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Exploring role of 5hmC as potential marker of chemoresistance

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ABSTRACT

Chemoresistance remains to be a common and significant hurdle with all chemotherapies. Tumors gain resistance by acquiring additional mutations. Some of the chemoresistance mechanisms are known and can be tackled. However, the majority of chemoresistance mechanisms are unknown. Our recent findings shed light on one such unknown mechanism. We identified a novel role for 5-hydroxymethycytosine (5hmC), an epigenetic mark on the DNA, in maintaining the integrity of stalled replication forks and its impact on genomic stability and chemoresistance.

Germline mutations in *Breast Cancer 1 (BRCA1)* and *Breast Cancer 2 (BRCA2)* genes are the major causes of hereditary breast cancer.[1] The protein encoded by these genes is known to play an important role in the repair of double-strand breaks (DSB) by homologous recombination (HR). Loss of HR renders BRCA1 and BRCA2-deficient cancer cells sensitive to Poly (ADP-ribose) polymerase inhibitors (PARPi). However, tumors eventually acquire mutations to gain chemoresistance. PARPi resistance in BRCA-deficient cells has been shown by multiple mechanisms. Some of the known mechanisms are associated with the restoration of BRCA functions such as homologous recombination and replication fork protection.[2–5] In our recently published study,[6] we investigated novel regulators of PARPi resistance using genome-wide siRNA screen in mouse embryonic stem cells (mESC). We found the loss of Ten Eleven Translocation 2 (TET2) to contribute to PARPi resistance in mESC expressing mutant BRCA2 as well as in a *Brca2*-deficient mouse mammary tumor cell line. Resultant chemoresistance was due to stalled replication fork (RF) stability, which has been shown to contribute to PARPi resistance.[4] *Tet2* knockdown conferred resistance to multiple PARPi and also to cisplatin in *Brca2*-deficient cells. *BRCA1*-deficient cells also exhibited PARPi resistance upon *TET2* knockdown by abrogating RF degradation.

TET2 is a metabolic enzyme that oxidizes 5-methylcytosine (5mC) to 5-hydroxymethycytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5CaC) during DNA demethylation.[7] Although 5hmC was detected in mammalian cells in 1972, its role as an epigenetic marker remains to be fully understood.[8] Initially, 5hmC was considered to be a demethylation intermediate of the cytosine cycle. Recent studies have shown its role in regulating gene expression, mammalian development.[9] In our study, we uncovered a novel role for 5hmC in maintaining replication fork stability. Using proximity ligation assay (PLA) we detected an increase in 5hmC on stalled replication forks. Our mechanistic studies revealed that the 5hmC-marked replication forks were degraded by base excision repair (BER) associated Apurinic/apyrimidinic endonuclease 1 (APE1). We, therefore, hypothesized that loss of TET2 may reduce 5hmC levels and protect stalled RF from APE1-mediated degradation. Conversely, treatment with Vitamin C, a known cofactor for TET2 or overexpression of TET2 may enhance global 5hmC levels and lead to APE1-dependent RF degradation. Indeed, vitamin C treatment enhanced fork degradation in BRCA2-deficient cells. Notably, TET2 overexpression increased global 5hmC levels significantly enough to cause RF degradation even in BRCA2-proficient cells. We showed that TET2-overexpression can increase genomic instability in U2OS cells. We observed an increase in radial structures in metaphase spreads of TET2 overexpressing U2OS cells, likely due to increased replicative stress.

