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From Cell Biology to Tissue Engineering

ATRX loss in adult supratentorial diffuse astrocytomas correlates with p53 over expression and IDH1 mutation and predicts better outcome in p53 accumulated patients

Li-Wei Shao[1]*, Yi Pan[1]*, Xue-Ling Qi[2]*, Yong-Xiao Li[1], Xiao-Long Ma[1], Wei-Ning Yi[3], Jing Zhang[1,4], Yan-Feng Zhong[1] and Qing Chang[1]

[1]Department of Pathology, School of Basic Medical Science, Peking University Third Hospital, Peking University, Beijing, China, [2]Department of Pathology, Beijing Sanbo Brain Hospital, Beijing, China, [3]Department of Epidemiology and Health Statistics, Peking University, Beijing, China and [4]Department of Neuropathology, Harborview Medical Center, University of Washington School of Medicine, Seattle, WA, USA

*These authors contributed equally to this work

Summary. Background: *IDH1/2* mutation, 1p/19q-codeletion and *MGMT* hypermethylation are well known molecular markers for gliomas. ATRX and p53 alterations are two lineage-specific genetic aberrations in diffuse astrocytic tumors. The aim of the present study is to clarify the significance of ATRX loss and its correlation with p53 overexpression, *IDH1/2* mutations, 1p/19q-codeletion and *MGMT* hypermethylation in supertentorial astrocytoma, and to determine the prognostic value of these factors in Chinese patients. Methods and Results: A total of 135 adult supertentorial astrocytomas were evaluated. ATRX loss was detected by immunohistochemistry (IHC) and was shown to be much less frequent in pGBs (3.5%) than in grade II, III astrocytomas and IV sGBs (31%). Direct sequencing and/or IHC analysis of the *IDH1R132H* gene mutation and p53 accumulation demonstrated correlation with age. Strong correlations were found between ATRX loss and *IDH1R132H* mutation, p53 overexpression as well as *MGMT* hypermethylation. 1p/19q-codeletion detected by fluorescence in situ hybridization (FISH) showed mutually exclusive with ATRX loss and p53 accumulation. In addition, patients with p53 overexpression combined with ATRX alterations demonstrated substantially longer survival than patients with wild-type ATRX. Conclusions: There may be interactions among these distinct molecules in astrocytoma development. ATRX loss may predict better clinical outcome in astrocytoma patients with p53 overexpression as compared to patients with wild-type ATRX. Tumors with astrocytoma phenotype accompanied by 1p/19q-codeletion and *IDH1R132H* mutation are mutually exclusive with ATRX and p53 alterations. Routine IHC can be used for evaluation of ATRX loss, p53 protein accumulation and IDH1R132H mutation, which may allow a means of classification of astrocytoma outcome.

Key words: ATRX, p53, IDH1, MGMT, Immunohistochemistry, Fluorescence in situ hybridization

Offprint requests to: Chang Q. and Zhong Y.F. Department of Pathology, School of Basic Medical Science, The Third Hospital of Peking University, Peking University Health Science Center, Xue Yuan Road 38#, Beijing, 100191, China. e-mail: qingchang@bjmu.edu.cn

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Introduction

Astrocytoma is one of the most common primary brain tumors in adults, and these tumors have traditionally been classified and graded on the basis of histologic features. In the WHO classification, lesions which are predominantly astrocytic include grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma) and grade IV (glioblastoma, GB). Glioblastoma has a dismal 5-year survival rate of 3%, and may arise *de novo* (primary GB) or evolve from lower grade astrocytomas (secondary GB). It has become increasingly clear that the WHO classification alone is insufficient for predicting patient outcome in these tumors (Weller et al., 2012).

Several prognostic markers have been developed for use in molecular classification of astrocytomas. Mutation(s) in isocitrate dehydrogenase (*IDH*) 1/2 are

strong prognostic markers, although reporting of occurrence of such mutation(s) has been exceedingly variable in different cohorts (Sanson et al., 2009; Yan et al., 2009; Qi et al., 2011; Pan et al., 2013). 1p/19q-codeletion, which was a valuable diagnostic and prognostic marker for oligodendroglioma, can also be detected in a subset of diffuse astrocytomas (Louis et al., 2014). Hypermethylation of the O6-methylguanine-DNA methyltransferase (*MGMT*) promoter has also proven to be useful, as it is not only associated with better patient prognosis, but also correlates with likelihood of increased benefit from chemotherapy with alkylating agents in glioblastoma (Komine et al., 2003; Hegi et al., 2005).p53 alteration is a well known astrocytic lineage tumor marker. However, in contrast to *IDH1*, 1p/19-codeletion and *MGMT*, prognostic application of p53 overexpression or mutation in astrocytomas has been controversial (Ständer et al., 2004; Momota et al., 2010).

The newest addition to molecular prognostic markers for tumors of astrocytic lineage is alpha-thalassemia/mental retardation X-linked (*ATRX*) (Wiestler et al., 2013). Mutation of this gene leads to fragile X syndrome and promotes malignant transformation in human tumors. ATRX loss is strongly associated with mutations in *IDH1/2* and *TP53* in astrocytomas of WHO grade II to IV (Liu et al., 2013) and almost mutually exclusive with 1p/19q-codeletion (Wiestler et al., 2013). It has also been shown to be associated with better prognosis in anaplastic astrocytomas with mutant *IDH* (Wiestler et al., 2013). However, ATRX status and its correlation with *IDH1/2* mutations, p53 overexpression, *MGMT* hypermethylation as well as 1p/19q-codeletion in astrocytoma has not been previously studied in a Chinese population. Moreover, the prognostic value of these factors in combination has not been well studied in the same cohort in any population, Chinese or otherwise.

It was reported that p53 overexpression and *IDH1* mutation have a predilection for location in astrocytomas (Nayak et al., 2004; Ellezam et al., 2012), We herein explored the potential linkage of ATRX, p53, IDH1/2 as well as 1p/19q and MGMT alterations in 135 adult Chinese patients with supratentorial astrocytoma. We also evaluated the prognostic value of ATRX alone or in combination with other molecular markers.

Materials and methods

Clinicopathologic data

Tumor tissue samples from 135 astrocytomas in patients of 18 years or older were obtained from the Third Hospital of Peking University, Beijing Sanbo Brain Hospital and Beijing Bo-Ai Hospital, Beijing, China. This study was approved by the Institutional Review Board of Peking University, Beijing, China (review reference number IRB00001052-14003). Each tumor was classified histologically based on WHO criteria for central nervous system tumors (Louis et al., 2007). Any sample showing apparent morphology of oligodendroglioma was not included in this cohort. All study samples were confirmed to consist of at least 80% tumor cells. Clinicopathologic Information is summarized in Table 1.

