Expression of the Pro-apoptotic Protein ARTS in Astrocytic Tumors

Correlation with Malignancy Grade and Survival Rate

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BACKGROUND. Apoptosis (i.e., programmed cell death) plays a major role in the development of astrocytic tumors, which are the most common tumors of the central nervous system. ARTS, a proapoptotic protein that is localized in the mitochondria, promotes apoptosis by functioning as an XIAP antagonist and a caspase activator.

METHODS. To investigate the role of ARTS in astrocytoma, the authors examined protein expression and apoptotic activity in 72 astrocytic tumors, which included low-grade astrocytomas, anaplastic astrocytomas, and glioblastomas.

RESULTS. Whereas normal astrocytes did not express the ARTS protein, astrocytoma cells strongly expressed ARTS, and the expression of this protein increased with increasing tumor grade. Furthermore, increased levels of ARTS were significantly associated with higher rates of apoptosis (as measured using the terminal deoxynucleotidyltransferase–mediated deoxyuridine triphosphate nick end-labeling [TUNEL] assay as well as an immunohistochemical staining assay for active caspase-3) in these tumors. Levels of two other apoptosis-related proteins, p53 and Bcl-2, also were examined using immunohistochemical methods; ARTS expression was found to be positively correlated with expression of the former and negatively correlated with expression of the latter, which is known to possess antiapoptotic activity.

CONCLUSIONS. The results of the current study suggest that ARTS levels reliably reflect the ability of cells to undergo apoptosis, which serves as a defense mechanism against the development and progression of astrocytoma. Furthermore, ARTS expression, when taken into consideration in combination with tumor grade, was the only independent predictor of survival identified in the current analysis. Thus, the authors conclude that ARTS may possess utility as a prognostic marker, as well as a therapeutic tool, for patients with astrocytoma. ***Cancer* 2004;101: 2614–21.** © *2004 American Cancer Society.*

KEYWORDS: apoptosis, ARTS, astrocytic tumors, survival rate.

Apoptosis (i.e., programmed cell death) is an active and well controlled cellular process that plays a major role in cell homeostasis. When deregulated, however, apoptosis can result in various pathologic conditions, including cancer.[1,2] In the central nervous system, the most commonly occurring malignancies are gliomas, which can be classified into four groups: pilocytic astrocytomas (Grade I), diffuse astrocytomas (Grade II), anaplastic astrocytomas (Grade III), and glioblastomas (Grade IV). According to Yuan and Yankner,[3] apoptotic cell death plays a significant role in the development of the central nervous system and also in the induction of central nervous system disorders.

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The apoptotic process is characterized by the activation of a family of cysteine proteases known as *caspases*. Caspases exist in live cells as latent zymogens and are activated via the cleavage of their prodomain during apoptosis. The caspase activation cascade begins with the activation of the 'initiator caspases' (caspase-8, caspase-9, and caspase-10) and continues with the activation of the 'executioner caspases' (primarily caspase-3, but also caspase-6, caspase-7, and others).[4] One of the main results of caspase activation is the fragmentation of genomic nuclear DNA into 200–280–base pair fragments, a phenomenon known as 'apoptotic laddering'.[5]

The role of apoptosis in astrocytic tumors and the possible exploitation of this role for prognostic and therapeutic purposes are extensively discussed in the literature. Numerous studies have demonstrated a positive correlation between apoptotic index and malignancy grade.[6–15] In contrast, others have found no correlation,[16,17] or even a negative correlation,[18,19] between apoptotic activity and grade of malignancy.

The involvement of several apoptotic proteins, such as p53, caspase-3, APO-1/Fas, and members of the Bcl-2 family, in astrocytoma has been thoroughly investigated,[10,13–25] with the expression of these proteins found to be associated with apoptosis. In 2000, our group isolated and characterized another apoptosis-related protein, ARTS, which initially was found to be essential for TGF-beta-induced apoptosis.[26,27] Recently, we found that ARTS also was involved in apoptotic processes induced by a variety of other proapoptotic triggers, including Fas, staurosporine, cytosine arabinoside (ara-C), and etoposide.[28] Under normal conditions, ARTS is localized in the mitochondria in living cells; however, proapoptotic stimuli cause it to be released into the cytosol, where it binds and inhibits XIAP.[28] As a result, XIAP levels grow smaller, leading to the activation of caspases and the induction of apoptosis.

