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REVIEW

Glioblastoma multiforme: the role of DSB repair between genotype and phenotype

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Glioblastoma is the most frequent primary brain tumor in adults. The average survival time of less than 1 year did not improve notably over the last three decades. The dismal prognosis of glioblastoma patients is largely due to the striking radioresistance of this tumor. Here, we attempt a combined view on the genetics, the repair mechanisms and the radioresistance of glioblastoma. Specifically, we address the role of DNA-PKcs and the novel potential end-joining factor KUB3 in maintaining the radioresistant phenotype, the interrelationship between genetic lesions and repair mechanisms, and new perspectives that emerge from the identification of glioblastoma stem cells.
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Genetic lesions

Glioblastoma arising from cells of glial lineage are the most frequent and malignant primary brain tumors in adults. The name glioblastoma multiforme denotes their very high cellular heterogeneity, their heterogeneous morphology and their complex chromosome aberrations. Glioblastoma can either develop *de novo* (>90%) as primary glioblastoma or through progression from low-grade glioma as secondary glioblastoma (Ohgaki and Kleihues, 2007). Although both types have several typical genetic lesions, conventional and array-based comparative genomic hybridization has recently demonstrated a number of common chromosomal aberrations, including extended deletions, gains of entire chromosomes or chromosome arms and amplifications, that is, increased copy numbers of chromosome portions (Koschny *et al*., 2002; Ruano *et al*., 2006). On average, the karyotype of primary glioblastoma shows 38 amplifications and 63 deletions, and the karyotype of secondary glioblastoma shows 53 amplifications and 83 deletions (Maher *et al*., 2006). To demonstrate the tremendous complexity of these aberrations, the following list summarizes prominent examples of amplified and gained regions commonly found in both types of glioblastoma: 1p31–1p22.3, 1q32.1–1q32.2, 1q44, 3p26.3, 4p12, 4q11–13.1, 5q31.2, 7p11.2, 7q22.2–32.1, 9p21.2, 9q34.3, 12q13.2–13.3, 12q13.3–14.1, 12q14.3–15, 15q26.3 and 19p13. Likewise, there are numerous deletions and in addition still other mutations.

Even in comparison to other human solid tumors, this karyotype is extremely aberrant leaving the question about the underlying molecular mechanisms. Although it is well accepted that misrepair of DNA double-strand breaks (DSBs) contributes to chromosome rearrangements, the detailed mechanisms remain to be elucidated. The inhibition of DNA-dependent protein kinase catalytic subunit (DNA-PKcs), a key player in nonhomologous end-joining (NHEJ) DSB repair, and ataxia telangiectasia mutated (ATM), the protein defective in A-T cells, suggested that the elimination of DNA-PK-dependent NHEJ in glioblastoma cells increases chromosome aberration levels (Virsik-Kopp *et al*., 2003), similar to what is observed in other cell types. Moreover, immortal rodent cells lacking DNA-PKcs show an increased propensity for gene amplifications (Mondello *et al*., 2001). As outlined below, the interrelationship between DSB repair and chromosome aberration may also work in the opposite direction: the altered karyotype of glioblastoma is likely affecting many cellular pathways including repair mechanisms.

Gene amplification and DSB repair

Gene amplification as a process leading to increased copy numbers of portions of the genome is a frequent manifestation of genomic instability in tumor cells. In glioblastoma, gene amplification is found in various chromosomal regions, including 7p13, 12q13–15, 7q31, 7q21, 4q11–12, 1q32.1 and 8q24.1 (Fischer *et al*., 1994; Muleris *et al*., 1994; Schröck *et al*., 1994; Maher *et al*., 2006). Extended gene amplifications are cytogenetically visible either as double minutes with extrachromosomal gene amplifications or as homogeneously staining regions with intrachromosomal gene amplifications. It was shown that intrachromosomal amplifications are initiated by DNA strand breaks at fragile sites (Coquelle *et al*., 1997). Fragile sites are specific loci that form gaps and constrictions in metaphase chromosomes after treatment under cell culture conditions that induce replication stress (Sutherland and Richards, 1995).

