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SHORT COMMUNICATION

Replication stress and oxidative damage contribute to aberrant constitutive activation of DNA damage signalling in human gliomas

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Malignant gliomas, the deadliest of brain neoplasms, show rampant genetic instability and resistance to genotoxic therapies, implicating potentially aberrant DNA damage response (DDR) in glioma pathogenesis and treatment failure. Here, we report on gross, aberrant constitutive activation of DNA damage signalling in low- and high-grade human gliomas, and analyze the sources of such endogenous genotoxic stress. Based on analyses of human glioblastoma multiforme (GBM) cell lines, normal astrocytes and clinical specimens from grade II astrocytomas ($n = 41$) and grade IV GBM ($n = 60$), we conclude that the DDR machinery is constitutively activated in gliomas, as documented by phosphorylated histone H2AX (γH2AX), activation of the ATM-Chk2-p53 pathway, 53BP1 foci and other markers. Oxidative DNA damage (8-oxoguanine) was high in some GBM cell lines and many GBM tumors, while it was low in normal brain and grade II astrocytomas, despite the degree of DDR activation was higher in grade II tumors. Markers indicative of ongoing DNA replication stress (Chk1 activation, Rad17 phosphorylation, replication protein A foci and single-stranded DNA) were present in GBM cells under high- or low-oxygen culture conditions and in clinical specimens of both low- and high-grade tumors. The observed global checkpoint signaling, in contrast to only focal areas of overabundant p53 (indicative of p53 mutation) in grade II astrocytomas, are consistent with DDR activation being an early event in gliomagenesis, initially limiting cell proliferation (low Ki-67 index) and selecting for mutations of p53 and likely other genes that allow escape (higher Ki-67 index) from the checkpoint and facilitate tumor progression. Overall, these results support the potential role of the DDR machinery as a barrier to gliomagenesis and indicate that replication stress, rather than oxidative stress, fuels the DNA damage signalling in early stages of astrocytoma development.

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Introduction

Malignant gliomas, particularly the most aggressive type, glioblastoma multiforme (GBM), are among the most devastating tumors, with generally poor response to therapy and dismal prognosis (Furnari *et al*., 2007). Despite recent insights into molecular pathways involved in GBM pathogenesis, gained through comprehensive genetic analysis during the pilot project of 'The Cancer Genome Atlas' initiative (The Cancer Genome Atlas Research Network, 2008) and other studies (Furnari *et al*., 2007; Zheng *et al*., 2008), better understanding of the molecular basis and biology of gliomas is needed to validate the emerging, and design new, targeted therapies. Rampant genomic instability and resistance to genotoxic treatment modalities including ionizing radiation and chemotherapy are among the hallmarks of GBM (Furnari *et al*., 2007), implicating the cellular DNA damage response (DDR) machinery (Jackson and Bartek, 2009; Luo *et al*., 2009) as an important factor in both pathogenesis and treatment response.

Recently, constitutive activation of DDR pathways, with the ensuing cellular senescence or cell death, has been identified as a biological barrier against activated oncogenes and tumor progression in early human lesions (Gorgoulis *et al*., 2005; Bartkova *et al*., 2005b, 2006; Di Micco *et al*., 2006; Malette and Ferbeyre 2007; Halazonetis *et al*., 2008). While oncogene-evoked replication stress was implicated in such DDR activation, and the concept validated for major types of human epithelial tumours (Gorgoulis *et al*., 2005; Bartkova *et al*., 2005b, 2007; Bartek *et al*., 2007), DDR activation was very low or absent in human testicular germ cell tumors (Bartkova *et al*., 2005a, 2007), and whether the DDR barrier operates in the

pathogenesis of brain neoplasms remains to be studied. Given the genetic instability, and the key role of radiotherapy and genotoxic chemotherapy in management of malignant gliomas (Furnari *et al*., 2007), here we studied the activation status of the DDR machinery in human glioma cell lines and clinical specimens from gliomas of various grades of malignancy, and examined the sources of the identified aberrant constitutive DNA damage signalling.

Results and Discussion

Immunohistochemical analysis of a series of 60 glioblastoma multiforme specimens showed that the DDR was commonly constitutively activated in these aggressive tumors, as indicated by the strongly positive γH2AX staining (the most widely used marker, as documented in: Gorgoulis *et al*., 2005; Bartkova *et al*., 2005a, b; Bonner *et al*., 2008), aberrant activation of the ATM-Chk2 kinase pathway and other DDR markers including focus formation by the 53BP1 (DiTullio *et al*., 2002) protein (Figures 1a and c). This was in contrast with the lack of DDR activation in the normal brain samples and only rare positive cells in brain tissues adjacent to the tumor areas on the same section (Figures 1a and d). Given that the GBM specimens examined in our study were surgically removed before the onset of radiotherapy or chemotherapy, these results indicated that the DDR machinery became activated because of some aberrant, stressful endogenous processes that threaten the genetic integrity and occur during the natural history of GBM.