ARTS is strongly expressed in the brain, a finding that suggests that this protein may play an important role in the nervous system.[27] Thus, in the current study, we set out to investigate the relation between ARTS protein expression and tumor grade in astrocytic malignancies.

MATERIALS AND METHODS

Study Population

We examined 72 paraffin sections that had been obtained from patients with astrocytic tumors diagnosed at the Department of Pathology, Rambam Medical Center (Haifa, Israel). The mean patient age was 50 years (standard deviation [SD], 19 years; range, 2–78 years). The study population consisted of 43 male patients (60%) and 29 female patients (40%). The distribution of tumor grades (according to the World Health Organization classification system[29]) was as follows: Grade I, $n = 7$ (9.7%); Grade II, $n = 12$ (16.7%); Grade III, $n = 11$ (15.3%); and Grade IV, $n = 42$ (58.3%). Grade I–II tumors were considered low-grade lesions, whereas Grade III–IV tumors were considered high-grade lesions. Survival rates were calculated using clinical follow-up data (follow-up duration: mean, 18.5 months; median, 9 months; range, 6–136 months).

Immunohistochemical Assays

Paraffin sections were stained with hematoxylin and eosin; pretreatment involved microwave heating for 5 minutes at 92 °C. Immunohistochemical staining for ARTS was performed using polyclonal anti-ARTS antibodies (1:100 dilution; Sigma, St. Louis, MO). Slides were incubated with a horseradish peroxidase–conjugated biotinylated secondary antibody according to the manufacturer's recommendations (HISTOSTAIN-PLUS Bulk Kit; Zymed Laboratories, South San Francisco, CA) and then developed using an aminoethyl carbazole substrate (Zymed Laboratories). Parallel sections were stained in similar fashion for Ki-67/MIB-1 (DAKO, Carpinteria, CA), p53 (clone DO-7; DAKO), Bcl-2 (clone 124, 1:80 dilution; DAKO), and active caspase-3 (1:1700 dilution; R&D Systems, Minneapolis, MN).

In each case, immunohistochemical findings were quantified by counting the number of stained cells out of a total of 1000 tumor cells. Results were presented as percentages of tumor cells that stained positively. For ARTS, Bcl-2, and p53, expression was considered to be positive when $> 25\%$ of the cells in a given sample stained positively for the marker in question. Proliferation index was calculated as the percentage of cells (out of a total of 1000) that exhibited positive staining for Ki-67.

Apoptosis Assays

Terminal deoxynucleotidyltransferase–mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay

Apoptotic activity was assessed using a TUNEL assay kit according to the manufacturer's instructions (In Situ Cell Death Detection Kit; Roche, Basel, Switzerland). Apoptotic index was calculated as the percentage (out of a total of 1000) of TUNEL-positive tumor cells.

Anti-active caspase-3 staining

Immunostaining with anti-active caspase-3 antibodies (1:1700 dilution; R&D Systems) was performed as described above.

Statistical Analysis

Comparisons between parametric groups were made using the Student t test for independent groups, whereas nonparametric groups were compared with one another using the Mann–Whitney U test. Equality of variances was assessed using the Levene test. Comparisons between paired groups were made using the paired Student t test.

Associations between categoric groups were assessed using the chi-square test or the Fisher exact test as appropriate. Univariate analysis of survival was performed using the log-rank test, and Kaplan–Meier survival curves were constructed. Multivariate analysis of survival involved application of the Cox proportional hazards method in forward-stepwise fashion. Two-tailed P values ≤ 0.05 were considered indicative of statistical significance.

