Glioblastoma Cells Containing Mutations in the Cohesin Component STAG2 Are Sensitive to PARP Inhibition

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Abstract

Recent data have identified STAG2, a core subunit of the multifunctional cohesin complex, as a highly recurrently mutated gene in several types of cancer. We sought to identify a therapeutic strategy to selectively target cancer cells harboring inactivating mutations of STAG2 using two independent pairs of isogenic glioblastoma cell lines containing either an endogenous mutant STAG2 allele or a wild-type STAG2 allele restored by homologous recombination. We find that mutations in STAG2 are associated with significantly increased sensitivity to inhibitors of the DNA repair enzyme PARP. STAG2-mutated, PARP-inhibited cells accumulated in G2 phase and had a higher percentage of micronuclei, fragmented nuclei, and chromatin bridges compared with wild-type STAG2 cells. We also observed more 53BP1 foci in STAG2-mutated glioblastoma cells, suggesting that these cells have defects in DNA repair. Furthermore, cells with mutations in STAG2 were more sensitive than cells with wild-type STAG2 when PARP inhibitors were used in combination with DNA-damaging agents. These data suggest that PARP is a potential target for tumors harboring inactivating mutations in STAG2, and strongly recommend that STAG2 status be determined and correlated with therapeutic response to PARP inhibitors, both prospectively and retrospectively, in clinical trials. Mol Cancer Ther; 13(3); 724–32. ©2013 AACR.

Introduction

Inhibition of PARP has emerged as a promising drug strategy for the treatment of cancers mutated for BRCA1/2 because of its ability to selectively kill cells through synthetic lethality (1, 2). More recently, PARP inhibitors have been shown to be effective in cells with defects in other genes involved in homologous recombination and the DNA damage response suggesting that PARP inhibitors may be effective in treating a wider range of tumors that do not have BRCA mutations (3–6). Identification of other tumor genotypes susceptible to PARP inhibition will expand the utility of these drugs.

The cohesin complex, named for its role in sister chromatid cohesion, is well conserved across organisms (7). In humans, the core mitotic complex consists of four subunits: SMC1A, SMC3, RAD21 (also known as SCC1 or MCD1), and one of two possible stromal antigen proteins (STAG1 or STAG2). Together, these four subunits can encompass newly replicated sister chromatids and hold them in close proximity (8). Beyond its well-known function in chromosome segregation, cohesin has several additional roles in the cell. Similar to other genes sensitive to PARP inhibition, defects in cohesin components affect both replication fork integrity and homologous recombination repair (7, 9, 10). Cohesin is recruited to sites of replication fork pausing and double-strand breaks (DSB) and has also been shown to promote replication fork restart and DNA repair through its interactions with other proteins (11–13). In addition, because of its ability to encircle sister chromatids, the cohesin complex is thought to promote error-free recombination repair with the neighboring undamaged DNA strand in the S-G2 phases of the cell cycle (10). Supporting the idea that cells mutated for cohesin genes might be sensitive to PARP inhibition, we have shown that knockdown of three of the cohesin core components (SMC1, SMC3, and RAD21) can render cells sensitive to the PARP inhibitor olaparib (14).

Recently, the cohesin gene, STAG2, was discovered to be highly mutated in glioblastoma, Ewing sarcoma, and melanoma cells (15). These mutations led to either truncation or functional inactivation of the STAG2 protein that is easily detected in cells or tissues by immunohistochemistry or Western blot analysis using antibodies. Given the previous data that knockdown of cohesin components results in PARP inhibitor sensitivity (14), we wanted to determine if tumor cells with...
STAG2 mutations were susceptible to PARP inhibition. Here, we show that glioblastoma cell lines with mutations in STAG2 are significantly more sensitive to PARP inhibitors than matched, isogenic STAG2 wild-type lines. This proliferation defect results in an accumulation of cells in G2 phase and genome instability. Furthermore, STAG2-mutated cell lines demonstrate an increased sensitivity when combinations of DNA-damaging chemotherapy agents and PARP inhibitors are used, providing a therapeutic rationale for PARP inhibitors either as a single agent, or in combination with other DNA-damaging agents, in STAG2-deficient tumors.