Immunohistochemical (IHC) analysis for expression of ATRX, IDH1R132H and p53

Formalin-fixed, paraffin-embedded sections from 135 astrocytic tumors were reviewed. Immunhistohisto-chemistry staining was performed using ATRX polyclonal rabbit antibody (dilution 1:500, product code HPA001906, Sigma-Aldrich, St. Louis, MO, USA), IDH1-R132H monoclonal mouse antibody (dilution 1:200, clone H09, Dianova, Hamburg, Germany) and p53 monoclonal mouse antibody (dilution 1:200, clone BP53.12, Invitrogen, Waltham, MA, USA). Replacement of primary antibody with TBS served as a negative control. A blocking step included incubation with 3% H_2O_2. 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used as the detection chromagen.

Staining patterns of ATRX, IDH1R132H and p53 were assessed by two neuropathologists (Q.C and YF.Z) independently and results were merged after a consensus score was obtained as previously described (Ikeguchi et al., 2002; Capper et al., 2010a; Wiestler et al., 2013). Briefly, strong cytoplasmic staining was scored as positive for IDH1R132H, while absence of, or weak staining was scored as negative (Capper et al., 2010a). Only nuclear staining was considered in evaluation of ATRX and p53. Tumors with more than 10% of cancer cells showing strong staining were classified as ATRX positive as described in a previous studiy (Wiestler et al., 2013). Endothelial cells and infiltrating inflammatory cells served as positive internal controls for ATRX. Cases with negative tumor cells in which vascular cells and neurons were not stained were not evaluated and were not used for further statistical analysis of ATRX. p53 immunostaining where more than 50% of tumor cells showed strong nuclear reactivity was scored as positive as previously described (Ikeguchi et al., 2002). In cases with heterogeneous immunoreactivity, areas with the strongest staining were scored.

Direct DNA sequencing for IDH mutations

IDH1/2 mutations were evaluated in 101 cases. Genomic DNA of FFPE samples and peripheral blood from normal control patients were extracted using the QIAampOR DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) and the Relax Gene Blood DNA System (Tiangen biotech, Beijing, China) according to the manufacturer's instructions. Forward and reverse primers were designed to amplify Exon 4 of IDH1 including codon 132 (Gene Bank, NM_005896.2) and

IDH2 including codon 172 (Gene Bank, NM_002168.2) with the polymerase chain reaction (PCR) assay. A 185 bp fragment of the IDH1 gene was amplified using the forward primer 5'-TGATGAGAAGAGGGTTGAG-3' and reverse primer 5'- TTACTTGATCCCCATAAGC-3' and a 293 bp fragment of IDH2 gene was amplified using the forward primer 5'-GCTGCAGTGGG ACCACTATT -3' and reverse primer 5'-TGTGGC CTTGTACTGCAGAG-3'. The annealing temperature for both genes was 56°C. PCR products were purified using the TIANgel Midi Purification Kit (Tiangen biotech, Beijing, China). All purified PCR amplifications and their PCR sense primers were screened for specific mutations. Results were manually interpreted and evaluated using Chromas software and BLAST from the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

FISH (fluorescence in situ hybridization)

Fluorescence In Situ Hybridization (FISH) was performed on unstained FFPE slides from 49 cases. Slides were deparaffinized and treated with proteinase K on VP2000 processor (Vysis, abbot, American) automatically, and were then denatured and treated with prediluted probes and hybridized using commercially available 1p36/1q21 and 19p13/19q13 Spectrum Red & Green-labeled Dual Color probes for 1p36 & 19q13 (red, Vysis, abbot, American) and 1q21&19p13 (green, Vysis, abbot, American), respectively. Values for each signal

Table 1. Clinicopathologic informations of astrocytomas.

Characteristic of astrocytoma	Median age (year)	Case No.	Percentage (%)	ATRX (-) Percentage (%)	p	P53 (+) Percentage (%)	p	IDH1(+) Percentage (%)	p	MGMT Percentage (%)	p	1p/19q(-) Percentage (%)	p
Total	48(19~82)	135	100	18.60(24/129)		52.8(66/125)		31.11(42/135)		60.47(52/86)		8.51(4/47)	
Gender													
Male	46(19~82)	79	56.30(76/135)	20(15/75)	0.5975	52.78(38/72)	0.9954	32.91(26/79)	0.5915	64.15(34/53)	0.3756	7.14(2/28)	1[a]
Female	51.5(21~82)	56	41.48(56/135)	16.67(9/54)		52.83(28/53)		28.57(16/56)		54.55(18/33)		10.53(2/19)	
Age(year)													
1949	38(19~49)	71	52.59(71/135)	30.88(21/68)	0.0002***	64.06(41/64)	0.0098**	43.66(31/71)	0.0009***	66(33/50)	0.216	4.55(1/22)	0.6965[a]
≥50	58(50~82)	64	47.41(64/135)	4.92(3/61)		40.98(25/61)		17.19(11/64)		52.78(19/36)		12(3/25)	
Grades													
II	39(22~69)	36	26.67(36/135)	33.33(12/36)	0.0075**[c]	41.38(12/29)	0.1598	61.11(22/36)	<0001***	68.18(15/22)	0.3908	6.67(1/15)	1[a]
III	46(20~68)	28	20.74(28/135)	32(8/25)		60.71(17/28)		50(14/28)		80(16/20)		30(3/10)	
IVsGB	45(29~57)	11	8.15(11/135)	20(2/10)		54.55(6/11)		27.27(3/11)		71.43(5/7)		0(0/4)	
IVpGB	56(19~82)	60	44.44(60/135)	3.45(2/58)		54.39(31/57)		5(3/60)		43.24(16/37)		0(0/18)	
ATRX	47.5(19~82)	130											
ATRX(-)	40(24~61)	24	18.60(24/129)			83.33(20/24)	0.0006***	79.17(19/24)	<0001***[c]	82.35(14/17)	0.0318*	0(0/10)	0.5644[b]
ATRX(+)	52(19~82)	105	81.40(105/129)			44.21(42/95)		21.90(23/105)		53.73(36/67)		10.81(4/37)	
NA	82	1											
P53	49(19~82)	125											
P53(+)	43.5(19~75)	66	52.8(66/125)	32.26(20/62)	0.0006***			36.36(24/66)	0.1253	68.09(32/47)	0.3089	0(0/8)	1[b]
P53(-)	53(20~82)	59	47.2(59/125)	7.02(4/57)				23.73(14/59)		57.14(20/35)		10.26(4/39)	
IDH1	48(19~82)	135											
IDH1(+)	41.5(27~65)	42	31.11(42/135)	45.24(19/42)	<0001***	63.16(24/38)	0.1253			84.38(27/32)	0.0005***	22.22(4/18)	0.0343*[a]
IDH1(-)	54(19~82)	93	68.89(93/135)	5.75(5/87)		48.26(42/87)				46.30(25/54)		0(0/29)	
MGMT	48(19~82)	115											
MGMT(+)	43.5(19~74)	52	60.47(52/86)	28(14/50)	0.0318*	61.54(32/52)	0.3089	51.92(27/52)	0.0005***			10.53(2/19)	1[b]
MGMT(-)	50.5(21~72)	34	39.53(34/86)	8.82(3/34)		50(15/30)		14.71(5/34)				6.25(1/16)	
NA	53(24~82)	29											
1p/19q	50(21~82)	49											
1p/19q (-)	52(41~58)	4		0(0/4)	0.5644[b]	0(0/4)	1[b]	100(4/4)	0.0343*[a]	66.67(2/3)	1[b]		
1p/19q (+)	50(21~82)	43		23.26(10/43)		18.60(8/43)		32.56(14/43)		53.13(17/32)			
NA	48(31~65)	2											

