High ATP2A2 expression correlates with better prognosis of diffuse astrocytic tumor patients

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Abstract. Novel molecular markers are required for defining subsets of diffuse astrocytic tumor patients with differing prognoses. Here, we examined ATP2A2 expression in 109 human diffuse astrocytic tumor samples (39 grade II diffuse astrocytoma (DA), 19 grade III anaplastic astrocytoma (AA), 51 grade IV glioblastoma) and its correlation with patient clinicopathologic characteristics. ATP2A2 expression significantly correlated with tumor grade and survival (P<0.05). High ATP2A2 expression was detected in 35.3% (18/51) of glioblastoma patients, compared to 61.5% (24/39) in grade II, and 52.6% (10/19) in grade III astrocytoma patients (P=0.043). The median survival was 45±5.3 (95% CI, 34.7-55.3) months in patients with high ATP2A2 expression and 16±5.0 (95% CI, 6.3-25.7) months in patients with low ATP2A2 expression (P<0.0001). Additionally, high grade astrocytoma patients with high ATP2A2 expression showed longer survival (median, 31.0±4.9 months, 95% CI, 21.4-40.7) than those with low ATP2A2 expression (median: 13.0±1.6 months, 95% CI, 9.9-16.1; P=0.027). Furthermore, both ATP2A2 overexpression and IDH1 mutation were detected in secondary glioblastoma, AA developed from DA and oligodendrogiomas with IDH1 mutation. The MTT assays showed that lentiviral ATP2A2 overexpression significantly suppressed the clonogenic growth of glioblastoma U251MG cells (P<0.05). Xenografts stably overexpressing ATP2A2 were markedly smaller in size 4 weeks post inoculation (P<0.05). Our findings identified high ATP2A2 expression in a subset of astrocytoma patients that was associated with better prognosis and ATP2A2 suppressed astrocytoma growth.

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Introduction

Astrocytoma, which originates from star-shaped glial cells, is the most commonly occurring primary brain tumor in adults. According to the World Health Organization (WHO) scheme, it represents a heterogeneous group of diseases with different degrees of malignancy from relatively indolent diffuse astrocytomas to highly aggressive glioblastoma multiforme (GBM) (1). Among these tumors, grade II astrocytomas are characterized by low level proliferative activity, but often recur, and some of the types tend to progress to higher grades of malignancy (1,2). The prognosis of patients with GBM, the most malignant astrocytoma, remains rather dismal. Even with the best standard of care currently available, these patients only have a median life expectancy of ~10.6 months after diagnosis (3). The current standard therapy for GBM patients includes maximal debulking surgery, radiation and adjuvant chemotherapy. Temozolomide, a current chemotherapeutic agent of choice for GBM patients, has yielded only very modest improvements in disease outcomes (4).

Considerable research efforts have been focused on the identification of genetic alterations in GBMs so as to help define subgroups of GBM patients with different prognosis. Several genes, including *TP53, PTEN, EGFR* and *IDH1*, are altered in gliomas. The *TP53/IDH1* mutation seems to occur early during the development of an astrocytoma (5), whereas the loss or mutation of *PTEN* and amplification of *EGFR* are characteristic of primary glioblastomas (6,7). The discovery of these genetic alterations helps the diagnosis of GBM and allows us to better define the prognosis of GBM patients and provides further characterization and understanding of gliomagenesis.

The *ATP2A2* gene encodes a protein called sarco (endo) plasmic reticulum Ca^{2+}-ATPase isoform 2 (SERCA2), which transports Ca^{2+} from the cytosol to the endoplasmic reticulum (ER) lumen. Mutations in *ATP2A2* cause Darier's disease, an autosomal dominant skin disorder characterized by loss of adhesion between epidermal cells and abnormal keratinization (8,9). Disturbances of Ca^{2+} homeostasis have been implicated in the development of many types of tumor, such

as colon cancer, liposarcoma, leukemia and head and neck squamous cancer (10-15). *ATP2A2* heterozygous mutant (*ATP2A2*$^{+/-}$) mice showed increased incidence of hyperkeratinized tumors in regions of stratified squamous epithelia, including the oral mucosa, tongue, esophagus, palate, skin, genitalia, and non-glandular mucosa of the stomach (16). The role of ATP2A2 in the oncogenesis of astrocytomas has not been elucidated. We hypothesized that, given the importance of Ca^{2+} homeostasis in maintaining the physiological processes, the expression of ATP2A2 could be altered in astrocytomas and the definition of such alterations may, as a prognostic marker, help identify subgroups of patients with a distinct prognosis, or, as a diagnostic marker, help distinguish astrocytomas of different grades.

Although ATP2A2 has recently been implicated in certain cancer types (19,20), no information is available on its expression in human astrocytoma tissues. In this study, we examined the expression of ATP2A2 in 109 human astrocytoma samples by immunohistochemistry and further analyzed the correlation between ATP2A2 expression and clinicopathological characteristics of these astrocytoma patients. We also investigated the effect of ATP2A2 overexpression on the growth of GBM cells *in vitro* and in a mouse xenograft model.

Materials and methods

Tissue specimens. In total, 109 formalin-fixed and paraffin-embedded surgically resected astrocytic tumor specimens (84 primary ones and 17 secondary GBM and 8 AA developed from DA) were acquired from the Department of Pathology, Changzheng Hospital, Second Military Medical University, Shanghai, China. Samples of oligodendrogiomas with IDH1 mutation (n=20) and pilocytic astrocytomas (PAs) (n=20) without IDH1 mutation were also acquired to further explore the relation between ATP2A2 expression and IDH1 mutation. These specimens were archived between January, 2007 and December, 2014. All analyzed brain tumors were subjected to consensus review by two neuropathologists (Wei-Qing Li, and Hui-Min Liu) according to the WHO Classification (1). Table I lists the types of brain tumors analyzed, including DA (n=39), AA (n=19) and GBM (n=51). There were 61 (60.0%) males and 48 (40.0%) females with a median age of 45 (range, 5-73) years. No patients had received preoperative chemo- or radiotherapy. The average period of follow-up was 27.6±20.5 (range, 1-90) months. Tumor size, location, extent of surgical resection, and postoperative consolidated treatment were recorded (Table I). In addition, normal brain tissue samples were obtained from patients undergoing decompressive craniectomy and patients undergoing selective temporal lobe resection for intractable epilepsy.

