



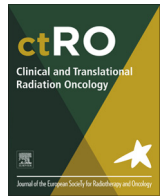
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## Original Research Article

## Profiling of a panel of radioresistant prostate cancer cells identifies deregulation of key miRNAs

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## ABSTRACT

**Background:** miRNAs are increasingly associated with the aggressive phenotype of prostate tumours. Their ability to control radiobiologically-relevant cellular processes strengthens their potential as novel markers of response to radiation therapy.

**Purpose:** To identify miRNAs associated with increased clonogenic survival following radiation exposure. **Material and methods:** The miRNA expression profiles of a panel of 22RV1 cells with varying levels of radiosensitivities (hypoxic H-22Rv1 cells, RR-22Rv1 cells derived from WT-22Rv1 cells through 2-Gy fractionated repeated exposure, the associated aged matched cells (AMC-22Rv1) and the WT-22Rv1 cell lines) were generated and cross-analysed to identify common miRNAs associated with a radioresistant phenotype.

**Results:** Increased clonogenic survival following irradiation was associated with significant modifications in miRNA expression pattern. miR-221 (up) and miR-4284 (down) in RR-22Rv1 and MiR-31 and miR-200c in AMC-22Rv1 were the most uniquely significantly deregulated miRNAs when compared to WT-22Rv1 cells. miR-200c ranked as the most downregulated miRNAs in hypoxic, when compared to RR-22Rv1 cells. miR-200a was the only differentially expressed miRNA between RR-22Rv1 and AMC-22Rv1 cells. miR-210 yielded the highest fold change in expression in H-22Rv1, when compared to WT-22Rv1 cells.

**Conclusion:** This study identifies candidate miRNAs for the development of novel prognostic biomarkers for radiotherapy prostate cancer patients.

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## Introduction

MiRNAs play a vital role in the regulation of cellular processes including cell cycle [1], DNA damage response [2], cell death [3] and hypoxia [4]. The expression of these small, non-coding RNAs that negatively regulate gene expression is increasingly reported to be modified in prostate cancer, and analysis of prostate cancer cell lines has identified several candidate miRNAs whose expression is modified following radiation exposure. (e.g. miR-34a [5], Let-7a and miR-17-92 cluster [6], miR-106b [7], miR-521 [8], miR-95 [9] and MiR-301a and miR-301b [10]). As a result, their prognostic potential has been proposed [11,12].

The engineering of radioresistant cell lines models can assist the evaluation of the function of specific genes and regulatory elements [13]. One known, easy to manipulate factor associated with radioresistance, is hypoxia [14,15]. But the selection of a cancer subpopulation with modified cell fate in response to radiation through exposure to a variety of fractionated radiation schedules is increasingly used to investigate the molecular response of cells to radiation [16]. Few of these models exist in prostate cancer and of the four commonly used prostate cancer cell lines (Du145, PC3, LnCap, 22Rv1), only one (22Rv1) may be representative of non-metastatic disease. [17]. Through the treatment of 22Rv1 prostate cancer cells with fractionated 2 Gy radiation to a cumulative total dose of 60 Gy, we have successfully selected for 22Rv1-cells with sustainable increased clonogenic survival following subsequent radiation exposure. Like models of other disease sites, this radioresistant RR-22Rv1 prostate cell line is enriched in S-phase

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cells, less susceptible to DNA damage, radiation-induced apoptosis and displays enhanced migration potential, when compared to wild type and aged matched control 22Rv1 cells [18].

This study hypothesises that a subset of miRNAs are involved in the radioresistant phenotype resulting from both hypoxic exposure and adaptation to repeated radiation exposure. The cross-evaluation of the changes in miRNAs expression profiles in wild type cells, hypoxic and fractionation-selected cells represents an alternative, novel approach to the identification of miRNAs associated with a radioresistant phenotype. Application of computational prediction of candidate miRNAs's gene targets has the potential to further accelerate the characterisation of the molecular radiobiology of cancer cells and enable the molecular classification of prostate cancer [19,20].

