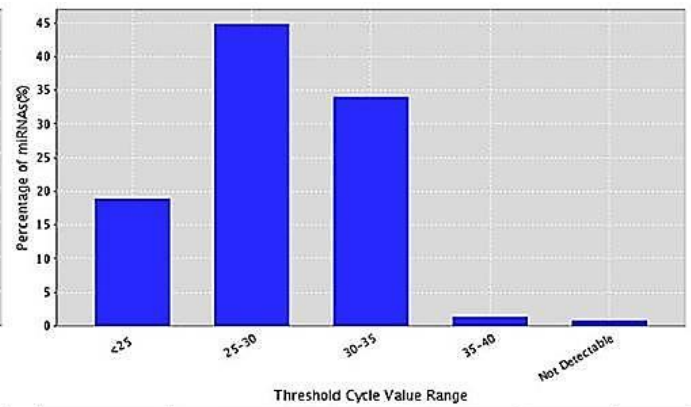
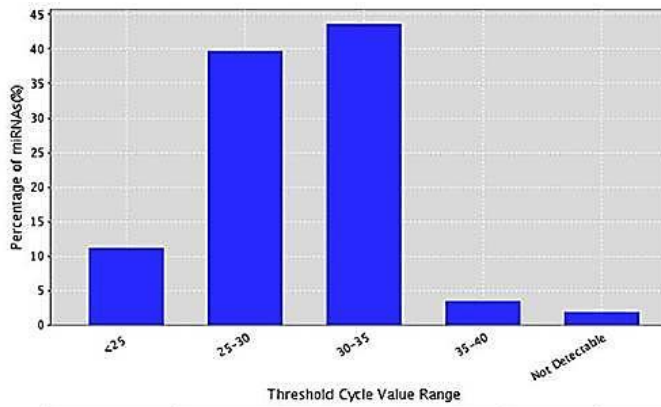