Replication stress can be induced by a specific concentration of aphidicolin (0.4 μM). Studies on HeLa cells

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show that aphidicolin treatment led to the recruitment of Rad51 into DNA damage-induced foci and to the formation of phosphorylated DNA-PKcs foci (Schwartz *et al*., 2005). Induced downregulation of Rad51, DNA-PKcs or ligase IV by small interfering RNA showed that NHEJ is required for fragile site stability specifically during replication stress, while homologous recombination (HR) is required both under normal conditions and during replication stress. Frequent colocalization of phospho-DNA-PKcs and Rad51 foci indicate that HR and NHEJ cooperate at the same DSB of a fragile site (Schwartz *et al*., 2005). These data suggest that reduced functionality of NHEJ and/or HR plays a central role for the process of gene amplification in glioblastoma.

Besides drugs like aphidicolin, physiological environmental factors like hypoxia are also strong inducers of common fragile sites causing genomic rearrangements and gene amplifications (Coquelle *et al*., 1998). Hypoxic tissue areas caused by necrosis and insufficient blood supply are typically found in glioblastoma. It is legitimate to postulate that in glioblastoma, hypoxia contributes to gene amplification via fragile site induction, likely involving an altered NHEJ or HR repair pathway.

Various mechanisms are potentially involved in the development of gene amplifications including breakage–fusion–bridge cycle, overreplication and the 'episome model'. The breakage–fusion–bridge cycle denotes a repetitive process consisting of breakage of a chromosome, followed by sister chromatid fusion and subsequent disruption of the sister chromatid fusion in anaphase (McClintock, 1941). Overreplication is caused by an unscheduled initiation of replication (Schimke *et al*., 1986). The 'episome model' proposes that episomes result from excision of small circular DNA that enlarges by overreplication or recombination, eventually becoming cytogenetically visible as double minutes (Ruiz and Wahl, 1988). Recently, palindromic sequences were suggested to be involved in the development of genomic instability, in particular, in the development of amplifications in cancer cells (Tanaka *et al*., 2005). However, the mechanisms by which palindromic sequences contribute to the initiation of amplification remain elusive for human cancer. Studies on gene amplification mechanisms in *Saccharomyces cerevisiae* indicate that the initial palindromic duplication does not require NHEJ function. Palindromic duplications were thought to initiate by a foldback replication mechanism (Rattray *et al*., 2005). In *Escherichia coli*, there is evidence that initial nonhomologous recombination events requiring proteins involved in DSB repair underlie stress-induced gene amplifications (Slack *et al*., 2006). Since DSBs in bacteria and yeast, in contrast to mammalian cells, are predominantly repaired by HR, mechanistic models developed for bacteria and yeast may not easily apply to mammalian gene amplification.

KUB3 as potential modulator of DSB repair

Double-strand break repair is not only crucial for the induction and/or development of gene amplifications, but vice versa, gene amplifications exert downstream effects on DSB repair. Glioblastoma are characterized by multiple gene amplifications as described above. A chromosome region on 12q is frequently amplified in glioblastoma and encompasses various genes crucial for the biology of this tumor including genes CDK4 and MDM2 (Reifenberger *et al*., 1996; Munnia *et al*., 2001). In addition, the amplified domain on chromosome 12q contains a gene encoding a Ku70-binding protein that was formerly termed KUB3 (Yang *et al*., 1999) and has been renamed XRCC6BP1. XRCC6BP1 shows amplification frequencies similar to CDK4 within the 12q amplicon and is overexpressed in glioblastoma-derived cell lines and in primary glioblastoma (Fischer *et al*., 2001). Binding of XRCC6BP1 to Ku70 suggested a role of this protein in NHEJ (Yang *et al*., 1999). Our preliminary results on the function of XRCC6BP1 in glioblastoma cell lines are consistent with the idea that XRCC6BP1 is involved in DSB repair (data not published). Glioblastoma that carry the endogenous amplification and overexpress XRCC6BP1 accordingly, likely exhibit altered NHEJ repair. It remains to be seen whether XRCC6BP1 overexpression impairs or promotes the efficiency of this repair pathway in glioma. A proposed feedback loop between XRCC6BP1 amplification and altered DSB repair is shown in Figure 1.