RESULTS

Correlation between ARTS Expression and Astrocytoma Grade

Aberrant apoptosis has been shown to play a major role in carcinogenesis.[30] We previously found that ARTS protein levels were indicative of the ability of cells to undergo apoptosis.[27] To investigate the role of ARTS in astrocytoma, we examined the expression of this protein in normal astrocytes and in astrocytoma cells at various stages of malignancy. Immunohistochemical staining of normal astrocytes did not reveal detectable levels of ARTS (Fig. 1A); this lack of staining was specific to astrocytes, as ARTS expression was evident in other types of cells, such as neurons and fibrillary neurophils. Unlike normal astrocytes, astrocytoma cells did express ARTS, and expression levels increased with increasing grade of malignancy (Fig. 1B–E); the proportion of ARTS-positive cells was significantly larger in high-grade (Grade III–IV) astrocytomas (68%) than in low-grade (Grade I–II) astrocytomas ($P = 0.04$) (Fig. 1E). In addition, ARTS staining in astrocytoma cells was cytoplasmic (Fig. 1B–D), despite our previous finding that ARTS was localized in the mitochondria[27]; thus, in tumor cells such as these, the ARTS protein appears to exist in its transformation state.

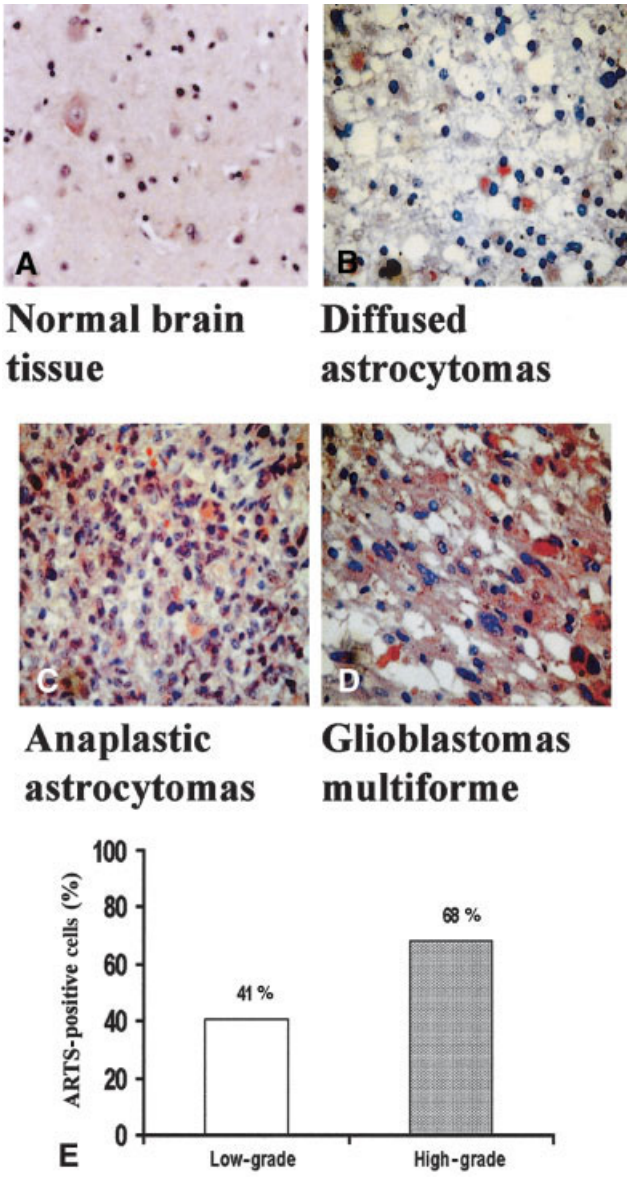
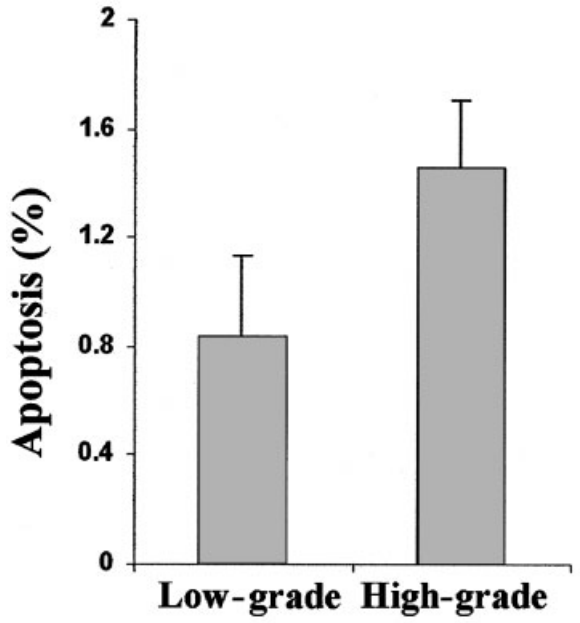
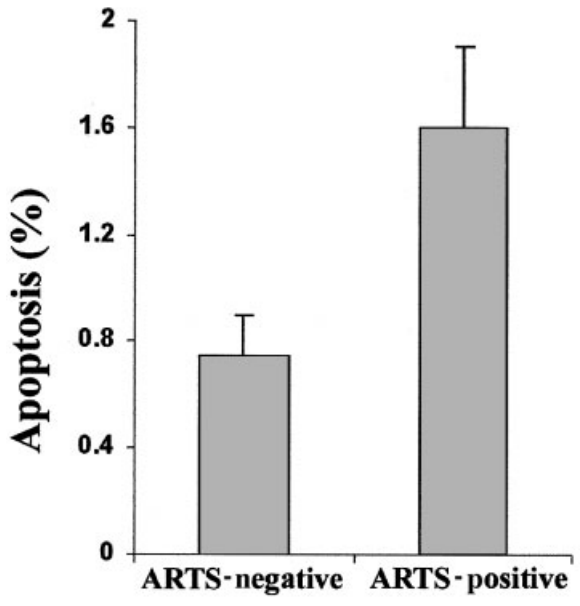