[a], continuous correction algorithm; [b], Fisher test; [c], grade II, III astrocytomas and sGBs compared with pGBs; [d], low grade (grade II) astrocytomas compared with high grade (III and sGBs) astrocytomas; ***, statistically significant difference, p<0.001; **, statistically significant difference, p<0.01; *, statistic difference, p<0.05. +, wild-type ATRX , p53 overexpression, IDH1 mutation, MGMT hypermethylation, no 1p/19q-codeletion; -, loss of ATRX, without p53 overexpression, wild-type IDH1, without MGMT hypermethylation, 1p/19q-codeletion. NA, not available.

and the ratios of red/green signals were reported in at least 200 nonoverlapping nuclei per specimen. The ratios of red/green signals were lower than 0.7 was regarded as 1p or 19q deletion.

Sodium bisulfite treatment of DNA

The sodium bisulfite reaction converts unmethylated cytosine in DNA to uracil, while leaving methylcytosine intact, allowing amplification of methylated and unmethylated alleles with specific primers by polymerase chain reaction (PCR). Genomic DNA from formalin-fixed, paraffin-embedded (FFPE) samples was extracted using the QIAampR DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) and 0.1-1.0 ug DNA samples were treated with sodium bisulfite using the BIsulFlash DNA Modification Kit (Epigentek, NY, USA) according to the manufacturers recommendations.

Methylation specific PCR (MSP) for MGMT methylation status

Methylation status of MGMT in 115 adult astrocytomas was evaluated with MSP. MGMT MSP primers used for the unmethylated reaction were 5'-TTTGTGTTTTGATGTTTGTAGGTTTT TGT - 3' (sense) and 5'- AACTCCACACTCTTCCAAAAAC AAAACA - 3' (antisense); for the methylated reaction, 5'- TTTCGACGTTCGTAGGTTTTCGC -3' (sense) and 5'- GCACTCTTCCG AAAACGAAACG -3' (antisense) were used. The annealing temperature for both sets of MSP primers was 59°C as previously described (Esteller et al., 1999). After thermocycling, PCR products were resolved with electrophoresis on 12% polyacrylamide and stained with ethidium bromide, followed by examination under UV illumination. CpGenome universal methylated DNA (Millipore, MA, USA) was used as a positive control for methylated MGMT alleles. DNA from the D283 medulloblastoma cell line (ATCC, VA, USA) was used as a negative control. All experiments were repeated at least twice.

Statistical analysis

The χ^2 test and Fisher test were used to compare distribution of molecular alterations.

Overall survival (OS) was defined as the time between diagnosis and patient death or last follow-up. Progression-free survival (PFS) was defined as the time between diagnosis and recurrence or last follow-up. The median follow up time was 24 months (1 to 132 months). To identify associations of clinical features and/or molecular alterations in astrocytomas and OS or PFS, univariate survival analysis was performed using the Kaplan–Meier estimator and log rank test. Multivariate survival analysis used a Cox proportional hazards model. Two-sided $p<0.05$ was considered significant. Analyses were carried out using SPSS version 20.0.

Results

Loss of ATRX expression, accumulation of p53 and codon 132 mutation in IDH1, as well as 1p/19q-codeletion and hypermethylation of *MGMT* were evaluated in 135 adult supratentorial astrocytomas. Clinicopathological information for each patient is listed in Table 1.

Loss of ATRX expression

Loss of ATRX expression as determined by immunohistochemistry was detected in 18.6% of tumors (24 of 129) (Fig. 1a,b), and showed distinct age dependence (~30.9% for those <50 vs. 4.9% for those ≥50; $p=0.0002$, χ^2 test, Table 1, Fig. 2a). ATRX loss was much less frequent in pGBs (3.5%) than in grade II (33.3%), III (32%) astrocytomas and grade IV (20%) sGBs, ($p=0.0075$, χ^2 test, Table 1, Fig. 3).

p53 overexpression and its association with ATRX alterations

The p53 monoclonal antibody (BP53.12) used in this investigation binds to an epitope between amino acids 1-45 at the N-terminus of both wild and mutant types of p53. IHC analysis of protein expression in these astrocytomas showed nuclear accumulation of p53 in 52.8% of tumors (66/125) (Table 1, Fig. 1c,d) and also distinct age dependence (~64.1% for those <50 vs. 41.0% for those ≥50; $p=0.0098$, χ^2 test, Table 1, Fig. 2b). No significant difference was identified in the frequency of p53 overexpression among tumor grades (Table 1, Fig. 3) . ATRX loss was much more common in astrocytic tumors with p53 overexpression (32.3%) than in astrocytomas without p53 alteration (7.0%) ($p=0.0006$, χ^2 test, Table 1, Fig. 2c).

