

## APLASIA RAS HOMOLOGOUS MEMBER I GENE AND DEVELOPMENT OF GLIAL TUMORS

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### ABSTRACT

The ARHI (aplasia Ras homologue member I, also known as DIRAS3) gene shows 60.0% sequence homology to the Ras proto-oncogene and was the first maternally-imprinted tumor suppressor gene identified in the Ras family. It is constitutively expressed from the paternal allele in normal breast, ovary, heart, liver, pancreas, thyroid and brain tissues, and is lost or markedly down-regulated primarily in breast, ovarian, pancreas and thyroid tumor tissues. We have investigated the expression, LOH (loss of heterozygosity) and methylation status of this gene in glial tumors and peripheral blood samples of 21 patients, and in seven normal brain tissue samples. Gene expression by real time reverse transcriptase polymerase chain reaction (RT-PCR) was found to be increased in 14 and decreased in seven of the 21 tumors. The LOH was detected by fragment analysis, using five labeled polymorphic mark-

ers specific for the 1p31 region, in two of the tumors. Methylation status of the CpG island I, II and III was evaluated using COBRA (combined bisulfite restriction analysis) and RFLP (restriction fragment length polymorphism) in 21 tumors and also a hypermethylated healthy volunteer as a positive control, revealed that only two tumors had hypermethylation in CpG island I (of which one also had LOH). These results suggest that LOH and hypermethylation may be one mechanism of silencing the ARHI gene expression and development of glial tumor development.

**Keywords:** Cancer; DNA testing; Genetic testing; Genes; Methylation

### INTRODUCTION

Glial tumors, take part in neuroepithelial tumors, are one of the most common primary human brain tumors originating from the astrocytes, oligodendroglial, ependymal or mixed glial cells [1]. Their development is a multi-step process that involves the accumulation of several genetic events such as activation of proto-oncogenes, expression of growth factors and their receptors or loss of expression tumor suppressor genes. Alterations of chromosome 1 that include deletion, LOH, amplification and hypermethylation, also play a role in the development of brain tumors. The LOH of 1p has been observed in most oligodendroglial and in some of the glioblastoma multiforme tu-

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mors (GBM) that originate from astrocytes [1-6]. All of these lead to the development of unregulated cell growth and differentiation.

Aplasia Ras homologue member I (ARHI), also known as DIRAS3, was the first tumor suppressor gene identified in the Ras superfamily [7]. Even though the maternally-imprinted ARHI gene shows 60.0% sequence homology to the Ras proto-oncogene, when it reexpressed, it inhibits proliferation and motility by blocking signal transducer and activator of transcription 3, up regulating p21WAF1/ CIP1 and inhibiting signal through Ras/Map [7-9]. The maternally-silenced ARHI gene is expressed only from the paternal allele and is constitutively expressed in normal ovary, breast, heart, liver, pancreas, thyroid and brain tissues. Expression is lost or markedly down regulated by LOH or hypermethylation in most cancers of ovary, breast, pancreas, thyroid, and brain [10-14].

We have evaluated the expression levels of the ARHI gene in 21 primary human glial tumor tissue and normal brain tissue samples with a real time reverse transcriptase polymerase chain reaction (RT-PCR) technique. To elucidate the possible cause of loss of expression, we also studied LOH in 21 tumors and peripheral blood samples of the patients by using fragment analysis with five highly polymorphic microsatellite markers and methylation profiles of 21 tumors using a hypermethylated healthy volunteer as a positive control by COBRA (combined bisulfite restriction analysis) and RFLP (restriction fragment length polymorphism) techniques.

## MATERIALS AND METHODS

**Materials.** This study involved 21 primary tumors consisting of 13 glioblastoma multiforme (GBM), WHO grade IV; five oligodendrogliomas (O), WHO grade III and two anaplastic astrocytomas (AA), WHO grade III and one anaplastic oligoastrocytoma (AOA), WHO grade III. The age range of the patients (13 male, eight female) were 21-72 years old. Primary tumor tissues were obtained from patients who underwent surgery, subtotal (seven patients) and gross total (14 patients) with their informed consent. For LOH analysis, peripheral blood samples from 21 normal healthy volunteers was also involved. We used RNA samples from normal brain tissues of the seven autopsies with their relatives' informed consent as reference tissue for mRNA expression analyses.

