

A genetic variant of *MDM4* influences regulation by multiple microRNAs in prostate cancer

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Abstract

The oncogene *MDM4*, also known as *MDMX* or *HDMX*, contributes to cancer susceptibility and progression through its capacity to negatively regulate a range of genes with tumour-suppressive functions. As part of a recent genome-wide association study it was determined that the A-allele of the rs4245739 SNP (A > C), located in the 3'-UTR of *MDM4*, is associated with an increased risk of prostate cancer. Computational predictions revealed that the rs4245739 SNP is located within a predicted binding site for three microRNAs (miRNAs): miR-191-5p, miR-887 and miR-3669. Herein, we show using reporter gene assays and endogenous *MDM4* expression analyses that miR-191-5p and miR-887 have a specific affinity for the rs4245739 SNP C-allele in prostate cancer. These miRNAs do not affect *MDM4* mRNA levels, rather they inhibit its translation in C-allele-containing PC3 cells but not in LNCaP cells homozygous for the A-allele. By analysing gene expression datasets from patient cohorts, we found that *MDM4* is associated with metastasis and prostate cancer progression and that targeting this gene with miR-191-5p or miR-887 decreases in PC3 cell viability. This study is the first, to our knowledge, to demonstrate regulation of the *MDM4* rs4245739 SNP C-allele by two miRNAs in prostate cancer, and thereby to identify a mechanism by which the *MDM4* rs4245739 SNP A-allele may be associated with an increased risk for prostate cancer.

Key Words

- ▶ *MDM4*
- ▶ microRNA
- ▶ single nucleotide polymorphism
- ▶ prostate cancer

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Introduction

Globally, prostate cancer is the second most common cancer in men (Siegel et al. 2014), with a significant proportion of cases attributed to heritable genetic conditions (Siegel et al. 2014) that influence not only cancer susceptibility but also its clinical course. *MDM4* is an oncogene that negatively regulates *p53* and several other tumour suppressor genes in prostate cancer and a range of

other cancers (Kadokia et al. 2002, Jin et al. 2008, Mancini et al. 2009, Miller et al. 2010). The A-allele of the *MDM4* rs4245739 SNP (A > C, GenBank accession no. AY207458), located in the 3' UTR, was originally shown to be associated with an increased risk of ovarian cancer. Patients homozygous for the A-allele not expressing the oestrogen receptor were shown to have a 4.2-fold

increased risk of recurrence and a 5.5-fold increased risk of tumour-associated death (Table 1; Wynendaele et al. 2010). Results of recent large-scale genome-wide association studies (GWAS) conducted by the Collaborative Oncological Gene-environment Study (COGS) also indicated that the rs4245739 SNP A-allele is associated with an increased risk of prostate and breast cancers (Table 1; Eeles et al. 2013, Garcia-Closas et al. 2013). The A-allele risk association for breast cancer has also been confirmed in a smaller scale study in Chinese populations (Table 1; Liu et al. 2013). Furthermore, this multi-cancer risk locus has also been identified in a smaller scale study of oesophageal squamous cell carcinoma in Chinese populations (Table 1; Zhou et al. 2013). Results of an *in silico* annotation of multiple prostate cancer risk loci indicated *MDM4* rs4245739 to be the causal (functional) SNP at this GWAS identified region, though no experimental evidence has yet been provided to demonstrate the prostatic function of this polymorphism (Hazelett et al. 2013). In ovarian cancer, Wynendaele et al. (2010) previously demonstrated a mechanism of microRNA (miRNA) regulation by which miR-191-5p showed a greater affinity for the *MDM4* rs4245739 SNP C-allele, resulting in higher levels of MDM4 expression from the risk-associated A-allele.

miRNAs are short approximately 19–24 nucleotide non-coding RNA molecules that post-transcriptionally regulate gene expression via mRNA binding (RNA interference). In cancers, miRNAs have been shown to affect several key oncogenic processes including apoptosis, migration and proliferation (Fonseca-Sanchez et al. 2013, Huang et al. 2013, Pinho et al. 2013). Interestingly, widespread dysregulation of miRNAs has been observed

in prostate cancer (Schaefer et al. 2010, Fendler et al. 2011, Wach et al. 2012), and these molecules have potential as both biomarkers and therapeutic targets/treatments (Lichner et al. 2013, Maugeri-Sacca et al. 2013). miR-191 is up-regulated in prostate, breast and colorectal cancers, though its role in these cancers has not been tested (Volinia et al. 2006, Xi et al. 2006, Hui et al. 2009). The capacity of other miRNAs to regulate MDM4 has been demonstrated in breast cancer, although these effects were not SNP-associated (Mandke et al. 2012, Hoffman et al. 2014). As MDM4 has also been proposed as a therapeutic target for several cancers (Garcia et al. 2011, Gembarska et al. 2012), it is important to further explore the MDM4-miRNA axis in prostate cancer.