## **Title Page**

**Title:** A Plasma Biomarker Panel of Four MicroRNAs for the Diagnosis of Prostate Cancer

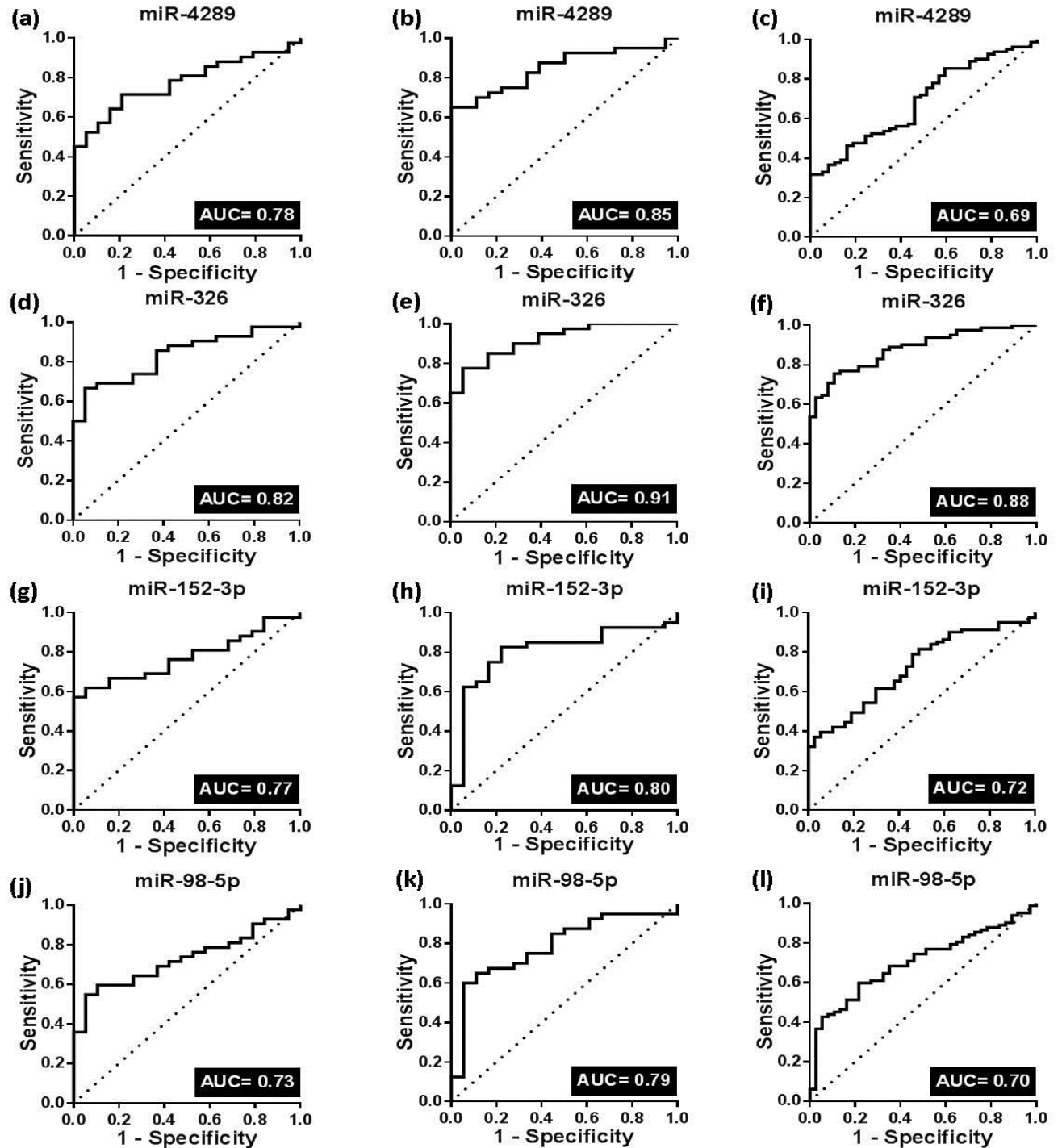
**Authors:** Farhana Matin, Varinder Jeet, Leire Moya, Luke A. Selth, Suzanne Chambers,  
Australian Prostate Cancer BioResource, Judith A. Clements, Jyotsna Batra\*



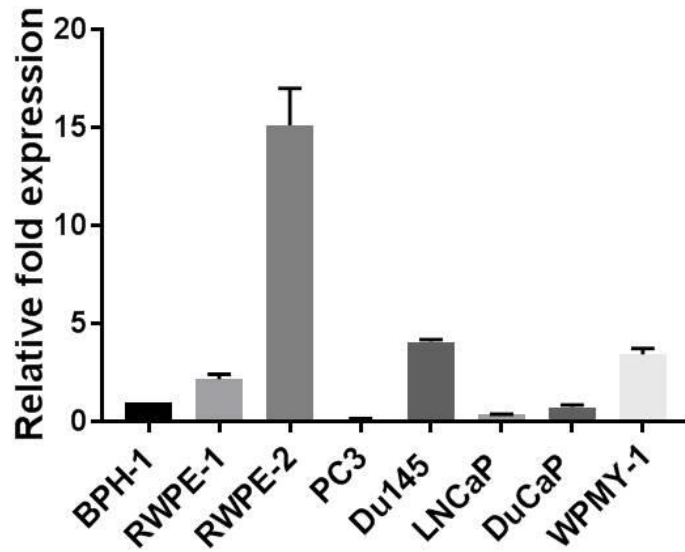
| CT Range                          | Distribution of CT Values |                | Average | ST DEV |
|-----------------------------------|---------------------------|----------------|---------|--------|
|                                   | Control pool-1            | Control pool-2 |         |        |
| <25                               | 39                        | 47             | 43      | 5.66   |
| 25-30                             | 158                       | 147            | 152.5   | 7.78   |
| 30-35                             | 162                       | 173            | 167.5   | 7.78   |
| Absent Calls                      | 25                        | 17             | 21      | 5.66   |
| Percent Distribution of CT Values |                           |                |         |        |
| <25                               | 10.16%                    | 12.24%         | 11.20%  | 0.01   |
| 25-30                             | 41.15%                    | 38.28%         | 39.71%  | 0.02   |
| 30-35                             | 42.19%                    | 45.05%         | 43.62%  | 0.02   |
| Absent Calls                      | 6.51%                     | 4.43%          | 5.47%   | 0.01   |