Parallel examination of 41 grade II astrocytomas, a class of less aggressive lesions that have an intrinsic tendency to progress into secondary GBM (grade IV), revealed even more pronounced DDR activation than seen in the GBM series, judged from the global γH2AX marker (Figures 1a, b and d). The signalling cascade of ATM-Chk2, known to primarily respond to DNA double-strand breaks, was activated in grade II astrocytomas as well as in GBM (Figure 1c, Supplementary Figure S1). Broadly reminiscent of the patterns seen in various epithelial tumors (Bartek *et al*. 2007), the proportion of γH2AX-positive cells was higher than that of cells with phosphorylated, activated forms of ATM or Chk2 within the individual tumor specimens (compare Figure 1d with Supplementary Figure S1). These results are consistent with the notion that also other types of DNA lesions, such as abnormal replication intermediates not converted into double-strand breaks, might underlie the observed γH2AX positivity and reflect the activity of the ataxia telangiectasia and rad-3-related kinase (ATR) that is activated by stress associated with DNA replication (Bonner *et al*., 2008; Cimprich and Cortez 2008). The extent of DDR signalling activation, as deduced from the γH2AX marker, did not strictly correlate with the degree of proliferation, as indicated by the significantly higher Ki67-positive, cycling fraction of cells in the GBM series, compared with a more moderate proliferation rate among grade II astrocytomas (Supplementary Figure S1c).

Interestingly, the overall degree of DDR activation (γH2AX) seen among gliomas was higher than that among a series of carcinomas –(*in situ*) of the breast, an early-stage carcinoma lesion that was examined here (Figure 1d) as a representative example of the epithelial tumors so far identified as displaying the aberrant constitutive activation of DNA damage signalling (Bartkova *et al*., 2005b; Bartek *et al*., 2007). We conclude from these results that the DDR machinery is activated constitutively, and to a high degree, during human glioma pathogenesis, analogous to epithelial cancers, but in contrast to testicular germ cell (Gorgoulis *et al*., 2005; Bartkova *et al*., 2005a, b, 2007; Nuciforo *et al*., 2007). Compared with other types of neoplasms, gliomas seem to be the tumor-type with very robust, if not the highest, degree of such spontaneous DDR activation.

Consistent with the clinical specimens, immunoblotting (Figure 2a) and immunofluorescence (Figure 2b) analyses showed variable degree of enhanced γH2AX, activation of the ATM-Chk2-p53 axis, and Chk1 phosphorylation under unperturbed culture conditions in GBM cell lines, in contrast to low-level DDR activation in normal human astrocytes (Figures 2a and b). Notably also the mutant p53 protein that is exclusively expressed in some GBM cell lines was constitutively phosphorylated on ser15, the site targeted by the DDR kinases (ATM and ATR) (Figure 2c). Whether such persistent phosphorylation may help stabilize the mutant p53 or even contribute to some gain-of-function properties of mutant p53 remains yet to be studied. Despite the constitutive activation, DNA damage signalling was further enhanced in response to ionizing radiation (Figures 2c and d), indicating that the DDR machinery is still capable of responding to exogenous genotoxic insults.

To assess the contribution of oxidative stress to the observed DDR activation, we compared two GBM cell lines displaying constitutive DDR signalling (p53-mutant U373MG and p53-wild-type A172), under standard (21%) versus low (3%) oxygen conditions, the latter mimicking the physiological oxygen tension in the brain (Li *et al*., 2005; Bristow and Hill 2008). In 3% oxygen conditions, the constitutive DDR signalling (γH2AX, phosphorylation of Chk2, Chk1 and Rad17) was reduced, but not eliminated (Figures 2a and 3b). Flow-cytometry measurement of 8-oxoguanine lesions showed a cell-line-dependent variation with the lowest level in normal astrocytes, and confirmed that under low-oxygen conditions the extent of oxidative damage in GBM cells was dramatically reduced, although not entirely eliminated (Figure 3c). Results consistent with the 8-oxoguanine measurements were obtained when the extent of oxidative stress was alternatively assessed by flow-cytometry estimation of thiol-reactive chloromethyl group derivates of reduced fluorescein to measure the level of reactive oxygen species (Figure 3d, right panel).