## Materials and methods

### 22Rv1 cell line panel

Newly acquired, authenticated human 22Rv1 (WT-22Rv1) prostate cancer cells (American Type Culture Collection) were cultured at 37 °C in 95% humidified air containing 5% CO<sub>2</sub> in RPMI cell culture medium containing L-glutamine (Lonza, Castleford, UK) with 10% foetal bovine serum (Gibco, Dublin, Ireland) and 1% pen/strep (Lonza). Fractionated 2 Gy X-rays doses (250 keV, 15 mA) using an RS225 cabinet (XStrahl, Surrey, UK) were delivered weekly to a cumulative dose of 60 Gy to generate the RR-22RV1 cell model, as described in [18]. Mock irradiated cells were cultured alongside to generate age-matched controls cells (AMC-22Rv1) [18]. 22Rv1 cells were exposed to hypoxia (0.5% O<sub>2</sub>, pO<sub>2</sub> < 2 mm Hg) in a 1000 *in vivo* hypoxic chamber (BioTrace, Bracknell, UK) for 24 h to generate the H-22Rv1 cell model.

### Clonogenic assays

Cell survival was evaluated using a standard colony forming assay [21]. To determine clonogenic survival under hypoxic conditions, cells were exposed to 0.5% oxygenated conditions in a 1000 *in vivo* hypoxic chamber (BioTrace, Bracknell, UK) for 24 h prior to hypoxic irradiation. The cells were returned to an aerobic incubator following irradiation and during colony formation. Colonies were counted using the ColCount instrument (Oxford Optronix Ltd, Oxford, UK).

### RNA extraction

RNA was extracted using Tri Reagent (Ambion) and quantified using the Qubit<sup>®</sup> Fluorometer 1.0 and the Qubit<sup>®</sup> RNA BR assay (Invitrogen) as per the manufacturer's instructions. Samples were prepared simultaneously across the panel to generate each independent biological replicates.

### Quantification of miRNA and target gene expression

cDNA synthesis of total RNA was performed using high-capacity cDNA reverse transcription kit (Applied Biosystems, Dublin, Ireland) and expression quantified using TaqMan<sup>®</sup> Gene Expression Master Mix (Applied Biosystems) with  $\beta$ -actin as endogenous control.

### miRNA profiling

miRNA was synthesised using the Universal cDNA Synthesis Kit II (Exiqon) and expression measured using the ExiLent SYBR<sup>®</sup> Green mastermix (Exiqon). The analysis of expression stability

across the panel identified hsa-miR-185p and hsa-miR-5p as the most robust endogenous controls. The expression profiles of 784 miRNAs were generated using the miRCURY LNA microRNA array system (Exiqon, Denmark). The data was normalised using the Lowess normalisation method and evaluated using unsupervised and supervised analysis. miRNAs associated with a statistically significant fold change in expression were deemed differentially expressed. The Pictar [22] and MirTarget 2 [23] programs were selected for prediction of miRNA targets.

### Transfection of miRNA mimic

Reverse transfection of hsa-miR-4284, the positive control hsa-miR-1 or scrambled non-targeting control miRVana mimics (Ambion, Dublin, Ireland), was performed using Lipofectamine RNAi Max transfection reagent (Ambion), as per manufacturer's instructions.

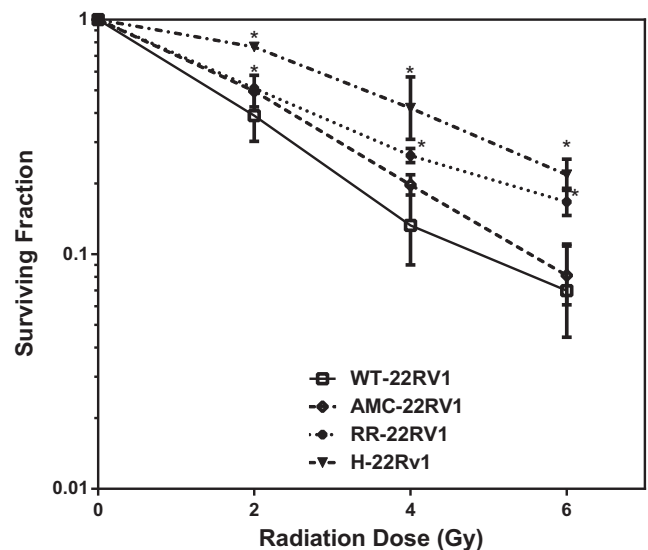
### Statistical analysis

Experiments were conducted with  $N = 4$ . Student t-tests were used to compare means between two groups, an ANOVA was used to compare means between two or more groups, as appropriate. The statistical analysis was performed using Prism, Version 5.01 (GraphPad Software Inc. CA). A  $p$ -value of <0.05 was considered statistically significant.