**DNA and RNA Extraction.** DNA was isolated from tumor tissue using the salting-out method [15]. Control DNA samples were prepared from peripheral blood lymphocytes of the same cases for LOH analyses. RNA was extracted from tumors and normal brain tissue samples using the SV Total RNA Isolation Kit (Promega, Madison, WI, USA) and the manufacturer's protocol.

**Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction.** Reverse transcription of total RNA was performed using TaqMan Reverse Transcription Reagents (Cat. N8080234; Applied Biosystems, Foster City, CA, USA). cDNA was synthesized from 2 µg of RNA using random hexamer and SuperScript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The amplification comprised one cycle at 25°C for 10 min. and one cycle at 48°C for 60 min. Real-time quantitative PCR was performed using two gene-specific primers (ARHI, 5'-TCT CTC CGA GCA GCG CA-3' and ARHI, 5'-TGG CAG CAG GAG ACC CC-3'), a labeled probe (5'-TGT CTT CTA GGC TGC TTG GTT CGT GCC-3') (5'-FAM; 3'-TAMRA) (9), 2 µL of reverse transcriptase reaction mixture and 12.5 µL of Master Mix (Applied Biosystems) on an ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems), according to the manufacturer's protocol. Ribosomal RNA was amplified in the same reaction. Both the rRNA primers and probes were obtained from PE Applied Biosystems (Cat. 4308329). Amplification used a touch-down PCR protocol beginning with 94°C for 5 min., followed by seven cycles at 95°C for 45 seconds, annealing temperatures starting at 62°C for 45 seconds (decreasing by 1°C/cycle) and 72°C for 90 seconds for extension. This step was followed by 40 cycles at 95°C for 45 seconds, 55°C for 45 seconds, 72°C for 90 seconds and a final extension at 72°C for 10 min. The expression level of the tumors was calculated by normalizing that of ARHI to that of the rRNA. Tumors that showed a lower ARHI level than normal brain tissue were interpreted as decreased. Expression results were analyzed by the comparative threshold cycle ( $\Delta\Delta C_t$ ) method according to the User Bulletin No. 2 (Applied Biosystems). All experiments were performed in duplicate.

**Bisulfite Treatment and Combined Bisulfite Restriction Analysis.** Twenty-one glial brain tumor samples were evaluated for loss of functional allele resulting from allele-specific methylation of CpG is-

land I, II and III. Bisulfite treatment of DNA was performed according to a modified standard protocol. The COBRA was performed as described in [16]. DNA from lymphocytes of a healthy volunteer was treated with Sss1 methyltransferase (New England Biolabs, Beverly, MA, USA), then subjected to bisulfite treatment as a positive control. After cleaning the modified DNAs using the GeneClean III Kit (Bio 101 system; Q-Biogen, Vista, CA, USA), 1  $\mu$ L (100 ng/ml) DNA was amplified in 50  $\mu$ L reaction mixtures containing 5  $\mu$ L of 10  $\times$  PCR buffer, 3  $\mu$ L of 25 mM MgCl<sub>2</sub>, 1.5  $\mu$ L of 40 mM dNTPs, 2.5  $\mu$ L of each primer (10 pmol/ $\mu$ L) and 0.2  $\mu$ L (2 U/ $\mu$ L) of Platinum AmpliTaq Gold DNA polymerase (Invitrogen). Primers were designed based on the nucleotide sequence of the ARHI gene submitted to GenBank (AF202543). Primers used for COBRA were: CpGI-F/R, 5'-GTA AGG GAG AAA GAA GTT AGA-3'/5'-TAC TAT CCT AAC AAA ACC CTC-3'; CpGII-F/R, 5'-GTT GGG TTA GTT TTT TAT AGT TGG TT-3'/5'-AAC CAA ACA ACC TAA AAA ACA AAT AC-3'; CpGIII-F/R, 5'-GTT TTT AAG TTT TAT AGG AAG ATT-3'/5'-ATA ATA TAC AAA AAA AAC ACA CC-3'. Amplification used 94°C for 5 min.; 35 cycles at 94°C for 50 seconds, 57°C for 1 min. (for CpG II); 60°C for 1 min. (for CpG I and III) and 72°C for 50 seconds and with a final extension at 72°C for 7 min. After amplification, the PCR products were digested with restriction enzyme *TagI* (New England Biolabs) for CpG I and *Bst*UI (New England Biolabs) for CpG II and III, (both enzymes recognize and cut methylated DNA). DNA samples were fractionated using 8.0% polyacrylamide gel. The gels were stained with ethidium bromide and the intensity of methylated alleles was calculated using DigiDoc 1000 software (Alpha Innotech, San Leandro, CA, USA). Results above 75.0% and 35.0-74.0% were interpreted as hypermethylation and partial methylation, respectively.

