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14. ABSTRACT
We proposed that *RNASEH2A* represents a novel type of gene, up-regulated in lethal prostate cancer to prevent catastrophic genomic instability and cell death and thereby acting to make prostate cancers resistant to treatment with radiation therapy. The major findings include (1) Expression of RNASEH2A in human prostate cancer cell lines. (2) Ability to modulate RNASEH2A expression genetically (3) Modulation of cell cycle, cell migration, transcription invasion and growth of prostate cancer cell lines with *RNASEH2A. (4)* Radio-resistance of prostate cancer cells that over express *RNASEH2A*. (5) Association of *RNASEH2A* with tumor grade. (6)Observation that RNASH2A expression does not independently predict lethal prostate cancer.(7)Observation that RNASH2A expression does predict radio-sensitivity and response to treatment in men who underwent radical prostatectomy and subsequently had post – operative radiation.

15. SUBJECT TERMS
High Risk prostate cancer, radio sensitivity, biomarker

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Introduction

Over the last two decades there have been major advances in both the detection and treatment of localized prostate cancer. Although this has resulted in a decrease in prostate cancer specific mortality, prostate cancer still remains the second leading cause of cancer related death among men. Thus, key issues in the management of prostate cancer today include the identification of men with aggressive disease and the development and improvement of therapies to treat lethal cancers. Currently, Gleason grade is the most potent forecaster of metastatic ability and prostate cancer specific mortality. As such, we have begun to investigate the molecular features of high risk prostate cancer by correlating Gleason grade with RNA based expression patterns. Our work has identified pathways of genome stability as enriched in high grade disease and specifically *RNASEH2A*, a putative chromosomal integrity determinant as one of the most strikingly over-expressed genes in aggressive prostate cancer (over 6 fold increase in Gleason 8 vs Gleason 6 tissue, $p < 1x10^{-6}$). In this proposal, we hypothesized that *RNASEH2A* is associated with lethal, high grade disease maintaining chromosomal stability. Together this proposal implicates *RNASEH2A* as a marker and modulator of radio-resistance in prostate cancer.

Key Words
Prostate Cancer
Genomic stability
Radiation resistance
RNASEH2A

OVERALL PROJECT SUMMARY

Previously, utilizing a genome wide screen for determinants of genomic stability we identified lossof ribonuclease H as being strongly associated with both increased chromosomal breaks and loss(1). RNASEH2A encodes the catalytic subunit of the RNASEH2 complex which functions as the primary source of RNASEH activity in mammalian cells(2). The roles of RNASEH2A include guarding the genome from aberrant ribonucleotide incorporation during DNA synthesis as the RNASEH2 complex can recognize single ribonucleotides embedded within the DNA:DNA duplexes and is critical for their removal(3). When not removed, RNA:DNA hybrids (RDHs), also called R loops, can form and create unstable single stranded DNA which induces DNA damage responses and, if unchecked, can lead to pronounced chromosomal instability(1, 3).

Suppression and overexpression of RNASH2A in prostate cancer cells. We demonstrate expression of RNASEH2A in multiple available prostate cell lines (Fig. 2) by quantitative PCR (qPCR) using commercially available primers for RNASEH2A and RNASEH1 (Sigma, USA), immunofluorescence and Western blot. All prostate cancer cell lines showed high levels of RNASEH2A expression (Fig. 2a), while primary cell lines showed barely detectable levels (PrEC). Next, we have generated stable suppressed cell lines by transduction with lentivirus particles containing validated RNASEH specific shRNAs in pLKO.1-puro (MISSION shRNA Lentiviral Transduction Particles, Sigma) or a non target shRNA control and selection with puromycin followed by confirmation by western and QT-PCR. Stable overexpression was achieved by transduction with viral particles containing RNASEH2A driven by a CMV promoter in the pG3.3 vector and selected for with blasticidin along with appropriate controls(4). Empty vector was used as a control, and expression was validated by Western blot, immunofluorescence and qPCR (Fig. 2A-D).