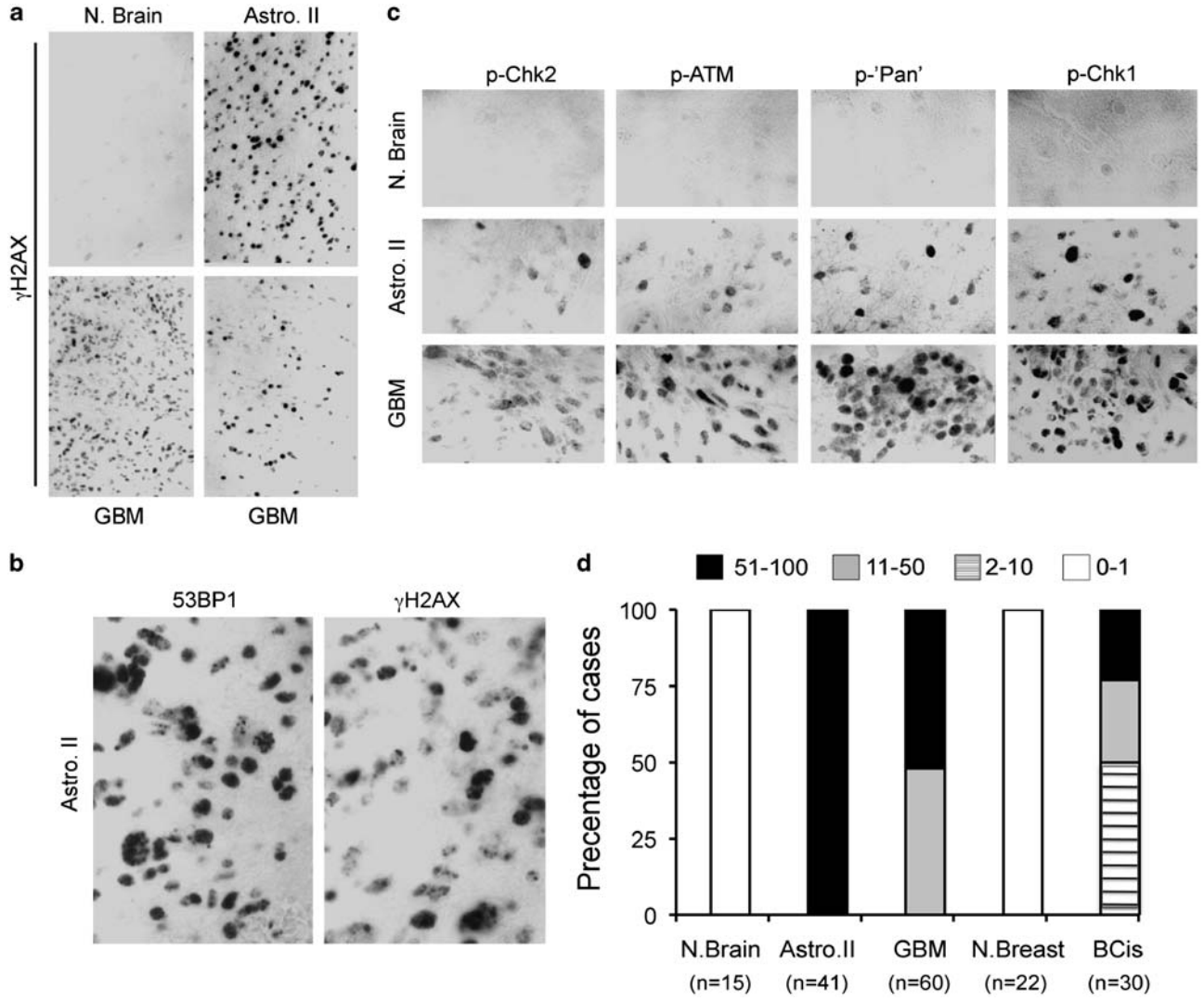

Figure 1 Immunoperoxidase staining of normal brain (N.Brain), astrocytoma grade II (Astro.II) and glioblastoma multiforme (GBM) for the indicated markers of activated DNA damage response (DDR): γH2AX (**a**); foci formation by 53BP1 and γH2AX (**b**); Thr68-phosphorylated Chk2 (p-Chk2), Ser1981-phosphorylated ATM (p-ATM), ATM/ATR phospho-substrates (p-'Pan'), Ser317-phosphorylated Chk1 (**c**); (**d**) graphical summary of constitutive γH2AX in brain tissue (N.Brain) and tumors (Astro.II, GBM), compared with normal breast (N.Breast) and breast carcinoma *in situ* (BCis), as assessed by semi-quantitative immunoperoxidase analysis based on the proportion of positive cells (Bartkova *et al*., 2005b). The four categories of marker positivity, indicated on the right (0–1; 2–10; 11–50; 51–100), reflect the percentage of positive cells within a lesion (Bartkova *et al*., 2005b). Specimens of formalin-fixed, paraffin-embedded normal brain ($n=15$) and gliomas resected before the onset of radiotherapy or chemotherapy: glioblastoma multiforme ($n=60$) and diffuse astrocytoma grade II ($n=41$), were obtained from the tissue banks of the Departments of Neuropathology and Pathology in University of Copenhagen and Olomouc, respectively. Within the GBM series, no obvious differences in staining patterns were observed between primary and secondary GBMs, and therefore the series was considered jointly as 'GBM'. Immunoperoxidase staining (with a panel of antibodies (Supplementary Table 1) and scoring of the resulting patterns by an experienced pathologist were performed as described (Bartkova *et al*., 2005b).

Given that the oxidative damage was almost eliminated (Figure 3c), while the DDR signalling decreased less dramatically (Figures 3a and b), we argued that the remaining constitutive DNA damage in GBM cells under low-oxygen conditions might reflect ongoing replication stress and ensuing DNA breakage (Halazonetis *et al*., 2008). Indeed, results from three independent assays performed to address this issue were consistent with such interpretation: (i) enhanced formation of single-stranded DNA (Figures 3a and d, left panel) detected by bromodeoxyuridine incorporation visualized under non-denaturing conditions (Raderschall *et al*., 1999); (ii) the γH2AX foci being most abundant in S-phase cells (Supplementary Figure S2) and (iii) constitutive presence of replication protein A foci (Figure 3a) indicative of replication fork collapse, single-stranded DNA formation and ongoing recombination repair (Raderschall *et al*., 1999).

Collectively, the results obtained with the cultured cells (Figures 2 and 3, Supplementary Figure S2) and clinical specimens (Figure 1) raised several predictions for the clinical samples, whether the observed DDR