Clinic and therapy

The aggressiveness of glioblastoma is not only reflected on the cellular level but also evident by its clinical behavior. While other common solid tumors frequently can be cured if caught at an early stage, small glioblastoma even after gross total resection recur and cause death within at best 2 years. On average, glioblastoma patients have a survival time of less than 1 year upon diagnosis. The severity of this tumor is also demonstrated by the disproportionate loss of live time caused by glioblastoma in comparison to other human tumors.

Treatment of glioblastoma includes maximal surgery and radiation therapy with parallel and adjuvant temozolomide (TMZ) (Stupp *et al*., 2005). Since glioblastoma very rarely metastasize outside the central nervous system, they appear as very attractive targets for radiotherapy. However, a major reason for the dismal prognosis of glioblastoma patients is the markedly augmented radioresistance of this tumor. Increased local radiation doses did not prove to be successful due to necrosis of the surrounding brain tissue. Sensitizing glioblastoma for radiation bears some promise but requires a better understanding of the molecular mechanisms responsible for radioresistance. The concurrent therapy with TMZ confers survival advantages only for a subgroup of glioblastoma patients (Stupp *et al*., 2007).

KUB3 as potential modulator of radioresistance

It remains to be clarified whether endogenous amplification and overexpression of XRCC6BP1 contributes to the radioresistant phenotype of glioblastoma. Although XRCC6BP1 is as of yet the only amplified and overexpressed gene involved in DSB repair in glioblastoma,