Effect of RNASEH2 expression on cell cycle, cell migration, transcription, invasive and xenograft.growth. LNCaP cells, both wild type and knocked down for RNASEH2A, were analyzed by using standard assays. Both delays of cell cycle (not shown), and a mild effect on

cell migration/invasivness (scratch assay, Boyden chamber assay) were observed with knockdown of RNASEH2A (Fig. 3A-D)(5). The animal studies were performed according to the protocols approved by the Animal Care and Use Committee at Johns Hopkins University. LNCaP (WT and shRNA knockdown RNASEH2A) or CRW22-RVI cells (WT or overexpressing RNASEH2A) were injected subcutaneously into SCID mice. As shown in Fig. 3, tumor growth was retarded in cells lacking RNASEH2A and accelerated in tumors with overexpressing of RNASEH2A (Fig. 3E). To determine if RNASEH2A affects transcription in the cells, RNA from LNCaP cells with and without suppressed RNASEH2A were processed, labeled, and hybridized to HG-U133 2.0 Plus (6) whole genome gene expression microarrays (Affymetrix) according to the manufacturer's protocols used at the Johns Hopkins Microarray facility. The results were confirmed by Western blot (not shown) and qPCR reactions (Fig. 3F). The results show that cells lacking RNASEH2A show suppressed expression of cell cycle associated genes and enhanced expression of histones (not shown), suggesting its role in cell cycle progression.

Inhibition of RNASEH2 complex affects cell growth. In order to assess the role of RNASEH2A on cell growth, we performed a series of experiments using standard MTT and clonogenic assays(1, 7) on prostate cancer cells with either genetically knocked down or overexpressed RNASEH2A or cells [AML cell lines (MV4-11, HL-60), breast cancer cell lines (MDA-231 and MCF-7) and prostate cancer cell lines (LNCaP and CRW22-RVI) with increasing concentrations of several selected RNASEH2A inhibitors (0.5–20 μM) at 37°C for 4h or 24, 42 and 72h. As shown on Figure 5, RNASEH2 inhibitors affected the relative absorbance of both MV4-11 (Fig. 4A) after only 4h of incubation, while an effect on prostate (Fig. 4B) and breast cells (not shown)is seen after 48h of incubation. Interestingly, the main effect is achieved at nM doses and further increase of drug concentration had no or little additional effect on cell viability suggesting that RNASEH2 inhibition in cells is achieved with low doses of drugs and that no significant additional targets exist for these drugs at higher doses. Similarly, cell with overexpressed RNASEH2A showed mild radioresistance in a clonogenic assay (Fig. 4C).

Association of *RNASEH2A* with lethal prostate cancer and radiation resistance. RNAseH2a expression in a radical prostatectomy cohort increases with increasing grade (Fig 5A). Overall in a cohort of men with a high risk disease and high likelihood of recurrence, expression of RNASEH2A was not associated with metastasis or prostate cancer death (fig 5B). However, in a subset of these men – who received post operative radiation, high expression of RNASEH2A was statistically associated with increased biochemical recurrence ($p=0.005$), metastasis ($p = 0.002$) and death ($p = 0.02$)(Fig 6A,B,C) On multivariate analysis incorporating PSA, Gleason score, Seminal vesicle invasion, Extraprostatic extension and surgical margin status, RNASH2A expression significantly predicted biochemical and metastatic free survival ($p <0.001$ for both). Thus a subset of men who underwent post-operative radiation with higher levels of of RNASH2A is associated with poorer outcomes in a statistically significant manner.

Key Research Accomplishments

- RNASH2A expression correlates with prostate cancer grade, additionally RNASH2A expression correlates with grades of Breast and Bladder cancer.
- RNASH2A expression is high in prostate cancer cell line and can be suppressed is shRNA complexes
- Cell proliferation and migration are inhibited with RNASH2A suppression or pharmacologic inhibition
- Xenografts expressing higher levels of RNASH2A exhibit increased growth

- RNASH2A expression confirs onlyh mild additional radio-resistance in cell line that already over- express RNASH2A.
- RNASH2A expression predicts radio-sensitivity and response to treatment in men who underwent radical prostatectomy and subsequently had post – operative radiation.