Herein, we investigated the miR-191-5p/*MDM4* rs4245739 interaction in prostate cancer as well as assessing the potential role of additional miRNAs that are predicted to target this sequence. We report herein that miR-191-5p and miR-887 have a specific affinity for the rs4245739 SNP C-allele in prostate cancer, presenting a mechanism by which the un-targeted A-allele may be associated with an increased risk of prostate cancer.

Materials and methods

Cell lines

Unless otherwise stated (see Acknowledgements), all cell lines were originally sourced from the American Type Culture Collection (ATCC) (www.atcc.org). Cell line authentication (STR profiling) was performed by either the

Table 1 *MDM4* rs4245739 SNP cancer association

Cancer	Reference	Outcome	Risk allele	Cases	Controls	OR/HR (95% CI)	P value
Ovarian	Wynendaele et al. (2010)	Recurrence	A	66	–	HR=4.2 (1.2–13.5)	0.02
Ovarian	Wynendaele et al. (2010)	Tumour-related death	A	66	–	HR=5.5 (1.5–20.5)	0.01
Prostate	Eeles et al. (2013)	Risk	A	25 074	24 272	1.1 (1.05–1.14)	2×10^{-11}
Breast	Garcia-Closas et al. (2013)	Risk	A	4193	35 194	1.14 (1.10–1.18)	2×10^{-12}
Esophageal	Zhou et al. (2013)	Risk (Jinan set)	C	540	550	OR=0.54 (0.35–0.82)	0.004
Esophageal	Zhou et al. (2013)	Risk (Huaian set)	C	588	600	OR=0.68 (0.45–0.99)	0.049
Breast	Liu et al. (2013)	Risk (Jinan Set)	C	1100	1400	OR=0.55 (0.4–0.76)	2.3×10^{-4}
		Risk (Huaian Set)				OR=0.41 (0.25–0.67)	3.1×10^{-4}

Oestrogen receptor-negative ovarian cancer patients homozygous for the *MDM4* rs4245739 A-allele were shown to have a 4.2-fold increased risk of recurrence and a 5.5-fold increased risk of tumour-related death. Larger scale genome-wide association studies have shown that the rs4245739 SNP A-allele is also associated with an increased risk of prostate and breast cancers. This multi-cancer risk locus has also been identified in a smaller scale oesophageal squamous cell carcinoma study in Chinese populations (two case/control sets – Jinan & Huaian) and a smaller scale breast cancer study in Chinese populations (two case/control sets – Jinan & Huaian) indicating that A/C and C/C genotypes were associated with a decreased risk. (OR, odds ratio; HR, hazard ratio). Ratios and 95% CIs were estimated by multivariate Cox regression and logistic regression.

Queensland Institute of Medical Research (Brisbane, Queensland, Australia) or DDC Medical (Fairfield, OH, USA).

miRNA target reporter vector assays

The miRNA-SNP online prediction tool, mirsnpscore available at www.bigr.medisin.ntnu.no/mirsnpscore (Thomas et al. 2011), was used to identify miR-191-5p, miR-887 and miR-3669 (Eeles et al. 2013) all predicted to preferentially target the *MDM4* rs4245739 SNP C-allele. To validate *in silico* predictions for miRNA-SNP affinity, miRNA target luciferase reporter vector assays were performed. Reporter vectors were constructed for the major and minor alleles of the *MDM4* rs4245739 SNP using the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) with *MDM4* 3'-UTR inserts pertaining to predicted miRNA binding (C allele insert 5'-GAACATAAAAATGCATTTATTC~~C~~GTTCACTTA-3', A allele insert 5'-GAACATAAAAATGCATTTATTC~~A~~GTTCACTTA-3') synthesised by Integrated DNA Technologies (Baulkham Hills, New South Wales, Australia). The prostate cancer cell line, PC3 (passage 8), was plated at a density of 50 000 cells/well on a 24-well plate and cultured overnight, transiently transfected with 50 ng vector and 60 nM *mirVana* miRNA Mimics (Life Technologies) using FuGENE transfection reagent (Promega), then analysed 24 h later using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase levels were normalised to Renilla luciferase co-expressed from the same vector. A negative control *mirVana* miRNA Mimic (Negative Control #1 – Life Technologies) was used for analysis alongside candidate miRNAs as was a perfect match positive control miRNA mimic (Customer-defined *mirVana* miRNA Mimic – Life Technologies) designed for the rs4245739 SNP, C-allele – 5'-GUGAACGGAAUAAAUGCAUUUU-3'. miR-331-3p which is not predicted to bind with *MDM4* was used as a second negative control. For a single experiment, each miRNA/vector treatment was cultured in triplicate, then each sample was assayed in duplicate. A total of three independent experiments were performed.