Materials and Methods

Materials and cell culture

Olaparib (AZD2281), veliparib (ABT-888), and rucaparib (AG014699) were purchased from Selleck Chemicals; temozolomide and camptothecin were purchased from Sigma-Aldrich. Antibodies used were anti-PAR (Trevigen), anti-STAG2 (Santa Cruz Biotechnology), anti-SMC1, anti-SMC3, anti-p531 Histone H3 (H3) (pH3), anti-53BP1 anti-GAPD, and anti-β-tubulin (all from Abcam). H4 and 42MGBA parental and STAG2 knock-in (KI) cell lines have been described previously (15). H4 and 42MGBA cell lines obtained from Solomon and colleagues were from the American Type Culture Collection and DSMZ, respectively, and were cultured in Dulbecco’s Modified Eagle Medium (DMEM) + 10% FBS at 37°C and 5% CO2 for 1 to 2 months at a time before reintitiation from early passage, frozen stocks. Cell lines were checked regularly for the presence or absence of STAG2 by Western blot analysis (Supplementary Fig. S1).

Cell counting experiments and clonogenic assays

To assess cell number by nuclei counting, cells were plated in a 96-well format with 6 technical replicates for each drug concentration. Twenty-four hours after plating, inhibitors or dimethyl sulfoxide (DMSO) were diluted into DMEM and added to wells. Cells were fixed in 3.7% paraformaldehyde after 4 to 5 days and then stained with Hoechst 33342 before nuclei were counted on a Cellomics Arrayscan VTI.

For clonogenic assays, cells were plated at single-cell density in 6-well dishes with three replicates per drug concentration. Drugs were added after 24 hours and cells were allowed to grow for 10 to 14 days; drug media were changed every 4 to 5 days. Colonies were then fixed and stained with 0.1% crystal violet in 95% ethanol for counting. Cell lines were all normalized to the DMSO control and compared using a two-tailed, matched Student t test. Error bars represent SEM.

Immunoblotting and flow cytometry

Cells were grown on coverslips with and without PARP inhibitor for 3 (H4) or 4 (42MGBA) days before fixation in 1:1 methanol:acetone and permeabilization in 0.1% Triton X-100. Coverslips were incubated with anti-53BP1 and anti-rabbit conjugated to Alexa Fluor 488 (Jackson Immunoresearch), before being incubated with propidium iodide and RNase A. Cell-cycle analysis was done using FlowJo. Cell lines were compared using a one-tailed, matched Student t test. Error bars represent SEM.

Results

STAG2-mutated glioblastoma cell lines are sensitive to PARP inhibition

To determine whether STAG2 mutation causes PARP inhibitor sensitivity, we used two paired sets of glioblastoma cell lines described by Solomon and colleagues (15): H4 (which has a 25-bp insertion in exon 12 of STAG2) and 42MGBA (which has a nonsense mutation in exon 20 of STAG2), which were each matched with STAG2 KI lines that have these mutations corrected via HR (H4 STAG2 KI and 42MGBA STAG2 KI, respectively). Using these two independent isogenic cell line pairs, we first looked at the proliferation of the H4 and 42MGBA cell lines in the presence of the PARP inhibitor, olaparib, and found that over a range of concentrations, both the H4 and 42MGBA STAG2-mutated cell lines showed significantly decreased cell number when compared with their STAG2 KI counterpart by nuclei-counting (Fig. 1A and B). STAG2-mutated cells treated with olaparib also resulted in fewer colonies compared with similarly treated STAG2 KI cells in clonogenic assays (Fig. 1C; Supplementary Fig. S2A). Finally, when STAG2 was knocked down by short hairpin RNA (shRNA) in HCT116 cells, these cells decreased proliferation in the presence of olaparib similar to the glioblastoma cell lines (Supplementary Fig. S2B and S2C). These results are consistent with our previous findings for siRNA-mediated cohesin knockdown and PARP inhibition (14), and suggest that decreases in cohesin—both the tripartite ring components and the SCC3 ortholog STAG2—sensitize cells to olaparib.
Figure 1. STAG2-mutated cell lines are more sensitive to PARP inhibitors. A, STAG2-mutated and STAG2 KI H4 glioblastoma cell lines were treated with increasing concentrations of olaparib in 96-well format and cell nuclei were counted after 4 days. B, 42MGBA glioblastoma cell lines were treated as in A and cell nuclei were counted after 5 days. C, clonogenic survival of H4 cells after olaparib treatment. The graph represents 3 technical replicates. D and E, STAG2-mutated and KI cell lines were treated with increasing concentrations of the PARP inhibitors veliparib (D) and rucaparib (E) as in A. **, P < 0.005; *, P < 0.01.
The cohesin complex contains one subunit each of SMC1, SMC3, and RAD21, as well as one of either STAG1 or STAG2. Excluding STAG2, we noted no difference in the levels of these proteins in the H4 parental and STAG2 KI cell lines (Supplementary Fig. S1A). Upon immunoprecipitation of RAD21, there was evidence of an increase in STAG1-containing complex in the STAG2-mutated line, suggesting compensation in the cells lacking STAG2 (Supplementary Fig. S1A). Examination of poly ADP-ribose (PAR) levels, a measure of PARP activity, in cells by Western blot analysis showed that PARylation in both the H4 and 42MGBA cell line pairs was high, and this was greatly decreased by treatment with the PARP inhibitor olaparib regardless of STAG2 status (Supplementary Fig. S1B and S1C). We also analyzed the protein levels of the core cohesin components SMC1, SMC3, and RAD21. As expected, the levels of SMC1, SMC3, and RAD21 were equivalent in untreated H4 STAG2-mutated and STAG2 KI cell lysates (Supplementary Fig. S1D). Interestingly, treatment with olaparib caused a decrease in the protein levels of these core components, and this decrease was much more pronounced in the H4 STAG2-mutated cell line. The reason for these low levels is currently not known but suggests that the accessory protein STAG2 stabilizes the cohesin ring components when they are challenged with PARP inhibitor.

