Radiosensitization of cancer stem cells in glioblastoma by the simultaneous inhibition of parallel DNA damage response pathways

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Glioblastoma is the most common primary brain cancer in adults, the standard treatment for which is surgical resection followed by radiotherapy and chemotherapy with temozolomide (an alkylating agent) (1). Glioblastoma cells extensively exhibit genetic and phenotypic intra-tumor heterogeneity, and the subclones of tumor cells have higher regenerative/stem cell activity than the other parts of glioblastoma tumor (2). Previous workers have shown that glioblastoma stem-like cells exhibit higher resistance to chemo- and radiotherapy than the differentiated bulk tumor cells (3-5). Therefore, analysis of the stem-like cells is essential to overcome the therapeutic resistance of glioblastoma.

Resistance to radiotherapy and chemotherapy arises from DNA damage response involving DNA repair and DNA damage checkpoint pathways. Both these pathways are regulated by serine/threonine protein kinases, ATM (ataxia telangiectasia mutated), and ATR (ATM and Rad3-related). Induction of double-strand DNA breaks activates ATM, which promotes DNA repair as well as activates DNA damage checkpoint. On the other hand, ATR is activated by the generation of single-stranded DNA due to stalled replication forks or processing of DNA double-strand breaks. ATR phosphorylates a downstream kinase CHK1, to activate the DNA damage checkpoint and to maintain the cell cycle arrest. In p53-deficient tumor cells, DNA damage checkpoint arrests the cell cycle at G2 phase in an ATR- and CHK1-dependent manner. PARP1 [poly (ADP-ribose) polymerase 1], which is activated by DNA single-strand breaks, synthesizes polymeric adenosine diphosphate ribose [poly (ADP-ribose)] chains to recruit multiple DNA repair proteins. A small-molecule inhibitor of PARP1, olaparib, has been developed and used for the treatment of a subset of ovarian cancer (6,7).

In glioblastoma, DNA damage response is activated constitutively and aberrantly (8). Bao *et al.* reported that the activation is higher in the glioblastoma cells that are positive for CD133, a marker for neural stem cells and brain cancer stem cells, than in CD133-negative cells and that the CD133-positive cells displayed a higher DNA repair capacity (3). However, the relationship and coordination between stemness and therapeutic resistance has not been clearly established yet (9,10). Radiosensitization increases the efficiency of radiotherapy by inhibiting DNA damage responses through pharmacogenic inhibition of the DNA damage response proteins (6). The inhibition of DNA damage responses also successfully improves the efficiency of chemo-and radiotherapy in glioblastoma tumors (11-14). Ahmed *et al.* have demonstrated successful radiosensitization not only in bulk tumors but also in glioblastoma stem-like cells (3,15,16).

In a recent issue of *Cancer Research*, Ahmed *et al.* reported radiosensitization of glioblastoma stem-like cells by parallel inhibition of the DNA damage response pathways (17). To

prove the relationship between stemness and radioresistance, Ahmed *et al.* generated two types of paired, primary patient-derived glioblastoma cell lines, one of which was enriched while the other was depleted of the stem-like cells, to generate glioblastoma stem-like cells and bulk tumor cells, respectively. The glioblastoma stem-like cells generated orthotopic tumors in mice and expressed neural stem cell markers (nestin, SOX2, CD133, Olig2, and CD15). These cell lines were irradiated with an ionizing radiation and the colony-forming ability was examined. Consequently, Ahmed *et al.* distinctively proved that glioblastoma stem-like cells are more radioresistant than bulk tumor cells. One of the notable points of this manuscript was that the authors compared isogenic glioblastoma stem-like cells and bulk tumor cells, and excluded other confusing cloning variants derived from the highly variable clonal nature of glioblastoma cells. In addition, Ahmed *et al.* showed that glioblastoma stem-like cells exhibited higher expression and activity of DNA damage response proteins than the isogenic bulk tumor cells. ATM- and ATR-dependent DNA damage checkpoints were found to be upregulated in glioblastoma stem-like cells. The expression of PARP1 and Rad50 was also found to have increased, suggesting a more effective DNA repair reaction. Moreover, the irradiated glioblastoma stem-like cells showed a more rapid and prolonged cell cycle arrest at the G2 phase than the isogenic bulk tumor cells, which was consistent with the upregulation of ATM- and ATR-dependent checkpoints.

