N⁶-Methylation of Adenosine of FZD10 mRNA Contributes to PARP Inhibitor Resistance

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Abstract

Despite the high initial response rates to PARP inhibitors (PARPi) in BRCA-mutated epithelial ovarian cancers (EOC), PARPi resistance remains a major challenge. Chemical modifications of RNAs have emerged as a new layer of epigenetic gene regulation. N⁶-methyladenosine (m⁶A) is the most abundant chemical modification of mRNA, yet the role of m⁶A modification in PARPi resistance has not been previously explored. Here, we show that m⁶A modification of FZD10 mRNA contributes to PARPi resistance by upregulating the Wnt/β-catenin pathway in BRCA-mutated EOC cells. Global m⁶A profile revealed a significant increase in m⁶A modification in FZD10 mRNA, which correlated with increased FZD10 mRNA stability and an upregulation of the Wnt/β-catenin pathway. Depletion of FZD10 or inhibition of the Wnt/β-catenin sensitizes resistant cells to PARPi. Mechanistically, down-regulation of m⁶A demethylases FTO and ALKBH5 was sufficient to increase FZD10 mRNA m⁶A modification and reduce PARPi sensitivity, which correlated with an increase in homologous recombination activity. Moreover, combined inhibition of PARP and Wnt/β-catenin showed synergistic suppression of PARPi-resistant cells in vitro and in vivo in a xenograft EOC mouse model. Overall, our results show that m⁶A contributes to PARPi resistance in BRCA-deficient EOC cells by upregulating the Wnt/β-catenin pathway via stabilization of FZD10. They also suggest that inhibition of the Wnt/β-catenin pathway represents a potential strategy to overcome PARPi resistance.

Significance: These findings elucidate a novel regulatory mechanism of PARPi resistance in EOC by showing that m⁶A modification of FZD10 mRNA contributes to PARPi resistance in BRCA-deficient EOC cells via upregulation of Wnt/β-catenin pathway.

Introduction

PARP inhibitors (PARPi) are synthetically lethal in cells with a dysfunctional homologous recombination (HR) pathway such as those with BRCA1/2 mutations (1). PARPi inhibitors such as olaparib have been approved by the FDA for treating BRCA1/2-mutated epithelial ovarian cancer (EOC) with substantial clinical benefits (1, 2). However, the mechanisms of resistance to PARPi remain to be fully elucidated.

Wnt signaling is initiated by binding of the Wnt ligand to its cognate frizzled receptor (3, 4). A key feature of the canonical Wnt signaling is stabilization of the downstream effector β-catenin. β-Catenin translocation to nuclei promotes the expression of β-catenin target genes such as CCND1 and FOSL1 through part-

Materials and Methods

Cell lines, culture conditions, and transfection

The ovarian cancer cell line PEO1 was cultured in RPMI1640 (Corning Life Sciences) supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin/streptomycin at 37°C supplied with 5% CO₂. PARPi-resistant PEO1 cells were published...
previously (15) and were developed by a continuous stepwise exposure to increasing concentration of the PARPi olaparib. The resistant PE01 cells were maintained and passaged in 5 μmol/L olaparib. The ovarian cancer cell line UWB1.289 was cultured in 1:1 RPMI1640/Mammary Epithelial Growth Medium (Lonza, catalog no. CC-3150) supplemented with 3% FBS at 37°C supplied with 5% CO₂. Viral packing cell 293FT was cultured in DMEM with 10% FBS and 1% penicillin/streptomycin at 37°C supplied with 5% CO₂. Cell lines were obtained from ATCC and were reauthenticated by The Wistar Institute Genomics Facility at the end of experiments using short tandem repeat profiling using AmpFLSTR Identifier PCR Amplification Kit (Life Technologies).

Mycoplasma testing was performed using LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich) every month. Transfection was performed using Lipofectamine 2000 (Life Technologies) on the QuantStudio 3 Real-Time PCR System (Life Technologies) on the QuantStudio 3 Real-Time PCR System (Life Technologies).