To ensure that the loss of survival we observed in STAG2-mutated cells was the result of PARP inhibition and not limited to the PARP inhibitor olaparib, we also treated H4- and 42MGBA-paired cell lines with two other PARP inhibitors: veliparib, an oral inhibitor shown to cross the blood–brain barrier (16), and rucaparib, a potent PARP inhibitors: veliparib, an oral inhibitor shown to cross the blood–brain barrier (16), and rucaparib, a potent PARP inhibitors: veliparib, an oral inhibitor shown to cross the blood–brain barrier (16), and rucaparib, a potent PARP inhibitor. Both of these inhibitors were more effective on STAG2-mutated cells when compared with STAG2 KI cells (Fig. 1D and E; Supplementary Fig. S2D and S2E). From these data, we conclude that mutations in the cohesin component STAG2 render cells more sensitive to PARP inhibition.

**PARP inhibition in STAG2-mutated cells is associated with an accumulation of cells in G2 phase and nuclear abnormalities**

As a more robust proliferation defect was observed in STAG2-mutated cells after treatment with PARP inhibitor, we next sought to determine whether this could be attributed to a specific phase of the cell cycle. Analysis of olaparib-treated H4 cells showed an accumulation of STAG2-mutated cells in the G2–M phase (Fig. 2A and B). Because STAG2-cohesin is involved in cohesion of sister chromatids in both G2 and mitosis up until their segregation, we also used an antibody to histone H3 phosphorylated at S10 (pH3) as a mitotic marker to differentiate between the mitotic and G2 cells. Staining with this marker showed very little difference in the percentage of mitotic cells in treated and untreated cells (Fig. 2A), indicating that STAG2-mutated cells treated with olaparib accumulate in G2.

Similarly, 42MGBA STAG2-mutated cells treated with olaparib also show an accumulation of cells in G2–M (Supplementary Fig. S3A and S3B). These results are consistent with H4 cells and suggest that glioblastoma cell lines treated with PARP inhibitor that are deficient for STAG2 show a more prolonged G2 delay when compared with those that express wild-type STAG2. It should be noted that both H4 and 42MGBA STAG2 KI cell lines show sensitivity and G2–M accumulation at higher concentrations of olaparib. As glioblastoma cell lines in general can have multiple mutations and chromosomal abnormalities, it is possible that these lines contain other defects in addition to STAG2 mutation. However, as growth differences are seen between mutated and KI cells across both H4 and 42MGBA lines, we believe that STAG2 function significantly contributes to PARP inhibitor sensitivity.

Given that we observed a consistent increase in both sub-G1 and >4N cells when STAG2-mutated lines were treated with olaparib (Fig. 2; Supplementary Fig. S3), we next looked for differences in other genome instability and cell death phenotypes. Accordingly, we observed a higher percentage of cells with micronuclei in STAG2-mutated, olaparib-treated cells (Fig. 3A; Supplementary Figs. S4, S5, and S6A). We also observed a higher incidence of chromatin bridges in these cells when compared with STAG2 KI cells (Fig. 3B; Supplementary Fig. S6B). Both of these phenotypes are consistent with these cells having higher genome instability. In addition, both H4 and 42MGBA STAG2–mutated cell lines had a higher fraction of fragmented nuclei when treated with PARP inhibitor (Fig. 3C; Supplementary Fig. S6C), which, along with higher percentages of sub-G1 cells in the flow cytometry profiles, suggests that these cells may be undergoing cell death. As we saw a large percentage of these fragmented nuclei (>12%) in 42MGBA cells treated with olaparib and acknowledged that fragmented nuclei can be a characteristic of apoptosis, we also looked for an increase in the levels of cleaved PARP, an indicator of apoptosis that is downstream of caspase-3/7 activation (19-21), in olaparib-treated cell lines. We did not, however, observe an increase in the levels of cleaved PARP (Supplementary Fig. S1E). Therefore, we believe that the olaparib-mediated cell death is unlikely to be apoptotic.