Further, Ahmed *et al.* showed that the ATR-CHK1 pathway is involved in the radioresistance of glioblastoma stem-like cells. The enhanced G2 checkpoint arrest was indicative of the role of ATR-CHK1 signaling in the radioresistance of glioblastoma stem-like cells. Therefore, Ahmed *et al.* inhibited the ATR-CHK1 pathway by pharmacogenic inhibition of CHK1, and examined its effect on radioresistance of glioblastoma cells. CHK1 inhibition abrogates DNA damage checkpoint-dependent cell cycle arrest at the G2 phase (18), and Ahmed *et al.* reported the observed abrogation in glioblastoma stem-like cells. CHK1 inhibition decreased the colony-forming ability in glioblastoma stem-like cells after treatment with ionizing radiation; a pronounced radiosensitizing effect was, however, observed in the bulk tumor cells. This observation raises a possibility that mechanisms other than the ATR-CHK1 pathway are involved in the survival and radioresistance of glioblastoma stem-like cells, which make the stem-like cells significantly more radioresistant than the bulk tumor cells.

Ahmed *et al.* also examined the mechanisms through which glioblastoma cells are radiosensitized by the inhibition of ATR-CHK1 pathway, and revealed that the ATR-CHK1 inhibition induces DNA double-strand breaks. In the glioblastoma stem-like cells, the exposure to CHK1 inhibitor and ionizing radiation significantly increased γ-H2AX, an indicator of DNA double-strand breaks. This is because the ATR-CHK1 pathway is essential for normal progression of DNA replication in the S phase, and the inhibition of ATR-CHK1 pathway results in the collapse of replication forks and generation of DNA double-strand breaks (7). Moreover, the exposure to CHK1 inhibitor and ionizing radiation induced a mitotic catastrophe, a catastrophic chromosome segregation, in the glioblastoma stem-like cells (17). After DNA damage induction, ATR- and CHK1-dependent cell cycle arrests are essential for correct segregation of sister chromatids in the following mitotic phase. Therefore, the abrogation of cell cycle arrest leads to a mitotic catastrophe and the generation of DNA double-strand breaks (7). A higher number of glioblastoma stem-like cells also showed a micronucleus (17), which could have originated during mitotic divisions, from the acentric chromosome or chromatid fragments caused by genome damage (19). In the glioblastoma stem-like cells, the increase in γ-H2AX was observed in S phase and in the mitotic phase (17), which can be understood as an effect of the inhibition of ATR-CHK1 pathway. In addition, Ahmed *et al.* observed a more pronounced increase in γ-H2AX, micronucleus, and mitotic catastrophe in the bulk tumor cells than in isogenic glioblastoma stem-like cells, suggesting that the glioblastoma stem-like cells have higher capacity of DNA double-strand break repair. In a previous report, Carruthers *et al.* showed that the glioblastoma stem-like cells have an increased capacity of ATM-dependent DNA double-strand break repair (15). These data indicate that the increased DNA repair capacity is correlated with the higher radioresistance of glioblastoma stem-like cells.

Finally, different DNA repair and DNA damage checkpoint pathways were targeted by pharmacogenic inhibitors, and the effect on radiosensitization was examined. DNA repair was targeted with the PARP inhibitor, olaparib, while the cell cycle checkpoint was targeted with the ATR inhibitor, VE821. Simultaneous inhibition of DNA repair and DNA damage checkpoint was achieved by the combination of the PARP inhibitor and ATR inhibitor. The combination of the PARP inhibitor and ATR inhibitor showed the most deleterious effect on the colony- and neurosphere-forming abilities after irradiation

with ionizing radiation in the glioblastoma stem-like cells. Ahmed *et al.*, therefore, concluded that the simultaneous inhibition of parallel DNA damage responses is essential to overcome the radioresistance of glioblastoma stem-like cells (17). Olaparib is effective against breast and ovarian cancer cells that show defect in one of the DNA repair pathways or homologous recombinational repair, arising from *BRCA1* or *BRCA2* mutation, and the cytotoxicity of olaparib is explained as an effect of its synthetic lethality (20). In the radiosensitization of glioblastoma cells by PARP and ATR inhibitors, the synthetic lethal mechanism is suggested to be involved.

In summary, glioblastoma is an example of a highly heterogeneous tumor (2), containing a subpopulation that is resistant to radio- and chemotherapy (3). The recent study by Ahmed *et al.*, as well as their accompanying studies, clearly showed that the glioblastoma stem-like cells are more resistant to radiotherapy than the isogenic bulk tumor cells (15,17). Ahmed *et al.* also showed that ATR-CHK1 pathway and the increased DNA repair play a role in the radioresistance of glioblastoma stem-like cells. Importantly, the maximum radiosensitization was only achieved by the simultaneous inhibition of ATR and PARP, which results in the inhibition of both DNA damage checkpoint and DNA repair pathways. The data presented strongly potentiate multiple-drug therapy with ATR and PARP inhibitors to enhance radio- and chemotherapy of glioblastoma.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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