Acquisition of all tissue specimens was approved by the local ethics committee at the authors' affiliated institution and was carried out in accordance with established institutional and national ethical guidelines regarding use of human tissues for research.

Immunohistochemical staining. Formalin-fixed, paraffin-embedded, 3-μm tissue sections were cut with 3 adjacent sections chosen from each sample for routine immunohistochemical staining. After deparaffinization in xylol and rehydration in gradient ethanol, sections were immersed in 1 μM EDTA buffer (pH 8.0) and microwaved for 20 min for antigen retrieval. Endogenous peroxidase was inactivated by 3% methanolic hydrogen peroxide solution for 30 min. Non-specific binding was blocked by incubation with non-immune serum for 30 min followed by overnight incubation at 4˚C with anti-ATP2A2 (Abcam, San Diego, CA, USA), anti-IDH1 and anti-GFAP antibodies (Maxin Biotechnology, Fujian, China). After staining with the DAB kit (Maxin Biotechnology), the slides were counterstained with hematoxylin.

For evaluating ATP2A2 and IDH1 mutation immunoreactivity, two experienced pathologists examined representative visual fields (x200 magnification) independently to identify positively stained tumor cells. The intensity of positive staining was scored using a scale from 0 to 3 (0 for no immunostaining, 1 for light-brown color, 2 for medium-brown color, and 3 for dark-brown color). The percentage of positive staining cells was also scored (0, no staining; 1, positive staining in <25% of the tumor cells; 2, positive staining in 25-75% of the tumor cells; and 3, positive staining in >75% of the tumor cells). The percentage of cells showing positive staining with the antibodies was calculated in 5 high-powered fields. The two scores were then multiplied, and the results were regarded as the expression score of the sample. All discrepancies in scoring were reviewed, and a consensus was reached. Samples were scored totally as follows: strong (+++, total score = 6), moderate (++, total score = 4-5), weak (+, total score = 1-3), and null (-, total score = 0). ATP2A2 was recorded as high expression (++ and +++), and low or negative expression (+ and -) according to the rate of labeled tumor cells and cytoplasm staining intensity.

Cell culture and lentiviral infections. Human glioblastoma cell line U251MG was obtained from the Cell Bank, Type Culture Collection, Chinese Academy of Sciences (CBTCCCAS, Shanghai, China) and cultured at 37˚C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

The lentivirus vector expressing ATP2A2, pcDNA3.1-SERCA2 ATPase, was constructed using the pcDNA3.1 (-)-3Flag-Myc-His expression system according to the manufacturer's instructions (Sunbio Medical Biotechnology, Shanghai, China). The recombinant lentiviruses were produced by co-transfecting human embryonic kidney (HEK) 293T cells using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). U251MG cells were infected with lentiviruses overexpressing ATP2A2 and then selected with 800 μg/ml G418 (Invitrogen) in complete medium for 20-30 days. Stable U251MG cells were maintained in α-MEM containing 10% FBS and 800 μg/ml G418. Total cellular RNA and proteins were extracted at 10 days after transfection for further analysis.

Quantitative real-time RT-PCR. For quantitative real-time RT-PCR, total cellular RNA was extracted with TRIzol (Invitrogen). Reverse transcription was performed using a Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. The expression of *ATP2A2* mRNA was

Table I. Clinicopathological characteristics of 109 diffuse astrocytic tumor patients.

Variables	N (%)
Gender	
Male	61 (60.0)
Age (years)[a]	
≥55	67 (61.5)
Tumor site	
Temporal lobe	16 (14.7)
Parietal lobe	46 (42.2)
Frontal lobe	31 (28.4)
Occipital lobe	16 (14.7)
Tumor size	
>4 cm	61 (56.0)
Extent of resection	
Total	91 (83.5)
Subtotal	18 (16.5)
Radiotherapy	
Yes	47 (43.1)
Chemotherapy	
Yes	60 (55.0)
WHO grade[b]	
II	39 (35.8)
III	19 (17.4)
IV	51 (46.8)

[a]Patient age refers to age at which the study sample was obtained. [b]Tumors were graded according to the World Health Organization (WHO) criteria.

normalized against β-actin mRNA. The comparative threshold cycle (ct) method was used, and the fold difference = $2^{-(\Delta ct \text{ of target gene} - \Delta ct \text{ of reference})}$. Quantitative real-time PCR was performed using the SYBR Premix Ex *Taq*™ kit (Takara, Kyoto, Japan) on the Takara TP800 System. The PCR was run at 95˚C for 15 sec, 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The sequences of the PCR primers used were as follows: ATP2A2, 5'-CTCGGATCCAACACTACAGGTGTTGAATGG-3' (sense), and 5'-CGGAATTCATGCGCAGTGATAAATTGAC-3' (antisense); β-actin, 5'-CGTGACATTAAG GAGAAGCTG-3' (sense), and 5'-CTAGAAGCATTTGCGGTGGAC-3 (antsense).

Western blot assays. Cellular lysates were prepared of U251MG cells using RIPA lysis buffer. Proteins were quantified using the Bradford method and samples were resolved on 10% SDS denatured polyacrylamide gel. Immunoblotting was performed as previously described (18) and the following antibodies were used: mouse anti-flag antibody (Sigma, St. Louis, MO, USA), rabbit anti-SERCA2 ATPase antibody (Abcam), and goat anti-mouse IgG and goat anti-rabbit IgG (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). The LabWorks™ Image Acquisition and Analysis Software (UVP) was used for densitometric analysis.

Cell proliferation and clonogenic assays. For cell proliferation studies, cells were plated onto 96-well plates at $2x10^3$ cells/well and cultured overnight to allow cell attachment. The number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide colorimetric assays at daily intervals (24, 48, 72, 96 and 120 h). For soft agar clonogenic survival assays, GBM cells were plated onto the top agar at $2.0x10^2$ cells per well and grew for 14 days at 37˚C. Colonies were visualized using the cell staining Giemsa solution (Chemicon, Millipore, Billerica, Danvers, MA, USA). An aggregate of ≥50 cells was considered a colony and the number of colonies was counted under the microscope. Each experiment was carried out in triplicate and at least three times independently.