Reagents and antibodies

Olaparib (catalog no: S1060), nucaparib (catalog no: S1098), niraparib (catalog no: S2741), and XAV939 (catalog no: S1180) were purchased from Selleckchem. Pyrinium pamoate (catalog no: 1592001) was purchased from Sigma-Aldrich. The following antibodies were obtained from the indicated suppliers: anti-β-actin (Sigma-Aldrich, catalog no. A5441, 1:10,000), anti-Vinculin (Santa Cruz Biotechnology, catalog no. sc-25336, 1:1000), anti-GAPDH (Millipore, catalog no. MAB374, 1:10,000), anti-Ki67 (Cell Signaling Technology, catalog no. 9449, 1:500), anti-cleaved caspase-3 (Cell Signaling Technology, catalog no. 9661, 1:1,000 for Western blot and 1:50 for IHC), anti-cleaved PARP p85 (Promega, catalog no. G7341, 1:1000), anti-FZD10 (Santa Cruz Biotechnology, catalog no. sc-33510, 1:2000), anti-BRCA1 (Millipore, catalog no. 07-434, 1:10,000), and anti-BRCA2 (Bethyl Laboratories, catalog no. A303-434A, 1:1000), anti-β-catenin (BD Biosciences, catalog no. 610155, 1:1,000), anti-α-HA (Cell Signaling Technology, catalog no. 2367, 1:1,000), and anti-v5 (Thermo Fisher Scientific, catalog no. R960-25, 1:1,000).

Immunoblotting

Protein was isolated with RIPA buffer [50 mmol/L Tris pH 8.0, 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 1 mmol/L phenylmethylsulfonylfluoride (PMSF)]. Protein concentration was measured using Bradford assay. Protein was separated on a SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore). Membranes were blocked with 5% nonfat milk (Bio-Rad) in TBS/0.1% Tween 20 (TBST), and then incubated sequentially with primary and secondary antibodies.

Chromatin fractionation was performed as described previously (16). Briefly, cells were washed once by PBS, trypsinized, and centrifuged at 1,000 rpm. The pellets were resuspended in 300 μL buffer A: 10 mmol/L HEPES-KOH, pH 8.0, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.34 mol/L sucrose, and 10% glycerol, pH 7.5, plus the EDTA-free Protease Inhibitor Cocktail (Roche), 1 mmol/L DTT, 0.1 mmol/L PMSF, 0.1% Triton X-100. Cells were incubated on ice for 5 minutes and pelleted at 1,300 g for 4 minutes at 4°C. The supernatant was obtained as the cytoplasmic fraction. The pellet was washed once in buffer A and then resuspended in 300 μL buffer B: 3 mmol/L EDTA, pH 8.0, and 0.2 mmol/L EGTA, pH 8.0, plus the EDTA-free Protease Inhibitor Cocktail, 1 mmol/L DTT, and 0.1 mmol/L PMSF. Samples were incubated on ice for 30 minutes and then centrifuged at 1,700 g for 4 minutes at 4°C. The supernatant was obtained as the nuclear fraction and resuspended in 1 × sample buffer.

Cycloheximide chase analysis

Cycloheximide (5 μmol/L) was added to cells, and then the cells were collected at 0, 4, 8, 16, and 24 hours posttreatment. The protein levels of FZD10 were examined by immunoblotting.

Lentivirus packaging and infection

Lentivirus was packaged using the Virapower Kit from Invitrogen according to the manufacturer's instructions as described previously (17). HEK293FT cells were transfected by Lipofectamine 2000 (Life Technologies). Lentivirus was harvested 48 hours posttransfection. Cells infected with viruses encoding the puromycin-resistant gene were selected using 1 μg/mL puromycin or 10 μg/mL blasticidin. pLKO.1-shFZD10 (1, TRCN0000008315; 2, TRCN0000008316), pLKO.1-shALKBH5 (1, TRCN0000064783; 2, TRCN0000064787), pLKO.1-shHuR (1, TRCN000017273; 2, TRCN000017274), pLKO.1-shGFZBP2 (1, TRCN0000149224; 2, TRCN0000148718) were obtained from the Molecular Screening Facility at The Wistar Institute (Philadelphia, PA). pLKO.1-shFTO (TRCN0000246250) and pLKO.1-shYTHDF2 (1, TRCN00001254410; 2, TRCN00001254411) were purchased from Sigma-Aldrich. For FTO and ALKBH5 expression vector, pLX304-FTO (clone ID# csebBroad304_15979) was obtained from the Molecular Screening Facility at The Wistar Institute (Philadelphia, PA). The pLenti-ALKBH5 (clone ID# NM_017758) was purchased from Applied Biological Materials Inc.