CONCLUSIONS

To date we have demonstrated *RNASEH2A* is associated with high grade prostate cancer but is not an independent marker of lethal disease in men undergoing radical prostatectomy. However in men who underwent radical prostatectomy and experienced a recurrence, RNASH2A expression is a independent marker of radio-resistance in prostate cancer and worse clinical outcome. RNASH2A appears to regulate cell growth. The precise role of RNASH2A in radiation sensitivity remains to be determined on a cellular level

Publications

"RNAseH2a – a putative "Non Oncogene Addiction" Gene target and marker for Radio sensitivity in High Risk Porstast cancer" Manuscript in preparation

Reportable Outcomes

No reportable outcomes have come from this work thus far

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APPENDICIES

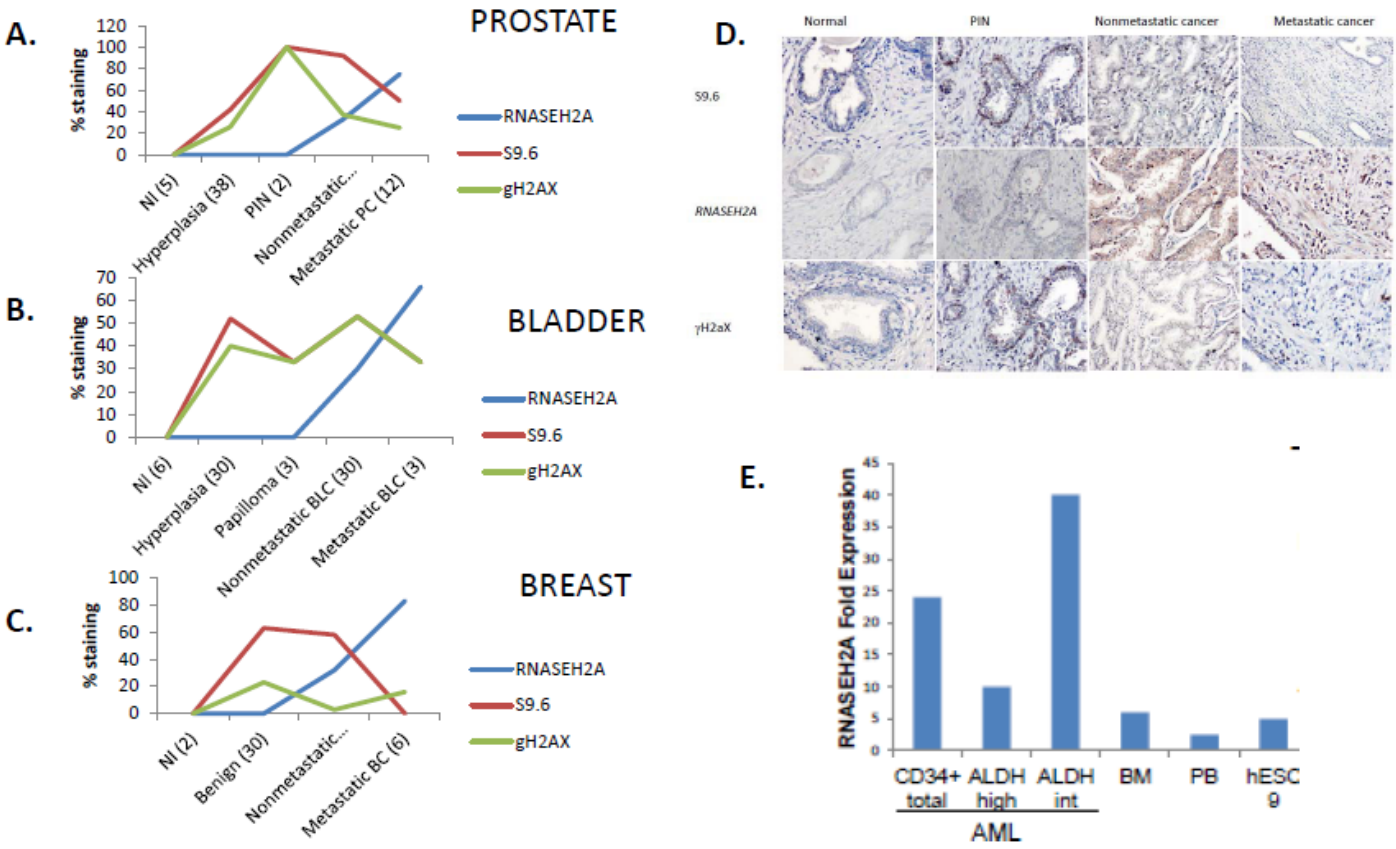


Figure 1. TMA Summary. Percentages of benign and malignant tissues staining for designated antigen by IHC, prostate (A), bladder (B), breast (C). (D)Example of IHC staining in prostate tissue (40x). (E) qPCR for RNASEH2A in leukemic stem cells (ALDH int) and normal counterpart (ALDH high), normal bone marrow (BM), peripheral blood (PB) and hESC9 cell line relative to normal prostate tissue.