Apoptosis assays. Cells were harvested 8 days after lentiviral infection and washed once with phosphate-buffered saline (PBS), trypsinized, and washed again in PBS with 2% FBS and resuspended in binding buffer containing 10 mM HEPES (pH 7.4); 2.5 mM $CaCl_2$, and 140 mM NaCl, and stained with Annexin V-R-PE and 7-AAD according to the manufacturer's protocol (Southern Biotech, Birmingham, AL, USA). Stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software, and the Mod-Fit program (Verity Software House Inc., Topsham, ME, USA) was used to analyze apoptosis. Each experiment was conducted in triplicate and at least three times independently.

Wound healing assays. The scratch assay was performed to investigate the effect of ATP2A2 overexpression on the migration of GBM cells. Briefly, cells were seeded in 6-well plates at a density of $2x10^5$ cells/well. When the cells were 90-100% confluent, the monolayer was scratched manually with a plastic pipette tip across the diameter of each plate, and after two washes with PBS, the wounded cellular monolayer was allowed to heal for 96 h. Images of central wound edges were taken at time 0 and at the indicated time-points using PowerShot G10 camera (Canon, Tokyo, Japan). Cell migration was observed by microscopy at 24, 48, 72 and 96 h.

Xenograft studies. Forty-five female BALB/c-nu mice, 7-8 weeks of age, were obtained from the Institute of Zoology, Chinese Academy of Sciences, Shanghai, China and kept under specific pathogen-free conditions in accordance with procedures and guidelines set by the Institutional Animal Care and Use Committee (IACUC) of the Second Military Medical University, Shanghai, China. The study protocol was approved by the local institutional review board. Each mouse was inoculated subcutaneously in the forelimb with $1x10^7$ U251 cells in 0.2 ml of medium. After 5 days, mice were randomized to receive twice weekly intratumoral injections (30 ml/mouse at 3-5 sites) of Lenti-ATP2A2 (pcDNA3.1-SERCA2 ATPase, $5x10^9$ TU/ml), or intratumoral injections of control lentiviruses ($5x10^9$ TU/ml), or vehicle control. Tumor size was measured every 3 days in two dimensions using a caliper, and tumor volume (mm^3) was calculated using the formula V = 0.5 x larger diameter x (smaller diameter)^2. Tumor volume was normalized

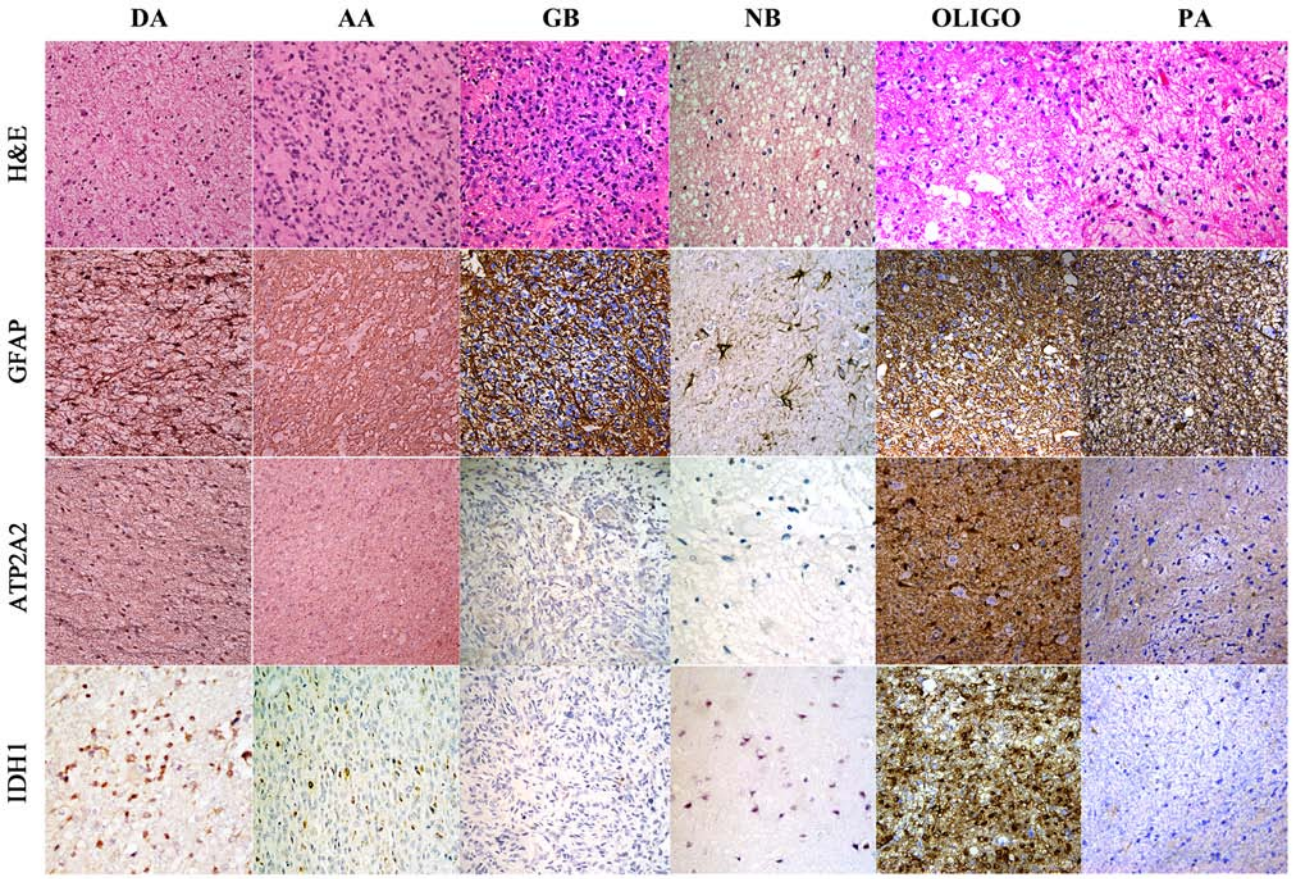

Figure 1. ATP2A2 expression in astrocytoma samples. Immunohistochemistry in normal brain shows that weak ATP2A2 is mainly expressed in some of the neurons, but not in the astrocytes. Stronger staining of ATP2A2 is noted in low-grade astrocytoma (DA) and oligodendrogioma rather than high-grade astrocytomas (AA and glioblastoma) and PA. DA, diffuse astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; NB, normal brain; OLIGO, oligodendrogioma; PA, pilocytic astrocytoma.

against the initial volume before intratumoral injection for plotting the curve of astrocytoma cell growth rate. Four weeks post injection, 6 mice from each group were randomly selected and sacrificed for weighing and photographing.