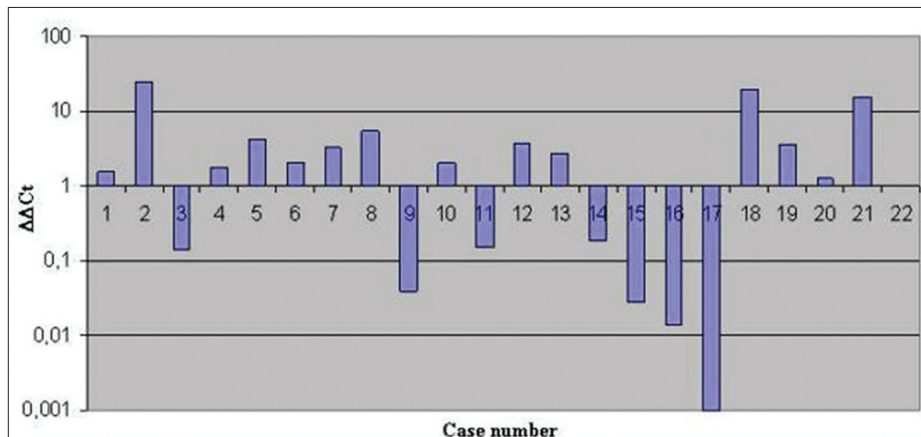
**Loss of Heterozygosity Analysis.** To study the allelic deletion at chromosome 1p31, the five highly polymorphic microsatellite markers D1S226, D1S488, D1S430, D1S207 and D1S2638 (<http://www.gdb.org>) were used. Primers for detection of LOH were as follows: D1S226-F/R, 5'-6-FAM-GCT AGT CAG GCA TGA GCG-3'/5'-6-FAM-GGT CAC TTG ACA TTC GTG G-3'; D1S488-F/R, 5'-6-FAM-GCA AAA CAG AGA CTT CAC CT-3'/5'-6-FAM-CTT CCA GGG ACT AGA ATG G-3'; D1S430-F/R, 5'-6-FAM-TCC AGA TTT AGT GTC ATT TCC C-3'/5'-6-FAM-CAC

TTA CAG TAA CAA GCC CCA G-3'; D1S207-F/R, 5'-6-FAM-CAC TTC TCC TTG AAT CGC TT-3'/5'-6-FAM-GCA AGT CCT GTT CCA AGT CT-3'; D1S2638-F/R, 5'-6-FAM-CTT GGA TTG GTG GGT ACTA-3'/5'-6-FAM-AGG TTT CAG GGT GGC T-3'. The PCR used 100 ng/mL of DNA in a 25  $\mu$ L reaction mixture that contained 2.5  $\mu$ L of 10  $\times$  PCR buffer, 0.2  $\mu$ L of 5 U/ $\mu$ L of Taq DNA polymerase (Invitrogen), 3  $\mu$ L of 25 mM MgCl<sub>2</sub>, 1.5  $\mu$ L of 20 mM dNTPs and 1.5  $\mu$ L of 10 pmol/ $\mu$ L of the primers mentioned above. Amplification used: initial denaturation at 94°C for 5 min.; 32 cycles at 94°C for 45 seconds, 50°C for 45 seconds and 72°C for 1 min. and with a final extension at 72°C for 7 min. for markers D1S226, D1S488, D1S207, D1S2638. For the D1S430 marker: initial denaturation at 94°C for 5 min.; 32 cycles at 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 1 min. with a final extension at 72°C for 7 min. The PCR products were diluted 30-fold, then 1.5  $\mu$ L was added to 23.5  $\mu$ L formamide with 0.5  $\mu$ L Genescan™ 500 LIZ™ size standards (Applied Biosystems). DNA fragments were separated by capillary electrophoresis and the signals were detected with DNA sequencer 310 (Applied Biosystems, Perkin-Elmer Corporation) The results were analyzed by Genescan collection and analysis software (Applied Biosystems, Perkin-Elmer Corporation). Scoring of LOH was performed by Genescan output. If the ratio of the normal and tumor DNA peak heights were  $\leq 0.5$  and  $\geq 1.5$ , it was interpreted as LOH of the long allele and of the small allele, respectively. The presence of LOH was calculated with a formula mentioned below.

