Alpha Internexin Expression Related with Molecular Characteristics in Adult Glioblastoma and Oligodendroglioma

Ja Hee Suh,1 Chul-Kee Park,2 and Sung-Hye Park1
1Departments of Pathology and 2Neurosurgery, Seoul National University Hospital, Seoul, Korea

INTRODUCTION

Gliomas, which include astrocytic tumors, oligodendrogliomas, mixed oligoastrocytomas, and ependymomas, are the most common primary malignancy of the central nervous system (CNS) and account for 78% of cases (1, 2). A wide spectrum of clinical behavior occurs among individual patients within each tumor group. Although chemotherapy, radiation therapy, and surgery have been attempted, improvements in patient survival have been limited. From a therapeutic point of view, the differential diagnosis among these entities is of clinical importance to predict biological behavior and to determine the optimal treatment protocol. However, distinguishing glial subtypes based on nuclear and cellular morphology alone is subjective, with significant interobserver variability, even among highly experienced neuropathologists (3, 4). Recent studies have suggested that these variable prognoses can be partly explained by different molecular profiles, especially detecting chromosomes 1p and 19q (1p/19q) as a specific molecular signature strongly associated with an oligodendrogial phenotype and a favorable prognosis (5). The glial subtypes have been recently been shown to be mediated by a specific translocation ([t(1;19)[q10;p10]).

The genetic characteristics, the 1p/19q codeletion, and epidermal growth-factor-receptor (EGFR) gene amplification are mutually exclusive in gliomas (6, 33).

Alpha internexin (INA) is a 66-kDa intermediate protein that maps to 10q24.33. It is a component of the primary neurofilament triplet proteins (NFTPs) of the central nervous system (CNS); a low-molecular-weight neurofilament subunit (68 kDa), middle-molecular-weight neurofilament subunit (160 kDa), and a high-molecular-weight neurofilament subunit (205 kDa). INA is also abundantly expressed in the peripheral nervous system. INA overexpression has been observed in neurodegeneration and neuronal cell death (7, 8). Ho and Liem (9) stated that the neurofilament-protein expression pattern is a good marker for identifying tumor cell origin and differentiation status. In recent studies, the relationship between tumor phenotype and genetic characteristics was examined based on a microarray and RNA extraction. Ducray et al. reported evidence of neuronal differentiation in oligodendrogliomas (ODGs) with a 1p/19q codeletion. In an immunohistochemical study, the most differentially expressed neuronal gene, INA, clearly distinguished the two groups of gliomas. That is, 1p/19q codeleted INA-expressing gliomas, and 1p/19q non-codeleted INA negative gliomas (10,
The aim of this study was to verify whether INA is strongly related to chromosomal 1p/19q status in a large series of glioblastomas (GBMs) and ODGs. The prognostic value of INA for gliomas was also evaluated.

**MATERIALS AND METHODS**

**Patients and Samples**
Cases were selected from pathologically proven low grade ODGs, anaplastic ODGs, and GBMs operated on at Seoul National University Hospital, Republic of Korea. A total of 230 patients who underwent a neurosurgical operation from July 1995 to June 2008 were included when complete clinical information and tissue paraffin blocks were available. Tumor histology was classified according to the 2007 WHO classification by neuropathologists (JH Suh and SH Park). Clinical information was extracted from the medical records, which showed secondary operations for recurrence, chemoradiation therapy status, and follow-up data.

**Tissue microarrays and immunohistochemical staining**
Each tumor tissue sample was fixed with formalin and embedded in paraffin. Representative paraffin blocks were selected and mounted on slides with hematoxylin and eosin (H&E) staining before they were prepared for the tissue microarray. Cores from representative areas of each tumor were marked on both an H&E stained tissue section and an original donor block. Three 2-mm diameter tissue cores were extracted from the marked area of each donor block and placed in 60 tissue cores. A 4-μm thick section was cut from each array block.