Statistical analysis. All statistical analysis and graphs were performed with the SPSS 22.0 analysis software (SPSS Inc., Chicago, IL, USA). The correlation between ATP2A2 expression and clinicopathologic characteristics was analyzed using the Kruskal-Wallis test and Chi-square test. The post hoc Dunn's test of multiple comparisons was used after the Kruskal-Wallis test to examine the sample contrasts between individual sample pairs. Besides, the correlation between ATP2A2 expression and IDH1 mutation in secondary glioblastoma and AA developed from DA was investigated by the McNemar test. The difference of ATP2A2 expression in oligodendrogliomas with IDH1 mutation and PAs without IDH1 mutation was investigated by Chi-square test. Survival was defined from the date of surgical diagnosis to death from any cause. Correlation of ATP2A2 expression with survival was determined using the Kaplan-Meier product-limit method, and differences between survival curves were tested using the log-rank test. For other experiments, differences between groups were measured by Student's t-test, and for comparing means of more than 2 groups, one-way ANOVA was used. P-values <0.05 were considered as statistically significant.

Table II. ATP2A2 immunoexpression based on staining intensity scores in diffuse astrocytic tumor according to tumor grade (n=109).

	Staining intensity scores of ATP2A2					
	- (n=32)	+ (n=30)	++ (n=32)	+++ (n=30)	Total	P-value
WHO grade						0.036
II	9	6	10	14	39	
III	1	8	7	3	19	
IV	19	14	9	9	51	

Tumors were graded by the World Health Organization (WHO) criteria.

Results

ATP2A2 is variably expressed in astrocytoma tissues and correlated with astrocytoma grade. We found weak staining of ATP2A2 in some of the neurons but not in the astrocytes of normal brains (Fig. 1). ATP2A2 was not expressed in 26.6% (29/109) of the patients, mildly positive in 25.7% (28/109), moderately positive in 23.9% (26/109) and strongly positive in 23.9% (26/109) of the patients (Table II). It was further

Table III. ATP2A2 immunoexpression in diffuse astrocytic tumor according to tumor grade (n=109).

	Low ATP2A2 expression	High ATP2A2 expression	Total	P-value
WHO grade				0.042
II	15	24	39	
III	9	10	19	
IV	33	18	51	

Table IV. ATP2A2 immunoexpression in low and high grade diffuse astrocytic tumor (n=109).

	Low ATP2A2 expression	High ATP2A2 expression	Total	P-value
Glioma grade				0.025
Low	15	24	39	
High	42	28	70	

examined whether ATP2A2 expression was associated with astrocytoma grades. ATP2A2 was positive in 76.9% (30/39) of grade II patients, 94.7% (18/19) of grade III patients and 62.8% (32/51) of grade IV patients (P=0.036, Table II). Moreover, 35.3% (18/51) of grade IV astrocytoma patients showed high positive staining of ATP2A2, which was significantly lower than that of grade II (61.5%, 24/39) and grade III (52.6%, 10/19) astrocytoma patients (P=0.042, Table III). The staining pattern of ATP2A2 in different grades of astrocytomas is illustrated in Fig. 1. As shown in Table IV, statistical analysis revealed a close correlation between ATP2A2 expression and WHO grade of astrocytomas (P=0.025). Furthermore, the results of the post hoc Dunn's test used after the Kruskal-Wallis test showed that the difference of ATP2A2 expression rate in grade IV and grade II was significant (P=0.015). However, there was no significant difference in the ATP2A2 expression between grade II glioma and grade III glioma (P=0.765), and grade III glioma and grade IV glioma (P=0.105). The above findings indicated that, despite variable expression of ATP2A2 in astrocytoma tissues, the most aggressive form of astrocytomas, GBM, exhibits noticeably lower expression of ATP2A2 compared to astrocytoma of lower grades. In addition, high ATP2A2 expression level is also seen in the patients with the secondary glioblastoma and AAs developed from DAs (56.0%, 14/25), which is similar to the relationship between IDH1 mutation and the secondary glioblastoma and AAs developed from DAs (44.0%, 11/25) (P=0.508). To further illustrate the correlation of high ATP2A2 expression and IDH1 mutation, ATP2A2 expression was also examined in oligodendrogiomas with IDH1 mutation and PAs without IDH1 mutation. The result supported that high ATP2A2 was more frequently observed in astrocytomas with IDH1 mutation (15/20) than in those without IDH1 mutation (3/20) (Chi-square test, P<0.001) (Fig. 1).

ATP2A2 expression correlates with survival of astrocytoma patients. The Kaplan-Meier distribution of survival is shown in Fig. 2A. The median survival was 45.0±5.3 (95% CI, 34.7-55.3) months in patients with high ATP2A2 expression and 16.0±5.0 (95% CI, 6.3-25.7) months in patients with low ATP2A2 expression (P<0.0001). There was a significant correlation between ATP2A2 expression and survival. We further analyzed the survival of high grade astrocytoma patients stratified by ATP2A2 expression. High grade astrocytoma patients with high ATP2A2 expression showed markedly longer survival (median, 31.0±4.9 months; 95% CI, 21.4-40.7) than those with low ATP2A2 expression (median, 13.0±1.6 months; 95% CI, 9.9-16.1) (P=0.027, Fig. 2B). Low grade astrocytoma patients with lower ATP2A2 expression showed significantly shorter survival (47.0±7.2 months, 95% CI, 32.8-61.2) than those with higher ATP2A2 expression (67.0±15.8 months, 95% CI, 36.0-98.0) (P=0.039, Fig. 2C). Besides, we examined whether the survival time was influenced by postoperative adjuvant therapy (radiotherapy and chemotherapy) in astrocytoma patients of different grades with different ATP2A2 expression levels (Fig.3). As shown in Fig. 3H, only in high grade astrocytoma patients with high ATP2A2 levels, those who received chemotherapy showed significantly longer survival (34±5.7 months, 95% CI, 22.7-45.3) than those who did not (19±6.7 months, 95% CI, 5.8-32.2) (P=0.021).