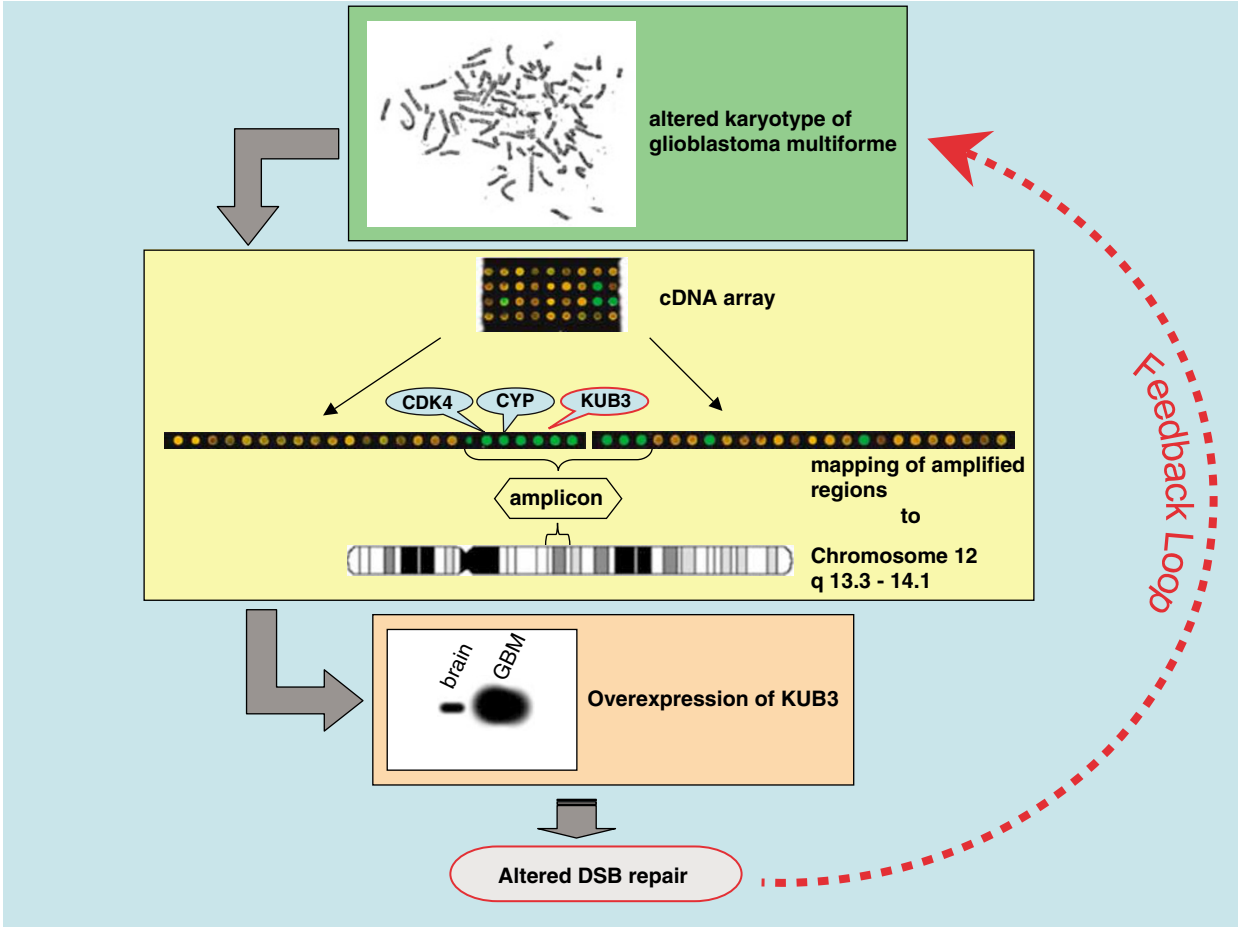

Figure 1 Proposed feedback loop between chromosome alterations and altered double-strand break repair: glioblastoma carry amplified genes on double minutes. Array comparative genomic hybridization mapped the amplified region to chromosome 12q13.3-14.1. Amplification results in overexpression of KUB3 (XRCC6BP1) and eventually in altered double-strand break (DSB) repair. Altered DSB repair may in turn impact the glioblastoma karyotype.

other DSB repair genes have been reported to be overexpressed in specific tissues. An example of an overexpressed DSB repair gene is DNA-PKcs in HL60 cells, a human promyelocytic leukemia cell line. Increased DNA-PKcs protein levels and enzyme activity lead to adriamycin resistance in HL60 cells (Shen *et al*., 1998). Using antisense RNA against DNA-PKcs, protein levels were reduced by 50%, significantly increasing sensitivity to adriamycin. Similar to these results, overexpression of other factors involved in NHEJ may improve DSB repair and resistance to DSB-inducing drugs or ionizing radiation. If such a scenario holds true for XRCC6BP1, glioblastoma with endogenous amplification and overexpression of XRCC6BP1, may exhibit a higher radioresistance than glioblastoma without this amplification.

DSB repair in MO59J, MO59K and other glioblastoma cell lines

Some general knowledge about repair mechanisms in higher eukaryotic cells stems from studies that compared the glioblastoma cell lines MO59J and MO59K. These two cell lines that were established from a single glioblastoma specimen, differ in their DNA-PKcs status, and show a strikingly different sensitivity to radiation and chemotherapeutic drugs, with DNA-PKcs-deficient MO59J cells being 30-fold more sensitive to radiation than MO59K cells (Allalunis-Turner *et al*., 1993). This 'pair' of cell lines was used to study DSB rejoining. It was shown that rejoining of DSBs is biphasic with a fast and a slow component. Deficiency of DNA-PKcs activity in MO59J cells reduced the fast rejoining component, underlining the role of DNA-PKcs for fast rejoining of DSBs by NHEJ (DiBiase *et al*., 2000). In contrast, the slow rejoining process likely involves a NHEJ subcomponent, which is regulated by ataxia telangiectasia mutated and Artemis (Riballo *et al*., 2004). In glioblastoma cells, the slow rejoining is very likely due to an error-prone backup NHEJ pathway (Wang *et al*., 2005). It remains to be seen to what extend these results apply to other glioblastoma-derived cell lines and to glioblastoma *in vivo*.

For the past decade, studies on DNA repair mechanisms in glioblastoma focused on tumor cells largely neglecting the situation in normal brain tissue. Recently,

clinically relevant radiation doses were applied to four glioblastoma cell lines (U373, U87, A7 and T898G) and to normal human astrocytes for comparison. Glioblastoma cell lines repaired DSBs more slowly and less efficiently than the normal human astrocytes (Short *et al*., 2007). A less efficient repair process may appear inconsistent with high levels of repair protein in glioma cells: Rad51, which is central in HR repair (Raderschall *et al*., 2002) and DNA-PKcs are highly expressed in various glioblastoma cell lines. It may, however, be an oversimplified view to expect that high levels of proteins involved in DNA repair pathways necessarily accompany efficient repair. Higher amounts of proteins like Rad51 may as well interfere with the stoichiometry of repair processes. This may also be the case for Ku70/Ku80. Overexpression of Ku70/Ku80 that was found in rat cells resulted in reduced DSB repair capacity (Kasten *et al*., 1999).