Immunohistochemical INA studies were conducted with antibodies purchased from Leica Microsystems (Newcastle, UK). Pre-treatment for antigen retrieval before staining with antibody was performed by autoclaving in 0.01 M citrate buffer, pH 6.0 and then placing the sections in 0.5% hydrogen peroxide/methanol for 10 min. Immunostaining was performed with an automated immunostainer, Dako TechMateTM 500 plus (Glostrup, Denmark) with INA antibody (1:200). Slides were incubated in diaminobenzidine, which served as the chromogen to visualize immunostaining. Finally, the slides were counterstained with Meyer’s hematoxylin.

We developed criteria for positive staining using strongly positive (2+) and weakly positive (1+) grading. Strong positivity was defined when positive tumor cells constituted more than 10% of all tumor cells in each core. Weak positivity was defined when positive tumor cells were confined to less than 10% of all tumor cells in the same field. Negative staining indicated that none of tumor cells was completely stained.

**Fluorescence in situ hybridization (FISH)**
The same tissue arrays were subjected to FISH analysis, which was performed in the following manner. Briefly, tumor sections were deparaffinized by dehydration and microwaving in citrate buffer, pH 6.0, for 10 min, digested in pepsin solution (4 mg/mL in 0.2 N NaCl pH 1.5) for 15 min at 37°C, rinsed in 2 × standard saline citrate (SSC) at room temperature for 5 min, and air dried. Dual-probe hybridization was performed for the 1p deletion analysis using a Spectrum Orange-labeled, locus-specific 1p probe and a Spectrum Green-labeled 1q probe (Vysis, Downers Grove, IL, USA). Similarly, a Spectrum Orange-labeled, locus-specific 19q probe and a Spectrum Green-labeled 19p probe (Vysis) were used for the 19q deletion analysis. Probes and target DNA were denatured simultaneously in a 73°C oven for 5 min, followed by an overnight incubation at 37°C. Slides were then washed in 1.5 M urea/0.1 × SSC at 45°C for 30 min and in 2 × SSC at room temperature for 2 min. After washing, the digoxigenin-labeled probes were detected using a rhodamine detection kit (Oncor, Gaithersburg, MD, USA). Nuclei were counterstained with 4, 6-diamidino-2-phenylindole and the anti-fade compound p-phenylenediamine. A Zeiss (Thornwood, NY, USA) Axioplan microscope equipped with a triple-pass filter (DAPI/FTC/orange; Vysis) was used to assess the number of FISH signals for each locus-specific FISH probe. Signals for each probe were counted under a microscope equipped with a triple-pass filter (DAPI/FTC/orange; Vysis) and an oil immersion objective. Approximately 60 non-overlapping nuclei were enumerated per hybridization.

To evaluate EGFR gene status, dual-color FISH analysis using the LSR EGFR Spectrum Orange/CEP 7 Spectrum Green Probe (Vysis) was performed in other paraffin sections from the same tissue array. Briefly, paraffin sections were deparaffinized, dehydrated in graded ethanol, and air dried. The sections were digested with protease K (0.5 mg/mL) at 37°C for 28 min. The slides were denatured at 76°C for 8 min, with an overnight incubation at 37°C in the HYBriteTM hybridization chamber (Vysis). Simultaneous probe/specimen denaturation at 76°C for 8 min was processed before hybridization. Slides were hybridized overnight at 37°C and washed in 2 × SSC/0.3% NP40 at 73°C for 5 min. The nuclei were counterstained with DAPI (1,000 ng DAPI/mL in antifade mounting solution; Vysis). The EGFR gene copy number was counted in 100 non-overlapping tumor cell nuclei in the paraffin sections. The mean signal number for the EGFR gene and CEP 7 was calculated for each case, followed by calculating the EGFR gene/CEP 7 ratios. The EGFR gene was considered amplified in individual cells when the EGFR control signal ratio was greater than 2. When the number of EGFR gene copies was more than 4 in at least 40% of tumor cells, high ploidy of the EGFR gene was recognized by applying a Colorado scoring system, as previously described (12).