IDH1R132H mutation and its association with ATRX loss

A total of 98 cases were analyzed for *IDH1R132H* mutation by both direct sequencing and IHC. The results of the former method overlapped strongly with those of the latter ($p<0.0001$), showing that IHC detection using IDH1R132H mutation-specific antibody accurately reflects the status of IDH1R132H mutation in tumors. Direct sequencing (Fig. 4) and/or IHC analysis of the *IDH1/2* gene and/or protein expression (Fig. 1e,f) in 135 adult astrocytomas showed that 31.11% of tumors (42/135) carried the IDH1R132H mutation, which occurred in 61.1%, 50% and 27.3% of grade II, III astrocytomas and sGBs, respectively. The frequency of this alteration was significant lower in pGBs ($p<0.0001$, Table 1, Fig. 3). No single IDH2 mutation was found in all astrocytomas. Moreover, patients with *IDH1R132H* mutated tumors were typically young (18-50 years) adults ($p=0.0009$, χ^2 test, Table 1, Fig. 2d). In addition, IDH1 mutations were much more common in astrocytic tumors with ATRX loss (79.2%) than in astrocytomas

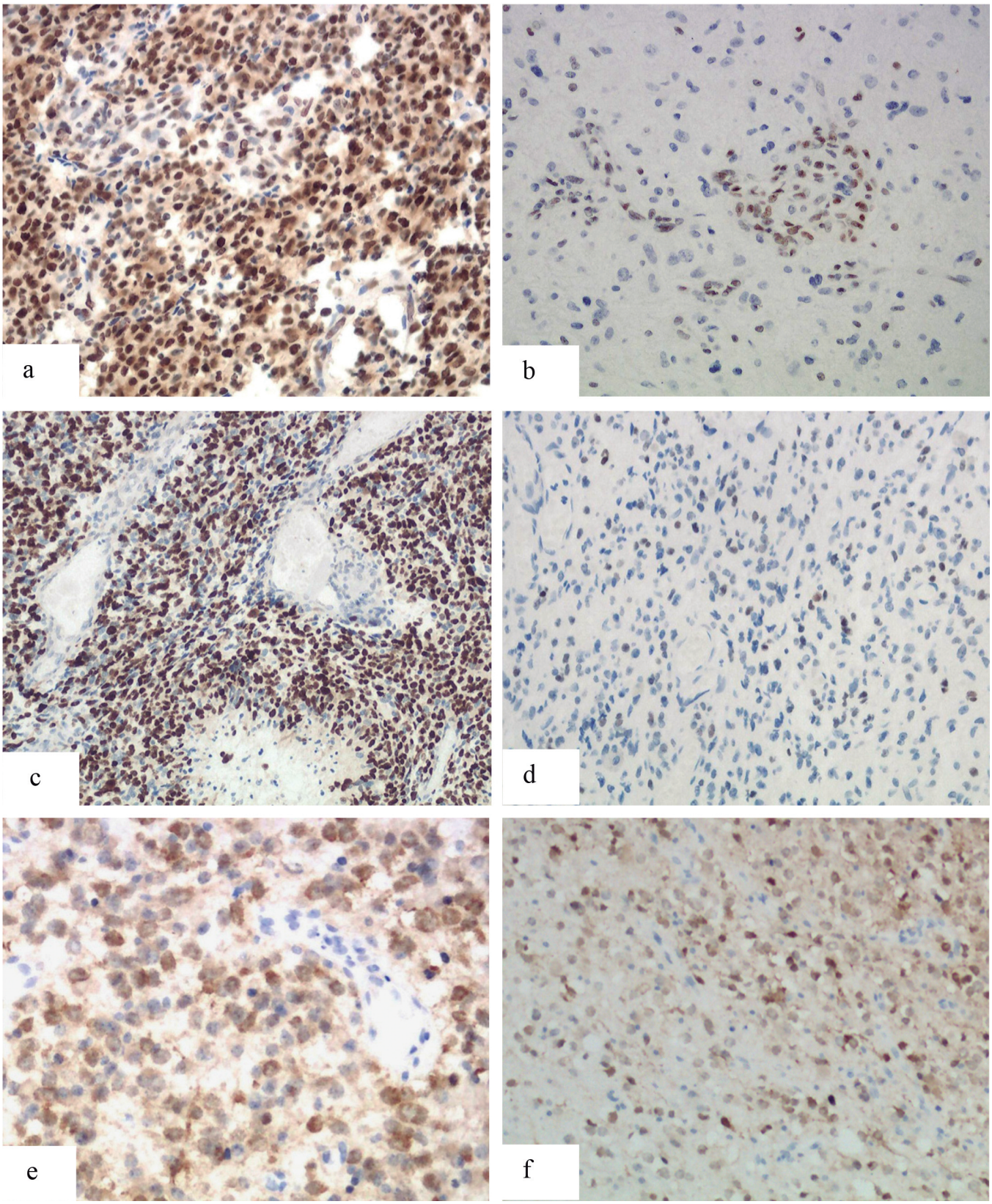

Fig. 1. Immunohistochemical (IHC) analysis of ATRX, p53 and IDH1R132H protein expression in supratentorial astrocytomas. **a.** Wild type ATRX identified in case A156. ATRX protein expression localized to the nucleus of glioblastoma cells. **b.** ATRX loss observed in case A140. ATRX protein expression was completely lost in glioblastoma cells, but was present in nuclei of endothelial cells. **c.** p53 overexpression detected in case A134. Strong p53 protein nuclear staining was identified in a majority of glioblastoma cells, but not in endothelial cells. **d.** No accumulation of p53 was identified in A120. p53 expression was identified only in a minority of tumor cell nucleus of glioblastoma. **e.** IDH1R132H mutated protein was identified in cytoplasm of glioblastoma cells. **f.** Normal brain adjacent to the upper right tumor. No IDH1R132H expression was found in astrocytes of the lower left part of the figure. a-c, x 200; f, x 100

expressing wild-type ATRX (21.9%) ($p<0.0001$, χ^2 test, Table 1, Fig. 2e).

1p/19q-codeletion and its relationship with ATRX loss

FISH analysis of 1p/19q in these astrocytomas showed 1p/19q-codeletion in 8.5% of tumors (4/47) (Table 1, Fig. 5). One of them (1/4) was Grade II, the others (3/4) were Grade III astrocytomas. All these four cases with 1p/19q-codeletion were tumors without ATRX loss (4/37). No one case carried 1p/19q-codeletion in tumors with ATRX loss (0/10) (Table 1). Thus, 1p/19q-codeletion was almost mutually exclusive with ATRX alterations. In addition, 4 of 47 (8.5%) cases showed loss on either 1p (1 case) or 19q (3 cases), in which only 1 case showing 19q loss coexisted with ATRX loss.

MGMT methylation status and its association with ATRX expression

Methylation specific polymerase chain reaction (MSP) analysis (Fig. 6) showed that 60.5% of astrocytomas (52/86) in this cohort harbored hypermethylated *MGMT* (Table 1). A similar percentage of grade II (68.2%), grade III (80.0%) astrocytomas, and sGB (71.4%) tumors exhibited hypermethylation (Table 1). No significant difference of the proportions of *MGMT* methylation was observed among grades (Table 1, Fig. 3). However, there was significant correlation of ATRX loss and MGMT hypermethylation in our cohort ($p=0.0318$, χ^2 test, Table 1, Fig. 2f).