| CT Range                          | Distribution of CT Values |                | Average | ST DEV |
|-----------------------------------|---------------------------|----------------|---------|--------|
|                                   | Patient pool-1            | Patient pool-2 |         |        |
| <25                               | 72                        | 73             | 72.5    | 0.71   |
| 25-30                             | 170                       | 174            | 172     | 2.83   |
| 30-35                             | 134                       | 128            | 131     | 4.24   |
| Absent Calls                      | 8                         | 9              | 8.5     | 0.71   |
| Percent Distribution of CT Values |                           |                |         |        |
| <25                               | 18.84%                    | 19.01%         | 18.92%  | 0.00   |
| 25-30                             | 44.27%                    | 45.40%         | 44.84%  | 0.01   |
| 30-35                             | 34.89%                    | 33.16%         | 34.03%  | 0.01   |
| Absent Calls                      | 1.99%                     | 2.43%          | 2.21%   | 0.00   |

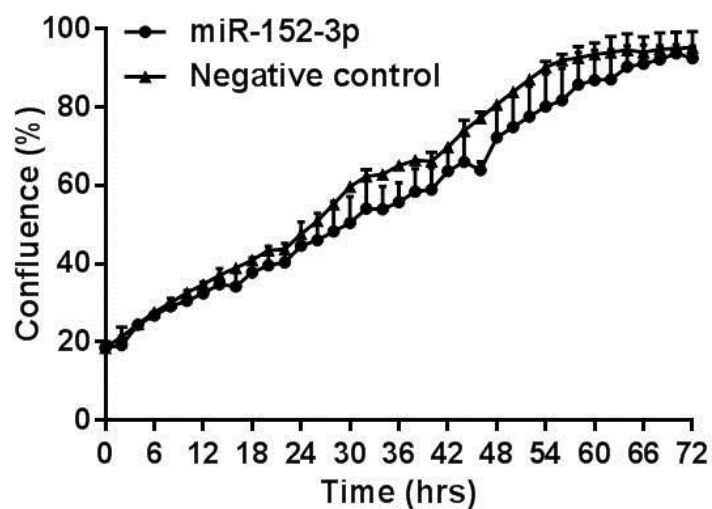
**Supplementary Figure-S1.** The distribution of miRNA  $C_T$  values between the healthy control and patient plasma pools during the screening study. In the control group, ~50% of the miRNAs had  $C_T$  values of <30, with 43.62% of miRNAs being in the 30-35  $C_T$  range, whereas in the patient group, ~65% of the miRNAs had  $C_T$  values <30, with 44.84% of miRNAs being in the 25-30  $C_T$  range. 5.47% miRNAs in the control group and 2.21% miRNAs in the patient group were undetected. The two patient pools are shown as an average of processed data from six pools. The raw unprocessed data from the miRNA PCR array is given in Supplementary Table-S1.