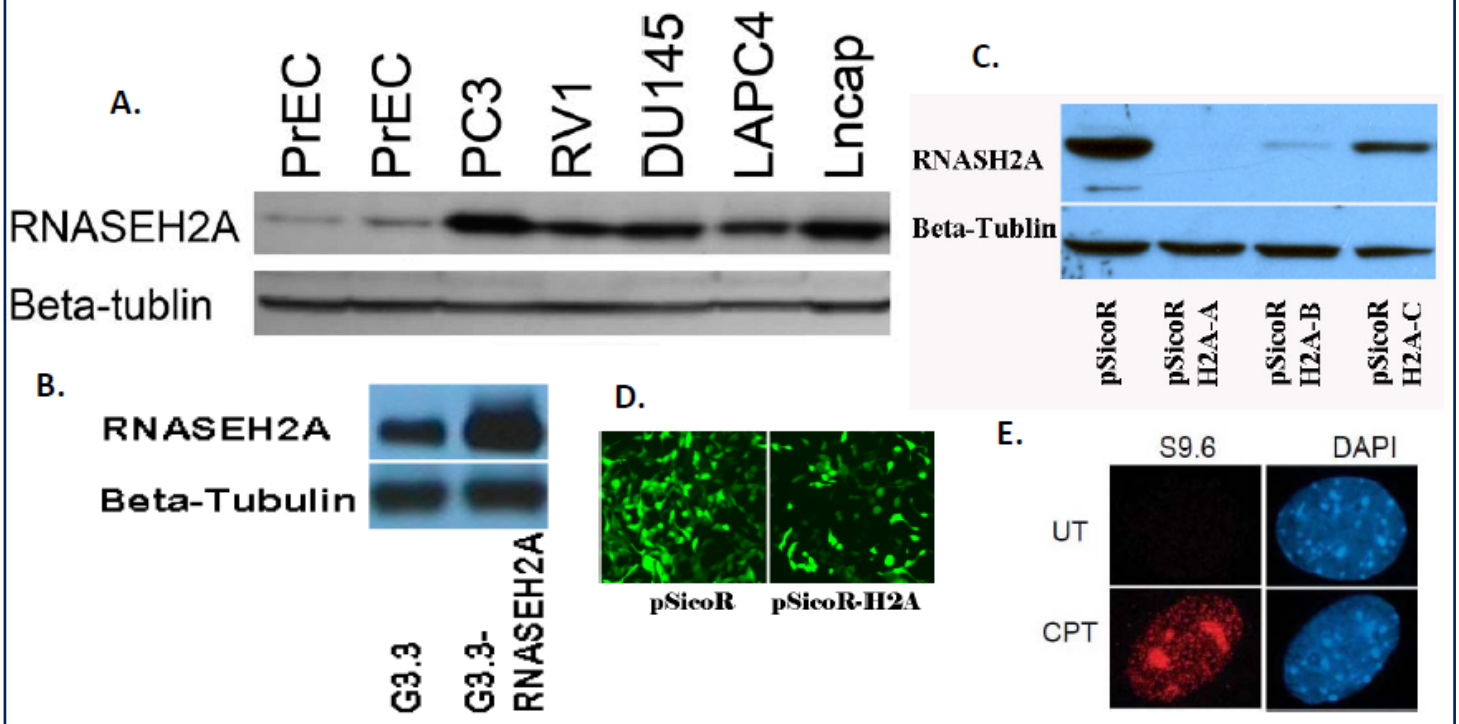


Figure 2. Expression of *RNASEH2A* in in human prostate cell lines (A), overexpression of *RNASEH2A* (B) and suppression of *RNASEH2A* (C) expression by Western and IF (D). (E) Detection of RNA:DNA hybrids by immunofluorescence (UT=untreated, CPT=campthotecin).