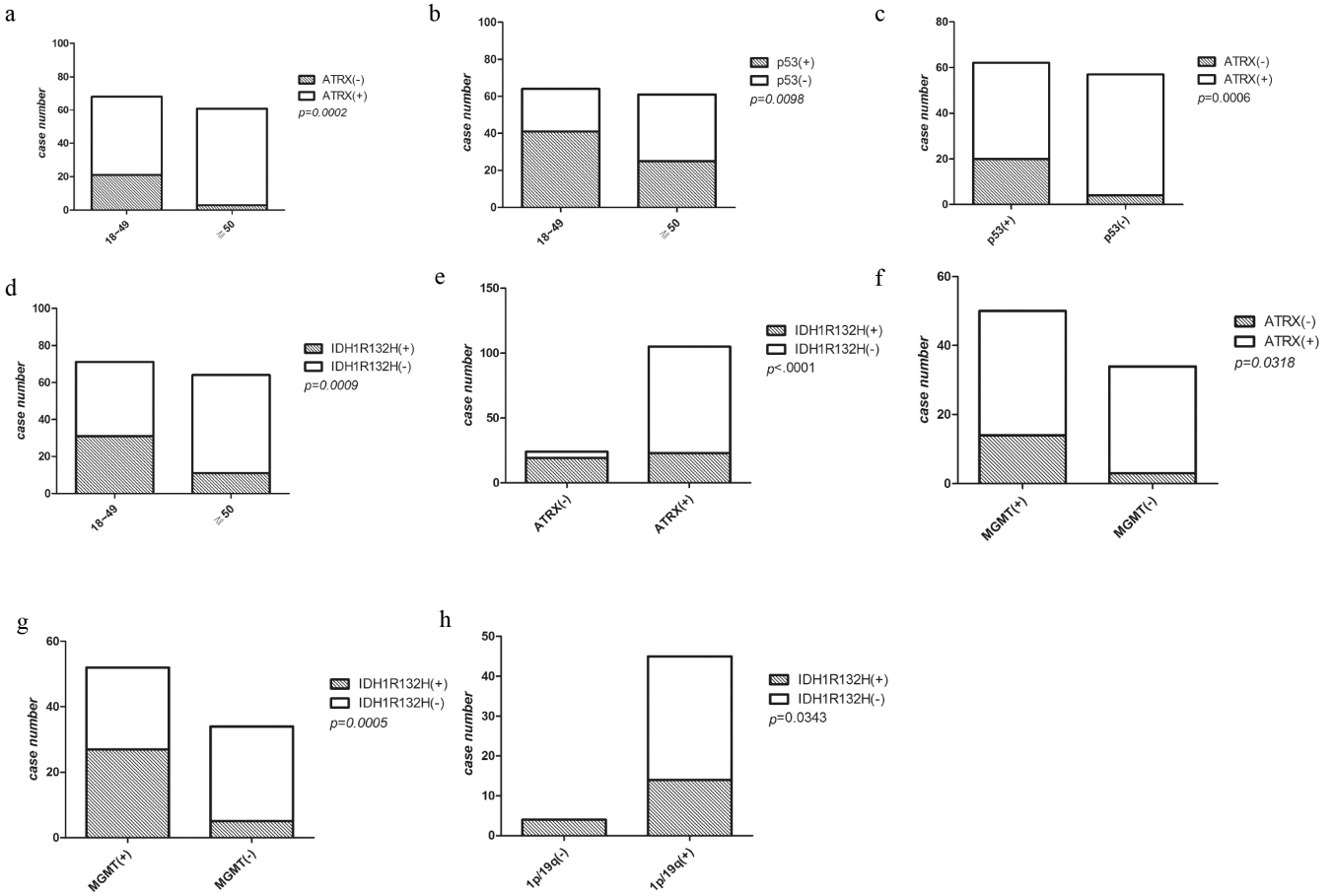

Fig. 2. Correlations among ATRX loss, p53 overexpression, MGMT hypermethylation as well as IDH1R132H mutation and 1p/19q-codeletion in whole population. The frequency of ATRX loss **(a)** and p53 overexpression **(b)** in young adults are significantly higher than those in older ones (p=0.0002; p=0.0098). **c.** ATRX loss was much more common in astrocytic tumors with overexpression of p53 than in astrocytomas without overexpression of p53 (p=0.0006). **d.** IDH1R132H mutation also showed distinct age correlation (p=0.0009). **e.** ATRX loss was much more common in astrocytic tumors with IDH1 mutations than in astrocytomas expressing wild-type IDH1 (p<0.0001). **f.** ATRX loss closely related with MGMT hypermethylation (p=0.0318). **g.** IDH1R132H mutation had a close relationship with both MGMT hypermethylation (p=0.0005) and 1p/19q-codeletion **(h)** (p=0.0343). ATRX (+), wild-type ATRX; ATRX (-), loss of expression of ATRX; p53 (+), overexpression of p53; p53 (-), no p53 overexpression; IDH1R132H (+), IDH1R132H mutation; IDH1R132H (-), wild-type IDH1; MGMT (+), MGMT hypermethylation; MGMT (-), MGMT unmethylation; 1p/19q (+), no codeletion; 1p/19q (-), codeletion.

Correlations among IDH1R132H mutation, MGMT hypermethylation, 1p/19q-codeletion and p53 overexpression

Remarkably, *IDH1R132H* mutation status appeared to influence *MGMT* methylation, as 51.9% of *MGMT* hypermethylated tumors showed *IDH1R132H* mutation, as compared with 14.7% of non-hypermethylation cases (p=0.0005, χ^2 test, Table 1, Fig. 2g). In addition, 1p/19q-codeletion was closely related with *IDH1R132H* mutation, as all four cases (4/4, 100%) with 1p/19q-codeletion coexisted with the mutation, while only 32.6% (14/43) of cases without the codeletion carried *IDH1R132H* mutation (p=0.0343, χ^2 test, Table 1, Fig. 2h). Furthermore, no 1p/19q-codeletion was identified in tumors with p53 overexpression (Table 1). There was no correlation of p53 overexpression and MGMT hypermethylation, as well as *IDH1* mutation (Table 1).

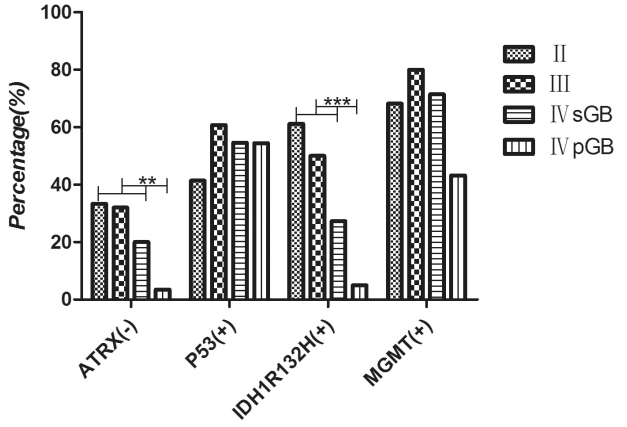

Fig. 3. Frequencies of molecular alterations in grade II, grade III astrocytomas, secondary glioblastomas (sGBs) and primary glioblastomas (pGBs). ATRX loss occurred much less frequently in pGBs than in grade II, grade III astrocytomas and sGBs. The IDH1R132H mutation occurred more frequenetly in grade II, III astrocytomas and sGBs than in pGBs. ATRX (L), loss of expression of ATRX; p53 (+), overexpression of p53; IDH1R132H (+), IDH1R132H mutation; MGMT(+), MGMT hypermethylation. ***, p<0.001; **, p<0.01; *, p<0.05.