Cell line genotyping

Genomic DNA was isolated from cultured cells using the DNA portion of the AllPrep DNA/RNA/Protein Mini Kit (Qiagen). The rs4245739 region was PCR amplified using the forward primer 5'-tattcatggaaggacggccatct-3' and the reverse primer 5'-gcctaagaactctctgacaggttg-3'. The PCR product was purified using the Wizard SV Gel and PCR Clean-up System (Promega – cat # A9282). Samples were

then sequenced at the Australian Genome Research Facility (St Lucia, Queensland, Australia) using the forward primer 5'-tgccagaagactaagaaggctgg-3'.

Western blotting

The prostate cancer cell lines LNCaP and PC3, and the ovarian cancer cell line OV90 were cultured under standard conditions. Cells were plated (passage 8) at a density of approximately 200 000 cells/well on a six-well plate, cultured overnight, and then transiently transfected with 30 nM of *mirVana* miRNA Mimics using Lipofectamine RNAiMAX transfection reagent (Life Technologies). Forty-eight hours later, total protein was isolated using SDS lysis buffer (1% SDS, 5% glycerol, 10 nM Tris, Roche Complete protease inhibitor), total protein concentration was assessed by the BCA method and 50 µg of total protein was run on a 12% resolving poly-acrylamide gel. Western blotting was performed using Millipore Immobilon FL PVDF membranes with a rabbit anti-MDM4 antibody (Bethyl Labs A300-287A (Bethyl Labs, Montgomery, TX, USA)) diluted 1:3000 and incubated at room temperature for 2 h. Normalisation was performed using either mouse anti β-actin (Abcam – ab8224 (Abcam, Cambridge, UK)) or rabbit anti β-actin (Abcam – ab25894) or mouse anti β-tubulin (Sigma – T4026). Western blots were imaged on an Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA) using fluorescently labelled secondary antibodies (Alexa Fluor 680 and 790 – Invitrogen) with protein band intensities analysed via densitometry using the Odyssey Imaging System software.

RT-qPCR analysis to assess *MDM4* expression

Total RNA was isolated using TRIzol Reagent (Life Technologies) and assessed for quality and quantity using a Nanodrop ND-1000 spectrophotometer. Samples (500 ng) of RNA were reversed transcribed using oligo dT primer. qPCR was then performed using the SYBR Green PCR Master Mix (Life Technologies) in triplicate with β-actin used as an endogenous quantitative normalisation control. Relative expression levels were calculated using the comparative threshold cycle (Ct) method. Primers were synthesised by Integrated DNA Technologies. Primer sequences for *MDM4* were as follows: forward primer 5'-tgaacatttcaccttgccacctg-3' and reverse primer 5'-caacatctgacagtgtctgacagga-3' as described previously (Wynendaele et al. 2010). Primer sequences for β-actin were as follows: forward primer 5'-gcgttacaccttcttgacaaaacct-3' and reverse primer 5'-gctgtcaccttcacgttcca-3'.

Analysis of miR-191-5p and miR-887 expression in human prostate tissue datasets

The expression of miR-191-5p and miR-887 in prostatic tissues was assessed in two datasets, one from The Cancer Genome Atlas (TCGA) and one from Memorial Sloan-Kettering Cancer Centre (MSKCC) (Taylor et al. 2010). For the TCGA dataset, normalised small RNA-seq data from 231 tissue samples (181 tumours and 50 normal prostate tissues), generated on an Illumina HiSeq platform, were downloaded from the web portal (<https://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp>). Expression values for candidate miRNAs for each sample were set to \log_2 of reads per million mapped miRNAs. The MSKCC Agilent microarray dataset, which comprises 99 tumours and 28 normal tissues, was processed as described previously (Selth et al. 2012).

RT-qPCR analysis to assess miRNA expression

Total RNA was extracted from cell lines using TRIzol reagent as described earlier in this study. To assess miRNA expression, RT and qPCR (in triplicate) were performed using the TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Assays (Life Technologies). Relative expression levels were calculated using the comparative Ct method with the small nuclear RNA RNU24 used as an endogenous quantitative normalisation control as it has been identified as being the most consistent option with the least variability for prostate cancer (Carlsson et al. 2010).

Alamar blue cell viability assay

PC3 cells (passage 8) were plated at a density of 10 000 cells/well on a 96-well plate (black, clear-bottom) and cultured overnight, then transiently transfected with 30 nM of *mirVana* miRNA Mimics using Lipofectamine RNAiMAX transfection reagent. Twenty-four hours after transfection, cells were treated with Alamar Blue Cell Viability Reagent (Life Technologies) according to the manufacturer's instructions and fluorescent measurements were taken using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). Positive and negative control miRNA mimics were used as described above.