**Statistical analysis**
Correlations between INA and 1p/19q codeletion status were
determined using Fisher’s exact test or Pearson’s chi square test. The Kaplan-Meier and log-rank methods were used to assess overall survival (OS) and progression-free survival (PFS) with compared strata. A Cox-regression test was used for the multivariate analysis. Statistical significance was defined as \( P < 0.05 \). The statistical analysis was performed with SPSS version 17.0 (Systat, Chicago, IL, USA).

**Ethics statement**

This study was approved by the institutional review board of Seoul National University Hospital (IRB registration number: H-1011-053-340). All of procedures were performed with the patient’s informed consent.

**RESULTS**

A total of 230 consecutive GBMs and ODGs, including anaplastic and low-grade tumors, were examined. The mean ages with standard deviations and gender compositions of patients are shown in Table 1. All tumors showed a male predominance (1.2:1 in ODGs, 1.5:1 in GBMs). The mean ages were 42.2-yr old and 47.5-yr old for the ODGs and GBMs, respectively.

Immunohistochemical staining and FISH were undertaken in 228 of the 230 studied cases; two cases were excluded because the available core was too small to analyze the immunohistochemical staining or we were unable to determine the FISH signal count. The results of the FISH and INA immunohistochemical staining are shown in Tables 2 and 3.

In the ODG group, 1p/19q codeletion was detected in 77% (97/122), and EGFR gene amplification in 6.6% (8/122) of cases, with 1p/19q codeletion occurring in 32 cases (29.6%) (Fig. 1). In the GBM group, 1p/19q codeletion was seen in six (97/122), and EGFR gene amplification in 6.6% (8/122) of cases, tochemical staining or we were unable to determine the FISH signal count. The results of the FISH and INA immunohistochemical staining are shown in Tables 2 and 3.

A Kaplan-Meier survival analysis revealed that INA expression was correlated with better PFS (28.9 vs 154.4 months \( P = 0.001 \)) and OS (37.8 vs 172.3 months \( P = 0.0001 \)) in all 230 tumors (Fig. 5). Patients in the GBM group with INA expression had significantly better OS (patients with strong INA expression: 116.3 months, patients with weak INA expression: 20.2 months) than patients with INA immunonegativity (14.6 months) \( (P < 0.001) \). PFS was also correlated with the INA immunohistochemical findings (Fig. 6). PFS was significantly correlated with INA positivity (\( P = 0.02 \)) in the ODG group (Fig. 7A). A strong positive INA finding was better for PFS (162.7 months) than weak INA positivity (76.6 months) and INA-negative staining (67.8 months) (Fig. 7B).

The median OS of patients with ODGs was calculated with a life table because only seven of 122 patients (5.7%) died from the disease, of whom three (2.4%) showed weak INA expression, and the remaining four cases were INA negative. The median OS of patients expressing INA (175 months) was significantly longer than that of INA negative cases (95.3 months, \( P = 0.01 \)) (Fig. 7A).

1p/19q codeletion was strongly related to better OS in pa-

**Table 1. Age and sex distribution of studied cases**

|                | LO (n = 49) | AO (n = 73) | GBM (n = 108) |
|----------------|-------------|-------------|---------------|
| Male           | 27 (55.1%)  | 40 (54.8%)  | 65 (60.2%)    |
| Female         | 22 (44.9%)  | 33 (45.2%)  | 43 (39.8%)    |
| Age (mean ± SD)| 40.63 ± 9.94| 43.82 ± 10.50| 47.45 ± 12.96|

LO, low grade oligodendroglioma; AO, anaplastic oligodendroglioma; GBM, glioblastoma; SD, standard deviation.

**Table 2. The result of the 1p/19q codeletion and EGFR amplification in oligodendroglial tumors and glioblastomas**

|                | ODG (n = 122) | GBM (n = 106) |
|----------------|---------------|---------------|
| 1p/19q codeletion | 97 (77.0%)   | 6 (5.5%)      |
| EGFR amplification | 8 (6.6%)      | 32 (29.6%)    |

ODG, oligodendroglioma; GBM, glioblastoma; EGFR, epidermal growth factor receptor.