ATP2A2 overexpression suppresses the clonogenic growth and migration of GBM cells. The above findings suggest that ATP2A2 may play a hitherto undiscovered role in the oncogenesis and development of astrocytomas. To decipher the role of ATP2A2 in astrocytoma genesis, we infected GBM U251MG cells with lentiviral vectors overexpressing ATP2A2 (Lenti-ATP2A2), which resulted in a 132% increase in the mRNA transcript levels and 163% increase in the protein levels of ATP2A2 (Fig. 4A and B). The MTT assays showed that ATP2A2 overexpression significantly suppressed the growth of U251MG cells 72 and 96 h post transfection (P<0.05 or <0.001), with a 15.2±0.7% reduction in the growth of U251MG cells at 96 h (Fig. 4C). Additionally, the clonogenic assays showed that ATP2A2 overexpression caused a 54.2±1.9% decrease in the number of colonies (Fig. 4D) [P=0.0004 vs. Lenti-green fluorescent protein (Lenti-GFP)]. We further investigated whether the growth-inhibitory effect of ATP2A2 was associated with induction of apoptosis of GBM cells. Our flow cytometric analysis revealed that ATP2A2 overexpression was associated with a significant increase in the percentage of apoptotic U251MG cells (Lenti-ATP2A2, 48.4±3.3% vs. Lenti-GFP, 1.7±0.2%, P=0.0008) (Fig. 4E).

Moreover, we sought to investigate whether ATP2A2 impacted on the migration of GBM cells. Our wound healing assays revealed that ATP2A2 overexpression markedly suppressed the migration of U251MG cells (Fig. 5).

ATP2A2 overexpression inhibits GBM xenograft growth in mice. It was examined whether ATP2A2 overexpression suppressed the growth of GBM xenografts in mice. We found that, compared with control xenografts expressing GFP, tumors stably overexpressing ATP2A2 were markedly

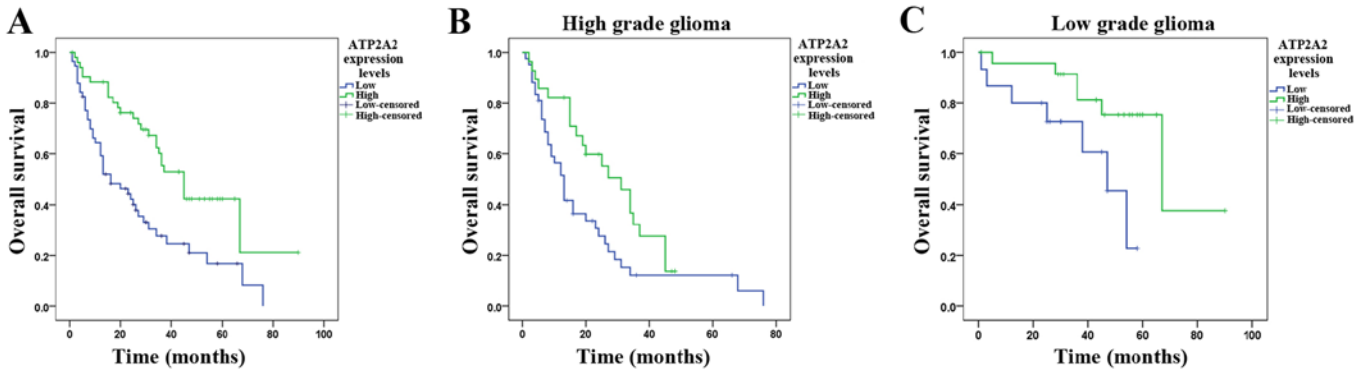

Figure 2. Kaplan-Meier postoperative survival curve for astrocytoma patients stratified by ATP2A2 expression. Low and high ATP2A2 expression is defined in Materials and methods. (A) The median overall survival of astrocytoma patients with high and low ATP2A2 expression is 45±5.3 (95% CI, 34.7-55.3) and 16±5.0 (95% CI, 6.3-25.7), respectively (log-rank test, P<0.0001). (B) High grade astrocytoma patients with high ATP2A2 expression show markedly longer survival (31±4.9 months, CI, 21.4-40.7) than those with low ATP2A2 expression (13±1.6 months, CI, 9.9-16.1) (P=0.027). (C) Low grade astrocytoma patients with lower ATP2A2 expression show significantly shorter survival (47.0±7.2 months, 95% CI, 32.8-61.2) than those with higher ATP2A2 expression (67.0±15.8 months, 95% CI, 36.0-98.0) (P=0.039).