Androgen and anti-androgen treatment of LNCaP cells

The androgen receptor-positive, LNCaP prostate cancer cell line was treated with androgen (10 nM dihydrotestosterone (DHT)) (Sigma-Aldrich), or therapeutic

anti-androgens (10 μ M bicalutamide) (Selleckchem.com, Waterloo, Australia) for 24 h as described previously (Lai et al. 2014). RNA was extracted from cells using Tri-reagent (Life Technologies), and reverse transcribed using superscript III (Life Technologies).

Statistical and other analyses

Unless otherwise stated, for all analyses, three independent experiments were conducted with results expressed as mean \pm s.d. and analysed using Student's *t*-test with a *P* value of <0.05 considered statistically significant. *MDM4* mRNA expression analysis (eQTL analysis) was conducted using the Genevar web tool (<http://www.sanger.ac.uk/resources/software/genevar/>).

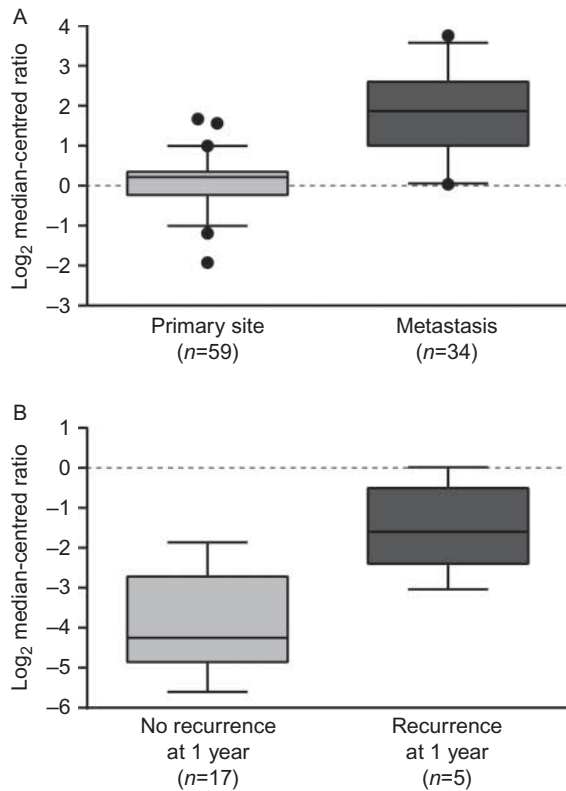
Results

MDM4 expression is associated with metastasis and recurrence after radical prostatectomy

Before functional analysis of the *MDM4* rs4245739 SNP, we mined data from existing databases to assess *MDM4* expression levels in relation to prostate cancer prognosis. Using OncoPrint (www.oncoPrint.org), a dataset comparing *MDM4* mRNA expression in 59 primary prostate cancer tissue samples with 34 metastatic samples (Grasso et al. 2012) revealed a 3.35-fold increase in *MDM4* expression ($P=1.75 \times 10^{-11}$) in the metastatic samples (Fig. 1A). A separate dataset comprising gene expression in primary epithelial cultures derived from tissue explanted from 17 patients undergoing radical prostatectomy (Nanni et al. 2006) was also analysed, revealing a 5.422-fold increase in *MDM4* mRNA ($P=0.002$) in patients whose cancer recurred biochemically 1 year after prostatectomy compared with patients with no recurrence, indicating a potential role of *MDM4* in the development of aggressive disease and relapse after treatment (Fig. 1B).

miR-191-5p and miR-887 have a higher affinity for the *MDM4* rs4245739 SNP C-allele

Using mirnpscore (www.bigr.medisin.ntnu.no/mirsnpscore; (Thomas et al. 2011)), *MDM4* rs4245739 SNP was predicted to affect the binding of three miRNAs to the *MDM4* 3'-UTR (Eeles et al. 2013). These included miR-191-5p, miR-887 and miR-3669, which were all predicted to have a higher affinity for the C-allele (Fig. 2A). Reporter vector assays were performed using PC3 cells to test the validity of these *in silico* predictions. miR-191-5p and

**Figure 1**

MDM4 expression is associated with metastasis and recurrence after radical prostatectomy. (A) OncoPrint was used to analyse datasets comparing *MDM4* mRNA expression for 59 primary prostate cancer tissue samples with 34 metastatic samples showing a 3.35-fold ($P=1.75 \times 10^{-11}$) increase in *MDM4* expression for metastasis (Grasso et al. 2012). (B) OncoPrint was used to analyse datasets for *MDM4* mRNA expression for primary epithelial cultures from tissue explanted from patients undergoing radical prostatectomy, revealing a 5.422-fold increase ($P=0.002$) in *MDM4* expression in primary cell cultures for patients who developed recurrence 1 year post prostatectomy compared with patients with no recurrence (Nanni et al. 2006).