Quantitative RT-PCR

RNA was extracted using TRIzol (Invitrogen) according to manufacturer's instruction, and then DNase treatment (RNaseasy columns by Qiagen) was performed. RNA expression was determined using the Taq Universal SYBR Green One-step kit (Bio-Rad Laboratories) on the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). The primers sequences are as follow:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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</thead>
<tbody>
<tr>
<td>FZD10</td>
<td>5'-CCTCACGCAGCTTCTTCATA-3'</td>
<td>5'-CCTCCCCTGGTTGTTTGCGG-3'</td>
</tr>
<tr>
<td>ALKBH5</td>
<td>5'-GTCATCCTCACTTTCCTCTC-3'</td>
<td>5'-GTCGCTGTTGTTGCGGATGACTT-3'</td>
</tr>
<tr>
<td>YTHDF2</td>
<td>5'-CCTCATTGGTGTTGTTGCGG-3'</td>
<td>5'-CCTCATTGGTGTTGTTGCGG-3'</td>
</tr>
<tr>
<td>BRAF</td>
<td>5'-GTCGCTGTTGTTGCGGATGACTT-3'</td>
<td>5'-GTCGCTGTTGTTGCGGATGACTT-3'</td>
</tr>
<tr>
<td>B2M</td>
<td>5'-CCTCATTGGTGTTGTTGCGG-3'</td>
<td>5'-CCTCATTGGTGTTGTTGCGG-3'</td>
</tr>
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m6A Modification Regulates PARP Inhibitor Resistance

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or 18s were used as an internal control. Each sample was run in triplicate.

m\(^6\)A immunoprecipitation and sequencing, and measurement of total m\(^6\)A

Cells were harvested at approximately 80% confluence. Total RNA was extracted and purified using miRNeasy Midi Kit (Qiagen, catalog no. 75142). Purified total RNA was fragmented in freshly prepared RNA fragmentation buffer (10 mmol/L Tris-HCl, pH 7.0, 10 mmol/L ZnCl\(_2\)). The fragmented RNA was validated by RNA electrophoresis and 5 μg of fragmented RNA of each sample was preserved as input control for RNA-seq. A total of 250 μg of fragmented RNA was subjected to m\(^6\)A immunoprecipitation using EpiMark N6-Methyladenosine Enrichment Kit (NEB, catalog no. E1610S) following manufacturer’s instructions. Briefly, N6-Methyladenosine antibody was coupled with protein G bead in reaction buffer for 30 minutes at 4°C, then fragmented RNA was incubated with the beads for 1 hour at 4°C. After incubation, the supernatant was discarded and the beads were washed twice in low salt reaction buffer and then washed twice in high salt reaction buffer. During this step, the RNA containing the m\(^6\)A modification will remain on beads. Enriched m\(^6\)A RNA was eluted with RT buffer (Qiagen, catalog no. 75142) and concentrated by Dynabeads MyOne Silane (Life Technologies, catalog no. 37002D). RNA was finally eluted with nuclease-free water and used for cDNA library preparation.

Library preparation and next-generation sequencing were performed by The Wistar Institute Genomics Facility. Libraries for RNA-seq were prepared using ScriptSeq complete Gold kit (Epicentre) following the manufacturer’s instructions. Libraries were pooled in equimolar concentration and then subjected to a 75-bp paired-end sequencing run on NextSeq 500, using Illumina’s NextSeq 500 middle output sequencing kit. RNA-seq data were deposited in GEO database (accession number: GSE119963).

For total m\(^6\)A measurement, total RNA was extracted from cells and m\(^6\)A content was measured by using EpiQuik m\(^6\)A RNA Methylation Quantification Kit (Epigentek, catalog no. P-9003) according to the manufacturer’s instructions.