## Results

### Radioresponse of the 22Rv1 cells panel

The radiation survival curves of the 22Rv1 cells panel were determined using clonogenic assays (Fig. 1). The surviving fractions of RR-22Rv1 and H-22Rv1 cells were significantly higher than that of the WT-22Rv1 cell line at all doses tested ( $p < 0.05$ ). A non-significant trend towards an increase in the clonogenic survival of AMC-22Rv1 cells, when compared to WT-22Rv1 was observed. H-22Rv1 cells were significantly more radioresistant than RR-22Rv1 at all doses tested ( $p < 0.05$ ). RR-22Rv1 cells were significantly more resistant than AMC-22Rv1 cells at 4 Gy and 6 Gy ( $p < 0.05$ ).



**Fig. 1.** Radiation survival curves and miRNA profiling of the 22Rv1 cell lines panel. Surviving fraction of the radiation resistant (RR-22RV1), wild type (WT-22RV1), 22Rv1 age matched control (AMC-22RV1) and hypoxic (H-22RV1) cells in response to single radiation doses of up to 6 Gy. Mean  $\pm$  SEM, \* $p \leq 0.05$ .

miRNA profiles of the 22Rv1 cells panel

The exposure of WT-22Rv1 to fractionated radiation leading to the emergence of an isogenic radioresistant cell line (RR-22Rv1) was associated with a significant change in the expression of 97 miRNAs (51 up- and 46 down) (adj.  $p < 0.05$ ) (Fig. 2). The prolonged culture of mock-irradiated WT-22Rv1 cells to generate an age-matched-control (AMC-22Rv1 cells) modified the expression of 65 (42 up, 23 down,) miRNAs. Only one miRNA (miR-200a) was significantly altered between the miRNA profile of this age-matched-control and that of the radioresistant RR-22Rv1 cells. The exposure of WT-22Rv1 to hypoxia lead to the modification in the expression of twelve microRNAs (11 up- and 1) (Fig. 2, Table 1). hsa-miR-210 displayed the highest significant fold change (3.711). miR-4284 was significantly down regulated (logFC =  $-1.090$ , adj.  $p = 0.04$ ). Dysregulation of six miRNAs was common in all three analyses: miR-141, miR-23b, miR24, miR-29a, miR-29c and miR-4284. The comparison of H-22Rv1 and RR-22Rv1 profiles yielded 207 differentially expressed miRNAs with miR-200c and miR-222 ranking as the most down and up regulated miRNAs.

miRNAs differentially expressed in radiation resistant cells

The list of miRNA differentially expressed in the two radioresistant 22Rv1 cell models (RR-22Rv1, H-22Rv1), when compared to the radiosensitive WT-22Rv1 cells were cross-examined. Eleven common significantly deregulated miRNA were identified. Five displayed opposite patterns of expression between the samples. Five were upregulated in both samples: miR-210, miR-23a, miR23b,

miR-24 and miR-29a. Only one, miR-4284, was down regulated in both models (logFC =  $-1.62$ , and  $-1.09$  in RR and H-22Rv1 respectively). miR-4284 was also significantly down regulated (logFC =  $-1.434$ ) in AMC-22Rv1 mock-irradiated 22Rv1. The expression patterns of miR-210 and miR-4284 were validated in independent samples by q-RTPCR (Fig. 3).

miR-4284 target gene prediction

Thirty potential miR-4284 target genes were predicted (Table 2). The impact of a change in miR-4284 expression levels following transfection of 22Rv1 cells with a miR-4284 mimic on the expression of 6 candidate targets genes was determined (Fig. 4). Pearson correlation identified a strong inverse relationship in the expression of miR-4284 and its predicted target genes *RLIM* ( $r = -0.9997$ ,  $p = 0.015$ ) and *RASGEF* ( $r = -0.9998$ ,  $p = 0.01$ ).