miR-887 induced a 27 and 28% decrease, respectively, in luciferase activity for the MDM4 C-allele construct. In contrast, these miRNAs did not regulate the A-allele construct, indicating that they have a specific affinity for the rs4245739 SNP C-allele (Fig. 2B). No significant changes were observed for miR-3669 with either SNP variant; therefore, this miRNA was not assessed further.

miR-191-5p and miR-887 affects MDM4 protein levels in C-allele-containing cell lines

We then tested whether miR-191-5p and miR-887 were able to affect endogenous MDM4 protein levels in C-allele-containing cell lines. Six model prostate cancer and six model ovarian cancer cell lines were genotyped for the rs4245739 SNP. All prostate cancer cell lines (LNCaP,

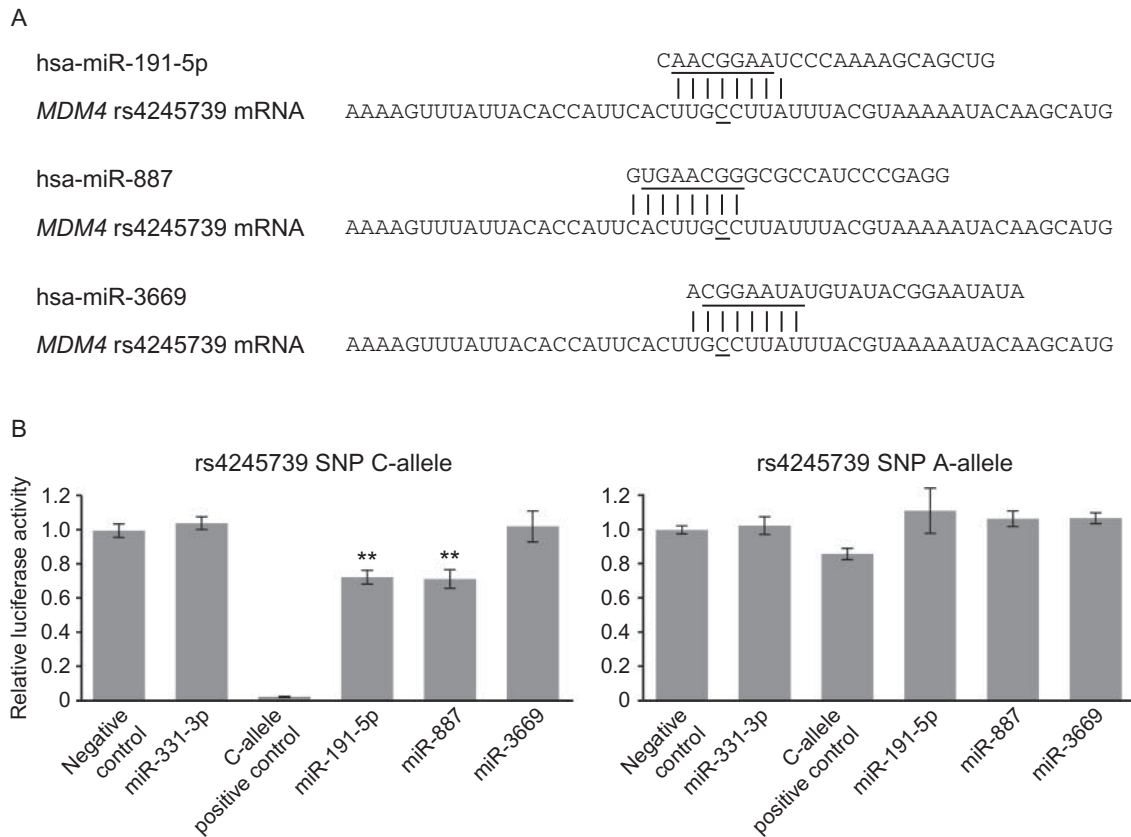
LAPC4, DUCaP, DU145 and 22RV1) were homozygous A/A for the rs4245739 SNP except PC3 cells, which were found to be heterozygous A/C. The ovarian cancer cell lines OAW42, OVMZ6, SKOV3 and OVCAR3 were homozygous A/A while OV90 and CAOV3 were found to be heterozygous A/C (Table 2). Interestingly, there were significantly lower MDM4 protein (Supplementary Figure 1A, see section on supplementary data given at the end of this article) and mRNA (Supplementary Figure 1B) levels in the heterozygous A/C lines (PC3 and OV90) compared with the lines homozygous A/A for the rs4245739 SNP.

