Specificity of S9.6 Antibody in the Detection of R-loops

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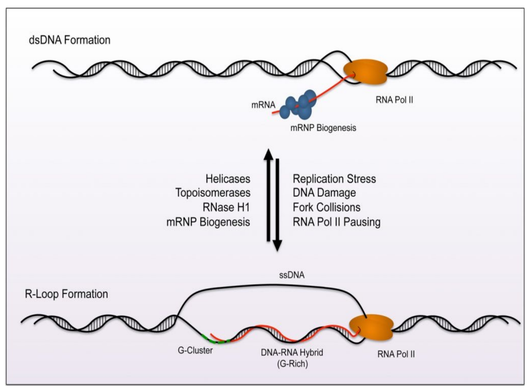
Background

- The ATR pathway plays a role in DNA damage repair as it regulates R-loops and other cellular processes.
- R-loops, specific DNA-RNA hybrids, form during transcription and require regulation.
- RNase H1 degrades the RNA of RNA-DNA hybrids and plays a key role in DNA replication and repair.
- Previous studies rely on immunodetection of R-loops by the S9.6 monoclonal antibody which specifically binds DNA-RNA hybrids.



- In this study, we tested the specificity of the S9.6 antibody's ability to detect R-loops using RNaseH1.
- We hypothesized that the RnaseH1 would digest the R-loop and that the S9.6 signal would be diminished.

Methods

Cell Culture	Highly transfectable derivative of human embryonic kidney 293 cells containing the SV40 T-antigen, 293T cell line, was used.
Transfection Treatment	Using CalFectin, we transfected RNaseH1 DNA (WKKD, D210N, WT) into 293T cells.
Immunofluorescence Microscopic Analysis	We used S9.6 antibody and DAPI to image DNA signals for R-loops and stain nuclei of SSP25 cell line with different shPTEN knockdown samples and ATRi's.
Western Blot	We separated and identified the RNaseH1 protein using the WT and its mutants. We used also used GFP as an additional tag.