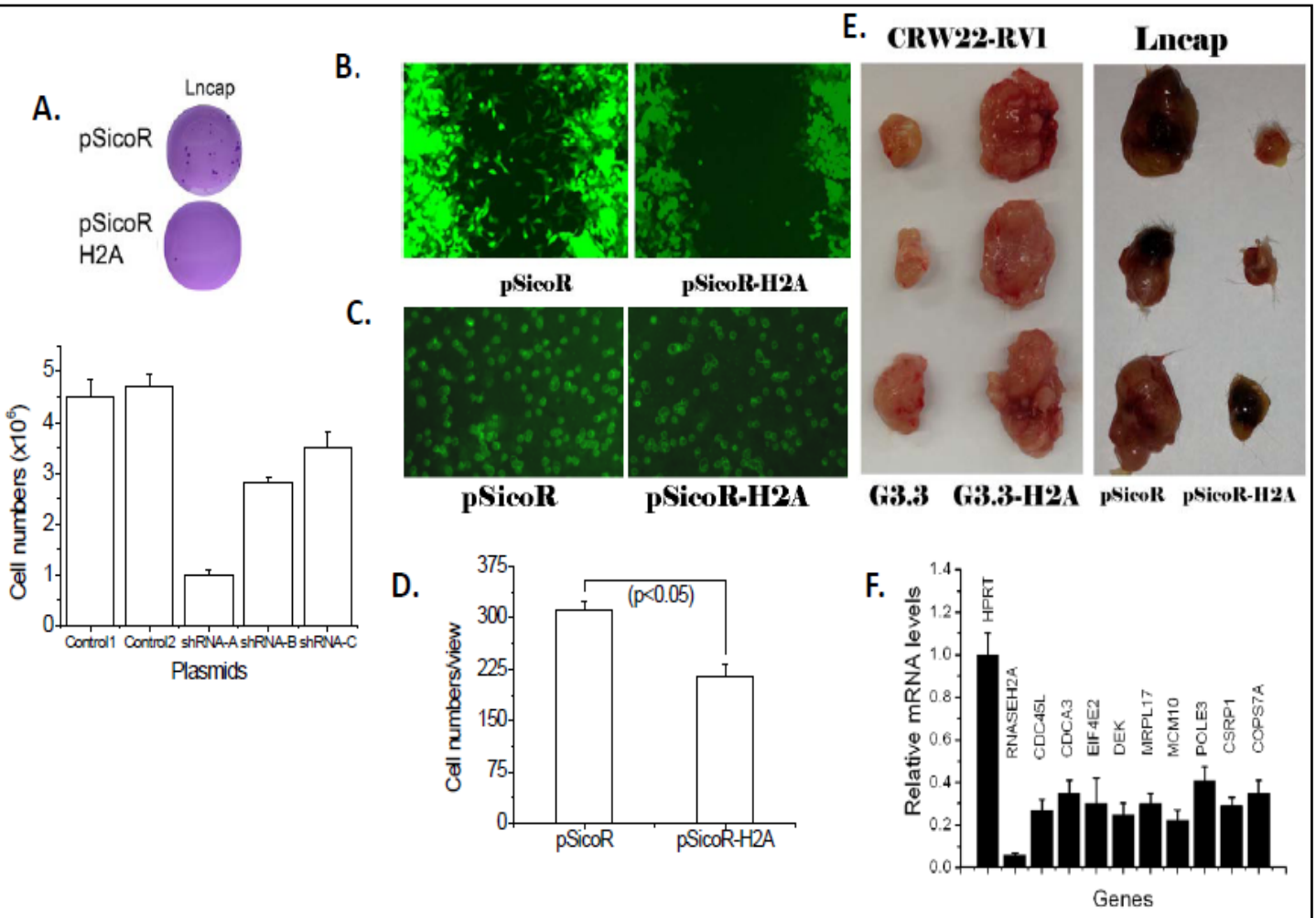


Figure 3. Cell proliferation is inhibited in LNCaP cells lacking *RNASEH2A* in a clonogenic assay (A). Cell migration assays: Skratch (B) and Boyden chamber assay (C, D) in cells with plasmid alone (left) or cells lacking *RNASEH2A* (right). (E) Xenografts of CRW22-RVI cells with plasmid alone (left) or overexpressed *RNASEH2A* (right) 3 weeks after innoculation and LNCaP cells with plasmid alone (left) or suppressing *RNASEH2A* expression (right) 6 weeks after innoculation. (F) RNA microarray cells lacking *RNASEH2A* shows suppression of genes involved in cell cycle regulation.