To directly assess the influence of miR-191-5p and miR-887 on MDM4 expression, these miRNAs were over-expressed in PC3 and LNCaP cells as models of A/C and A/A genotypes respectively. miRNA mimic transfection led to over-expression of miR-191-5p and miR-887 by > 5000-fold as analysed by RT-qPCR. Over-expression of miR-191-5p and miR-887 resulted in a 38% (P value=0.037) and 52% (P value=0.035) decrease in MDM4 protein, respectively, in PC3 cells (Fig. 3A), whereas no decrease in MDM4 protein was observed following miRNA over-expression in LNCaP cells, which are homozygous A/A (Fig. 3B). The miRNA mimics did not affect *MDM4* mRNA levels in PC3 cells (Supplementary Figure 1C), indicating that they act to inhibit protein translation rather than causing mRNA degradation.

Though miR-191-5p affinity for the C-allele had been reported previously in ovarian cancer, a role for miR-887 had not been tested (Wynendaele et al. 2010). We demonstrated that miR-191-5p along with miR-887 can reduce MDM4 protein levels in an ovarian cancer model using the OV90 cell line heterozygous A/C for the rs4245739 SNP (Supplementary Figure 1D). Collectively, these findings indicate that miR-191-5p and miR-887 preferentially target the *MDM4* rs4245739 SNP C-allele in prostate and ovarian cancers.

miR-191-5p and miR-887 are up-regulated in prostate cancer

Prostatic expression of miR-191-5p has been reported previously, with levels shown to be up-regulated in prostate cancer compared with normal controls (Volinia et al. 2006). To further assess the biological relevance of miR-191-5p and to gain insight into miR-887 in prostate cancer, we first assessed prostatic expression for these miRNAs in a large cohort from TCGA comprising 181 tumours and 50 benign prostate tissues. In this RNA-seq dataset, both miRNAs were readily detectable in benign

**Figure 2**

miR-191-5p and miR-887 directly target the *MDM4* rs4245739 SNP C-allele. miR-191-5p, miR-887 and miR-3669 were predicted by mirnspcore to preferentially target the *MDM4* rs4245739 SNP C-allele. (A) Schematic showing the mRNA sequence for *MDM4* rs4245739 with the C SNP underlined and miRNA sequences showing the miRNA-mRNA nucleotide affinities around the SNP and miRNA seed regions. (miRNA nucleotides 2–8 underlined, referred to as the seed region are thought to be most important for binding affinity) (hsa, *Homo sapiens*). (B) Reporter vector assays using PC3 cells detected 27% ($P=0.0011$) and 28% ($P=0.0026$)

decreases in luciferase levels for miR-191-5p and miR-887, respectively (compared with the negative control miRNA), for the rs4245739 C-allele while no significant changes were observed for the A-allele. (miR-331-3p not predicted to bind with *MDM4* was used as a second negative control). (The perfect match positive control miRNA mimic for the C-allele as expected showed some affinity for the A-allele as only one nucleotide was mismatched. The mismatched nucleotide being found in the seed region is the probable reason for the significantly weaker affinity). Mean \pm s.d., $n=3$ (** $P<0.01$).

and malignant tissues, with miR-191-5p being expressed at considerably higher levels (average 208.5 reads/million mapped miRNA reads; s.d. 111.9; range 32.0–691.1) compared with miR-887 (average 2.3; s.d. 1.7; range 0.25–13.4) (Fig. 4A). In the full TCGA dataset, miR-191-5p was significantly elevated in the tumours compared with normal tissues, but miR-887 was present at similar levels in the two tissues. An examination of a subset of the cohort comprising patient-matched tumour and benign samples ($n=50$ of each) revealed that both miRNAs were elevated in cancer tissues (Fig. 4B). We also examined the prostatic expression of miR-191-5p and miR-887 in another large publicly available cohort from the MSKCC (Taylor et al. 2010), where miR-887 was found to be expressed at higher levels in metastatic tissues compared with normal tissues or primary tumours (Fig. 4C).

In contrast to the TCGA cohort, there was no significant difference in miRNA expression in tumours compared with a patient-matched set of benign samples (Fig. 4D). Collectively, these results indicate that both miR-191-5p and miR-887 are expressed in the normal and malignant prostate, highlighting their potential to play an important role in regulating prostatic *MDM4* expression. To further validate these findings, the expression of miR-191-5p and miR-887 was examined in a range of prostatic cell lines. As expected from the patient data, RT-qPCR demonstrated that miR-191-5p and miR-887 were expressed in these lines (PC3, LNCaP, 22RV1 and RWPE1).

We also assessed if there was any negative correlation between miR-191-5p/miR-887 and *MDM4* mRNA expression in TCGA and MSKCC cohorts, but no such correlation was observed.