**Table 3. The result of the INA immunostaining in oligodendroglial tumors and glioblastomas**

|                | ODG (n = 122) | GBM (n = 106) | Total (n = 228) |
|----------------|---------------|---------------|----------------|
| Strong positive | 65 (53.3%)    | 7 (6.5%)      | 72 (31.3%)     |
| Weak positive   | 33 (27%)      | 30 (27.8%)    | 63 (27.4%)     |
| Total           | 98 (80.3%)    | 37 (34.9%)    | 135 (59.2%)    |

INA, alpha-internexine; ODG, oligodendroglioma; GBM, glioblastoma.
DISCUSSION

For the prognostic and predictive values of the adult gliomas, oligodendroglial phenotype is sufficient to determine a treatment option (13). The oligodendroglial phenotype indicates a better prognosis and more chemosensitivity than astrocytic tumors, but the histological diagnosis is subjective and suffers from interobserver variability and discrepancies (2, 14). 1p/19q codeletion status, which is related to an unbalanced t(1;19) (q10;p10) translocation, is mutually exclusive with EGFR gene amplification (15) and is a diagnostic, prognostic, and predictive marker for ODGs. In our study, none of the 1p/19q codeleted cases showed EGFR amplification, and all cases revealed that EGFR amplification was not consistent with 1p/19q codeletion. However, either 1p or 19q deleted cases were present among the EGFR amplified cases; two 19q-deleted and one 1p-deleted GBM, and two 1p-deleted and one 19q-deleted ODG. In our series, patients with a 1p/19q codeletion had a better prognosis than did patients with the 1p/19q non-codeletion whether the tumors were oligodendroglial or astrocytic. 1p and
19q deletions are common in ODGs (75%-85%), but not in GBMs, which have a lower frequency of 1p/19q codeletion (5%-10%). In contrast, EGFR amplification is common in GBMs (25%-35%), but low in ODGs (6.6%) (16, 17). Comparative ge-

Fig. 2. INA immunostaining pattern in oligodendrogliomas (A-C) and glioblastomas (D-F). (A, D) negative, (B, E) weak positive (positive in < 10% of tumor cells), (C, F) strong positive (positive in ≥ 10% of tumor cells) (magnification, × 400).

Fig. 3. Correlation with alpha-internexin (INA) and 1p/19q codeletion in oligodendrogliomas. INA immunostaining result is well correlated with 1p/19q codeletion in oligodendroglioma group.

Fig. 4. Correlation with INA and 1p/19q codeletion in glioblastomas. INA immunostaining result is well correlated with 1p/19q codeletion in glioblastoma group.
Table 4. The relation between INA expression, 1p/19q deletion status and EGFR amplification

| Tumors  | INA positive | INA negative | Total |
|---------|--------------|--------------|-------|
| ODG     | Codeletion   | No codeletion|       |
| (n = 122)| 94 (100%) | 7 (25%) | 28 |
| EGFR    | Amplification| No amplification| |
| (n = 122)| 1 (12.5%) | 7 (87.5%) | 8 |
| ODG     | 100 (87.7%) | 14 (12.3%) | 114 |
| GBM     | Codeletion   | No codeletion|       |
| (n = 106)| 6 (100%) | 31 (30.4%) | 102 |
| EGFR    | Amplification| No amplification| |
| (n = 106)| 9 (23.7%) | 29 (76.3%) | 38 |
| GBM     | 21 (36.8%) | 36 (63.2%) | 57 |

INA, alpha-internexine; ODG, oligodendroglioma; GBM, glioblastoma; EGFR, epidermal growth factor receptor.

Fig. 5. Kaplan-Meier survival curves of 230 tumor patients in relation with INA expression. (A) Overall survival (OS) in 230 tumor patients in relation with INA expression. (B) Progression-free survival (PFS) in 230 tumor patients in relation with INA expression.