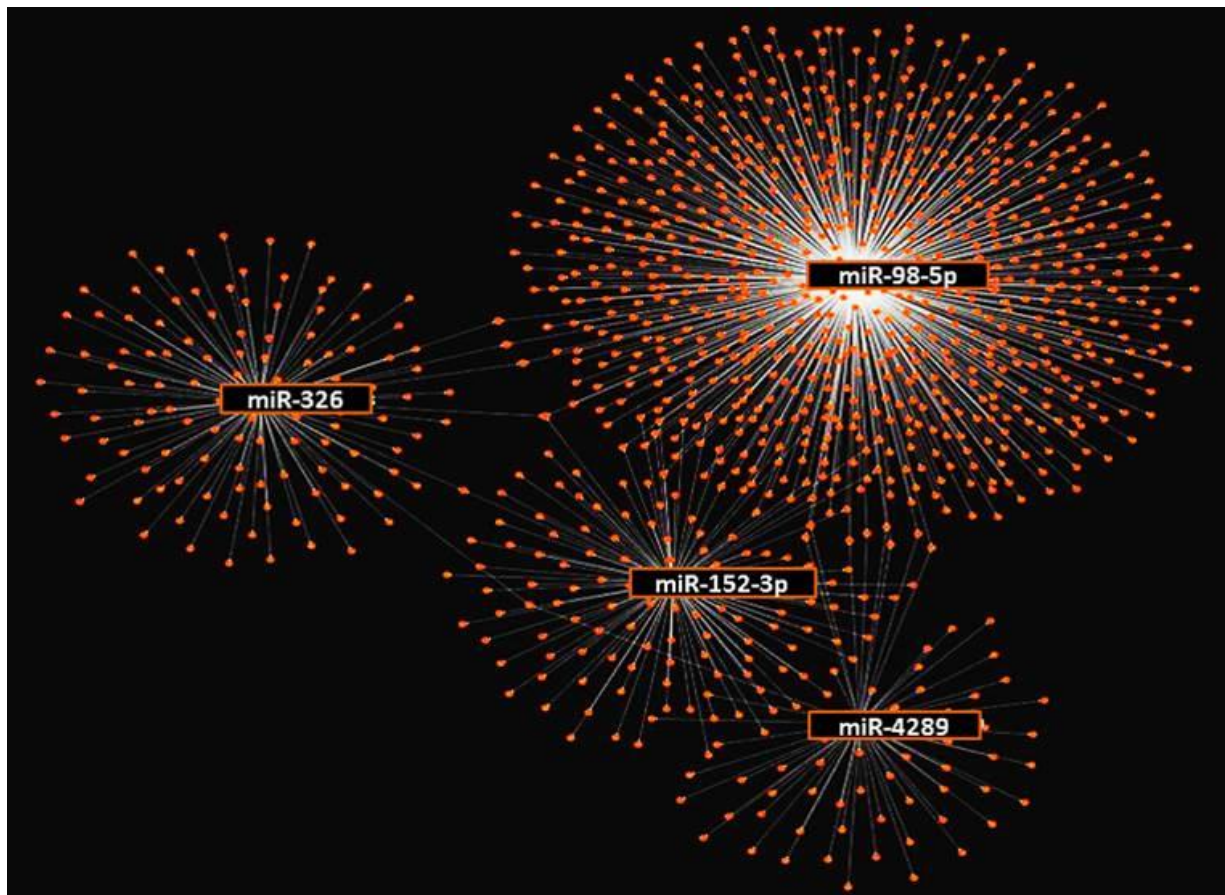
**Supplementary Figure-S2.** ROC curve analysis in the discovery (a, d, g, j), validation (b, e, h, k) and combined (c, f, i, l) cohorts comparing the ability of each miRNA in the panel to identify prostate cancer patients. A combined measure of the sensitivity and specificity of each miRNA in all three cohorts is represented by the Area under the curve AUC ( $p \leq 0.05$ ). The diagonal reference line reflects the performance of the diagnostic test i.e. whether a test yields the positive or negative results by chance or due to a relation with the true disease status.



**Supplementary Figure-S3.** Endogenous expression of miR-152-3p in prostate cell lines. Data was normalised to the housekeeping gene RNU6 and further normalised relative to BPH-1 to determine fold change.



**Supplementary Figure-S4.** Overexpression of miR-152-3p using miRNA mimics in PC3 cells did not affect their proliferative capacity when compared with the negative control treated cells as measured as a change in percentage confluence by the IncuCyte live cell imaging system over a period of 72 hours.