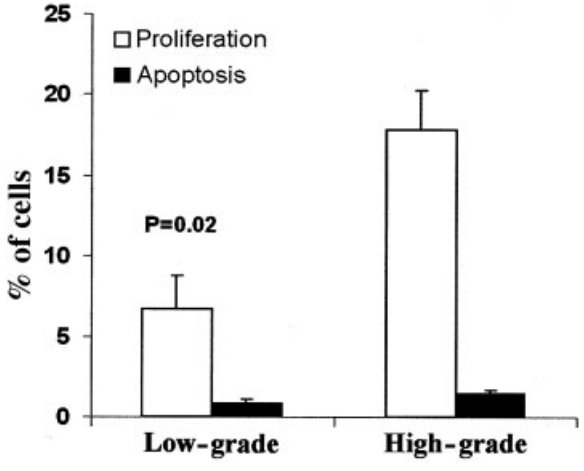
FIGURE 1. Correlation between ARTS protein expression and astrocytoma grade. Immunohistochemical staining of astrocytoma cells and normal brain tissue was performed using polyclonal anti-ARTS antibodies. (A) Lack of ARTS staining in normal astrocytes. (B) ARTS staining in low-grade astrocytoma. (C,D) Elevated ARTS expression in high-grade astrocytoma. (E) Correlation between ARTS expression and tumor grade. High-grade astrocytomas contained a significantly larger proportion of ARTS-positive cells compared with low-grade astrocytomas ($P = 0.04$).