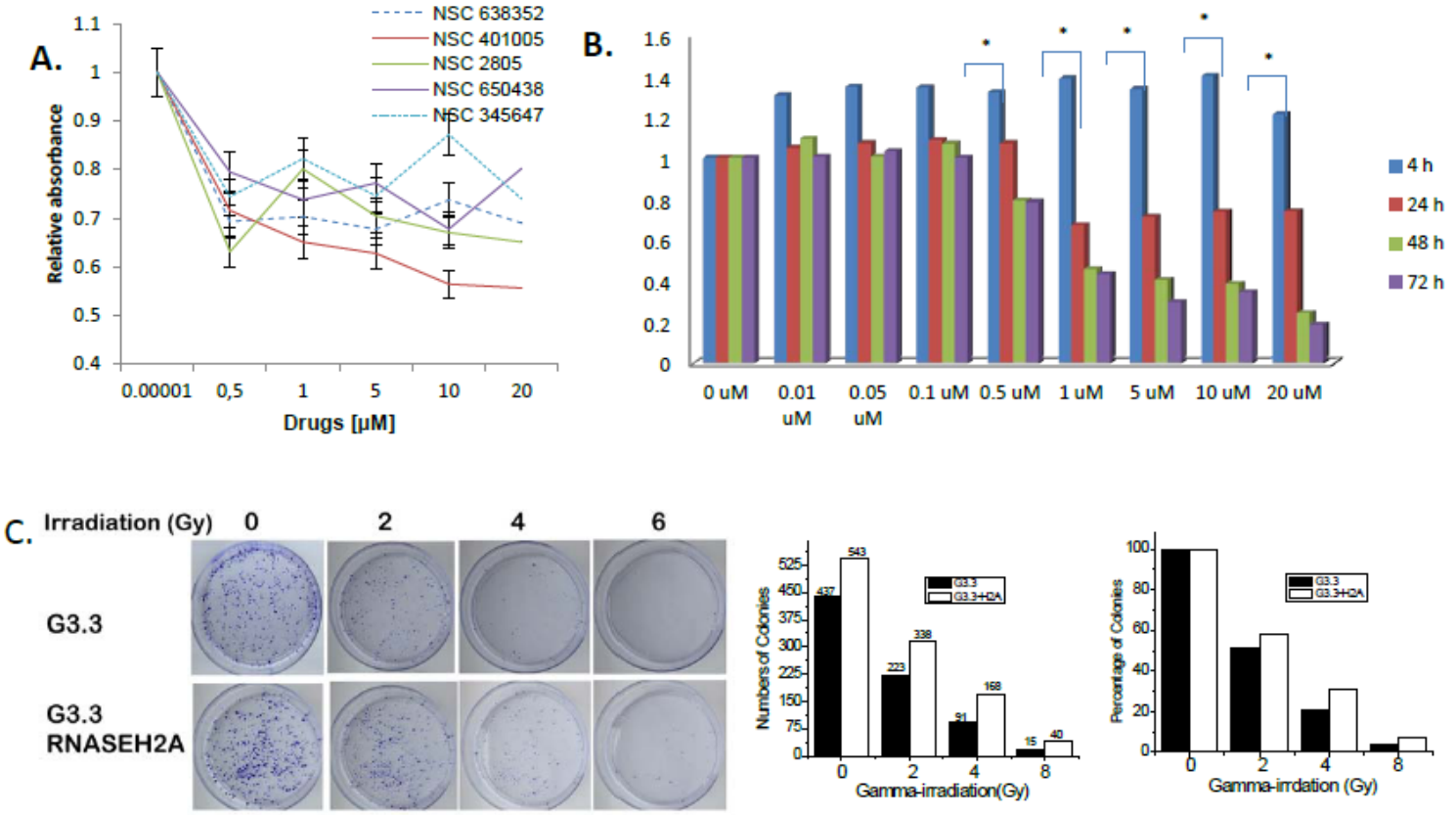


Figure 4 Effect of RNASEH Inhibitors on MV4-11 and LNCaP cells, and effect of radiation on cells overexpressing *RNASEH2A*. (A) Dose curves of 5 different inhibitors on MV4-11 growth (4h) in a MTT assay. (B) Effect of RNASEH2 inhibitor NSC401005 on LNCaP cell line, MTT assay. (C) Cells overexpressing RNASEH2A show resistance to radiation in a clonogenic assay (left=assay, middle panel number, right % of colonies at designated exposure).