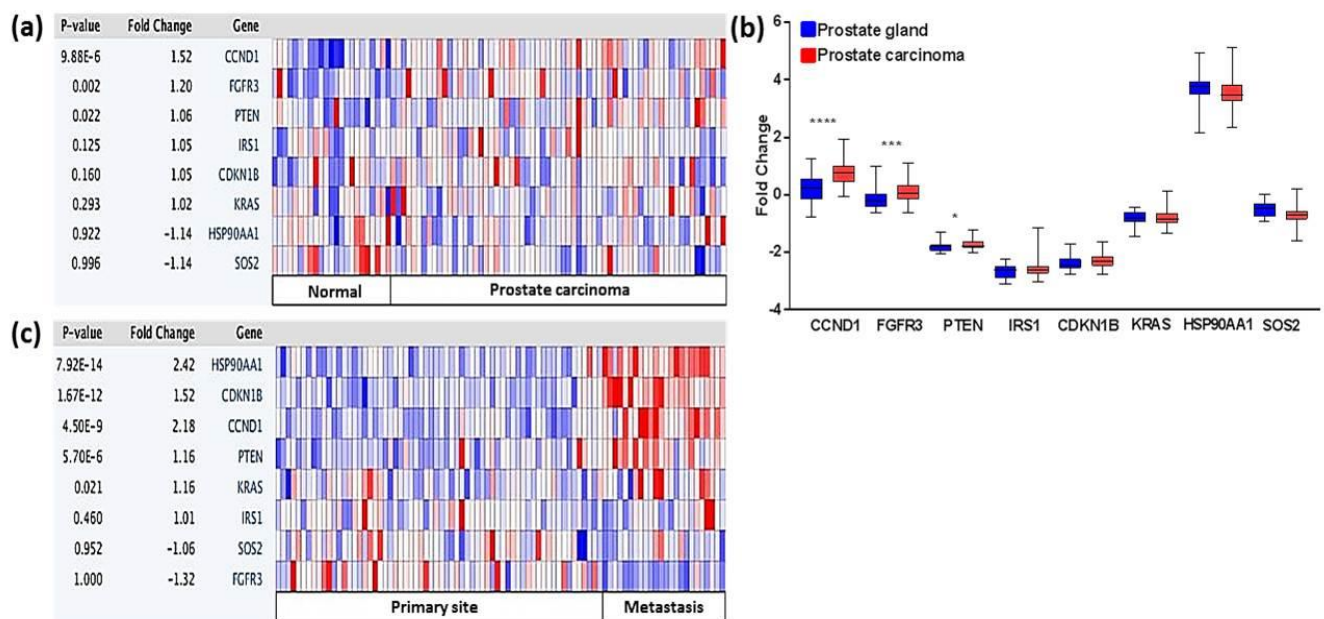


**Supplementary Figure-S5.** *In silico* pathway analysis using the miRNet platform generated a total of 261 candidate pathways and 1055 target genes (represented as round orange nodes) of the 4 miRNAs (default cut-off  $p \leq 1.00$ ).