High Levels of ARTS Expression and Apoptotic Activity in High-Grade Astrocytoma

Analysis of astrocytes in normal brain tissue adjacent to tumor tissue revealed negative TUNEL findings as well as negative ARTS staining. We previously found that increased levels of ARTS induced apoptotic cell death in various types of cells, including neuronal cells (unpublished data).[27,28] In the current study, we performed the TUNEL assay to investigate whether astrocytoma cells, which also expressed elevated levels of ARTS, had increased apoptotic activity. High-grade astrocytomas were found to contain significantly more TUNEL-positive cells (mean $\pm$ SD, 1.45 $\pm$ 0.25) compared with low-grade astrocytomas (mean $\pm$ SD, 0.83 $\pm$ 0.3; $P = 0.036$) (Fig. 2A). In addition, ARTS-positive







cells exhibited much higher levels of apoptotic activity compared with ARTS-negative cells, and the presence of a direct positive correlation between apoptotic index and percentage of ARTS-positive cells was confirmed ($P = 0.038$) (Fig. 2B). Another apoptosis assay, which involved the staining of samples with anti-active caspase-3 antibody, yielded similar results ($P = 0.034$). Furthermore, the increased proportion of active caspase-3-positive cells in high-grade astrocytomas was significantly correlated with higher levels of ARTS expression ($P = 0.02$). Taken together, these findings suggest that the elevated expression of ARTS in astrocytic tumors contributes to an increase in apoptotic response.

Decreased Apoptosis/Proliferation Index Ratio in High-Grade Astrocytomas

Normal cells are known to maintain a balance (or *homeostasis*) between rates of proliferation and apoptosis. In contrast, tumor cells exhibit an imbalance between these two processes, favoring proliferation over apoptosis.[30] In the current study, proliferation rates were evaluated by staining astrocytoma samples with an anti-Ki-67 antibody, and this analysis revealed that the proliferation index was almost 3 times greater in high-grade gliomas (proliferation index, 17.8) compared with low-grade gliomas (proliferation index, 6.7). Furthermore, although the rate of apoptosis also was elevated in high-grade astrocytomas, this elevation was not sufficient to counteract the concomitant increase in proliferative activity (Fig. 2C). This finding suggests that a decreased apoptosis-to-proliferation ratio is a feature of high-grade astrocytomas. Furthermore, the observation of significant positive correlations between ARTS expression and both apoptotic activity and astrocytoma grade suggests that this protein is actively involved in apoptosis and disease progression.

FIGURE 2. Correlation between apoptosis index and astrocytoma grade and between apoptosis index and ARTS expression. (A) In situ cell death detection (terminal deoxynucleotidyltransferase–mediated deoxyuridine triphosphate nick end-labeling [TUNEL]) assay in low-grade (Grade I–II) vs. high-grade (Grade III–IV) astrocytomas. Apoptosis rate is significantly higher in high-grade astrocytomas ($P = 0.036$). (B) Apoptosis rates in samples with high ARTS expression levels are considerably higher than apoptosis rates in samples with low ARTS expression levels ($P = 0.038$). (C) Although apoptotic index is elevated in high-grade astrocytomas compared with low-grade tumors, the increase in proliferation index in these high-grade tumors outpaces the increase in apoptotic index (low-grade: $P = 0.02$; high-grade: $P < 0.0001$).

