 5 – Representative examples of ras p21 and beta-actin protein expression assessed by Western blot analysis in tumour and normal brain specimens. The G12V H-ras-transformed 208F rat fibroblast cell line and normal endometrial tissue protein extracts were used as a positive control of ras p21 expression.

normal brain tissues. The decreased K- and H-*ras* mRNA levels observed in tumours are consistent with our finding of the absence of activating *ras* mutations and along with the absence of detectable ras protein support the lack of *ras* activation in brain gliomas. Interestingly, transcript levels of N-*ras* were not found to differ significantly between tumour and normal samples. In addition to the lack of N-*ras*-activating mutations, we can only speculate that N-*ras* plays a less crucial role in brain tumour formation than the other two *ras* oncogenes. This hypothesis is reinforced by our finding that K- and H-*ras* transcript levels are positively correlated in glioblastoma multiforme but not in normal brain specimens. The latter suggests a possible synergistic role of K- and H-*ras* in the malignant transformation processes of the brain.

Furthermore, the most prominent event in glioblastoma multiforme tumours is the down-regulation of all three *ras* genes. Down-regulation of the *ras* family genes was also the most frequent event in anaplastic astrocytomas (grade III). However, fibrillary astrocytomas (grade II) exhibited significant down-regulation only in K-*ras* transcript levels. Based on the fact that anaplastic astrocytomas (grade III) are considered to be more malignant than fibrillary astrocytomas (grade II), our findings, if verified in a larger set of specimens, would suggest that down-regulation of the three *ras* genes could be used as a molecular marker of brain malignancy. Genetic markers for primary glioblastoma formation such as the *APOD* gene that may have a crucial role in malignant astrocytoma formation are shown by MacDonald and colleagues[28] using microarray analysis. Similarly, our findings probably indicate that the down-regulation of the three *ras* genes comprises a genetic marker for glioblastomas versus non-malignant astrocytomas in the case of primary glioblastoma formation.

Studies have shown a number of genetic markers for grade III and IV astrocytomas according to the WHO classification in relation to tumour progression. Van den Boom and colleagues[29] reported many genes such as *VEGFA*, *COL4A1*, *COL4A2*, *COL5A2* and *FOXM1* with distinct expression patterns in WHO grade II astrocytomas, as compared to WHO grade III astrocytomas and/or GBMs. According to Rickman and colleagues,[30] the transcript levels of several genes such as *FOXG1B*, *FOXM1* and *ZYX* differ significantly in high-grade and low-grade gliomas. Another possible marker, according to Reddy and colleagues,[31] is *PEBF1* which had higher expression levels in GBMs than in lower-grade tumours. Our results, if expanded to a larger set of samples, may indicate the down-regulation of *ras* genes as a possible genetic marker of tumour progression for grade III/IV astrocytomas versus grade II astrocytomas. Specifically, down-regulation of the three *ras* genes is associated with grade III/IV astrocytomas, while only K-*ras* down-regulation is associated with grade II tumours. Consequently, the progressive down-regulation of the three *ras* genes is an indication of tumour progression.

In the tissue-pair analysis, which was performed in 12 glioblastoma tissue pairs, the results were somewhat different compared to those of the non-pair analysis. Specifically, the incidence of K- and H-*ras* down-regulation was higher than that found by means of non-pair analysis. N-*ras* over-expression appeared to be the prevailing event of glioblastoma tumourigenesis, while N-*ras* transcriptional down-regulation was implied by the non-pair analysis. This, however, is in accordance with a previous report of N-*ras* over-expression in five glioblastoma multiforme cell lines compared to that in normal brain specimens.[32] Furthermore, K-*ras* mRNA was not positively correlated with H-*ras*, perhaps due to the small number of glioblastoma tissue pairs used ($N = 12$) compared to non-pair-analysis ($N = 21$). Conclusively, there are discrepancies in the results provided by the two analytical techniques. We believe that the pair-wise comparison of inter-patient samples is the most appropriate way to evaluate the mRNA expression and co-expression profile of certain genes in brain tumours as opposed to normal samples, because both tumours and normal tissue specimens are derived from the same patient. In this analysis, the molecular portrait of each tumour was analysed based on the molecular portrait of a normal sample of the same patient taking into consideration the unique molecular profile of each individual.

K- and H-*ras* mRNA down-regulation in glioblastoma multiforme failed to provide prognostic information about patient outcome and survival according to the Kaplan-Meier analysis.

In our investigation, we found that wild-type *ras* genes are transcriptionally down regulated in human brain gliomas, while activating mutations are absent, suggesting the loss of *ras* onco-suppressor properties, leading to the formation of malignant astrocytomas. *Ras* genes have been demonstrated to possess a dual role in human malignancies.[33–36] They can act as oncogenes when they are activated or may have tumour-suppressive functions. Expression of wild-type *ras* has been shown to inhibit the action of the mutant counterpart,[34] thus reversing the malignant phenotype of cells. Our findings reinforce previous reports supporting the onco-suppressive role of *ras*. We can only speculate that the mechanism through which wild-type *ras* can act as a tumour suppressor, is the activation of certain signalling pathways that may lead to apoptosis and senescence. The apoptotic and pro-apoptotic effects from *ras* family members have been exhibited in a number of studies indicating the involvement of several external signals[37,38] or activation of pathways such as the Raf/MAP kinase.[39] Furthermore, *ras* activates the Raf kinase signalling cascade, resulting in the promotion of senescence which comprises an alternative mechanism to prevent proliferation of cells that have acquired a malignant phenotype.[40] The mechanism through which wild-type *ras* can activate certain signalling pathways that may lead to apoptosis and senescence, inhibiting in that way the action of the mutant counterpart, remains to be elucidated.

In conclusion, our results provide evidence of K- and H-*ras* involvement in glioma carcinogenesis through transcriptional down-regulation, whereas codon 12 *ras*-activating mutations and ras p21 protein expression are absent. Based on the non-pair analysis, K- and H-*ras* transcript levels were positively correlated suggesting a synergistic role in glioblastoma formation, whereas N-*ras* is hypothesised to play a less crucial role in brain tumour formation.

Conflict of interest statement

None declared.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.12.028.

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