Results

Decreased S9.6 Signal in Cells with BAY1895344 ATRi and PTEN Knockdown

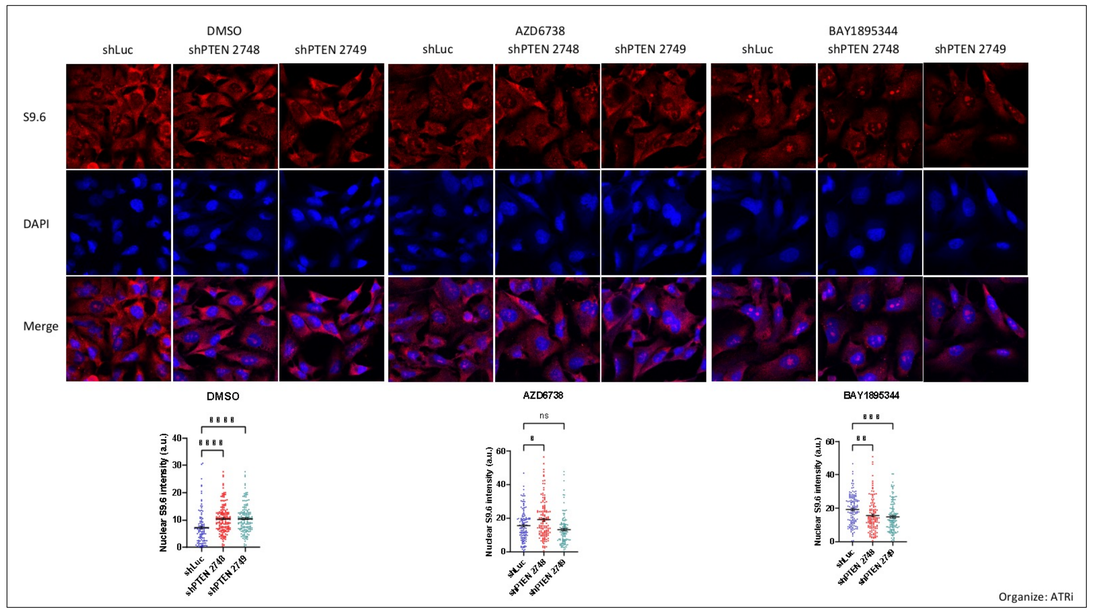


Figure 1. Immunofluorescent analysis shows that the S9.6 antibody can accurately detect S9.6 signals. The analysis also shows that there was a decrease in S9.6 signal intensity in the ATRi BAY1895344 with both PTEN knockdown samples. In ATRi AZD6738, there was no significance in shPTEN 2749. There was significance in shPTEN 2748. The control, DMSO, showed that there was an increase in S9.6 signal intensity in both shPTEN samples.

Increased Nuclear S9.6 Intensity in PTEN Knockdown

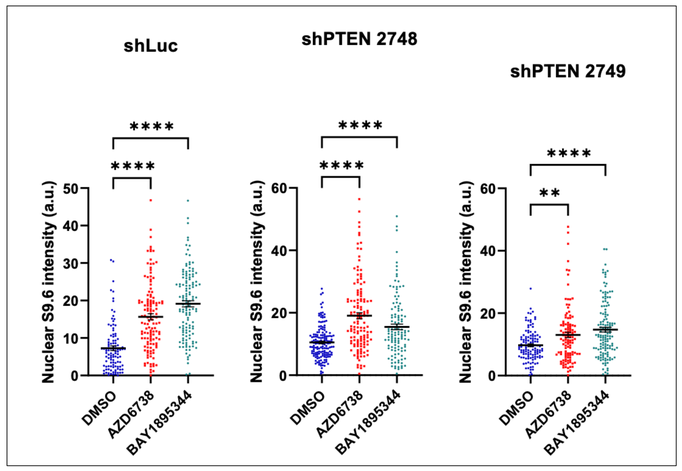


Figure 2. Immunofluorescent analysis shows that there is an increase in S9.6 signaling intensity in shPTEN 2748 and 2749 knockdown samples.

Increased Expression of RNaseH1 in Mutations and WT

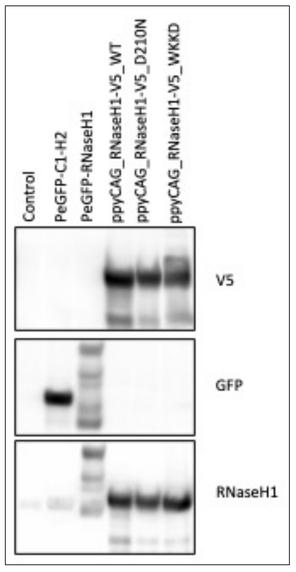


Figure 3. Western blot analysis shows the expression of RNaseH1 in all RNaseH1 samples except for the one containing the GFP plasmid. RNaseH1 diminished the expression of GFP in PeGFP-RNaseH1. V5 resulted in the overexpression of RNaseH1.

Conclusions

- Immunofluorescent analysis can use the S9.6 antibody and DAPI to accurately detect S9.6 signals and stain nuclei.
- Immunofluorescent analysis shows that ATRi BAY1895344 decreases the nuclear intensity of the S9.6 signal in shPTEN.
- Immunofluorescent analysis shows that shPTEN increases the nuclear intensity of the S9.6 signal.
- The western blot showed the expression of RnaseH1 was detected in all RnaseH1 mutants and in the WT except for the one containing the GFP plasmid.

Future Directions

- We were unable to run a final immunofluorescence analysis analysis using RNaseH1 and S9.6 on the SSP25 cell line. That would be the next step in investigating the specificity of the S9.6 in the detection of R-loops with RNaseH1 in cholangiocarcinoma.
- Antibody engineering in the enhancement of hybrid/dsRNA selectivity and exploring related underlying mechanisms remains an active area of research.

References and Funding

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2. Bou-Nader C, Bothra A, Garboczi DN, Leppla SH, Zhang J. Structural basis of R-loop recognition by the S9.6 monoclonal antibody. *Nat Commun*. 2022;13(1):1641. doi:10.1038/s41467-022-29187-7

Summer Undergraduate Research Program (G. Peng)