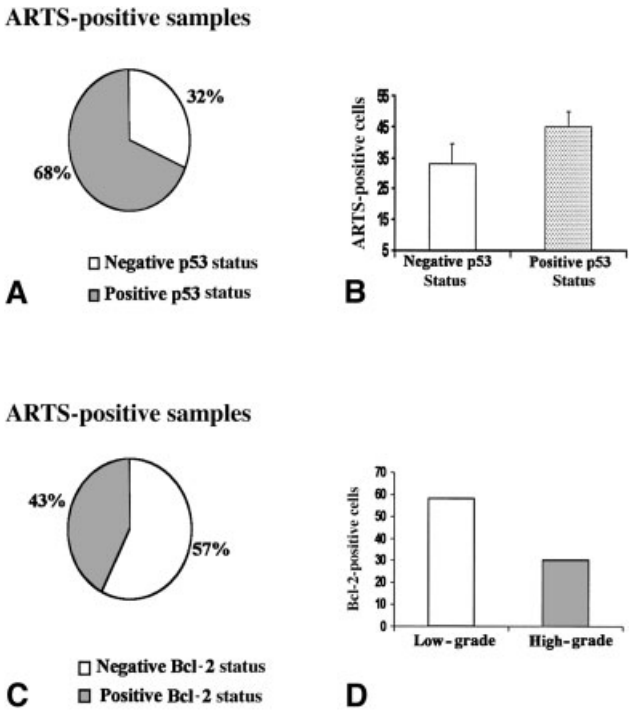


FIGURE 3. p53 and Bcl-2 participate in cooperation with ARTS to promote apoptosis in astrocytomas. p53 and Bcl-2 expression levels in astrocytoma cells were evaluated by immunohistochemical methods, using anti-p53 and anti-Bcl-2 antibodies, respectively. (A) The number of ARTS-positive samples expressing p53 was significantly higher than the number of ARTS-positive samples not expressing p53 ($P = 0.035$). (B) p53-positive samples contained a higher percentage of ARTS-positive cells than did p53-negative samples ($P = 0.03$). (C) The number of ARTS-positive samples not expressing Bcl-2 was significantly higher than the number of ARTS-positive samples expressing Bcl-2 ($P = 0.024$). (D) Compared with high-grade astrocytomas, low-grade astrocytomas contained a higher percentage of Bcl-2-positive cells ($P = 0.02$).

Association of ARTS, p53, and Bcl-2 in the Promotion of Apoptosis in Astrocytomas

To establish the contribution of apoptosis to the malignant progression of astrocytomas, we examined the expression of two other apoptosis-related markers, p53 and Bcl-2, using immunohistochemical methods. We found that 68% of all ARTS-positive samples also had positive p53 status, whereas the remaining 32% did not express p53 ($P = 0.035$) (Fig. 3A); in addition, an even stronger correlation between these two markers ($P = 0.009$) was documented in high-grade astrocytoma samples. Thus, overall, there was a direct positive correlation between p53 expression and ARTS expression in astrocytic tumors ($P = 0.03$) (Fig. 3B).

We also found that elevated ARTS expression was associated with decreased levels of the antiapoptotic marker Bcl-2. Similarily, we observed a significant negative correlation between Bcl-2 expression and grade of malignancy ($P = 0.02$) (Fig. 3D). Furthermore, 57% of all ARTS-positive samples had negative Bcl-2 status, whereas only the remaining 43% expressed Bcl-2 ($P = 0.024$) (Fig. 3C). Multivariate analysis revealed that Bcl-2 expression and ARTS expression, respectively, were negatively and positively correlated with tumor grade ($P = 0.02$).

Overall, on the basis of these findings, it appears that ARTS operates in connection with p53 and Bcl-2 to promote apoptosis in astrocytoma cells.

Association between ARTS Expression and Survival in Patients with Astrocytoma

In the current study, we found that the proapoptotic protein ARTS was involved in the transformation of astrocytes to maliganant astrocytoma cells and that ARTS expression, like p53 expression, increased with increasing tumor grade. To investigate the potential association between ARTS expression and survival in the study cohort, we performed a univariate analysis of survival. This analysis revealed that ARTS expression was negatively correlated with survival ($P < 0.0019$) (Fig. 4A)—i.e., the survival of patients whose tumors strongly expressed ARTS was significantly shorter than that of patients whose tumors weakly expressed ARTS. Furthermore, ARTS expression, when taken into consideration in combination with tumor grade, was the only independent predictor of survival identified on Cox multivariate analysis of patients with astrocytoma (Fig. 4B). Overall, we demonstrated that ARTS levels were correlated with survival rates for patients with low-grade astrocytomas as well as for patients with high-grade disease (Fig. 4B). Thus, ARTS expression levels may serve as a useful prognostic marker for patients with astrocytoma.

DISCUSSION

Apoptosis induced by ischemic conditions has been reported to play an important role in gliomas (predominantly glioblastomas).[6,10,11] Apoptotic cells are found around necrotic foci and adjacent to areas of high proliferative activity.[10] Investigation of the role of apoptotic proteins in glioma could yield findings that are useful with respect to both prognosis and treatment. Thus, our primary goal in the current study was to examine the expression of ARTS, a proapoptotic mitochondrial protein,[27] in gliomas of various grades. Specifically, we assessed potential correlations between ARTS expression and apoptosis rate, grade of malignancy, and patient survival.

