Investigating potential indicators of replication stress as biomarkers to stratify patients for ATRi sensitivity

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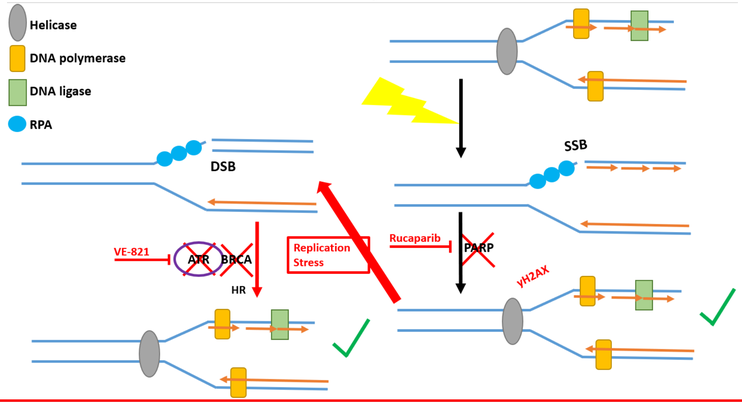
INTRODUCTION

- The DNA damage response (DDR) is a network of proteins responsible for sensing DNA damage and signaling this to cell cycle checkpoints and DNA repair pathways.
- Ataxia telangiectasia and Rad3-related (ATR) is a key kinase in the DDR, responsible for sensing replication stress (RS) and signaling this to the S and G2/M checkpoints, and DNA repair pathways such as homologous recombination repair (HRR).
- Replication stress is high in cancer cells driven by the urge to proliferate due to loss of G1 checkpoint control and over-expression of oncogenes, making them highly dependent on their S and G2/M checkpoints and therefore ATR.
- In addition anticancer agents such as those that block the repair of endogenous damage (e.g. PARP inhibitor, rucaparib) increase replication stress resulting in the conversation of single strand breaks (SSB) in the DNA into double strand breaks (DSB).
- ATR inhibitors (ATRi) such as VE-821 are therefore likely to be effective in cancers with high levels of replication stress, however a biomarker to identify these cancers has not yet been developed.
- RPA is a protein which binds to sections of single stranded DNA, yH2AX is a marker of DSB, and TIAR granules have all been shown to be markers of replication stress.

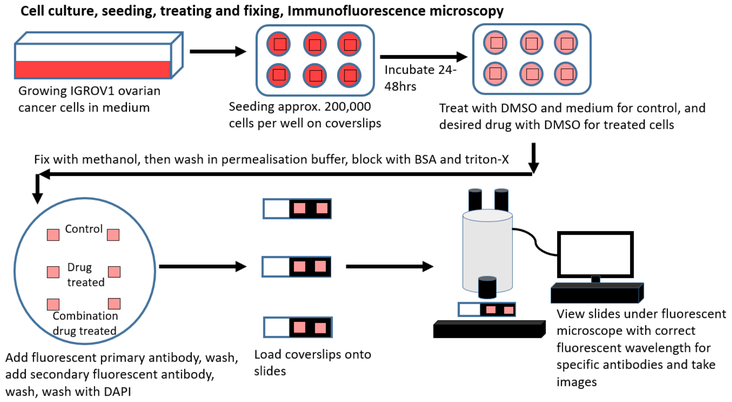


PROJECT AIMS

1. Optimise antibodies against TIAR, pHH3 (as a marker of cells in mitosis), RPA, pRPA and yH2AX
2. To see if RPA or pRPA and yH2AX can be used as markers of replication stress
3. To see pHH3 positive cells containing TIAR granules can be used an indicator of replication stress

METHODS

- The potential of antibodies against pHH3, TIAR, RPA and yH2AX was assessed using fluorescent microscopy in IGROV1 cancer cells

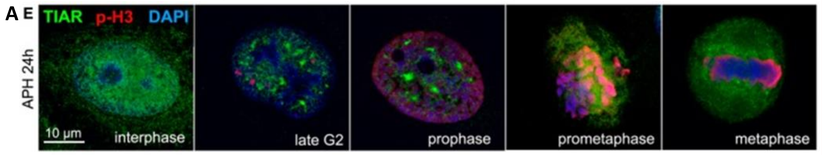


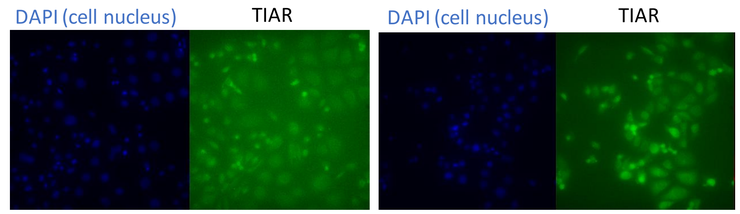
- Where the TIAR antibody could not be effectively optimized for immuno-fluorescent detection via microscopy, western blotting was performed to look at the levels of TIAR protein present.