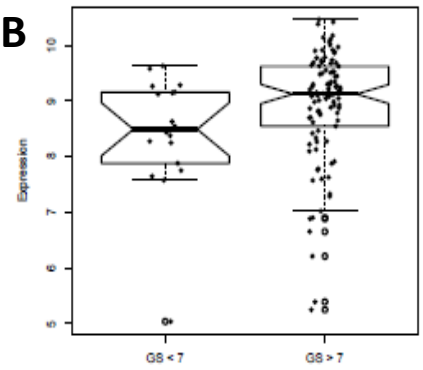
A

	BCR	MET	PCSM	GS > 7	SMS	Adj RTx	Late RTx
%	53	31	14	41	57	10	31

Table 1: The percentage of biochemical recurrence (BCR), metastatic progression (MET), prostate cancer specific mortality (PCSM), Gleason score (GS), positive surgical margins (SMS), adjuvant radiation therapy (Adj RTx), and salvage radiation therapy (Late RTx) events in the dataset (n = 235).

- Metastatic progression is defined by a positive bone or CT scan.

B



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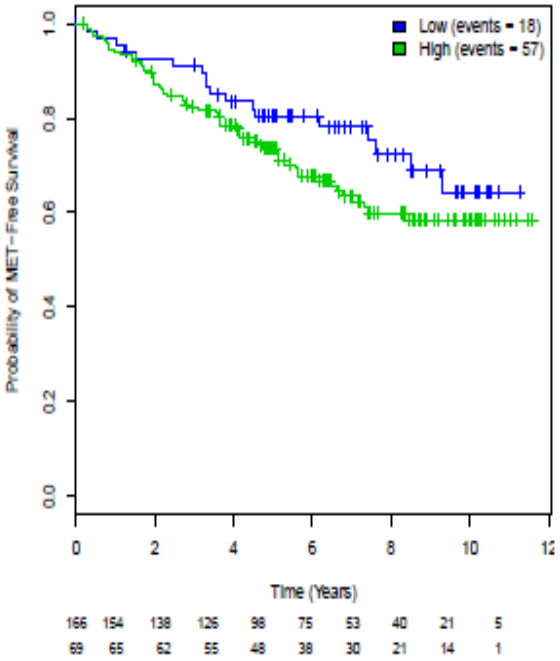

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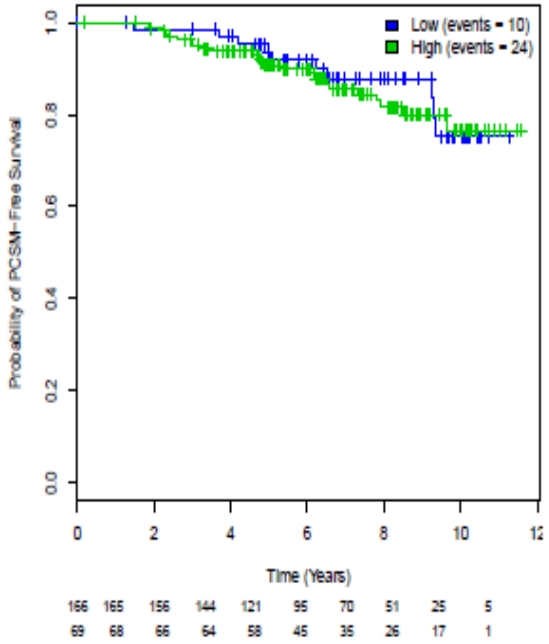


Figure 5. RNASEH2A expression increases with increasing grade. (a)Clinical cohort and follow up events. (B) Distribution of RNASEH2A In low and high gleason grade. (C) Kaplan Meier curve showing differences in metastatic free survival in patients with high and low RNASH2a expression. (p = 0.158) (D) Kaplan Meier curve showing differences in Prostate cancer specific mortality free survival in patients with high and low