Bioinformatics analysis

Raw sequencing data was aligned using bowtie2 against hg19 version of the human genome and RSEM v1.2.12 software was used to estimate raw read counts and RPKM using Ensemble transcriptome. EdgeR (18) was used to estimate significance of differential expression between input RNA samples. Gene expression changes were considered significant if passed FDR < 5% thresholds. HOMER was used to generate bigwig files and call m\(^6\)A peaks (FDR < 5%, at least 4-fold) in resistant versus parental cell lines and perform motif analysis. Most enriched consensus motif (ATGACGCTK, 73% of all FDR < 5% peaks) was used for additional peak filtering. Only peaks that passed FDR < 1% threshold and contained the motif were considered significant and used for further analysis. m\(^6\)A signal fold difference in resistant versus parental cell lines was corrected by the difference in input RNA levels to account for overall gene expression changes effect on m\(^6\)A signal. Genes that were significantly upregulated at least 2-fold and had a significantly higher m\(^6\)A peak of at least 2-fold in resistant versus parental cells in 5’ or 3’ UTR were reported. Normalized average signal around genes transcription start site and 3’ UTR were derived from bigwig files using bigWigAverageOverBed tool from UCSC toolbox (19) with mean0 option using 20-bp bins.

Colonies formation assay

Colonies formation was performed as described previously (17). Briefly, cells were cultured in 12-well, 24-well, or 96-well plates with different seeding number according to the growth rate. Medium was changed every 3 days with appropriate drug doses for 12 days or until control wells became confluent. Colonies were washed twice with PBS and fixed with 10% methanol and 10% acetic acid in distilled water. Fixed colonies were stained with 0.05% crystal violet. Analysis was performed using NIH ImageJ software.

Flow cytometry

The Annexin V FITC and PI Kit (Thermo Fisher Scientific, catalog no. V13242) was used following the manufacturer’s instructions. Briefly, cells were washed by PBS, trypsinized, and suspended in annexin V binding buffer. Cells were stained with annexin V and PI for 15 minutes and then analyzed. Analysis was performed using FlowJo version 7 software module.

For cell-cycle analysis, the cells were fixed, treated with bovine RNase, and then stained with propidium iodide (Sigma-Aldrich) for 15 minutes at room temperature. Samples were subjected to analysis using the Becton Dickinson LSR18 machine and FlowJo version 7 software module.

Wnt/β-catenin TCF/LEF reporter assay

The TOP FLASH reporter plasmid and LSV40 Renilla plasmid were cotransfected into cells using Lipofectamine 3000. After 48 hours, luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega) and Victor X3 2030 Multilabel Reader (Perkin Elmer) according to the manufacturer’s instructions. Data were normalized based upon control Renilla luciferase activity. Each group was repeated four times.

Dual HR and nonhomologous end-joining reporter assay

The HR and nonhomologous end-joining (HR/NHEJ) assay was performed as described previously (20). Briefly, cells were plated in 6-well plates at a 50% confluence 1 day before transfection. Cells were transfected with 500 ng of pLCN-double-strand break (DSB) Repair Reporter DNA damage response (DDR), 500 ng of pCAGGS DRR mCherry Donor EF1 BFP plasmid, and 500 ng of pCABacel plasmid with Lipofectamine 2000. Analysis was performed seventy-two hours after transfection. pCABacel plasmid or pCAGGS DRR mCherry Donor EF1 BFP plasmid alone were used as a control. The following plasmids were used: pLCN-DSB Repair Reporter (DDR; Addgene catalog no.: 98895) pCAGGS DRR mCherry Donor EF1a BFP (Addgene catalog no.: 98896), and pCABacel (Addgene catalog no.: 26477).

Xenograft PARPi-resistant ovarian cancer mouse models

The protocols were approved by the Wistar Institutional Animal Care and Use Committee (IACUC). A total of 2 × 10^6 PARPi-resistant PEO1 cells were suspended in 200-μL PBS:Matrigel (1:1) and unilaterally injected subcutaneously into the right dorsal flank of 6- to 8-week-old female immunocompromised NOD/SCID gamma (NSG) mice. When the average tumor size reached approximately 100 mm³, the mice were then randomized into four groups and treated with vehicle control, olaparib (50 mg/kg), XAV939 (5 mg/kg), or a combination daily for...
18 days. The indicated doses were determined on the basis of previous studies (15, 21). Olaparib was suspended in 10% 2-hydroxypropyl-β-cyclodextrin solvent (Sigma-Aldrich), and XAV939 was suspended in 4% DMSO/96% Corn oil (Sigma-Aldrich). Tumor size was measured three times a week. Tumor size was evaluated using the formula: tumor size (mm$^3$) = $\frac{d^2 \times D}{2}$, where $d$ and $D$ are the shortest and the largest diameter. At the end of the experiments, tumors were surgically dissected, and the weight of tumors were measured as a surrogate for tumor burden or followed for the survival of tumor-bearing mice. The Wistar IACUC guideline was followed in determining the time for ending the survival experiments (tumor burden exceeds 10% of body weight).