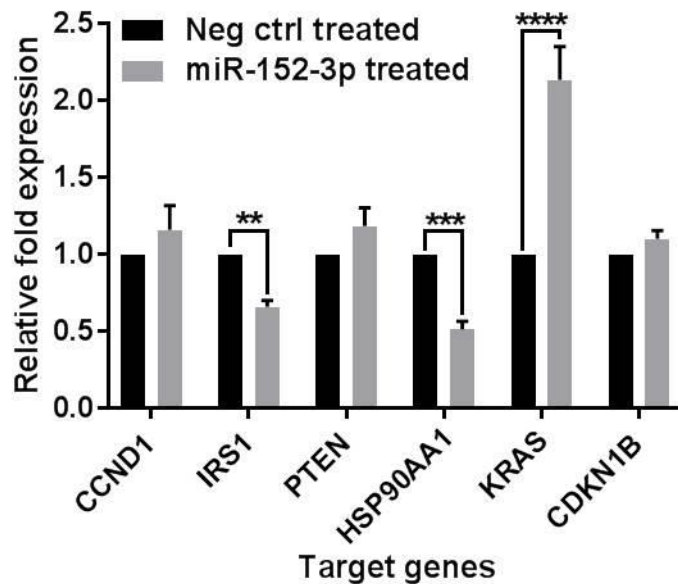


| Entrez Gene Id | Gene Symbol | KEGG_PATHWAYS_IN_CANCER | KEGG_PROSTATE_CANCER | KEGG_GLIOMA | KEGG_ENDOCYTOSIS | KEGG_MELANOMA | KEGG_REGULATION_OF_ACTIN_CYTOSKELETON | KEGG_ENDOMETRIAL_CANCER | KEGG_NON_SMALL_CELL_LUNG_CANCER | KEGG_FOCAL_ADHESION | KEGG_NEUTROPHIL_SIGNALING_PATHWAY | KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY | KEGG_DORSO_VENTRAL_AXIS_FORMATION | KEGG_CHRONIC_MYELOID_LEUKEMIA | KEGG_VIRAL_MYOCARDITIS | KEGG_PROGESTERONE_MEDIATED_OOCYTE_MATURATION | KEGG_MAPK_SIGNALING_PATHWAY | KEGG_ERBB_SIGNALING_PATHWAY | KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION | KEGG_ALLOGRAFT_REJECTION | KEGG_MELANOGENESIS | Entrez Source | Gene Description                                                                         |
|----------------|-------------|-------------------------|----------------------|-------------|------------------|---------------|---------------------------------------|-------------------------|---------------------------------|---------------------|-----------------------------------|------------------------------------------------|-----------------------------------|-------------------------------|------------------------|----------------------------------------------|-----------------------------|-----------------------------|------------------------------------------|--------------------------|--------------------|---------------|------------------------------------------------------------------------------------------|
| 3480           | IGF1R       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | insulin-like growth factor 1 receptor                                                    |
| 3845           | KRAS        |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog                                     |
| 595            | CCND1       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | cyclin D1                                                                                |
| 5728           | PTEN        |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | phosphatase and tensin homolog                                                           |
| 6655           | SOS2        |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | son of sevenless homolog 2 (Drosophila)                                                  |
| 7039           | TGFA        |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | transforming growth factor, alpha                                                        |
| 1027           | CDKN1B      |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | cyclin-dependent kinase inhibitor 1B (p27, Kip1)                                         |
| 3320           | HSP90AA1    |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | heat shock protein 90kDa alpha (cytosolic), class A member 1                             |
| 2261           | FGFR3       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | fibroblast growth factor receptor 3                                                      |
| 2247           | FGF2        |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | fibroblast growth factor 2 (basic)                                                       |
| 7471           | WNT1        |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | wingless-type MMTV integration site family, member 1                                     |
| 7482           | WNT2B       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | wingless-type MMTV integration site family, member 2B                                    |
| 7855           | FZD5        |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | frizzled family receptor 5                                                               |
| 3105           | HLA-A       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | major histocompatibility complex, class I, A                                             |
| 3107           | HLA-C       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | major histocompatibility complex, class I, C                                             |
| 3135           | HLA-G       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | major histocompatibility complex, class I, G                                             |
| 137492         | VPS37A      |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | vacuolar protein sorting 37 homolog A (S. cerevisiae)                                    |
| 79720          | VPS37B      |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | vacuolar protein sorting 37 homolog B (S. cerevisiae)                                    |
| 3696           | ITGB8       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | integrin, beta 8                                                                         |
| 3678           | ITGA5       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | integrin, alpha 5 (fibronectin receptor, alpha polypeptide)                              |
| 8976           | WASL        |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | Wiskott-Aldrich syndrome-like                                                            |
| 8986           | RPS6KA4     |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | ribosomal protein S6 kinase, 90kDa, polypeptide 4                                        |
| 7529           | YWHA8       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide |
| 3667           | IRS1        |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | insulin receptor substrate 1                                                             |
| 51513          | ETV7        |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | ets variant 7                                                                            |
| 890            | CCNA2       |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | cyclin A2                                                                                |
| 81579          | PLA2G12A    |                         |                      |             |                  |               |                                       |                         |                                 |                     |                                   |                                                |                                   |                               |                        |                                              |                             |                             |                                          |                          |                    | S             | phospholipase A2, group XIIA                                                             |