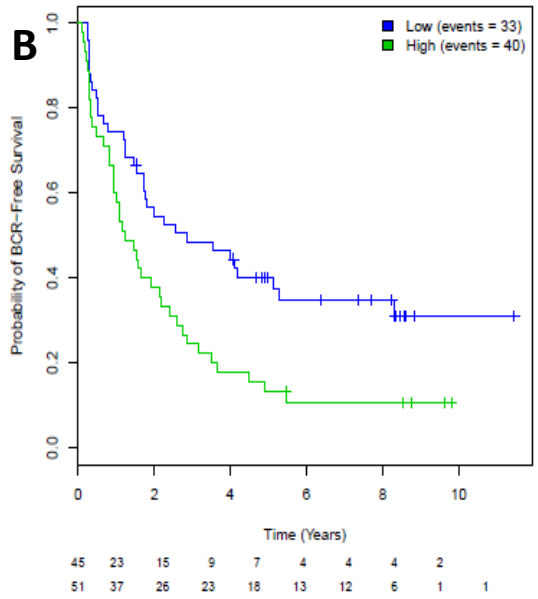
A

	BCR	MET	PCSM	GS > 7	SMS	Adj RTx	Late RTx
%	76	48	21	38	71	25	77

Table 1: The percentage of biochemical recurrence (BCR), metastatic progression (MET), prostate cancer specific mortality (PCSM), Gleason score (GS), positive surgical margins (SMS), adjuvant radiation therapy (Adj RTx), and salvage radiation therapy (Late RTx) events in the dataset (n = 96).

- Metastatic progression is defined by a positive bone or CT scan.

B



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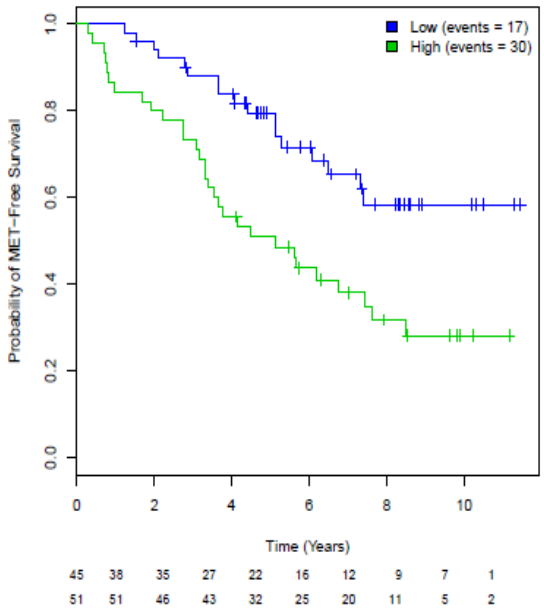

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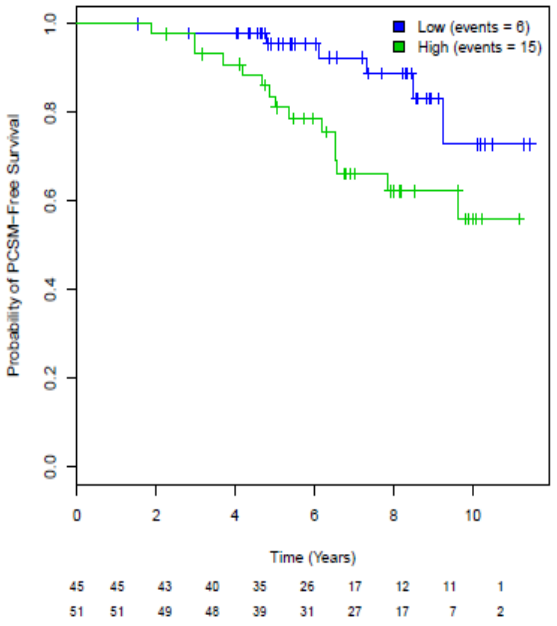

Figure 6. RNASEH2A is associated with radiation resistance.
(a)Clinical cohort and follow up events of men receiving post procedure radiation. Kaplan Meier curves showing differences in biochemical free survival (B), metastasis free survival (C), prostate cancer specific survival (D) in patients who had a radical prostatectomy and subsequently underwent post-operative radiation based on high and low RNASH2a expression. (p = 0.005, p = 0.002, p = 0.024)