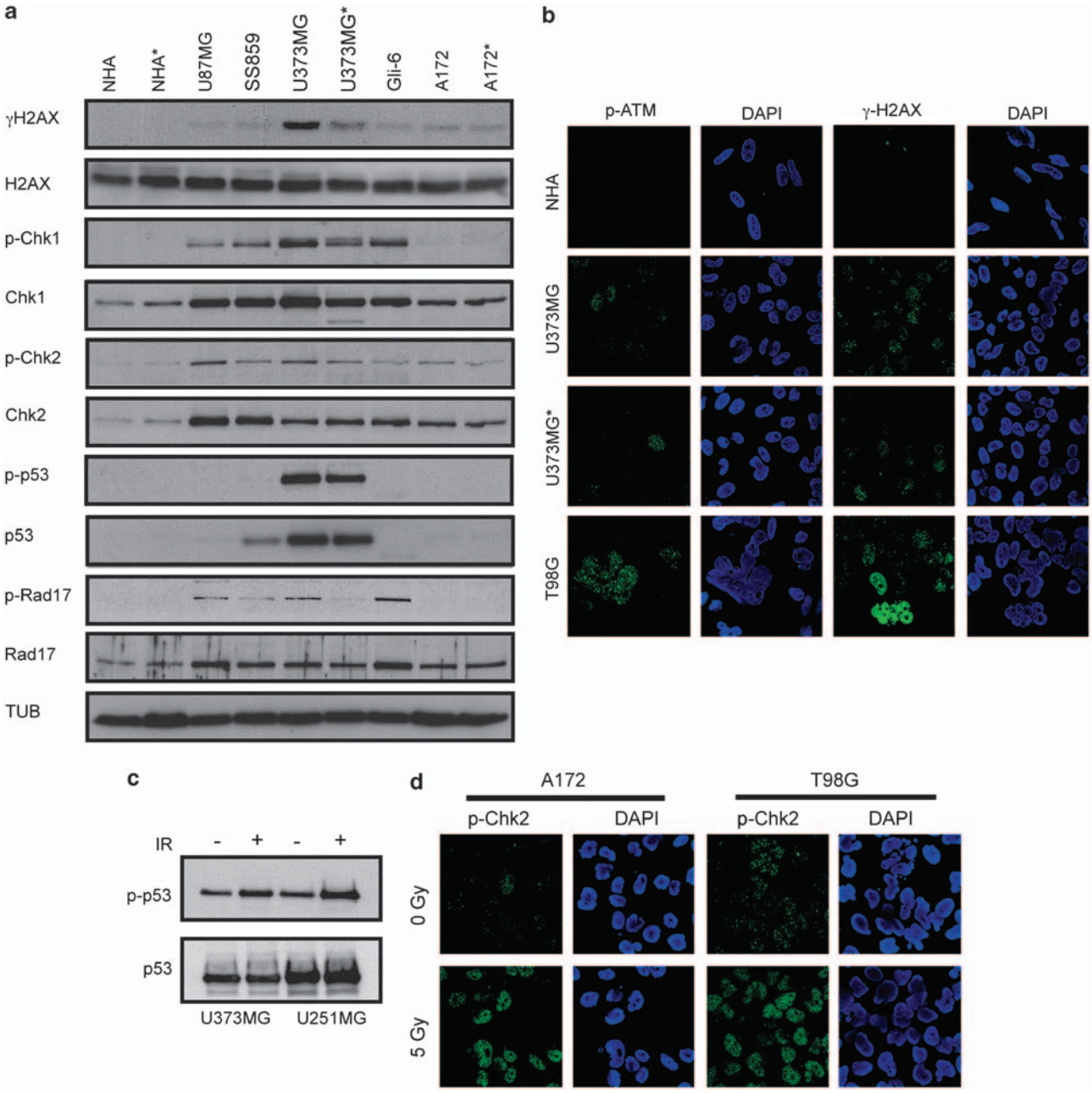

Figure 2 DNA damage response (DDR) is constitutively activated in human glioma cell lines as documented for the indicated markers by immunoblotting (**a**, **c**) and confocal immunofluorescence microscopy images (**b**, **d**). (**a**) Whole cell extracts from U87MG, U373MG and A172 (European collection of cell cultures) SS859, Gli-6 and normal human astrocytes (NHA) (Lonza) were separated by 8 or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by immunoblotting, using the antibodies listed in Supplementary Table 1. NHA, U373MG and A172 cells were also cultured in parallel in the atmosphere of 3% oxygen (further marked as NHA*, U373MG*and A172*) to mimic physiological hypoxia (Li *et al*., 2005). α-tubulin served as a loading control (TUB). (**b**) Immunofluorescence staining patterns of spontaneously activated ATM (p-ATM) and γH2AX, using antibodies listed in Supplementary Table 1 were detected by secondary antibody coupled to Alexa Fluor 488 (Molecular Probes, Invitrogen, Paisley, UK) and dried coverslips mounted in a 4′,6-diamidino-2-phenylindole-containing medium (Vector Laboratories Inc., Burlington, Canada), as described (Bekker-Jensen *et al*., 2005). Confocal images were acquired with equal settings and processed with Zen 2008 software (Carl Zeiss MicroImaging, Inc., Jena, Germany). (**c**) U373MG, U251MG cells and (**d**) A172, T98G cells were treated with ionizing radiation of 5 Gy (dose rate 2.18 Gy/min by an X-ray generator, Pantak, Berkshire, UK; HF160; 150 kV; 15 mA) or left untreated and analyzed 1 hour (**c**) or 30 min (**d**) after irradiation, as described (Knizetova *et al*., 2008). Further increase of p-p53 Ser15 and p-Chk2 Thr68 after ionizing radiation (5 Gy) over the already enhanced basal activation was observed in both GBM cell lines.

activation *in vivo* also reflect constitutive oxidative damage and replication stress. Immunohistochemistry analysis showed almost no 8-oxoguanine in normal brain tissues, low level among grade II astrocytomas, in contrast to commonly high oxidative damage in GBM (Figure 4a). Taken together with the maximal DDR signalling (γH2AX) in grade II astrocytomas, and the fact that also some 8-oxoguanine-low regions of GBM