Since its recognition as an epigenetic mark, the role of 5hmC has been established in regulating gene expression. Although we did not observe any changes in expression of genes associated with RF stability in *Tet2* knockdown mESC and mouse mammary tumor cells, we cannot completely rule out the indirect effects of TET2 knockdown on gene expression and its impact on RF stability. Mass spectrometric quantitation suggested a global increase in 5hmC levels and PLA detected higher 5hmC levels on replication fork in *BRCA2* deficient cells, possibly due to higher intrinsic DNA damage in cells. Inhibition of APE1 resulted in partial rescue of RF degradation in BRCA2-deficient cells. This provided direct evidence to support a physiological role played by APE1 in stalled RF degradation when 5hmC levels are high. Surprisingly, when BRCA2-deficient cells were treated with Vitamin C, APE1 inhibition completely protected RF degradation but MRE11 nuclease inhibition did not. We hypothesize that the affinity of MRE11 may be reduced to RF with elevated 5hmC levels. It is also possible that BER-associated proteins may prevent MRE11 localization to stalled RF in BRCA2-deficient cells. Future studies will be focused on assessing the binding affinity of MRE11 to DNA substrates with varying 5hmC levels.
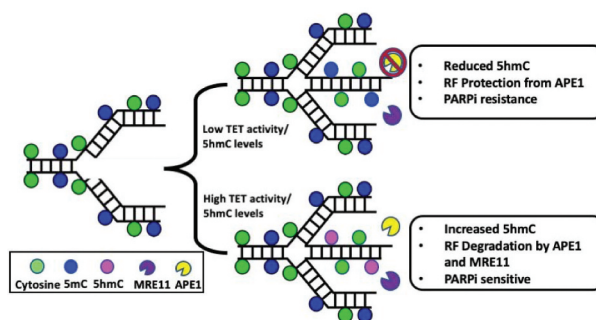

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Figure 1. Impact of 5hmC levels on the fate of DNA replication forks. MRE11 is the primary nuclease, while Apurinic/apyrimidinic endonuclease 1 (APE1) is the secondary nuclease responsible for replication fork (RF) degradation in *BRCA2*-deficient cells. TET proteins oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). Reduction in 5hmC levels by either *TET* knockdown or low activity prevents APE1-mediated RF degradation results in Poly (ADP-ribose) polymerase inhibitor (PARPi) resistance. Conversely, increasing 5hmC levels on RF by Vitamin C treatment augments RF degradation by APE1 endonuclease rendering cells sensitive to PARPi.

Our recent study clearly demonstrated that APE1 endonuclease degrades stalled RF in a 5hmC dependent manner. 5hmC is further oxidized by TET proteins to 5fC and 5caC and these are processed by glycosylases leading to the generation of abasic sites on the DNA. These abasic sites are substrates for APE1 endonuclease. Since the loss of TET2 reduces 5hmC levels, we hypothesize that this will also reduce the number of abasic sites on the DNA, including stalled forks in BRCA-deficient cells. Fewer abasic sites may render stalled forks less susceptible to APE1-mediated degradation, which can contribute to PARPi resistance. Our findings are supported by a recent study that found Thymine DNA glycosylase (TDG) loss to confer PARPi resistance. PARP trapping mechanism of chemoresistance was ruled out but the exact mechanism was not elucidated in this study.[10] We speculate that loss of TDG prevents the generation of abasic sites thus rendering RF immune to degradation by APE1.

In conclusion, our findings suggest that the stability of RF is dependent on the levels of 5hmC on nascent strand (Figure 1). 5hmC production gets stimulated in response to DNA damage.[11] Thus, any tumor with elevated intrinsic DNA damage such as *BRCA1/BRCA2* mutated tumors should have higher 5hmC levels. Our findings can be exploited for therapeutic purposes. We propose that treating PARPi resistant BRCA-deficient cells, that are defective in HR but have acquired resistance due to RF stability, treatment with Vitamin C may enhance RF degradation and re-sensitize the cells to PARPi. Also, 5hmC levels are greatly reduced in tumor samples in comparison to surrounding tissue, because tumor microenvironment exhibits hypoxic conditions which diminishes TET activity.[12] Thus, low 5hmC levels in the tumors can be associated with chemoresistance. If such a distinct variation in 5hmC levels exists, then tumors can be categorized based on 5hmC levels. 5hmC levels can be tested in tumor biopsies from patients and suitable treatment option can be made available. If Vitamin C-mediated re-sensitization of BRCA-deficient cells to PARPi is successful, it can be used as an adjuvant to PARPi therapy in other HR defective tumors.

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