**PARP inhibitor sensitization is characterized by increased levels of DNA damage**

As PARP inhibitor-treated cells showed a delay in G2 phase and eventual genome instability, we hypothesized that these cells may be responding to increased DNA damage. To further examine this, we stained cells for the DNA damage response protein 53BP1, which rapidly forms foci upon DNA damage, and found that more STAG2-mutated cells have >5 53BP1 foci after olaparib treatment than similarly treated STAG2 KI cells (Fig. 3D; Supplementary Fig. 6D). In fact, at 2.4 μmol/L olaparib, the STAG2-mutated H4 line had an increase of approximately 10% of cells with >5 53BP1 foci over its STAG2 KI counterpart (Fig. 3D). This is remarkably similar to the...
approximate 10% increase in G2 cells seen at the same concentration over the same time period (Fig. 2B). Together, these results suggest that the lower survival rate of STAG2-mutated cells after treatment with PARP inhibitor may be due to increased levels of DNA damage, which leads to accumulation of cells in G2, genome instability, and cell death.

**Combining PARP inhibition with camptothecin or temozolomide is more synergistic in STAG2-mutated glioblastoma cells**

PARP inhibitors have been used extensively to potentiate the toxicity of several chemotherapeutic agents by increasing the DNA damage of these agents (6). Because our paired cell lines showed an increase of 53BP1 foci, a marker for DNA damage, in STAG2-mutated cells, we wanted to determine the synergistic effect of PARP inhibition with DNA-damaging agents in STAG2-mutated and KI cells. To this end, we tested the effect of the topoisomerase I poison camptothecin, which causes DNA lesions in replicating cells, alone and in combination with olaparib. We found that STAG2-mutated H4 cells were more sensitive than H4 STAG2 KI cells to camptothecin even in the absence of olaparib (Supplementary Fig. S7A). In combination with low doses of olaparib, however, cells were sensitized to a much lower dose of camptothecin than when camptothecin was used alone with a large differential seen between STAG2-mutated and STAG2 KI cells (Fig. 4A). Furthermore, 42MGBA cells were significantly more sensitive than 42MGBA STAG2 KI cells when treated with both camptothecin and olaparib (Supplementary Fig. S7B).

We also used a combination of olaparib and temozolomide, an alkylating agent that has previously shown robust synergy with PARP inhibitors and is currently used to treat glioblastomas. These two drugs together
had similar results to those with olaparib and camptothecin, showing increased sensitivity in STAG2-mutated lines compared with STAG2 KI lines (Fig. 4B). As both camptothecin and temozolomide are known to be involved in generating lesions that affect DNA replication and repair, our results suggest that the response to these lesions involves not only PARP activity, but also STAG2.

Discussion

The concept of synthetic lethality holds the promise of chemotherapeutics that specifically target tumor cells for killing. Key to the development of synthetic lethal therapeutics is the identification of synthetic lethal interactions between mutations frequently observed in tumors and small molecule inhibitors. PARP inhibitors are a promising class of small molecules that are currently in multiple clinical trials for cancer. The goal of this study was to determine if mutations in the cohesin complex gene STAG2, which is frequently mutated in several tumor types and is easily assayed using immunohistochemistry, resulted in sensitivity to PARP inhibitors. In this study, we demonstrated that STAG2-mutated glioblastoma cell lines were more sensitive to PARP inhibition than paired cell lines that contained wild-type STAG2. This increase in sensitivity in STAG2-mutated cells was characterized by increased DNA damage, an accumulation of cells in G2 phase, and nuclear abnormalities such as chromatin bridges, micronuclei, and fragmented nuclei. PARP inhibition also increased the sensitivity of STAG2-mutated cells to the topoisomerase poison camptothecin and the DNA alkylating agent temozolomide, suggesting that
PARP inhibitors could be used in combination with DNA-damaging agents to cause STAG2-mutated tumor cell killing.

Cohesin and cohesin-associated genes are frequently mutated in a number of solid tumor types and leukemias (15, 22–25). More specifically, loss of STAG2 expression has been shown by immunohistochemistry to be common in a significant number of solid tumor types, including glioblastoma (19%), Ewing sarcoma (21%), and melanoma (19%; ref. 15). STAG2-truncating mutations have also recently been found in bladder cancer (26–28). A large majority of glioblastoma, melanoma, and Ewing sarcoma tumors had little intratumoral heterogeneity (15), suggesting that sensitivity to PARP inhibitors may be especially relevant in these tumor types.