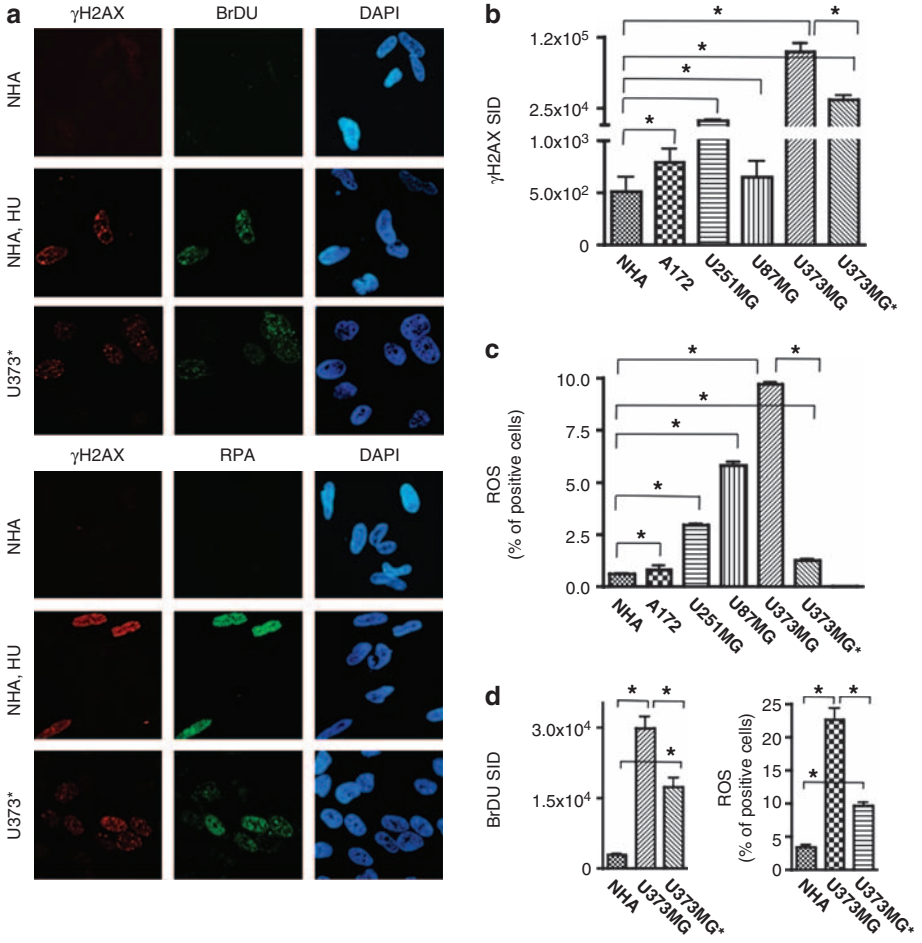

Figure 3 Constitutive activation of DNA damage response (DDR) is a consequence of sustained replication-stress signalling and the presence of oxidative lesions in astrocytes and glioma cell lines. NHA and U373MG* cells were grown on coverslips and treated with hydroxyurea (2 hours, 3 mM, Sigma-Aldrich Denamrk A/S, Broendby, Denmark) or left untreated. For bromodeoxyuridine (BrdU) and replication protein A staining, pre-extraction was performed to facilitate visualization of the chromatin bound protein (Raderschall *et al*., 1999; Bekker-Jensen *et al*., 2005). After staining (see Supplementary Table 1 for antibodies), the coverslips were dried, mounted in a DAPI-containing medium (Vector Laboratories), and examined using the LSM 510 META/Imager.Z1 (Plan-Apochromat $\times 63/1.40$ oil DIC M27 objective, Zeiss). Confocal images were acquired with equal settings and processed with Zen 2008 software (Zeiss). For evaluation of signal-integrated density (SID), images were acquired through Zeiss Axioplan II fluorescence microscope (PLAN-Neofluar $\times 40/1.3$ oil-immersion objective and photographed by a digital camera (Cool Snap). The exposition time, the binning, the setting of the microscope and the UV light source were kept constant for all samples. Further image processing and analysis was performed as described (Mistrik *et al*., 2009). (**a**) Replication stress (single-strand DNA detected by BrdU incorporation visualized under non-denaturing conditions: top panel; and by RPA foci formation: lower panel) persistent in U373MG* cells cultured in low oxygen, compared with untreated or hydroxyurea (HU)-treated normal astrocytes (NHA), relative to DDR signalling (γH2AX foci). (**b**) Quantification of γH2AX SID in NHA and GBM cell lines. (**c**) Glioma cells were stained with OxyDNA Assay Kit (Calbiochem, CA, USA) following manufacturer's instructions to detect 8-oxoguanine (8-OxoG) residues using FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA). The levels of 8-oxoguanine in selected cell lines were expressed as fold increase over the control samples, from three independent experiments. (**d**) Quantification of single-strand DNA by measuring SID of BrdU foci (incorporated BrdU detected under non-denaturing conditions, left panel) and level of reactive oxygen species (ROS, right panel) was measured by flow cytometry (as percentage of positive cells over unstained control). Cells (NHA, U373MG and U373MG* were detached using trypsin and stained with CM-H2DCFDA probe according to manufacturer's instructions (Molecular Probes, Invitrogen). The **b**, **c**, **d**: graphs show mean values from 2-3 experiments with indicated standard error bars. Asterisks indicate significance values $P<0.0001$.

showed high γH2AX signalling (Figure 4a), these results indicated that sources of DNA damage other than oxidative stress likely contribute to the DDR activation in gliomas, and may in fact represent the major source of the observed constitutive DNA damage signalling. Our findings that both γH2AX (Figure 1) and Chk1 activation (Figure 1c and data not shown) were massively enhanced in both grade II gliomas and most GBM, are consistent with ongoing replication stress (Gorgoulis *et al*., 2005; Bartkova *et al*., 2005b; Halazonetis *et al*., 2008) throughout the process of glioma development.