Prognostic implications of p53 overexpression and ATRX loss

It is well known that *MGMT* hypermethylation and *IDH1* mutation predict better outcome of glioma patients, and it is recognized that these two molecular events are usually associated with each other (Sanson et al., 2009; Juratli et al., 2012). Results from our study confirmed this association (p<0.001, log-rank test). *IDH1* mutation is also a good independent prognostic marker in astrocytomas for both OS and PFS (p=0.0002 and 0.0010, repectively, log-rank test), especially in grade III tumors. In addition, patients with tumors showing p53 accumulation combined with ATRX loss demonstrated substantially longer survival times than patients with wild-type ATRX (median OS: 79 vs. 16 months, p=0.0391, log-rank test; median PFS, 60 vs. 16 months, p=0.0344, log-rank test, Fig. 7). There was no significant difference in survival time in patients with positive versus negative p53 overexpression (median OS: 32 vs. 37 months, p=0.739, log-rank test; median

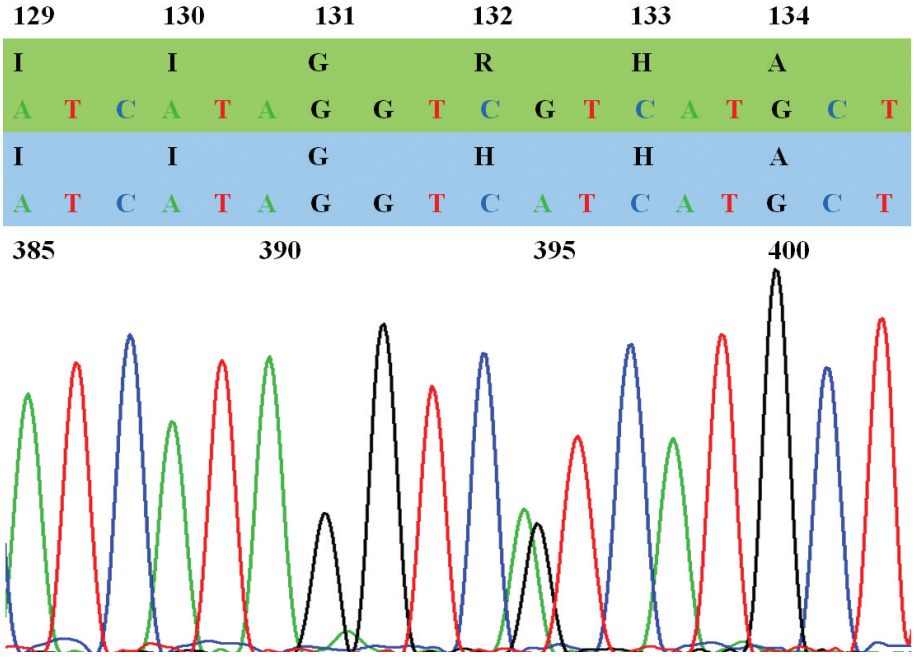

Fig. 4. IDH1 mutation detected by direct sequencing. IDH1R132H (CGT→CAT) was detected in A31.

PFS: 48 vs. 36 months, p=0.678, log-rank test). Although case numbers were too limited to get significant results, patients with 1p/19q-codeletion were likely related with better prognosis (Fig. 8). Multivariate analyses with the Cox proportional hazards model demonstrated that ATRX loss increased predictive value for patient outcome when comparisons included p53 and MGMT alterations, showing p=0.0371, and p=0.0308, for OS and PFS, respectively (Table 2).

Discussion

In this study, we investigated ATRX expression and its prognostic value in association with p53, *IDH1* mutation, 1p/19q-codeletion and *MGMT* hypermethylation in 135 adult supratentorial astrocytic tumors. We