Using Northern blot analysis, we previously demonstrated that ARTS was strongly expressed in normal brain tissue.[27] Surprisingly, although high levels of ARTS were found in neurons and fibrillary neurophils

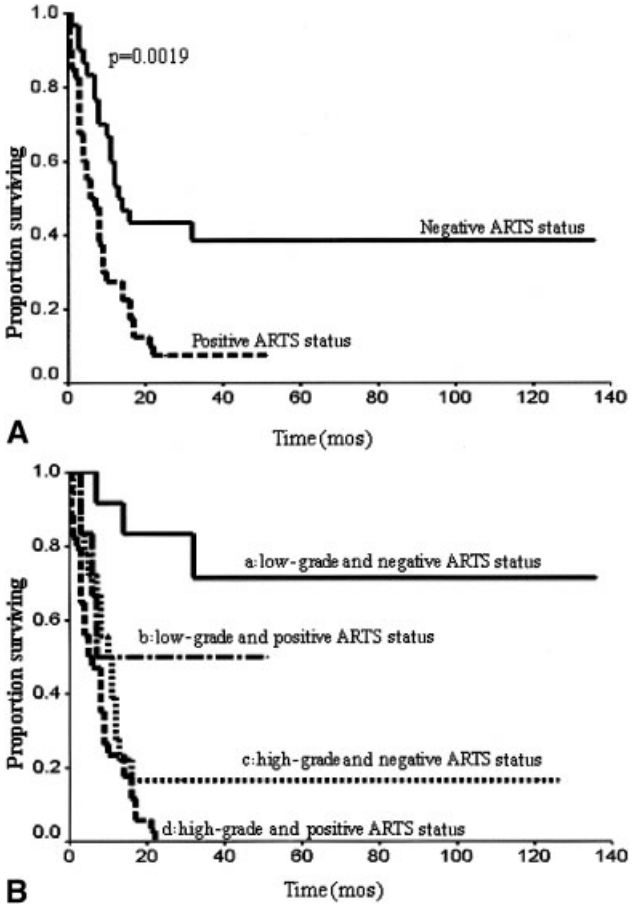


FIGURE 4. Survival rates for patients with high ARTS expression levels were significantly lower than those for patients with low ARTS expression levels. (A) Cox univariate analysis revealed that ARTS expression was negatively correlated with survival ($P < 0.0019$). (B) Cox multivariate analysis revealed that ARTS expression, when taken into consideration in combination with grade of malignancy, was significantly correlated with patient survival (log-rank test: a vs. d, $P < 0.0001$; a vs. c, $P = 0.0008$; a vs. b, $P < 0.13$).

in normal brain tissue, this protein was not expressed in normal astrocytes. In contrast, malignant astrocytoma cells exhibited high levels of ARTS expression, with these levels being directly and positively correlated with tumor grade (Fig. 1B–D). Consequently, ARTS expression may possess utility as a diagnostic and prognostic tool for patients with astrocytoma.

Significant positive correlations were found between grade of malignancy and both ARTS expression and apoptotic activity. The correlation between apoptosis and tumor grade has been reported in several other studies.[31–36] Although the observed increase in apoptosis rate in high-grade tumors was not dramatic, it appears to be significant for the following reasons: 1) the finding was confirmed using two separate apoptosis assays (TUNEL and active caspase-3 staining); and 2) levels of the proapoptotic proteins ARTS and p53 increased with increasing tumor grade, whereas levels of the antiapoptotic protein Bcl-2 decreased. We believe that the inherent limitations of our apoptosis assays, as well as the phagocytosis of apoptotic cells by neighboring cells,[37] hindered the assessment of apoptotic activity in the current study.

Increased apoptotic activity is associated with higher tumor grade in a number of malignancies, including breast, endometrial, and thyroid carcinoma. Soini et al.[38] suggest that the increased apoptotic activity observed in high-grade malignancies may result from a hypoxic process occurring in the core of the tumor. Thus, elevated apoptosis rates may be reflective of the increased area occupied by transformed cells in advanced stages of disease.