## RESULTS

We found decreased ARHI gene expression in seven of the tumors (33.3%). Three of these (samples 3, 11 and 14) were GBM (23.1%), three (samples 15, 16 and 17) were O (60%) and one (sample 9) was AA. We found increased ARHI gene expression in 14 of the tumors (66.7%) of which 10 (samples 4, 5, 6, 8, 10, 12, 13, 18, 19 and 20) were GBM (77.0%), two (samples 1 and 2) were O (40.0%), one (sample 7) was AA and one (sample 21) was AOA (Figure 1).

The LOH analysis revealed allelic loss in two tumors which were O (samples 15 and 17) (Figure 2, Table 1). Both of these also showed decreased ARHI gene expression in real time RT-PCR.



**Figure 1.** Results of real time RT-PCR in 21 primary glial tumors (bar 1-21) and the mean expression level of seven normal brain tissues as reference (bar 22). Bars below 1 threshold value represent samples with decreased ARHI gene expression.

To determine whether epigenetic events contributed to ARHI silencing, we studied promoter methylation of for CpG island I, II and III from the tumors. Only two (samples 9 and 17) had hypermethylation in CpG island I (ure 3 and Table 2). Both of these also showed decreased ARHI gene expression as measured by real time RT-PCR.

$$\frac{\text{the area of normal allele II}}{\text{the area of normal allele I}}$$

LOH=

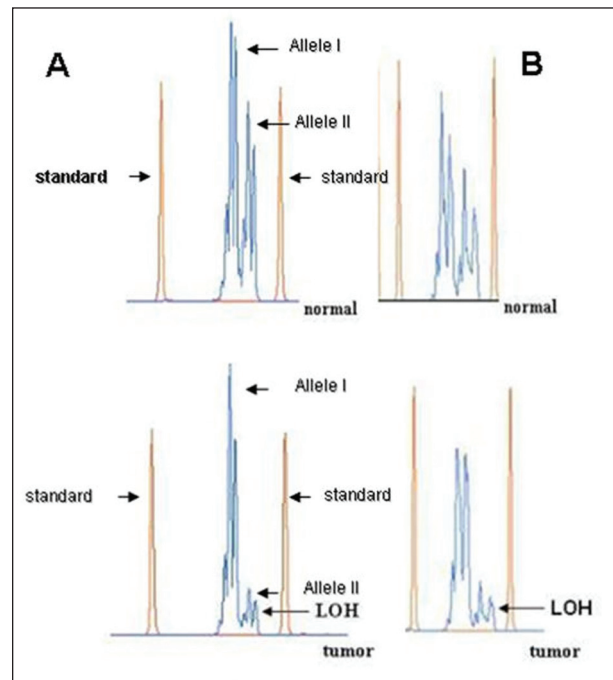
$$\frac{\text{the area of tumor allele II}}{\text{the area of tumor allele I}}$$

**DISCUSSION**

The ARHI gene, localized at 1p31 and maternally imprinted, was studied in several tumor types and was found to have expression alterations [13]. Reduced expression of ARHI was observed in 70.0-80.0% of breast and ovarian, 84.6% of thyroid, and 42.3% of well-differentiated pancreatic endocrine cancers [10-13]. Lack of expression of ARHI gene is strongly associated with development of oligodendrioglial tumors [14].

Our results demonstrated reduction of ARHI expression in seven of 21 glial tumors (33.3%). Since LOH of the functional paternal allele is considered one of the mechanisms that lead to decreased ARHI expression [13], we performed LOH analysis in 21 glial tumors and found LOH in two of 21 (9.5%)

samples. Although the number of cases were insufficient, the cases with LOH were all oligodendroglial tumors. Five oligodendroglial tumors were included in this study and two with LOH of five oligodendroglial tumors also showed decreased ARHI expression in real time RT-PCR. That is why the frequency (40.0%) is so important. These results indicate that LOH may be a primary cause for reducing ARHI



**Figure 2.** Loss of heterozygosity analyses for the ARHI gene. a) LOH of D1S488 in tissue15. b) LOH of D1S430 in tissue 17.