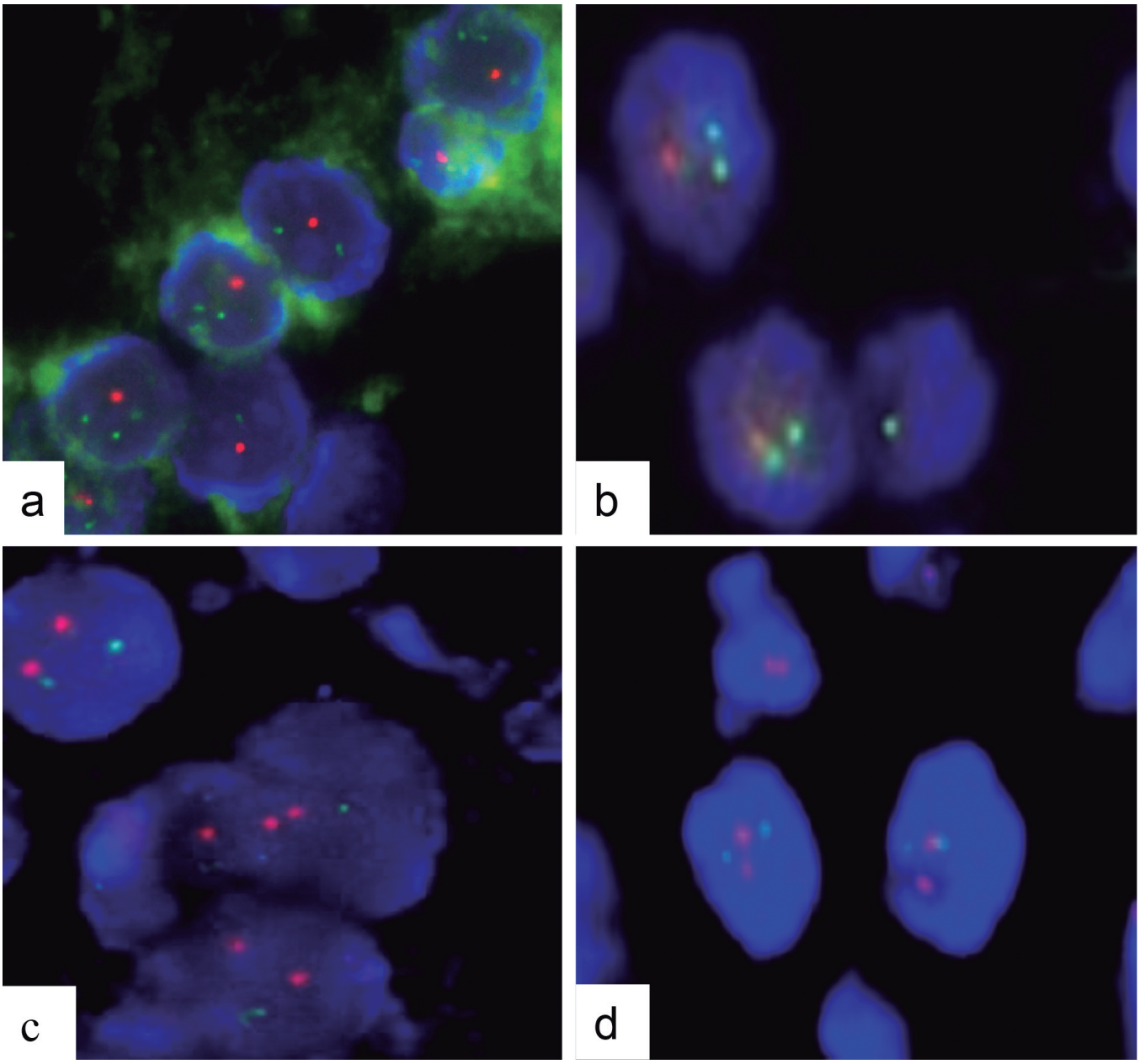

Fig. 5. 1p/19q-codeletion detected by FISH (fluorescence in situ hybridization) with 1p36/1q21 and 19p13/19q13 Dual Color probes. The ratios of red/green signals lower than 0.7 were regarded as 1p or 19q deletion. Co-deletion was identified on both **(a)** 1p and **(b)** 19q in A31. No deletion was identified on either **(c)** 1p or **(d)** 19q in A11.

identified strong correlations between loss of ATRX and *IDH1R132H* mutation, p53 protein overexpression as well as *MGMT* hypermethylation. 1p/19q-codeletion was almost mutually exclusive with both ATRX loss and p53 overexpression, but was closely related with *IDH1R132H* mutation. In addition to *IDH1R132H* mutation, we found loss of expression of ATRX may be a significant prognostic marker for astrocytoma patients with p53 protein accumulation.

ATRX alterations which may be identified by IHC are believed to be lineage-specific genetic aberrations in diffuse astrocytic tumors (Liu et al., 2013; Wiestler et al., 2013) and mutually exclusive with 1p/19q-codeletion, the molecular hallmark of oligodendroglioma

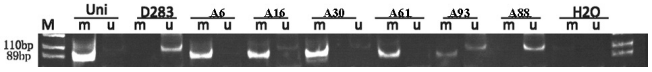

Fig. 6. MGMT methylation status of astrocytomas detected by methylation specific PCR. Hypermethylation of MGMT was detected in Ab, A16, A30, A61 and A93, but was not detected in A88. M, marker; m, hypermethylated; u, unhypermethylated; Uni, universal methylated DNA as positive control; H_2O, negative control.

(Kannan et al., 2012). We found ATRX loss in 31% of Chinese adult astrocytoma (grade II, III and sGBs) patients by IHC, which was a little bit lower than what has been reported in another cohort employing IHC (33%) (Jiao et al., 2012). A subset of tumors showing 1p/19q-codeletion which was mutually exclusive with ATRX alterations may contribute to the lower frequency of ATRX loss in the current cohort of astrocytomas. The frequency of *IDH1R132H* mutation observed in the present study with IHC and/or direct sequencing is somehow lower in grade 2, 3 astrocytomas and sGBs (27.3 to 61.1%) than the frequency reported in western countries (60% to 90%) (Yan et al., 2009; Hartmann et al., 2009; Watanabe et al., 2009; Capper et al., 2010b). The differences between our findings and those in North American and European studies may be due to racial differences in genetic background. This effect of racial background has in fact been described previously for *IDH1* mutation in another Chinese glioma cohort (Qi et al., 2011). The results of *IDH1R132H* mutation analyzed by direct sequencing overlapped strongly with results of IHC in our study, indicating that IHC detection using *IDH1R132H* mutation specific antibody can accurately reflect the status of *IDH1R132H* mutation in tumors. Nonetheless, despite this lower prevalence of

Table 2. Cox multivariate analyses with the Cox proportional hazards model.

	Variables in the Equation--OS				Variables in the Equation--PFS			
	p value	HR	95.0% CI		p value	HR	95.0% CI	
			Lower	Upper			Lower	Upper
MGMT	0.1043	0.499	0.215	1.155	0.2219	0.603	0.268	1.357
ATRX	0.0371	0.203	0.045	0.909	0.0308	0.192	0.045	0.858
p53	0.4303	1.407	0.602	3.287	0.6413	1.220	0.529	2.817

OS, overall survival; PFS, progression free survival; HR, hazard rate ratio; CI, confidence interval.

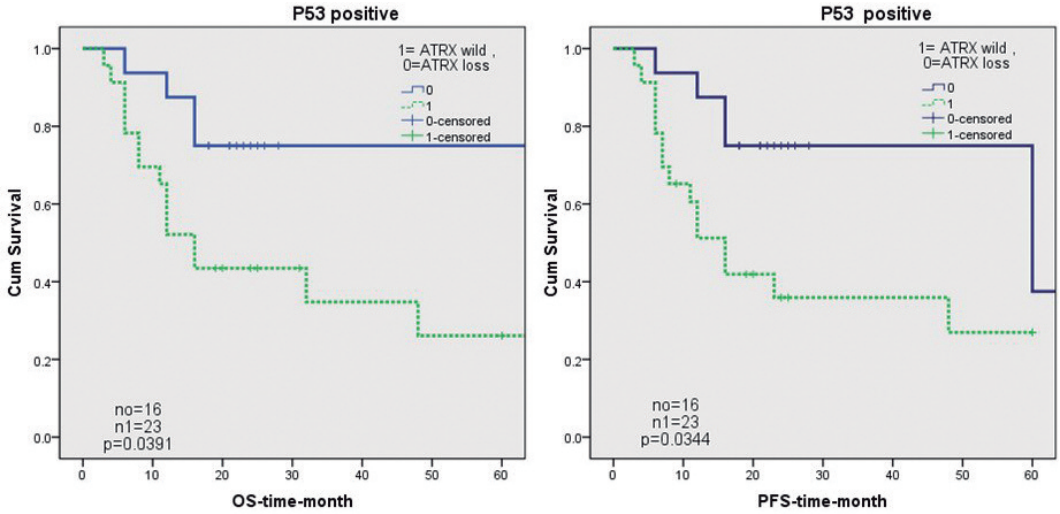

Fig. 7. Prognostic implications of p53 overexpression and ATRX loss in whole population. Patients with tumors showing p53 accumulation combined with ATRX loss demonstrated substantially longer survival times than patients with wild-type ATRX, with median OS and PFS of 79 vs. 16 months (p=0.0391) and 60 vs. 16 months (p=0.0344), repectively.