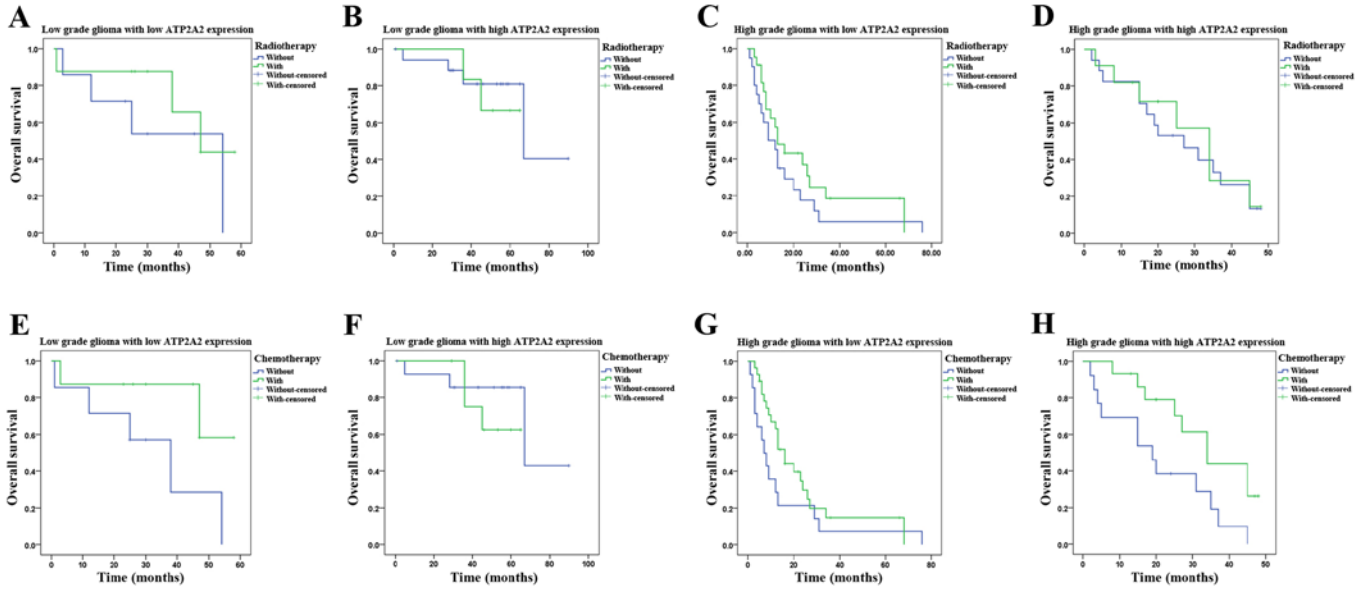

Figure 3. Association of postoperative adjuvant therapy with overall survival in astrocytoma patients of different grades stratified by ATP2A2 expression. Only in high grade astrocytoma patients with high ATP2A2 levels, those who received chemotherapy showed significantly longer survival (34±5.7 months, 95% CI, 22.7-45.3) than those who did not receive chemotherapy (19±6.7 months, 95% CI, 5.8-32.2) (P=0.021).

smaller in size 3 weeks after xenograft implantation (Fig. 6). The mean tumor volume was 81.6±21.5 mm^3 in xenografts overexpressing ATP2A2 and 238.2±65.2 cm^3 in the control xenografts respectively at week 4 post inoculation (P<0.05).

Discussion

Comprehensive elucidation of genetic alterations in astrocytomas may provide novel therapeutic targets, and diagnostic as well as prognostic markers for the disease. Furthermore, detailed delineation of prognostic markers for astrocytomas, especially GBMs, may lead to the identification of subsets of astrocytoma patients who may have better prognosis in response to adjuvant or neoadjuvant chemotherapies or targeted therapies. Here, we characterized the expression of ATP2A2 in astrocytoma tissues and documented for the first time that ATP2A2 showed altered expression in astrocytoma tissues. We found that ATP2A2 was expressed in the majority (73.4%, 80/109) of the patients and more than half (62.8%, 32/51) of grade IV astrocytoma patients expressed ATP2A2. Two thirds (61.5%, 24/39) of grade II astrocytoma patients exhibited high ATP2A2 expression while only approximately one third (35.3%, 18/51) of grade IV astrocytoma patients showed high ATP2A2 expression. Our statistical analysis further revealed a close correlation between ATP2A2 expression and WHO grade of astrocytomas. These findings implicate ATP2A2 participates in the oncogenesis in astrocytomas.

ATP2A2 acts as a critical component of several signaling pathways that have been demonstrated to be aberrant in astrocytoma. Genetic alterations occur frequently in the RTK/