Many types of cells lose the expression of proapoptotic proteins while transforming into malignant cells; this loss of expression provides a selective advantage to such cells.[30] We previously noted a loss of ARTS expression specifically in lymphoblasts in the majority of patients with acute lymphoblastic leukemia.[39] Furthermore, cells that had lost ARTS expression could not undergo apoptosis in response to treatment with the chemotherapeutic agent ara-C, and transfection of these cells with an ARTS expression vector restored their ability to undergo apoptosis. Thus, although ARTS expression is absent in malignant leukemic lymphoblasts but present in astrocytoma cells, in both malignancies, it appears that ARTS expression levels reflect the ability of tumor cells to undergo apoptosis. In fact, increased ARTS expression is correlated with elevated apoptosis rates in astrocytoma cells. We therefore suggest that the absence of ARTS expression in normal astrocytes is consistent with the absent or low levels of apoptotic activity reported in these cells.[40,41] Also noteworthy was our finding of an elevated proliferation index in high-grade gliomas, which supports the theory that the net balance between apoptosis and proliferation plays a critical role in tumor progression.[2] We also found that the apoptosis/proliferation index ratio was lower in high-grade gliomas than in low-grade gliomas (Fig. 2C). These results suggest that the increase in proliferative activity outpaces the increase in apoptosis rates in high-grade astrocytomas, leading to disease progression.

The apoptosis/proliferation index ratio has been identified as a significant prognostic marker for patients with glioblastoma.[7,12,13] Kuriyama et al.[7] reported that among patients with glioblastoma, those who had higher apoptosis/proliferation index ratios had a more favorable prognosis compared with those who had lower apoptosis/proliferation index ratios.

This finding, which confirms that increased apoptotic activity can successfully stem the growth of astrocytoma cells, is consistent with our results. Thus, because ARTS expression levels were found to be significantly higher in tumors with a higher apoptotic index, this protein could serve as a basis for the development of therapeutic tools for increasing rates of apoptosis and thereby preventing the proliferation of glioma cells.

To obtain additional information on the ARTS-mediated apoptotic pathway in astrocytomas, we assessed the expression of two other apoptosis-related proteins, the tumor suppressor p53 and the antiapoptotic protein Bcl-2. Mutations in the *p53* tumor suppressor gene occur during the early stages of astrocytoma tumorigenesis,[25] and some of the resulting mutant p53 proteins can lead to apoptosis as efficiently as wild-type p53 can.[42,43] This may be the case in astrocytic tumors, as we found that higher levels of p53 expression were associated with increased apoptotic activity.

Bcl-2 is a mitochondrial protein that inhibits apoptosis by blocking the release of cytochrome C into the cytosol.[44] This protein has been identified as a direct target in p53-mediated apoptosis.[16] We found that elevated levels of ARTS expression were correlated with decreased Bcl-2 levels, which in turn were associated with apoptosis (unpublished data). The significant negative correlation found in the current study between Bcl-2 expression and ARTS expression, together with the significant positive correlation found between p53 expression and tumor grade, suggests that these apoptotic regulators are, to some extent, coordinated with each other during the apoptotic response against astrocytoma cells.

Because ARTS appears to be an important participant in the apoptotic response against astrocytoma cells, we hypothesized that ARTS levels could serve as a prognostic marker for patients with astrocytoma. In agreement with this hypothesis, we found that survival rates for patients with low ARTS expression levels were much higher than those for patients with high ARTS expression levels. In addition, multivariate analysis revealed that ARTS expression, when taken into consideration together with grade of malignancy, was a strong predictor of survival for patients with astrocytoma. Overall, our results suggest that ARTS acts in coordination with p53 and Bcl-2 to promote apoptosis arising as a defense mechanism against malignant disease. Thus, ARTS may possess utility as both a prognostic marker and a therapeutic tool for patients with astrocytoma.

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