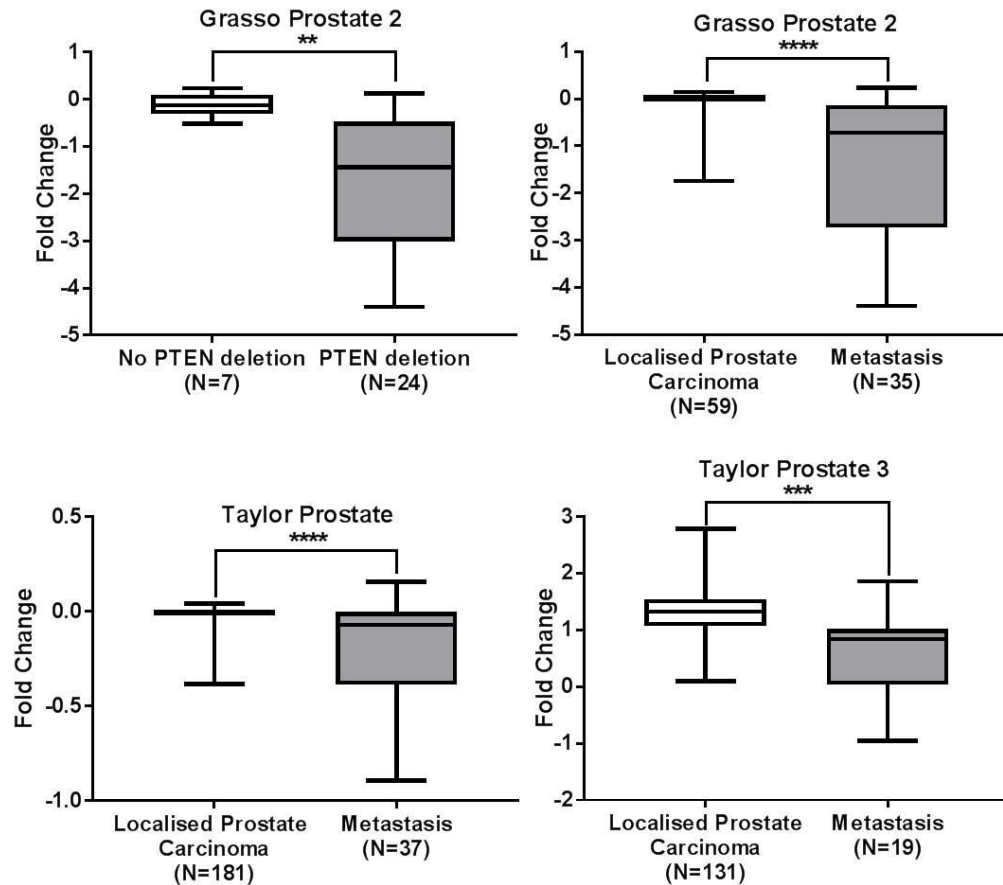
**Supplementary Figure-S6.** Of the eight target genes of miR-152-3p, six i.e. KRAS, CCND1, PTEN, SOS2, CDKN1B and HSP90AA1 (red rectangle) overlapped with GSEA where prostate cancer signalling was found to be the second most important pathway among the top 20 KEGG pathways.



**Supplementary Figure-S7.** Oncomine data analysis of miR-152-3p targets to determine their expression in normal vs prostate carcinoma (a/b), and in primary vs metastatic cancer (c) in the Yu Prostate Cancer Dataset.



**Supplementary Figure-S8.** qRT-PCR analyses of six target genes of miR-152-3p in LNCaP cells after treatment with non-targeting negative control and miR-152-3p mimics for 72 hours. miR-152-3p induced a reduction in IRS1 ( $p= 0.0014$ ) and HSP90AA1 ( $p= 0.0007$ ) mRNA, and increased the expression of KRAS ( $p< 0.0001$ ) in LNCaP cells. Data was normalised to the housekeeping gene RPL32 and further normalised relative to the non-targeting negative control to determine relative fold expression. The differences in target gene expression between the negative control and miR-152-3p treated cells were assessed using an Unpaired t test,  $N=3$  ( $p\leq 0.05^*$ ,  $p\leq 0.01^{**}$ ,  $p\leq 0.001^{***}$  and  $p\leq 0.0001^{****}$ ).