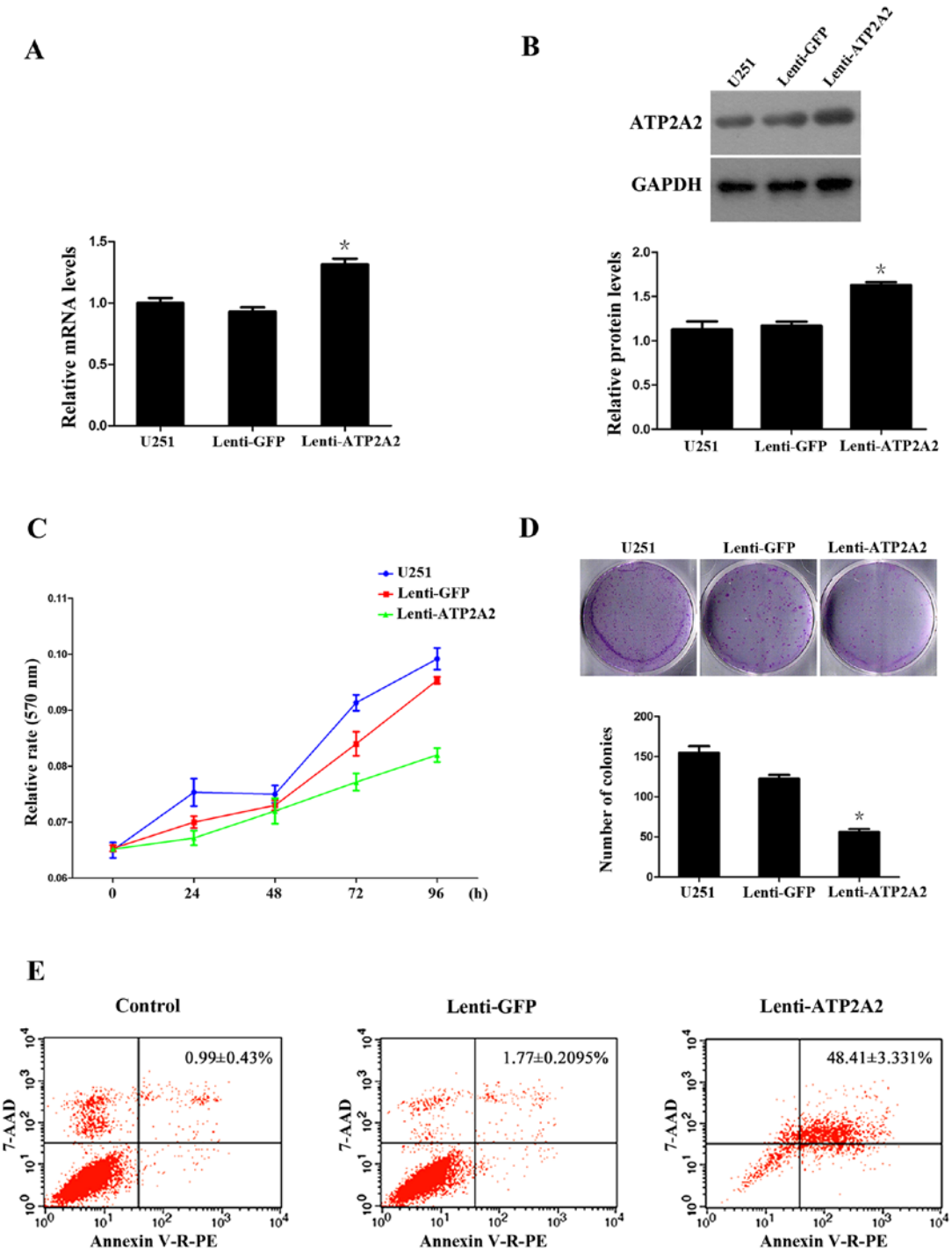

Figure 4. ATP2A2 overexpression was determined by western blot and real-time RT-PCR assays. U251MG cells were transfected with lentiviral vectors overexpressing ATP2A2 (Lenti-ATP2A2) or control vector (Lenti-GFP). The *ATP2A2* mRNA transcript levels (A) and ATP2A2 protein levels (B) were significantly increased in U251MG cells transfected with Lenti-ATP2A2. Cellular proliferation was examined by the MTT assays (C) and soft agar assays (D). Apoptosis of U251 cells was examined by flow cytometry (E). Data in (A-D) are shown as mean ± SD of at least three independent experiments. Data in (E) are representative of at least three independent experiments. *P<0.05 vs. Lenti-GFP or non-transfected controls.

RAS/PI-3K signaling pathway, the p53 signaling pathway and the RB signaling pathway (17). RAS signaling and calcium signaling converge at Raf1 (18) while calcium plays a pivotal role in p53 signaling (19) and Ca^{2+} and CaM-dependent signaling is required for RB phosphorylation (20,21). ATP2A2 expression is downregulated in cancer of the lung and head and neck squamous cell cancer, but upregulated in the colorectal cancer, liposarcoma and prostate cancer (15,22-25). Our demonstration of aberrant expression of ATP2A2 in astrocytoma tissues further adds ATP2A2 in a growing list of malignant cancers in humans. Besides, high ATP2A2 expression was associated with better overall survival of astrocytoma patients. Our study also evaluated whether ATP2A2 could be used as a prognostic marker for identifying a subset of astrocytoma patients responding preferentially to particular therapies. The result indicates that high grade astrocytoma patients

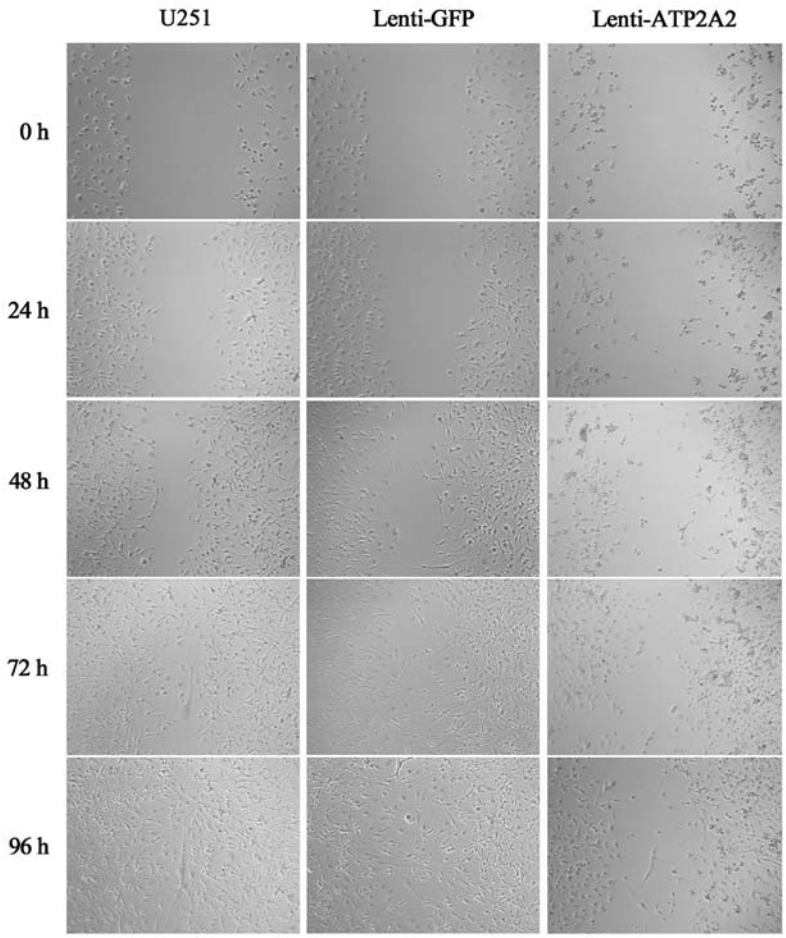

Figure 5. Effects of ATP2A2 overexpression on GBM cell invasion and migration. U251MG cells were transfected as described in Fig. 4 and wound scratch assays were performed as detailed in Materials and methods. Images of migrated cells were taken at 0, 24, 48, 72 and 96 h and these images are representative of at least 3 independent experiments.