IHC staining

IHC staining was performed as described previously (15, 17) on consecutive sections from xenografted tumors dissected from control or treated immunocompromised NSG female mice, using Dako EnVision+ system following the manufacturer’s instructions. Expression of the stained markers was scored using a histologic score (H-score).

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 7 (GraphPad) for Mac OS. Experiments were repeated three times unless otherwise stated, and the representative results were shown. Quantitative data are expressed as mean ± SEM. For IC$_{50}$ differences Z-test with 95% confidence intervals were used. A two-tailed t test was used to identify significant differences in comparisons unless otherwise stated. Combination index (CI) was analyzed by Compsyn software. CI value indicate: <1 synergism, = 1 additive effect, and >1 antagonism. For all statistical analyses, the level of significance was set at 0.05.

Data availability

All sequencing data have been deposited in the Gene Expression Omnibus (GEO) under accession GSE119963.

Results

m$^A$-modified FZD10 is upregulated in PARPi-resistant cells

To identify transcripts regulated by m$^A$ in PARPi resistance, we profiled m$^A$ distribution at the transcriptome level in...
BRCA2-mutated parental and PARPi-resistant ovarian cancer PEO1 cells (15) by RNA immunoprecipitation followed sequencing using an anti-m^6^A antibody (Supplementary Fig. S1A–S1D). Consistent with previous reports (7–10), m^6^A modification was enriched in both 3' and 5' UTR regions at the transcriptome level (Fig. 1A). However, the overall m^6^A pattern in the transcriptomes was not significantly different between parental and resistant cells (Fig. 1A). Likewise, there was no significant difference in total m^6^A-modified mRNA between parental and resistant cells (Supplementary Fig. S1E).

We next analyzed the genes that were differentially modified by m^6^A between parental and resistant cells. The top gene whose m^6^A modification was increased in PARPi-resistant cells was FZD10 (Fig. 1A). m^6^A levels at the 3' UTR region of the FZD10 mRNA was significantly increased in resistant cells (Fig. 1B). The increase in m^6^A-modified FZD10 was confirmed by anti-m^6^A immunoprecipitation and quantitative (q)RT-PCR analysis of the m^6^A-immunoprecipitated RNAs (Fig. 1B). The increase in m^6^A-modified FZD10 correlated with an increase in FZD10 mRNA in resistant cells (Fig. 1C). These results suggest that m^6^A modification may stabilize FZD10 mRNA. Consistently, we observed an increase in FZD10 protein in resistant cells (Fig. 1C). Indeed, RNA stability analysis revealed that FZD10 mRNA is significantly stabilized in resistant cells (Fig. 1D). However, there is no significant difference in FZD10 protein stability between parental and resistant PEO1 cells (Supplementary Fig. S1F and S1G). Together, we conclude that m^6^A modification is increased in the 3' UTR region of the FZD10 gene when the BRCA2-mutated PEO1 cells developed resistance to PARPis.

**Inhibition of FZD10 and Wnt signaling overcomes PARPi resistance**

Given that FZD10 is a receptor in the canonical Wnt/β-catenin signaling (4), we next determined whether the Wnt/β-catenin signaling is altered in PARPi-resistant cells. Notably, nuclear β-catenin levels were increased in PARPi-resistant cells and this correlates with an increase in the expression of the Wnt/β-catenin target genes in these cells (Fig. 2A; Supplementary Fig. S2A and S2B). Consistently, the TCF/LEF reporter activity was higher in resistant cells (Fig. 2A). To determine the role of the upregulated FZD10 in PARPi sensitivity in the resistant cells, FZD10 was knocked down in PARPi-resistant cells (Fig. 2B). FZD10 knockdown decreased the Wnt/β-catenin target gene expression in PARPi-resistant cells (Supplementary Fig. S2C and S2D). This correlated with a decrease in the IC50 of two different PARPis olaparib and rucaparib in FZD10 knockdown cells (Fig. 2B).
Supplementary Fig. S2E). Consistently, inhibitors of the Wnt/β-catenin signaling, namely XAV939 (22) and pyrvinium pamoate (23), were more effective in suppressing the growth of PARPi-resistant cells (Fig. 2C).