Discussion

Cellular adaptation to both hypoxia and radiation exposure is associated with a modification in miRNAs expression pattern that has the potential to yield the identification of novel predictive biomarkers of radiotherapy response [24–26]. Our analysis of the alteration in the expression of miRNAs in 22Rv1 cells exposed to hypoxia identified 12 differentially expressed miRNAs. These include previously reported miR-210 [27,28] and miR-141 [29] and indicate potential additional regulatory roles for miR-19, miR-23, miR-27, miR-29 and miR-30, whose expression was related to prostate cancer tumorigenesis [30–33]. Radioresistant

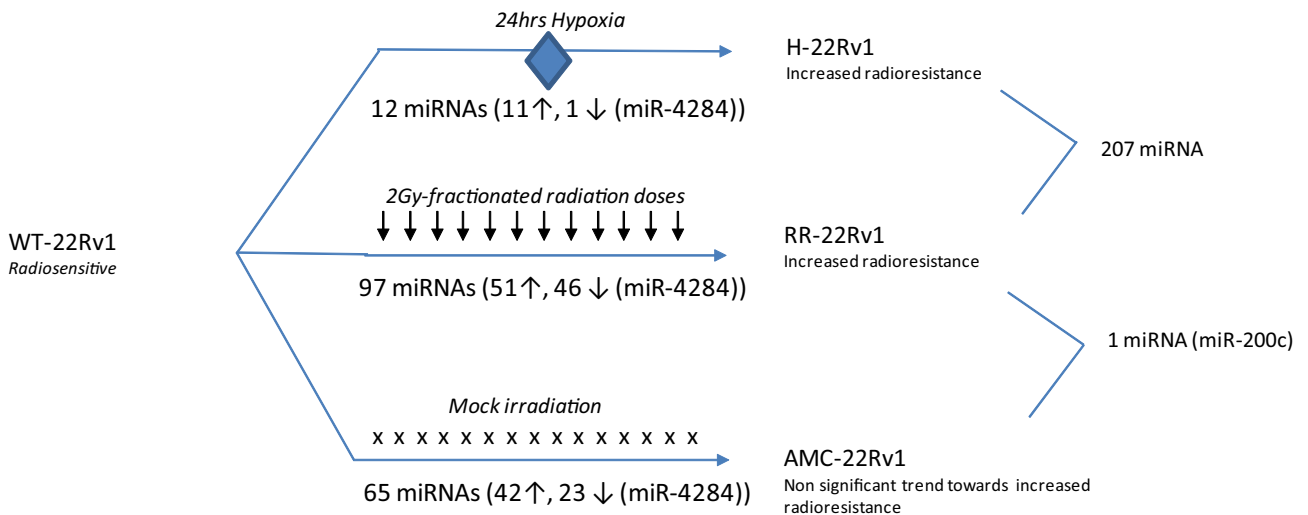
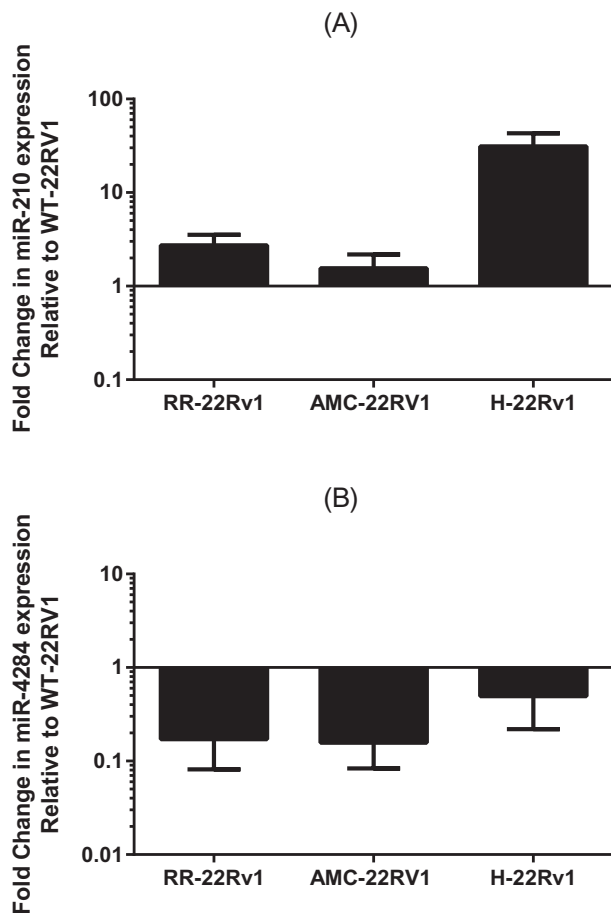


Fig. 2. Schematic representation of miRNAs profiles analysis.