IDH1R132H mutation, several features observed in our cohort are consistent with what has been reported in other studies, including 1) close relationship of loss of ATRX expression with *IDH1* mutation; 2) both alterations are observed more frequently in young adults; 3) both of the latter alterations occur much more frequently in grade II, III astrocytomas and sGBs than in pGBs (Kannan et al., 2012; Liu et al., 2013). This suggests ATRX may play a significant role in IDH1 driven astrocytic tumorigenesis as previously noted, and ATRX alterations, like IDH1 mutation, occur at an early stage of tumor development rather than during tumor progression (Yan et al., 2009; Kannan et al., 2012; Jiao et al., 2012; Liu et al., 2013).

p53 overexpression is another lineage-specific genetic marker in diffuse astrocytic tumors (Liu et al., 2013). p53 alteration which is observed commonly in various glial tumors has been suggested to be an early event in astrocytic tumorigenesis and possibly a molecular marker which predicts malignant progression (Momota et al., 2010). The correlation of ATRX with p53 in our cohort is consistent with an earlier report where DNA sequencing was employed to detect mutant TP53 (Liu et al., 2013), supporting the concept that use of p53 staining can be a surrogate for mutational DNA analyses in these tumors. Like ATRX loss, p53 overexpression was also mutually exclusive with 1p/19q-codeletion, while 1p/19q-codeletion had close relationship with IDH1R132H mutation in the present study. Our observations were consistent with other reports (Figarella-Branger et al., 2011; Wiestler et al., 2013), supporting that glioma with astrocytoma phenotype accompanied by 1p/19q-codeletion and IDH1R132H mutation, without ATRX loss as well as p53 accumulation, is a distinct molecular subtype of gliomas, as recommended by International Society of Neuropathology-Haarlem Consensus Guidelines (Louis et al., 2014).

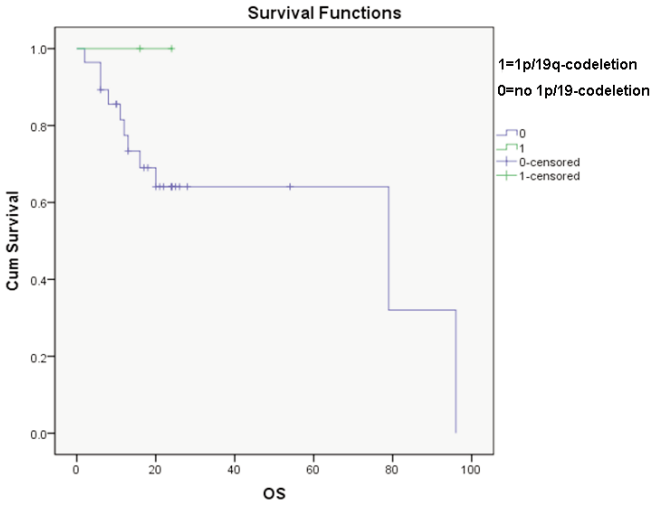

Fig. 8. Prognostic implications of 1p/19-codeletion in 30 patients. Patients with tumors carrying 1p/19-codeletion (2 patients) demonstrated better prognosis than patients without codeletion (28 patients), although no significant difference could be identified due to limited case number.

One of the interesting discoveries of the present investigation is the demonstration of a close relationship between ATRX aberration and MGMT hypermethylation. It is known that ATRX has sequence homology with DNMT3A, DNMT3B, and DNMT3L, three proteins involved in DNA methylation and as a SWI/SNF family member, ATRX may rearrange promoter region chromatin to facilitate gene expression (Otani et al., 2009). Thus, our findings provide a potential link between ATRX loss and MGMT hypermethylation in astrocytomas, although the precise mechanisms involved need to be investigated further. Our result comes from a cohort of supratentorial astrocytomas. It may be the reason why different correlations of ATRX loss and MGMT hypermethylaiton were found between our study and others' in which tumor location was not clarified and oligodendrogliomas were also involved (Wiestler et al., 2013). Larger cohorts of astrocytomas within the same location are needed to support our findings.

The most important observation in the current investigation is the fact that while neither p53 overexpression nor ATRX loss alone had a significant effect on prognosis of the whole population, ATRX loss is correlated with a strongly favorable outcome in adult astrocytomas with co-existing p53 overexpression. Concurrence of these two molecular alterations suggests that there may be a link between p53 and ATRX mediated tumorigenesis. The precise mechanism of interaction of these genes remains to be investigated, however, it is known that both these genes and their proteins are involved in epigenetic regulation. For example, p53 recruits histone-modifying enzymes to bring about a permissive chromatin configuration (Saldaña-Meyer et al., 2011), and inactivation of p53 is correlated with aberrant methylation of downstream genes (Yoda et al., 2014). As a SWI/SNF family member, ATRX loss alters imprinted genes in the postnatal brain (Otani et al., 2009; Kernohan et al., 2010). Thus, it was postulated that p53 alterations may interact with ATRX at various levels, including epigenetic regulations of both DNA and chromatin structure, to influence tumor development and progression. The main limitation of the survival analysis of our cohort is not to differentiate patients between groups, which was due to the limited case number and relatively short periods of follow up time. It may be the reason why no correlation of ATRX loss and good prognosis in astrocytoma patients carrying IDH1 mutation was found in our study, in contrast with a previous report (Wiestler et al., 2013). Further prospective studies are warranted to support our results.

In summary, our study is the first to show that loss of ATRX expression is closely linked to p53 accumulation

and *IDH1R132H* mutation as well as MGMT hypermethylation in a cohort of adult supratentorial astrocytomas in Chinese populations. Moreover, ATRX loss may predict better clinical outcome in astrocytoma patients with p53 overexpression as compared to patients with wild-type ATRX. Glioma with astrocytoma phenotype accompanied by 1p/19q-codeletion and IDH1R132H mutation is a distinct molecular subtype of tumors. Immunohistochemical detection of ATRX loss combined with p53 overexpression and *IDH1R132H* mutation holds promise as a technique for diagnosis and tumor classification in astrocytic tumorigenesis, facilitating future individualization of target therapy.

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Conflict of interest. The authors have no conflict of interest.

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