Table 2 Genotyping results for the *MDM4* rs4245739 SNP in model prostate cancer and ovarian cancer cell lines

Cancer	Cell line	A/A	A/C	C/C
Prostate	LNCaP	✓		
Prostate	PC3		✓	
Prostate	22RV1	✓		
Prostate	Du145	✓		
Prostate	LAPC4	✓		
Prostate	DUCaP	✓		
Ovarian	OAW42	✓		
Ovarian	OVMZ6	✓		
Ovarian	SKOV3	✓		
Ovarian	OVCAR3	✓		
Ovarian	OV90		✓	
Ovarian	CAOV3		✓	

Model cell lines for prostate and ovarian cancers were genotyped for the rs4245739 SNP. Results are expressed as either homozygous A/A or heterozygous A/C or homozygous C/C.

miR-191-5p and miR-887 inhibit PC3 cell growth

If targeting of MDM4 by miR-191-5p and miR-887 is an important pathway regulating growth in prostate cancer, it would be expected that these miRNAs would act as growth suppressors in prostate cancer. To test this concept, we performed Alamar Blue cell viability assays, using PC3 cells transfected with miRNA mimics. Both miR-191-5p and miR-887 were able to induce a reduction in PC3 cell viability similar to the *MDM4* siRNA positive control, indicating that these two miRNAs can act as tumour suppressors in this cell line (Fig. 5).

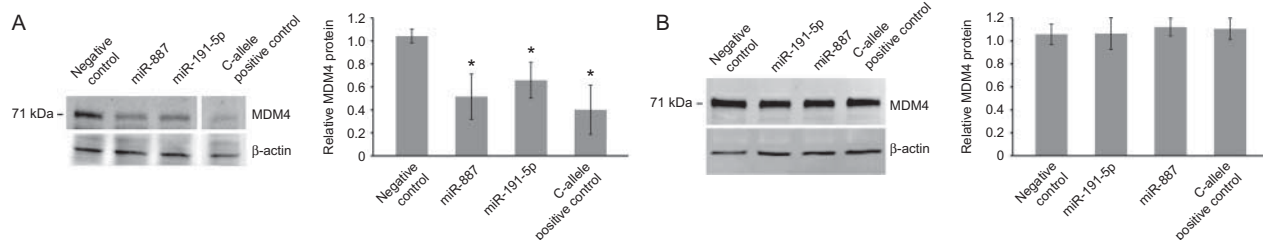
Correlation analysis of the rs4245739 genotype and *MDM4* expression

To assess the biological relevance of the *MDM4* rs4245739 SNP, we conducted a correlation analysis of

its genotype with *MDM4* mRNA expression using various publicly available datasets. Using data from the HapMap3 project (for lymphoblastoid cell line expression), we were able to show for two distinct populations (Caucasians of northern and western European descent and Mexican ancestry) (Stranger et al. 2012) a correlation between *MDM4* mRNA expression and SNP genotype, with the highest expression levels in subjects with the homozygous A/A genotype and the lowest in those with homozygous C/C (European $P=2.7 \times 10^{-5}$, Mexican $P=0.0236$). We also assessed lymphoblastoid cell line expression data from a large nuclear family study (of British decent), which allows analyses focused on heritable expression traits (Liang et al. 2013). A strong correlation of *MDM4* mRNA expression with SNP genotype was observed, which again indicated that the highest expression was associated with the A/A genotype ($n=400$, $P=1.82 \times 10^{-23}$). A third dataset derived from a study in 856 twins (Grundberg et al. 2012) also displayed a similar correlation for *MDM4* mRNA expression with SNP genotype in multiple tissues (highest expression for the A/A genotype – $P=\text{adipose } 1.44 \times 10^{-5}$, lymphocyte 2.16×10^{-18} , skin 1.71×10^{-7}).

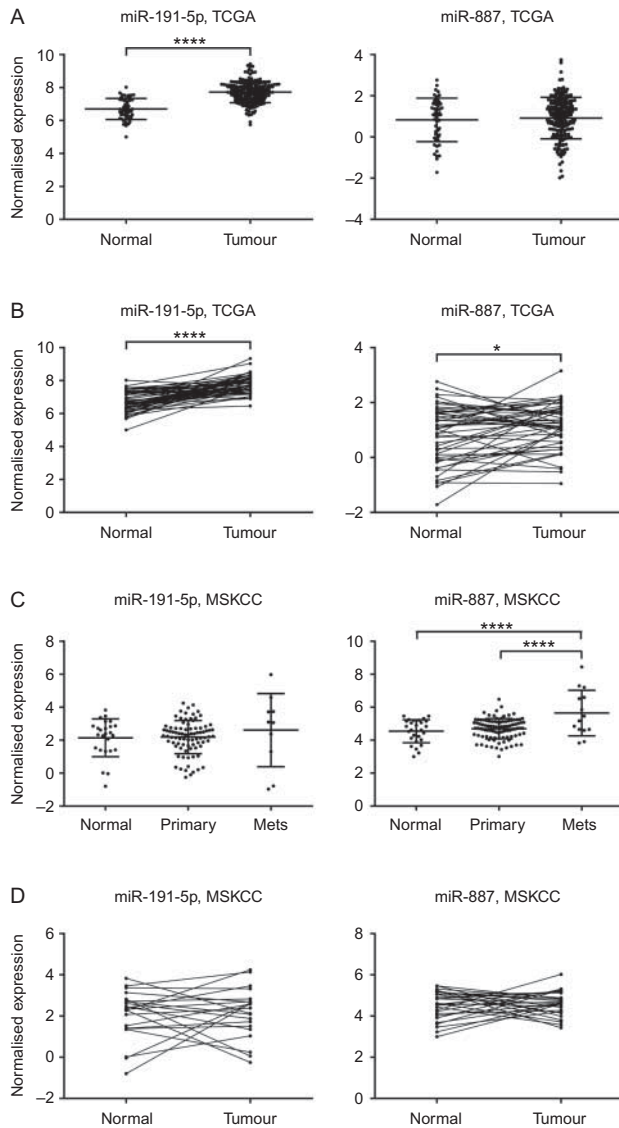
miR-191-5p and miR-887 are not androgen-regulated in LNCaP cells