Table 1 List of top 10 most differentially expressed miRs according to adj.  $p$ . Value in the 22Rv1 panel.

Rank	RR-22Rv1 vs WT-22Rv1 (fold change)	RR-22Rv1 vs AMC-22Rv1 (fold change)	AMC-22Rv1 vs WT-22Rv1 (fold change)	H-22Rv1 vs WT-22Rv1 (fold change)	H-22Rv1 vs RR-22Rv1 (fold change)
1	miR-130a-3p (2.9)	miR-200a ( $-0.983$ )	miR-141-3p ( $-3.2$ )	hsa-miR-210 (3.6)	hsa-miR-141-3p ( $-4.6$ )
2	miR-141-3p ( $-3.35$ )		miR-130a-3p (3.19)	hsa-miR-27a-3p (1.49)	hsa-miR-200c-3p ( $-4.7$ )
3	miRPlus-A1086 (3.6)		miR-29a-3p (2.31)	hsa-miR-23a-3p (1.47)	hsa-miR-106b-5p ( $-2.3$ )
4	miR-3607-3p ( $-1.8$ )		miR-3607-3p ( $-2.04$ )	hsa-miR-19a-3p (1.26)	hsa-miR-130a-3p (2.9)
5	miR-222-3p (2.76)		miR-222-3p (2.4)	hsa-miR-29c-3p (1.22)	hsa-miR-210 ( $-2.55$ )
6	miR-29a-3p (2.26)		miR-31-5p (2.65)	hsa-miR-29a-3p (1.18)	hsa-miR-19b-3p ( $-2.1$ )
7	miR-4521 (2.15)		miRPlus-A1086 (3.0)	hsa-miR-141-3p (1.26)	hsa-miR-25-3p ( $-1.9$ )
8	miR-221-3p (1.95)		miR-4521 (2.19)	hsa-miR-30b-5p (1.4)	hsa-miR-222-3p (3.1)
9	miR-3607-5p ( $-1.5$ )		miR-200c-3p ( $-3.5$ )	hsa-miR-23b-3p (1.3)	hsa-miR-29c-3p (2.2)
10	miR-4284 ( $-1.6$ )		miR-3607-5p ( $-1.76$ )	hsa-miR-19b-3p (1.2)	hsa-miR-26a-5p ( $-2.25$ )



**Fig. 3.** Validation of miR-210 and miR-4284 expression patterns in radioresistant 22Rv1 cells. Fold change in mRNA expression of (A) miR-210 and (B) miR-4284 in RR-22Rv1, AMC-22Rv1 and H-22Rv1 relative to WT-22Rv1 cells.

22Rv1 cells generated from chronic exposure to fractionated radiation displayed changes in the expression of 97 miRNAs. Ranking according to statistical significance identified miR-130-3p, miR-141, miRPlus-A1086, miR-3607 and miR-222 as the most differentially expressed miRNAs, when compared to radiation naïve, wild type cells. Reports exist for involvement of miR-141 [34], miR-3607 [35] and miR-222 [36] in the radioresponse of cancer cells. MiR-130 overexpression promoted clonal expansion of gastric cancer cells [37]; miRPlus-A1086 was implicated in the mechanism of action of combination Temozolimus and Bevacizumab in metastatic melanoma [38].

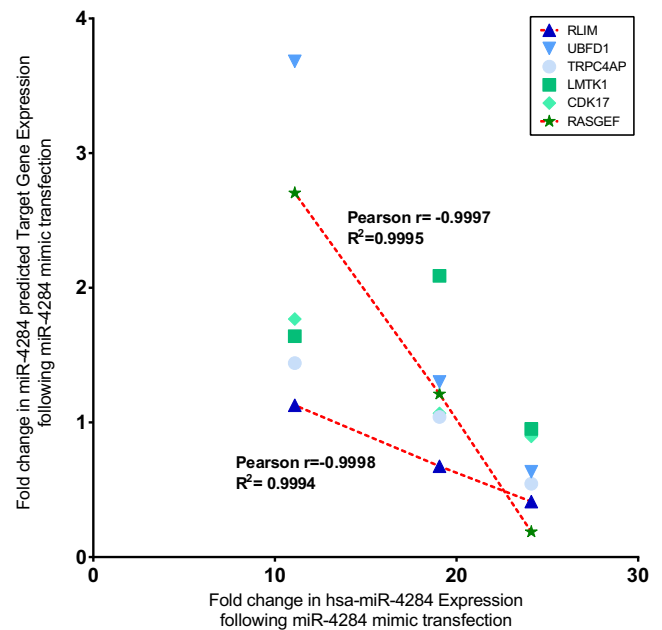
We hypothesised that the emergence of a radioresistant phenotype following both fractionated radiation and hypoxic exposure is regulated by a common set of miRNAs. This novel approach identified six candidate miRNAs differentially expressed in both the hypoxic and isogenic radiation resistant 22Rv1 models, when compared to the more radiosensitive WT-22Rv1 cell line: miR-210, miR-23a, miR23b, miR-24, miR-29 and miR-4284. miR-210 and miR-4284 were independently validated, and the potential target genes of miR-4284 were predicted. miR-200a, associated with more aggressive prostate cancer [39–41], was the only miRNA differentially expressed between RR-22Rv1 and AMC-22Rv1 cells.