Radioresistance of MO59J and MO59K cell lines

Analysis of glioblastoma cell lines MO59J and MO59K also contributed to the understanding of the radioresistant phenotype reported for many glioblastoma. Early evidence linked the radiosensitivity of MO59J cells to the absence of DNA-PKcs in these cells (Lees-Miller *et al*., 1995). Experiments introducing a fragment of human chromosome 8 that harbors a copy of the DNA-PKcs gene into MO59J cells reversed the radiation-sensitive phenotype (Hoppe *et al*., 2000). However, these studies do not explain the exceptional radioresistance of MO59K cells.

Low-dose radiation of MO59J induces massive autophagic cell death (Daido *et al*., 2005). Autophagic cell death is a form of programmed cell death that is characterized by the formation of double-membrane structures in the cytoplasm. While most of the DNA-PKcs-proficient MO59K cells survived after low-dose radiation, inhibition of DNA-PKcs activity by antisense oligonucleotides caused MO59K cells to undergo autophagic cell death upon low-dose radiation. While DNA-PKcs expression appears to protect glioblastoma cells from undergoing autophagy, the radioresistance does not necessarily imply efficient DSB repair of this tumor. By contrast, and as mentioned above, several radioresistant glioblastoma cell lines have been shown to inefficiently repair DSBs (Short *et al*., 2007). Inefficient DSB repair is likely due to the specific genetic background of this tumor: High-grade glioma frequently bear TP53 mutations (Ohgaki and Kleihues, 2007). Glioblastoma cells with mutated TP53 show no significant G_1 checkpoint response but largely depend on G_2 activation for their radioresistant phenotype (Short *et al*., 2007).

Summarizing the limited data on DNA repair mechanisms in radioresistant glioblastoma, it has to be acknowledged that the radioresistance cannot easily be linked to efficient DSB repair, and that likewise, high levels of the repair proteins Rad51 and DNA-PK cannot necessarily be associated with improved DSB repair. As demonstrated for the TP53 phenotype, in understanding DNA repair and radioresistance in glioblastoma, the genetic background of this tumor must be considered. Additional proteins like the recently described NHEJ factor NHEJ1 (XLF) will further refine our picture of DSB repair and radioresistance of glioblastoma. As shown in a recent cDNA microarray analysis aimed to identify upregulated genes associated with radioresistance (Otomo *et al*., 2004), expression profiling is a promising way to complement our picture of the proteins involved in DSB repair.

Cellular effects of temozolomide

As indicated above TMZ is commonly used for the treatment of several human tumors including glioblastoma. TMZ as methylating agent generates several methylated DNA adducts including the minor alkylation product O^6-methylguanine, which is the most potent killing lesion and a powerful trigger of apoptosis (Kaina *et al*., 1991). Because O^6-methylguanine mispairs with thymine during DNA replication, mismatches recognized by the DNA repair system lead to multiple rounds of futile mismatch repair and finally cause DSBs. Cells lacking O^6-methylguanine mismatch repair do not recognize TMZ-induced DNA mismatches and are resistant to TMZ-induced apoptosis (Friedman *et al*., 1997). Notably, cells with mutated DNA-PKcs were more sensitive to TMZ-induced apoptosis. Since both p53 wild-type and p53 mutant glioma cells undergo apoptosis in response to TMZ, TP53 is not necessarily required for TMZ-induced apoptosis (Roos *et al*., 2007). Inhibition of DNA-PKcs may offer a way to improve both the efficiency of TMZ therapy and radiotherapy.