**Table 1.** Results of LOH Analysis of ARHI in Glial Tumors by Using Polymorphic Microsatellite Markers (D1S226, D1S488, D1S430, D1S207, and D1S2638)

Tumor	Histology	Sex-Age	D1S226	D1S488	D1S430	D1S207	D1S2638
1	O	M-46	Normal	Normal	Normal	Normal	Normal
2	O	F-22	Normal	Normal	Normal	Normal	Normal
3	GBM	M-44	Normal	Normal	Normal	Normal	Normal
4	GBM	M-72	Normal	Normal	Normal	Normal	Normal
5	GBM	M-62	Normal	Normal	Normal	Normal	Normal
6	GBM	F-56	Normal	Normal	Normal	Normal	Normal
7	AA	F-59	Normal	Normal	Normal	Normal	Normal
8	GBM	F-68	Normal	Normal	Normal	Normal	Normal
9	AA	M-55	Normal	Normal	Normal	Normal	Normal
10	GBM	M-76	Normal	Normal	Normal	Normal	Normal
11	GBM	M-57	Normal	Normal	Normal	Normal	Normal
12	GBM	M-58	Normal	Normal	Normal	Normal	Normal
13	GBM	F-71	Normal	Normal	Normal	Normal	Normal
14	GBM	M-61	Normal	Normal	Normal	Normal	Normal
15	O	M-33	Normal	<b>LOH</b>	Normal	Normal	Normal
16	O	M-42	Normal	Normal	Normal	Normal	Normal
17	O	F-43	Normal	Normal	<b>LOH</b>	Normal	Normal
18	GBM	M-21	Normal	Normal	Normal	Normal	Normal
19	GBM	F-60	Normal	Normal	Normal	Normal	Normal
20	GBM	M-55	Normal	Normal	Normal	Normal	Normal
21	AOA	F-55	Normal	Normal	Normal	Normal	Normal

O: Oligodendroglioma; GBM: glioblastoma multiforme; AA: anaplastic astrocytoma; AOA: anaplastic oligoastrocytoma.

gene expression in oligodendrioglial tumors. Our results agree with those in other studies [10,11,14].

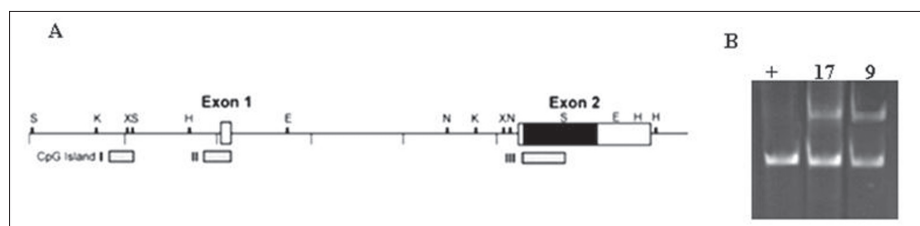
In addition to LOH, expression of ARHI from the paternal allele can also be down-regulated by hypermethylation [17]. In previous studies, hypermethylation of ARHI CpG island I, II and III, were observed in 35.0% of breast cancer and in 95.0% of oligoden-

drioglial tumors associated with 1p deletion [9,14]. We observed hypermethylation in only two of the 21 glial tumor samples (9.5%). One of these was oligodendrioglial, and the other one was AA. The mRNA level of ARHI in oligodendrioglial tumor with LOH on 1p was significantly lower than that of astrocytoma without LOH on 1p. This result indicates that there could be an association between ARHI transcriptional repression and allelic LOH profile on 1p. Although hypermethylation of CpG island II was associated with complete loss of ARHI expression in breast cancer [9], we found only hypermethylation of CpG island I in glial tumors. Our result is in line with a previously published study [14]. Thus, it seems that hypermethylation of CpG island I can also be responsible for down-regulation of ARHI expression.

We also found increased ARHI expression in 14 glial tumors, 10 of which were GBM tumors but

**Table 2.** Aaplasia Ras homologue member I methylation status in two of 21 glial tumors

Tumor	Methylation (%)
9	76.5
17	82.4
Plus control	100.0



**Figure 3.** a) Structural organization of the ARHI gene. b) Methylation status of the ARHI gene in two of the 21 glial tumors. Samples 9 and 17 show decreased ARHI gene expression by quantitative real-time RT-PCR and hypermethylation for CpG island I; + control, positive control for hypermethylation.

have no explanations for the mechanism behind this. We speculate that increased levels of growth factors (EGF, PDGF), their receptors (EGFR, PDGFRA) in tumor microenvironment or constitutive activation of PI3K, MAPK, STAT3 pathways may be responsible for our results [14].

We conclude that decreased ARHI expression may play a role in development of glial brain tumors. However, LOH and methylation analysis need to be evaluated in larger number of samples before ARHI can be considered to be a prognostic marker in glial tumors.

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