The up-regulation of miR-210 under hypoxic conditions in both malignant and normal cells is well-established [27,28] and associated with poor prognosis in multiple cancers including breast [27], melanoma [27] colorectal [42] and non-small cell lung cancer [43]. In prostate cancer, its serum levels, alongside those of miR-220c, miR-141, miR-200a and miR-375, may indicate metastatic disease

**Table 2**

Predicted gene targets of hsa-miR-4284 ranked according to the aggregate score generated using the Pictar and MirTarget computational prediction tools.

Symbol	Pictar Score	MirTarget score	Aggregate score
RLIM	38	99	137
UBFD1	39	82	121
TRPC4AP	23	95	118
LMTK2	27	89	116
CDK17	21	95	116
ADAMTS6	26	89	115
ZMYM3	20	94	114
ZNRF3	24	89	113
NFAT5	30	81	111
ZNF292	13	91	104
RASGEF1A	14	90	104
ERC2	17	85	102
ZDHHC9	29	73	102
UHMK1	37	63	100
XPO1	5	94	99
TBL1XR1	9	90	99
ELP5	14	83	97
FIZ1	14	82	96
FAM134B	5	91	96
LRRC15	25	56	81
PHF2	20	57	77
BACH2	22	54	76
NAV2	19	57	76
SH2B1	10	62	72
CELF5	18	54	72
N4BP2L2	6	65	71
AHDC1	3	66	69
ARHGAP32	13	54	67
HK1	9	56	65
TEX2	11	50	61



**Fig. 4.** Correlation between miR-4284 expression and target gene expression. The fold change in miR-4284 expression following transfection of 22Rv1 cells with a miR-4284 mimic when compared to untransfected controls are plotted against the measured fold change in expression of predicted miR-4284 target genes in individual sample replicates. The relationship was analysed using a Pearson correlation: Pearson  $r$  coefficient and  $R^2$  values are presented.

[44,45]. Knockdown of miR-210 in human hepatoma xenograft enhanced the anti-tumour effects of radiotherapy [46] and may prevent the development of radiation enteropathy [47]. But miR-210 inhibition failed to enhance clonogenic survival in irradiated PC3 prostate cancer cells [48].



miR-4284 was the only miRNA commonly down regulated amongst radiation resistant models and AMC-22Rv1 cells, associated with a non-significant trend towards acquisition of age-related radioresistance. Transfection of RR-22Rv1 cells with a hsa-miR-4284 mimic indicated that over expression results in the negative regulation of two computationally predicted target genes: *RLIM* and *RASGEF1A*. This interaction may participate to the radioresistant phenotype of these cells through regulation of LIM homeodomain transcription factors [49], telomere length [50] and random X chromosome inactivation [51] (*RLIM*) and specificity for the RAP2 [52], KRAS, HRAS and NRAS [53] members of the RAS superfamily (*RASGEF1A*), whose association with oncogenesis [54–57] and radiotherapy resistance is well documented [58–65]. The functional validation of miR-4284, *RLIM* and *RASGEF1A* to radioresistance is warranted.

Analysis of the miRNA profiles of a panel of 22Rv1 prostate cancer cells with varying radiosensitivities identified novel potentially key miRNAs to the improved diagnosis and prognosis of prostate cancer.

### Declaration of interest.

All authors declare no conflict of interest.

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