RESULTS I

Optimisation of TIAR:

- It had been suggested(1) that a TIAR antibody can show TIAR granules which can work to be a potential indicator of replication stress.
- However the antibody used in this paper has been discontinued, so a different antibody against TIAR was used
- Despite optimisation efforts, the new TIAR antibody was ineffective, and TIAR granules were not visualised by immuno-fluorescent microscopy.

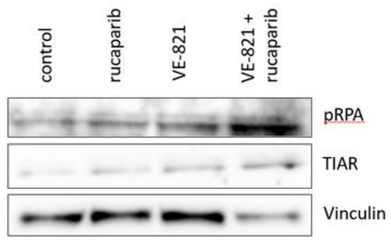




Cells were stained with TIAR antibody after no treatment (control) or treatment with rucaparib to induce replication stress and TIAR granule formation, as reported in (1), shown in A. B. Pan nuclear and cytosolic staining was observed in contrast to the TIAR granules observed in the cells either in control or rucaparib treated cells. This is a representative of one repeat and 1 optimisation repeated. Different antibody dilutions and pre-extractions achieved similar unspecific binding and poor images.

Analysis of TIAR by western blotting:

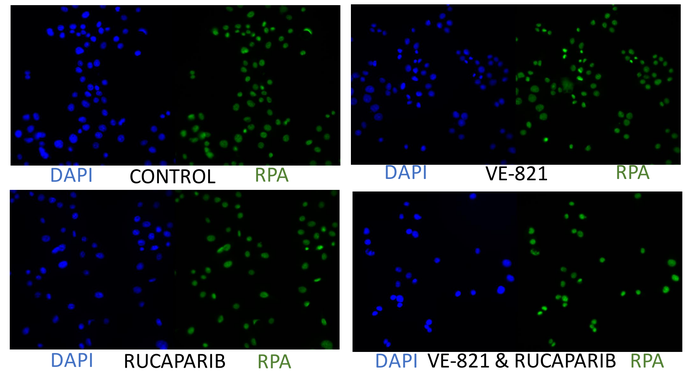
- As IF was unsuccessful at visualising TIAR – western blotting was tried

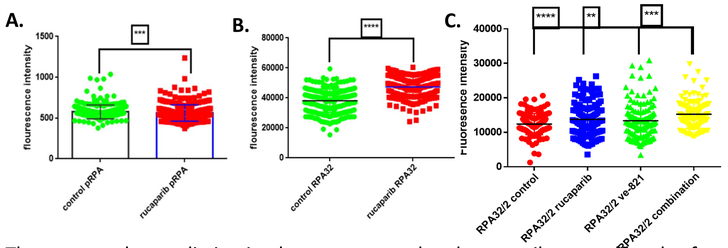


There was an increase in both TIAR and pRPA protein expression in the presence of either VE-821 or rucaparib compared to control, and the most TIAR and pRPA was seen in the combination of treatments. This would suggest analysis of either protein would be indicative of replication stress.

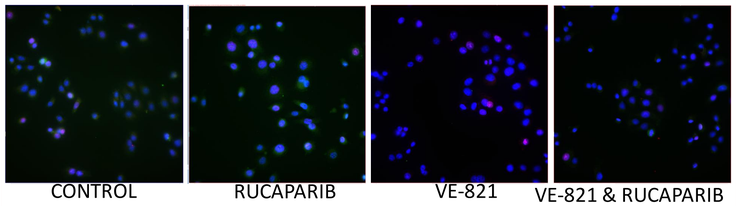
RESULTS II

- The western blot results with pRPA antibody suggested that RPA would be indicative of replication stress
- Two antibodies against RPA antibodies were therefore also optimized for immuno-fluorescent microscopy – RPA32 and pRPA32, then fluorescent intensity (FI) of the RPA signal in the nuclei was analysed.





There was a clearer distinction between control and rucaparib treated levels of RPA (as seen in the western blot) when using the RPA32 antibody (B) compared to pRPA32 antibody (A) therefore this was used to assess levels of replication stress in the different treatments shown above with the FI levels with treatments measured (C).



Shown above is the merged data of yH2AX(red) with RPA(green) in the nuclei of differently treated cells (blue) with an increase in yH2AX in each compared to control but this hasn't been quantified.

REFERENCES

1. Lafarga V, Sung H, Haneke K, Roessig L, Pauleau A, Bruer M et al. TIAR marks nuclear G2/M transition granules and restricts CDK 1 activity under replication stress. EMBO reports. 2018;20(1)

ACKNOWLEDGEMENTS

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CONCLUSIONS

- The TIAR antibody currently available was not effect for visualisation of TIAR via immunofluorescence, but was effective via western blotting
- Concluded concentration 1:4000 is best for pRPA32 and 1:1000 is best for RPA32/2 however RPA32 is better for measuring FI
- Fluorescence intensity data for RPA32 shows that combination induces the most RPA hence DNA damage
- The yH2AX shows numbers of DSB increase with the addition of drugs

Additional comments

Rucaparib treated cells tend to be much smaller than control
RPA tends to cluster more where denser DAPI/nucleic material does not