We next explored changes in HR and activities, two alternating DNA DSB repairing pathways using a dual HR and NHEJ reporter assay (Fig. 2D; ref. 20). Consistent with previous reports (1), we observed a significant increase in HR activity in PARPi-resistant cells (Supplementary Fig. S2F). As a control, NHEJ activity was not significantly affected in PARPi-resistant cells (Supplementary Fig. S2G). However, the observed increase in HR activity was not due to an increase in S-phase of the cell cycle and FZD10 knockdown did not significantly affect S-phase of the cell cycle in the resistant cells (Supplementary Fig. S2H and S2I). Notably, FZD10 knockdown significantly decreased HR activity without affecting NHEJ activity (Fig. 2D; Supplementary Fig. S2J). Together, we conclude that inhibition of FZD10 suppresses the Wnt/β-catenin signaling and sensitizes the PARPi-resistant cells to PARPi.

**Downregulation of FTO and ALKBH5 contributes to FZD10 mRNA upregulation**

Because FZD10 m^6^A upregulation contributes to FZD10 upregulation, we next determined the changes in expression of m^6^A methyltransferase and demethylase in parental and resistant cells. Notably, both m^6^A demethylases FTO and ALKBH5 are downregulated in resistant cells compared with parental cells (Fig. 3A). As a control, the expression of m^6^A methyltransferase METTLE14 and METTLE3 expression was downregulated in resistant cells (Supplementary Fig. S3A). Given FZD10 m^6^A modification was increased in resistant cells, we determined whether downregulation of FTO and/or ALKBH5 is sufficient to upregulate FZD10 expression (Supplementary Fig. S3B). Indeed, knockdown of either ALKBH5 or FTO in PEO1 parental cells increased the expression of FZD10 and a combination of ALKBH5 and FTO knockdown further increased FZD10 expression (Fig. 3B). This suggests that downregulation of both ALKBH5 and FTO contributes to FZD10 upregulation. Similar upregulation of FZD10 by knockdown of ALKBH5 and FTO was also obtained in BRCA1-mutated UWB1.289 ovarian cancer cells (Supplementary Fig. S3C–S3E), suggesting that the observed effects are not a cell line-specific effect. Notably, the observed FZD10 upregulation correlates with an increase in Wnt target gene expression and an increase HR activity without affecting NHEJ activity (Fig. 3C; Supplementary Fig. S3F–S3H), which was accompanied by a decrease in sensitivity to olaparib (Fig. 3D). Conversely, ectopic expression of ALKBH5 and FTO in the resistant cells increased olaparib sensitivity (Supplementary Fig. S3I–J). We next determined the expression of the m^6^A readers namely HuR, IGF2BP2, and YTHDF2 (11–13) in parental and resistant cells. Notably, expression of both YTHDF2 and HuR was decreased in the resistant cells, while IGF2BP2 expression was increased in the resistant cells (Supplementary Fig. S3K). In addition, knockdown of any of the readers decreased FZD10 expression (Supplementary Fig. S3L–S3N).
Given that \textit{YTHDF2} promotes the decay while \textit{HuR} and \textit{IGF2BP2} stabilize the m\textsuperscript{6}A-modified mRNA \cite{11-13}, these results suggest that \textit{IGF2BP2} plays a role downstream of the m\textsuperscript{6}A-modified \textit{FZD10} mRNA. Together, we conclude that downregulation of m\textsuperscript{6}A demethylases \textit{ALKBH5} and \textit{FTO} contributes to \textit{FZD10} upregulation in PARPi-resistant cells.

**Wnt signaling inhibitor XAV939 and olaparib are synergistic in suppressing PARPi-resistant cells in vitro and in vivo**

We next determined that olaparib and XAV939 were synergistic in suppressing the growth of PARPi resistant cells (Fig. 4A). In contrast, there was no synergy between olaparib and XAV939 in parental cells (Fig. 4A). Notably, XAV939 alone induced apoptosis of PARPi-resistant cells as evidenced by an increase in Annexin V-positive cells and an upregulation of cleaved PARP p85 and cleaved caspase-3 (Fig. 4B). As a control, olaparib alone did not affect markers of apoptosis in PARPi-resistant cells. However, a combination of XAV939 and olaparib significantly increased markers of apoptosis compared with XAV939 alone (Fig. 4B).