Finally, if the activated DDR machinery should provide a barrier against glioma progression, such

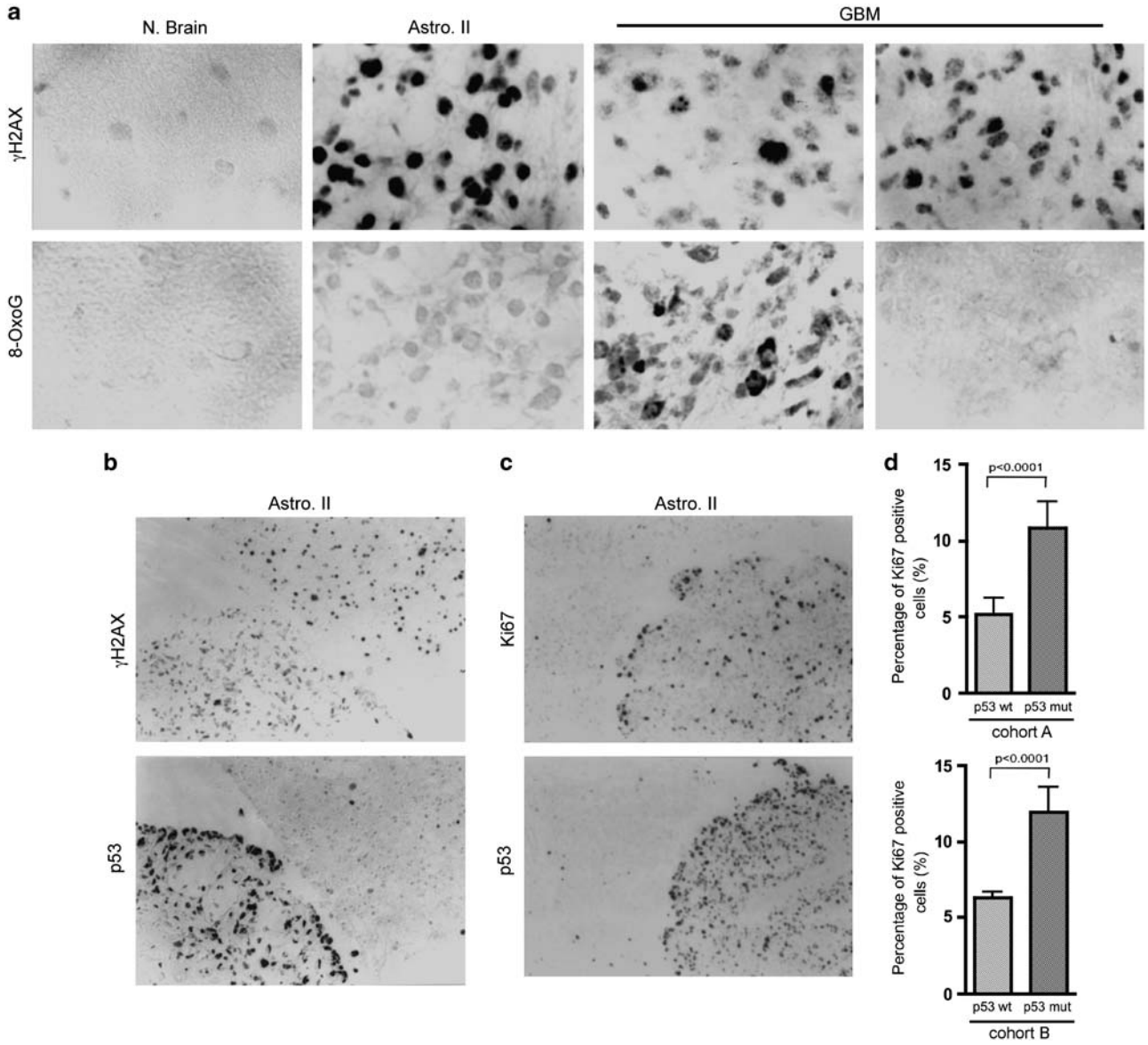

Figure 4 Immunoperoxidase staining of normal brain (N.Brain), astrocytomas grade II (Astro.II) and glioblastomas (GBM) for the indicated markers: (**a**) γH2AX and 8-oxoguanine on parallel sections; (**b**) γH2AX and p53 on parallel sections; while both areas of this tumor show aberrant constitutive DNA damage signalling (γH2AX), only one is strongly p53-positive, indicative of p53 mutation. (**c**) Ki-67 and p53 on parallel sections; note the higher proliferation rate (more Ki-67-stained cells) in the region with strongly p53-positive (p53-mutant) cells. For details of clinical specimens and immunoperoxidase staining, see legend to Figure 1. The antibodies and their specifications are listed in Supplementary Table 1. (**d**) Graph summary of Ki-67 index in grade II astrocytoma regions with 'wild-type' versus 'mutant' p53 patterns within the same lesion containing both patterns in distinct areas of the section (cohort A, different regions from five cases); and analogous comparison of Ki-67 index between all cases of astrocytoma grade II with exclusively 'p53-wild-type' pattern, versus those cases that showed the pattern of 'mutant p53' (cohort B, $n = 35$).

DNA damage signalling should precede the occurrence of for example, p53-mutant clones (Gorgoulis *et al*., 2005; Bartkova *et al*., 2005b; Halazonetis *et al*., 2008) that are being selected for, and initially show a focally-restricted growth, in lower-grade astrocytomas (Furnari *et al*., 2007). Testing this hypothesis, we found that parallel astrocytoma sections showed the predominant patterns of activated DDR (γH2AX) but normal p53 (with only scattered p53-high nuclei). Importantly, restricted areas in some astrocytoma samples showed highly abundant p53 (a 'p53-mutant-clonal' pattern) while DDR was globally activated in the entire lesion (Figure 4b). This, together with the fact that over-abundant 'mutant' p53 was always found only in GBM with high constitutive DDR activation, supported the notion that DDR activation precedes, and likely selects for, the occurrence of more malignant glioma clones with p53 mutations.

Another prediction of the active DDR providing a barrier to glioma progression, and p53 mutations being possibly a route to escape from the constrains of activated DDR, is that the proliferation rate of tumor