Fig. 6. Kaplan-Meier survival curves of glioblastoma patients in relation with INA expression. (A) Overall survival in glioblastomas related with INA expression. (B) Progression-free survival in glioblastomas related with INA expression.
which has implications for INA overexpression. INA overexpression leads to the accumulation of neurofilaments. Recently, the pathophysiology of INA in brain tumors is beginning to be known as a neurodegenerative disease. INA is helpful to distinguish neuroblastomas from other small round cell tumors (23). INA is expressed in the majority of medulloblastomas and atyp-

**Table 5. Univariate analysis of glioblastomas and oligodendroglioma**

| Tumors                        | Variables | No. of death (recurrence)*/No. of observed (%) | Median OS (month) | 95% CI lower-upper | P value |
|-------------------------------|-----------|-----------------------------------------------|-------------------|---------------------|---------|
| Glioblastoma (n = 108)       | Age       |                                               |                   |                     |         |
|                               | < 50      | 37/63 (58.7)                                 | 23.6              | 17.8                | 29.4    | 0.014  |
|                               | > 50      | 31/45 (68.9)                                 | 15.8              | 12.8                | 18.9    |         |
|                               | INA       |                                               |                   |                     |         |
|                               | Negative  | 68/71 (95.8)                                 | 16.2              | 13.7                | 18.7    | 0.001  |
|                               | Weak positive | 27/30 (90.0)                               | 20.2              | 13.1                | 27.3    |         |
|                               | Strong positive | 4/7 (57.1)                                | 116.3             | 20.0                | 186.6   |         |
|                               | 1p 19q    |                                               |                   |                     |         |
|                               | Codeletion | 1/6 (16.7)                                  | 103.5             | 81.0                | 126.0   | 0.001  |
|                               | No codeletion | 68/102 (66.7)                           | 16.9              | 13.6                | 20.2    |         |
|                               | EGFR      |                                               |                   |                     |         |
|                               | Amplification | 29/39 (74.4)                              | 22.9              | 18.8                | 27.1    | 0.030  |
|                               | No amplification | 38/69 (55.0)                           | 15.0              | 11.6                | 18.5    |         |
| Oligodendroglioma (n = 122)  | Age       |                                               |                   |                     |         |
|                               | < 50      | 20/96 (20.8)                                 | 127.9             | 109.2               | 146.7   | 0.207  |
|                               | > 50      | 8/26 (30.8)                                  | 97.5              | 9.2                 | 185.7   |         |
|                               | INA       |                                               |                   |                     |         |
|                               | Negative  | 9/21 (4.76)                                  | 67.8              | 46.7                | 88.6    | 0.001  |
|                               | Weak positive | 10/38 (26.3)                              | 76.6              | 54.1                | 99.0    |         |
|                               | Strong positive | 5/63 (7.9)                                | 162.7             | 146.3               | 179.1   |         |
|                               | 1p 19q    |                                               |                   |                     |         |
|                               | Codeletion | 18/94 (19.1)                                 | 142.7             | 121.7               | 171.4   | 0.017  |
|                               | No codeletion | 10/28 (35.7)                             | 94.5              | 16.0                | 172.9   |         |
|                               | WHO grade |                                               |                   |                     |         |
|                               | II        | 7/49 (14.3)                                  | 153.2             | 79.4                | 56.7    | 0.009  |
|                               | III       | 21/73 (28.8)                                 | 115.0             | 72.3                | 239.5   |         |
|                               | EGFR      |                                               |                   |                     |         |
|                               | Amplification | 6/8 (73)                                   | 112.8             | 45.3                | 63.2    | 0.001  |
|                               | No amplification | 22/114 (19.3)                           | 26.8              | 14.7                | 38.9    |         |

*No. of death for glioblastoma and No. of recurrence for oligodendroglioma. INA, alpha-internexin; EGFR, epidermal growth factor receptor; CI, confidence interval.
The expression of proneural genes characterizes a group of malignant gliomas, but did not predict the absence of a 1p/19q codeletion. INA expression highly predicted the presence of 1p/19q codeletion in immunostaining. These results suggest that INA, alpha-internexin; EGFR, epidermal growth factor receptor; CI, confidence interval.