This is consistent with the observed synergy between olaparib and XAV939 in suppressing the growth of PARPi-resistant cells (Fig. 4A). In contrast, XAV939 did not significantly increase apoptosis induced by olaparib in parental PEO1 cells (Supplementary Fig. S4A and S4B).

We next transplanted PARPi-resistant PEO1 cells into NSG mice. When the average xenograft tumor size reached approximately 100 mm\textsuperscript{3}, mice were randomized into four groups with 12 mice in each of the groups for the following treatments: vehicle control, olaparib, XAV939, and a combination of olaparib and XAV939. After 18 days of treatment, half of the mice from each of the groups were euthanized (Fig. 4C). Compared with vehicle controls, XAV939 significantly suppressed the growth of the xenografted PARPi-resistant tumors (Fig. 4C). Notably, a combination of olaparib and XAV939 exhibited significantly greater...
tumor-suppressive effect as compared with either XAV939 or olaparib alone (Fig. 4C, Supplementary Fig. S4C). The doses of olaparib and XAV939 used in this study did not significantly affect the body weight of treated mice (Supplementary Fig. S4D), suggesting that effective combination doses can be achieved without added toxicity. XAV939 alone significantly improved the survival of mice bearing PARPi-resistant tumors (Fig. 4C). However, the survival of mice treated with a combination of XAV939 and olaparib was significantly longer than those treated with XAV939 alone (Fig. 4C).

We next performed IHC staining for Ki67, a cell proliferation marker, and cleaved caspase-3, an apoptosis marker, on the consecutive sections of the dissected tumors (Fig. 4D). Both XAV939 alone and a combination of XAV939 and olaparib significantly decreased Ki67 expression and increased cleaved caspase-3 expression (Fig. 4D). In addition, XAV939 alone or in combination with olaparib significantly decreased the expression of the Wnt/β-catenin target genes such as FOSL1 and CCND1 (Fig. 4D). Taken together, we conclude that XAV939 synergizes with olaparib in suppressing the growth of PARPi-resistant tumors. This activity correlates with a decrease in cell proliferation and an increase in apoptosis, accompanied by downregulation in Wnt/β-catenin signaling by XAV939.

Discussion

Here, we show that downregulation of m6A erasers FTO and ALKBH5 contribute to PARPi resistance by increasing m6A modification in FZD10 mRNA to upregulate Wnt signaling. This study focuses on FZD10 regulated Wnt signaling due to the fact that FZD10 showed the highest degree of increase in m6A modification. However, it is possible that FTO and ALKBH5 may also contribute to PARPi resistance through regulating m6A modification in genes implicated in Wnt-independent pathways.

Restoration of HR activity by mechanisms such as genetic reversion of truncating mutations (1, 24) contributes to PARPi resistance in BRCA1/2-mutated cancer cells. Here we show that knockdown of FZD10 decreases HR activity in PARPi-resistant cells while upregulation of FZD10 increases HR activity in parental cells. Thus, FZD10-regulated Wnt pathway contributes to an increase in HR activity. These results suggest that Wnt signaling is a novel regulator of HR activity. This is consistent with a preprint finding that Wnt signaling upregulates HR activity (25). Interestingly, the observed upregulation of HR activity is independent of BRCA2 genetic reversion (25).

In summary, our findings revealed that m6A modification represents a novel mechanism of PARPi resistance. In addition, we showed that upregulation of Wnt signaling, at least in part, contributes to m6A modification–mediated PARPi resistance. Our results suggest that inhibition of the Wnt/β-catenin pathway represents a potential strategy to overcome PARPi resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: T. Fukumoto, H. Zhu, R. Zhang


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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Fukumoto, H. Zhu, S. Karakashev, N. Fatkhutdinov, P. Liu, A.V. Kossenkov, L.C. Showe, L. Zhang, R. Zhang

Writing, review, and/or revision of the manuscript: T. Fukumoto, N. Fatkhutdinov, A.V. Kossenkov, L.C. Showe, S. Jean, R. Zhang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Zhu, A.V. Kossenkov

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