Stem cells

Our present knowledge about DSB repair and radioresistance in glioblastoma largely stems from few cell lines that allow only a very narrowed view on the underlying mechanisms in the different cell types of this complex tumor. Among the various cell types, tumor stem cells are most central for the devastating growth of glioblastoma despite therapy. Tumor stem cells possess the capacity to self-renewal leading to daughter cells with the same predisposition for replication as the parental cells, and the capacity to recapitulate the generation of a growing tumor (Clarke, 2004). These capacities have been shown for CD133+ cells isolated from glioblastoma (Galli *et al*., 2004). CD133+ is a surface marker found on normal neural stem cells. A small number of approximately 100 CD133+ glioblastoma cells are sufficient for the formation of glioblastoma in immunodeficient mice. The resulting primary xenograph consisting of a minority of CD33+ cells and a majority of CD133− cells is a phenocopy of the patient's tumor (Singh *et al*., 2004).

The evidence for a role of stem cells in the pathogenesis of glioblastoma leaves the question about the

role of DNA repair in tumor stem cells. First studies on hematopoietic cells demonstrate that deficiencies in DNA damage repair limit the function of stem cells. Mice with deficiencies in excision repair, telomere maintenance and NHEJ showed under stress reduced self-renewal, increased apoptosis and functional exhaustion of hematopoietic stem cells (Rossi *et al*., 2007). Likewise, a mouse strain carrying a hypomorphic mutation in DNA ligase IV, which is essential for NHEJ, showed impaired stem cell function in tissue culture and transplantation (Nijnik *et al*., 2007). These results indicate that accumulation of unrepaired DSBs likely decreases the self-renewal capacity of stem cells.

Notably, CD133+ glioblastoma cells preferentially activate the DNA damage checkpoint in response to radiation (Bao *et al*., 2006). They also repair ionizing radiation-induced damage more efficiently than CD133− cells and likely represent the cell population that is responsible for the radioresistance of glioblastoma (Figure 2). Targeting the checkpoint response in CD133+ glioblastoma cells may help to overcome the radioresistance of this tumor. As indicted above for hematopoietic stem cells, further studies may confirm a rate-limiting role of DNA repair for the functionality of glioblastoma stem cells.

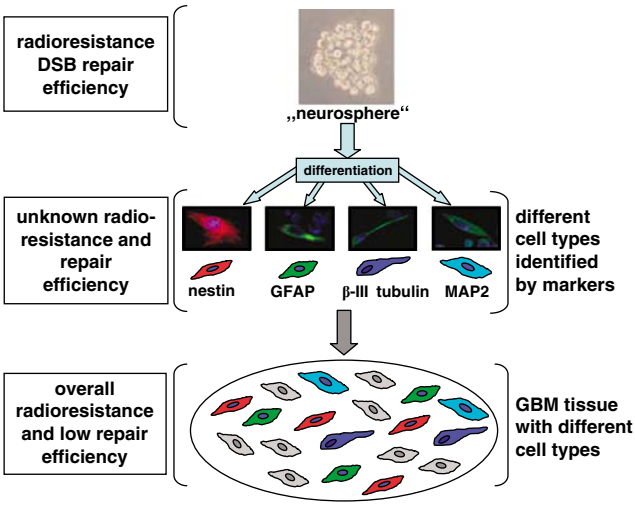

Figure 2 Radioresistance and DNA repair efficiency: glioblastoma multiforme (GBM) stem cells shown as 'neurospheres' in cell cultures are likely radioresistant and efficient in double-strand break (DSB) repair. GBM stem cells can differentiate into various cell types with unknown radioresistance and efficiency in DSB repair. While the various cell types of GBM differ in their DSB repair efficiency, the entire tumor shows an overall radioresistant phenotype.

Summary

The present data indicate that a reduced functionality of NHEJ enhances the development of chromosome aberrations including gene amplifications in glioblastoma. As shown for XRCC6BP1, amplified and overexpressed genes can be involved in DSB repair. In a possible feedback loop, impaired NHEJ causes gene amplification that leads to overexpression of repair genes, which in turn may further impair the NHEJ functionality in glioblastoma (Figure 1). Alternatively, amplified repair genes may improve NHEJ functionality. In the latter case, glioblastoma with XRCC6BP1 amplification likely have a more stable karyotype.

An unsolved enigma is the relationship between the radioresistance of glioblastoma and the low-efficient repair. However, low-efficient repair has as of yet been demonstrated for only four glioblastoma. Since glioblastoma stem cells have recently been associated with both efficient repair and radioresistance, various cell types in glioblastoma may differ in their radioresistance and their capacity to repair DSBs (Figure 2).

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