**Supplementary Figure-S9.** (a) Oncomine data analysis of PTEN copy number deletion in prostate carcinoma metastasis in the Grasso Prostate 2 Cancer Dataset ( $p=0.0011$ ). (b) Copy number deletion of PTEN in localised vs metastatic prostate carcinoma in the Grasso Prostate 2 Cancer Dataset ( $p<0.0001$ ). (c) Copy number deletion of PTEN in localised vs metastatic prostate carcinoma in the Taylor Prostate Cancer Dataset ( $p<0.0001$ ). (d) Expression of PTEN in localised vs metastatic prostate carcinoma in the Taylor Prostate 3 Cancer Dataset ( $p=0.0001$ ). Unpaired Mann-Whitney U tests were performed to identify differences in PTEN expression levels ( $p\leq 0.05^*$ ,  $p\leq 0.01^{**}$ ,  $p\leq 0.001^{***}$  and  $p\leq 0.0001^{****}$ ).

**Supplementary Table-S1. (Attached as an Excel file)** The expression level (Ct value) of each miRNA in the PCR-array during miRNA screening from pooled plasma samples.



| Genes Over-Expressed in  |                 |                 |
|--------------------------|-----------------|-----------------|
| Position in PCR array    | Mature ID       | Fold Regulation |
| A06                      | hsa-miR-152-3p  | 2.8014          |
| D21                      | hsa-miR-590-5p  | 3.3123          |
| I01                      | hsa-miR-98-5p   | 2.9646          |
| L24                      | hsa-miR-301a-3p | 4.3104          |
| M05                      | hsa-miR-4289    | 2.6138          |
| M22                      | hsa-miR-1260b   | 2.7533          |
| M24                      | hsa-miR-326     | 2.706           |
| Genes Under-Expressed in |                 |                 |
| Position in PCR array    | Mature ID       | Fold Regulation |
| B08                      | hsa-miR-4732-5p | -2.5856         |
| K11                      | hsa-miR-671-3p  | -2.7969         |
| L07                      | hsa-miR-675-3p  | -2.5589         |
| M01                      | hsa-miR-181d-5p | -2.5826         |

**Supplementary Table-S2.** Of the 372 cancer-associated miRNAs, 11 deregulated miRNAs with a fold regulation cut off of 2.5 were shortlisted for validation in the discovery cohort.

**Supplementary Table-S3. (Attached as an Excel file)** The clinical characteristics of patients and healthy controls in the discovery and validation cohorts.

**Supplementary Table-S4. (Attached as an Excel file)** miRNA expression in prostate tumour (N=52) and adjacent non-malignant (N=52) tissues in the TCGA dataset.

**Supplementary Table-S5. (Attached as an Excel file)** The miRNet web-based platform generated a total 1055 target genes (default cut-off  $p \leq 1.00$ ) of which 65 genes were targeted by miR-4289, 120 genes by miR-326, 131 genes by miR-152-3p and 739 genes by miR-98-5p.

**Supplementary Table-S6. (Attached as an Excel file)** The miRNet web-based platform generated a total of 261 candidate pathways (default cut-off  $p \leq 1.00$ ). Of the 261 pathways, 25 pathways were significant ( $p \leq 0.05$ ) with the prostate cancer pathway being eleventh and the viral carcinogenesis pathway being the topmost in the list.

**Supplementary Table-S7. (Attached as an Excel file)** Ingenuity pathway analysis (IPA) generated a total of 275 canonical pathways for the 131 miRNet predicted targets of miR-152-3p. Prostate cancer signalling was the sixth canonical pathway in the list and

corresponded to 8 deregulated target genes i.e. FGFR3, IRS1, SOS2, HSP90AA1, KRAS, CDKN1B, CCND1 and PTEN of miR-152-3p ( $-\log p = 5.75$ ).

**Supplementary Table-S8. (Attached as an Excel file)** Analysis of molecular function of miR-152-3p target genes confirmed their involvement in cellular processes such as cell cycle, cell morphology, cell growth and proliferation and cell movement.

**Supplementary Table-S9. (Attached as an Excel file)** IPA generated 1055 upstream regulators of the eight target genes of miR-152-3p.