One explanation for the high frequency loss of STAG2 expression is the location of STAG2 on the X chromosome, meaning that only a single mutation is needed to inactivate it. Furthermore, unlike the core cohesin components SMC1A, SMC3, and RAD21, which are essential for cell survival, somatic cells have a mitotic STAG2 paralog, STAG1, which may share a level of functional redundancy with STAG2. STAG1 can also form a functional cohesin complex, but unlike STAG2, it has not been found to be lost or mutated in glioblastoma lines (15). The presence of STAG1 may explain why truncating mutations and loss of expression of STAG2 are well tolerated in cells. In support of this, we have found that more STAG1 associates with the cohesin complex in STAG2-mutated cells compared with STAG2 KI cells in immunoprecipitation experiments (Supplementary Fig. S1A). We show here, however, that STAG1 is not sufficient for survival in STAG2-mutated cells upon exposure to PARP inhibitors (Fig. 1), and suggest that STAG2 status in tumors may be a marker for PARP inhibitor sensitivity.

The cohesin complex is multifunctional and has a known role in G2 DNA repair that has mainly been attributed to its physical ability to hold sister chromatids in close proximity after replication to allow efficient error-free homology searching before recombination or template switching (10, 29). Consistent with DNA repair function, cohesin components have been found to localize to DNA DSBs in human cells (11, 30). Furthermore, Bauerschmidt and colleagues have demonstrated that depletion of SMC1 impairs the repair of radiation-induced DSBs as measured by the increase in γH2AX and 53BP1 foci in G2 cells (30). Our results show that in the absence of PARP inhibition, STAG2-mutated cells have slightly increased 53BP1 foci compared with STAG2 KI (Fig. 3D; Supplementary Fig. S6D), suggesting that loss of STAG2 results in increased DNA damage. Inhibition of PARP activity increased the 53BP1 foci differential between the STAG2-mutated and KI cells further and also led to the formation of micronuclei and fragmented nuclei (Fig. 3; Supplementary Fig. S6). This suggests that prolonged or less accurate DNA repair in STAG2-mutated cells after PARP inhibitor treatment can result in accumulation of cells in G2 phase, genome instability, or cell death.

Figure 4. Combinations of PARP inhibitor and known chemotherapeutics are more effective in STAG2-mutated H4 cells. Paired H4 cell lines were treated with either camptothecin (CPT, A) and olaparib or temozolomide (TMZ, B) and olaparib and cell number was determined after 4 days using nuclei counting. **, $P < 0.005.$
shown to immunoprecipitate with a PAR antibody after treatment with the DNA alkylating agent, methylnitro-nitrosoguanidine (MNNG), suggesting an interaction of PAR and cohesin under certain conditions (37). Our results and those of others that have been obtained using PARP inhibitors on cells depleted of cohesin components (14, 38, 39) provide additional evidence of a link between cohesin and PARP activity.

Several chemotherapeutic agents, including temozolomide and campothecin, are currently in clinical trials with PARP inhibitors as it has been proposed that combining PARP inhibition with DNA-damaging agents will exacerbate their effects (40). Both temozolomide and topoisomerase poisons like campothecin show increased toxicity in tumors when combined with PARP inhibitors (41–44), and we confirm this synergy in our glioblastoma cell lines (Fig. 4; Supplementary Fig. S7). Several reports have suggested that the potentiation of DNA-damaging agents by PARP inhibition results in an increased need for DSB repair and homologous recombination. For example, combining either temozolomide or campothecin with PARP inhibitors leads to an increase in DSBs (45, 46). Furthermore, resistance to temozolomide and veliparib in HCT116 cells has been attributed, at least in part, to an increase in Rad51-dependent homologous recombination (47). Other reports have shown that sensitization to alkylating agents by PARP inhibitors is enhanced in cells downregulated for homologous recombination and DSB repair pathway components (48, 49). Our results show that STAG2 deficiency is another condition that can further sensitize cells to combinations of PARP inhibitors and DNA-damaging agents. Given its mutation or loss of expression in approximately 20% of glioblastomas as well as several other tumor types (15), STAG2 shows potential as a marker of sensitivity not just to PARP inhibitor monotherapy, but also to combination therapy with camptothecin or temozolomide. Consequently, the status of STAG2 in tumors should be considered as PARP inhibitors move forward in clinical trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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