Table 6. The result of multivariate analysis in glioblastomas related with overall survival

| Variables                | P value | Hazard ratio | 95.0% CI Lower | 95.0% CI Upper |
|--------------------------|---------|--------------|----------------|---------------|
| Age                      | 0.060   | 0.619        | 0.376          | 1.020         |
| INA                      | 0.000   | 0.326        | 0.178          | 0.596         |
| 1p 19q codeletion         | 0.976   | 0.130        | 0.000          | 0.000         |
| EGFR amplification       | 0.699   | 0.904        | 0.540          | 1.511         |

Table 7. The result of multivariate analysis in oligodendroglioma related with progression-free survival

| Variables                | P value | Hazard ratio | 95.0% CI Lower | 95.0% CI Upper |
|--------------------------|---------|--------------|----------------|---------------|
| 1p 19q codeletion         | 0.016   | 0.111        | 0.018          | 0.665         |
| WHO grade                | 0.801   | 0.865        | 0.279          | 2.677         |
| Age                      | 0.583   | 0.791        | 0.343          | 1.827         |
| INA                      | 0.003   | 0.170        | 0.053          | 0.548         |
| EGFR amplification       | 0.003   | 0.129        | 0.033          | 0.498         |

INA, alpha-internexin; EGFR, epidermal growth factor receptor; CI, confidence interval.

INA expression rather than 1p/19q codeletion can be done quickly from a simple biopsy, is inexpensive, and does not require any special equipment. Our data revealed that the sensitivity of INA expression for 1p/19q codeletion in both ODGs and GBMs was 100%. When INA was strongly positive in tumor cells (more than 10% of the cells expressed INA), only two cases were absent the 1p/19q codeletion of 65 INA strongly positive ODGs. So, expression of INA indicated a 96.9% chance of a 1p/19q codeletion in the ODG. Only one case in the GBM group showed no 1p/19q codeletion in seven INA positive cases, which represents an 85.7% chance of a 1p/19q codeletion in that group. The 16.2% positive predictive value in GBMs was lower than that of ODGs (91.3%) because only six cases revealed strong INA positivity in immunostaining. These results suggest that INA expression highly predicted the presence of 1p/19q codeletion, but did not predict the absence of a 1p/19q codeletion. The expression of proneural genes characterizes a group of malignant gliomas with a better prognosis than the “mesenchymal” subtype (26). In our study, the survival analysis based on 1p/19q codeletion and EGFR amplification status agreed with previously reported results (27, 28).

INA expression was found to be significantly more frequent in ODGs than in GBMs (11), which were also reproduced in our study. Additionally, our results revealed that INA-positive GBMs had a better OS and PFS and that INA expressing ODGs had a better PFS than did INA-negative gliomas (10, 11). Moreover, INA was significant in the multivariate analysis controlling for factors such as age, 1p/19q status, EGFR amplification, and the WHO grading system. As we could not find a glioma study in the English literature with a multivariate analysis including INA expression with other factors, the present study could be novel.

INA expression in both ODGs and GBMs suggests that the progenitor cells of both neuron and oligodendrocyte lineages, so neuronal and glial differentiation could be implicated in gliomagenesis (29). Because the process of the transition from nonneoplastic glial cells to GBM is unknown, more in-depth studies are required for a clear understanding of gliomagenesis. However, INA is not expressed in normal glial or neuronal cells but in axons, suggesting that INA expression in glial tumors should be considered an abnormal feature.

A reasonable positive cut-off value for the immunohistochemical analysis of INA staining is required in a future study. Previous studies set the cut off at 10%, but more variable cut-off values should be attempted.

We showed that INA immunoeexpression could be a possible candidate as a surrogate marker for 1p/19q codeletion, good prognosis and long term survival. Further perspective studies are needed to identify INA as a prognostic and predictive factor.

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