cells in the low-grade astrocytoma lesions with persistent DDR signalling and still wild-type p53 would be lower compared with regions of p53-mutant clones emerging within such lesions. Indeed, direct evaluation by Ki-67 staining on grade II astrocytoma tissue sections parallel to those stained for p53 revealed a highly significantly enhanced Ki-67 index in the areas with the over-abundant (mutant) p53 (Figures 4c and d), consistent with the notion that p53 mutation provides a proliferative advantage in the face of constitutive DDR signalling.

While mutations of p53 have classically been regarded as being more frequent among the secondary GBM lesions that develop from the lower-grade gliomas (Furnari *et al*. 2007), more recent data indicate that p53 is also targeted very frequently among the primary GBM (Zheng *et al*., 2008). These results are consistent with our present data on widespread activation of the DDR machinery in human lower-grade gliomas as well as GBM, and with the concept of DNA damage checkpoint activation as a selection pressure for outgrowth of p53-defective tumor clones (Halazonetis *et al*., 2008).

Overall, our results document that DNA damage checkpoint signalling is aberrantly and constitutively activated from low-grade gliomas up to GBM, in a large fraction of cells that is unlikely to be limited to, but may encompass, the glioma stem cells (Bao *et al*., 2006). These findings support the possible biological role of the DDR machinery in limiting the expansion of nascent malignant clones with unstable genomes (Bartek *et al*., 2007; Halazonetis *et al*., 2008). What are the genetic lesions that lead to the enhanced replication stress and constitutive DDR activation in gliomas remains to be determined, however aberrant activities of tyrosine kinase receptor class oncogenes, and loss of some tumor suppressors such as retinoblastoma protein, are among the top candidates for such events. Finally, our data are relevant for the current efforts to design individualized treatments by radiation or chemotherapy combined with checkpoint or DNA repair inhibitors (Zhou and Bartek, 2004; Bao *et al*., 2006; Helleday *et al*., 2008; Martin *et al*., 2008; Dungey *et al*., 2009; Jackson and Bartek 2009; Mukherjee *et al*., 2009). For example, our present findings of high levels of 8-oxoguanine in subsets of GBM cases might prove useful in selecting patients for treatment with inhibitors of poly ADP ribose polymerase, an enzyme involved in the base excision pathway that is required to repair such oxidative lesions (Helleday *et al*., 2008; Martin *et al*., 2008; Jackson and Bartek 2009; Mukherjee *et al*., 2009).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)