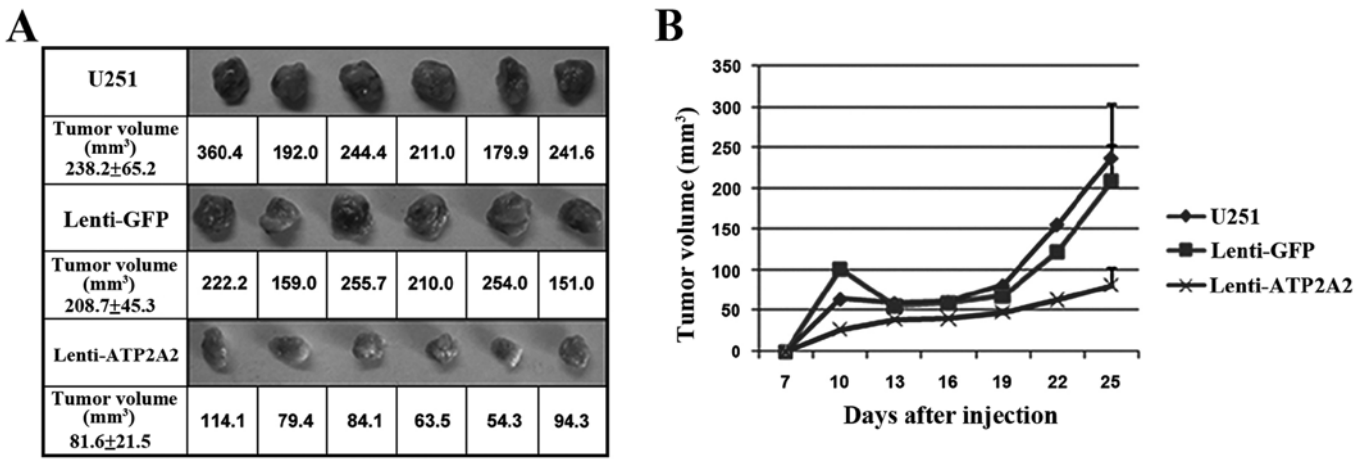

Figure 6. ATP2A2 overexpression suppresses xenograft tumor growth. (A) The effect of ATP2A2 overexpression on xenograft tumor growth was examined in SCID mice. U251MG cells stably overexpressing ATP2A2 were injected into the mice. Four weeks after injection, the tumors were dissected and captured. (B) Tumor size was measured every three days and data are shown as mean ± SD. $^{*}P<0.05$ vs. Lenti-GFP or non-transfected controls.

with high ATP2A2 expression level are more sensitive to the postoperative chemotherapy. Another substantial result of our study is that high ATP2A2 expression shared a similar distribution pattern with IDH1 mutation in secondary GBM, AA developed from DA, oligodendroglioma and PA, suggesting that ATP2A2 may possess a similar prognostic value as IDH1 in astrocytoma (26). We hypothesized that the alteration of energy metabolism due to IDH1 mutation (27) may affect the expression and function of ATP2A2. The aforementioned outcomes are consistent with findings from previous studies

on the role of ATP2A2 in other tumor types (22,23), implying that ATP2A2 expression may be a potential molecular marker for predicting the prognosis of astrocytoma patients.

In this study, we provide further evidence that increased ATP2A2 expression inhibited the clonogenic growth of GBM cells *in vitro*, probably via induction of apoptosis. Additionally, we observed that ATP2A2 overexpression depressed the migration of GBM cells in an *in vitro* wound healing assay. Moreover, tumor xenograft growth was noticeably suppressed by intratumoral injection of lentiviral vectors overexpressing ATP2A2. This may partially explain the survival benefit of high ATP2A2 expression in astrocytoma patients. However, it still needs to be further validated by additional studies.

Sarco (endo)plasmic reticulum (SER) Ca^{2+} ATPases are a highly conserved family of Ca^{2+} pumps. SERCA2 is implicated in certain cancers. Another isoform, SERCA3, encoded by the *ATP2A3* gene, has been shown to be down-regulated in colon cancer, lung adenocarcinoma and head and neck squamous cell cancer (11,28,29). Both proteins are involved in maintaining Ca^{2+} homeostasis, which is critical to the normal functioning of a plethora of cellular processes ranging from cellular proliferation to apoptosis by modulating cellular signaling pathways (30), and the aberration of some of these Ca^{2+}-mediated signaling pathways is implicated in tumorigenesis and tumor progression, such as metastasis, invasion and angiogenesis (31). ER Ca^{2+} levels remain undefined in astrocytoma cells. Astrocytoma cells are known to suffer from low grade of ER stress (32). Agents affecting ER Ca^{2+} homeostasis such as flavonoids could activate ER stress and induce cell death in glioma cells (33). Kovacs *et al* (34) found that compared with normal astrocytes, GBM cells exhibit higher resting cytosolic Ca^{2+} levels. It remains to be elucidated how ATP2A2 induces apoptosis of GBM cells in terms of ER stress. We speculate that in astrocytoma, alteration in ATP2A2 expression may contribute to tumorigenesis by interfering with the balance between cytosolic and ER Ca^{2+} level, thereby affecting certain Ca^{2+} signaling pathways, especially the RTK/RAS/PI-3K signaling, p53 signaling and RB signaling pathways (17). However, the exact mechanisms whereby ATP2A2 is involved in oncogenesis and development of astrocytomas require further investigation. In some other studies, the intervention of ATP2A2 expression, either upregulating or inhibiting, could exert influence on the progression of various malignancies (14,35), indicating that ATP2A2 may also function as a therapeutic target for astrocytoma treatment.

In conclusion, we present here direct clinical evidence that ATP2A2 is variably expressed in astrocytoma tissues and its expression correlates with tumor grade, and secondary glioblastoma and AA developed from DA has a propensity to overexpress ATP2A2. Importantly, patients with high ATP2A2 expression show better overall survival, thus identifying a subset of astrocytoma patients with differing prognosis. Furthermore, ATP2A2 overexpression suppresses growth of astrocytoma cells. Although the precise correlation of the altered ATP2A2 expression with astrocytoma grade, molecular subtypes or clinical parameters and the underlying mechanism requires further investigation, our findings implicate ATP2A2 in gliomagenesis and suggest that ATP2A2 may serve as a prognostic marker identifying astrocytoma patients with differing prognoses.

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