Given the critical role of androgen signalling in prostate cancer, we tested whether miR-191-5p and/or miR-887 were androgen regulated in the androgen-dependent LNCaP cell line. We did not observe any significant change in the expression of either of the miRNAs in LNCaP cells upon treatment with androgen and/or anti-androgen (Fig. 6).

**Figure 3**

miR-191-5p and miR-887 induce MDM4 protein reduction in PC3 cells heterozygous rs4245739 A/C but not in LNCaP cells homozygous for the A-allele. (A) Western blot analyses in PC3 cells revealed 52% ($P=0.035$) and 38% ($P=0.037$) decreases in MDM4 protein following over-expression of miR-887 and miR-191-5p respectively. (B) No decrease in MDM4 protein was observed following miR-887 and miR-191-5p over-expression in LNCaP cells

or treatment with the C-allele perfect match positive control miRNA mimic. For (A) and (B), representative western blots are shown (left panels). All four treatments shown for the western blot shown in (A) were derived from the same membrane. Right panels show results of densitometry analysis with mean \pm s.d., $n=3$ ($*P<0.05$).

**Figure 4**

Expression of miR-191-5p and miR-887 in prostate cancer. (A and B) Expression of miR-191-5p and miR-887 in normal prostate tissue and tumour samples from the full TCGA cohort (A) or a subset of the cohort with patient-matched normal and tumour tissues (B). (C and D) Expression of miR-191-5p and miR-887 in normal prostate tissue, tumour samples and metastases from the full MSKCC cohort (C) or a subset of the cohort with patient-matched normal and tumour tissues (D). Statistically significant differences were assessed using an unpaired *t*-test (A), paired *t*-tests (B and D) or a one-way ANOVA with Tukey's multiple comparison test (C) (* $P < 0.05$, **** $P < 0.0001$).

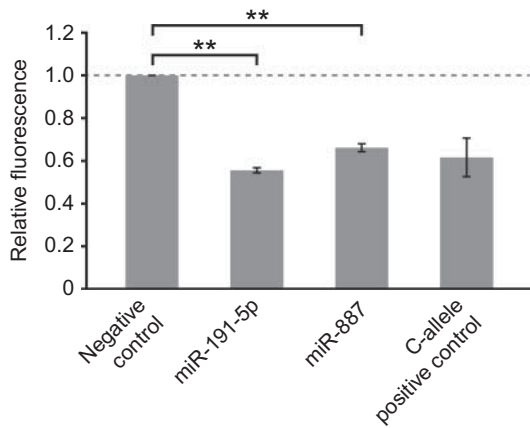
Discussion

MDM4 contributes to cancer susceptibility and progression through its capacity to negatively regulate a range of genes with tumour-suppressive functions. Hence, MDM4 expression levels are a key modulator of these effects.

In this study, we found that increased *MDM4* expression is associated with metastasis and recurrence after radical prostatectomy, highlighting the significance of this gene in prostate cancer (Nanni *et al.* 2006, Grasso *et al.* 2012). To determine how the *MDM4* rs4245739 SNP allele 'A' may be associated with an increased risk of prostate cancers, we assessed the interaction of miR-191-5p and miR-887 with both the alleles of the *MDM4* rs4245739 SNP and found that both these miRNAs have a specific affinity for the C-allele in prostate cancer. We demonstrated that both miRNAs are able to induce a decrease in MDM4 protein expression in PC3 cells (heterozygous A/C) but not in LNCaP cells (homozygous A/A). Homozygous C/C cell lines for prostate cancer were unavailable, which was not surprising considering the minor allele frequency of approximately 0.28 for the C-allele and the increased frequency of the A-allele in prostate cancer as it is associated with an increased risk. However, it has been previously revealed that in esophageal squamous cell carcinoma homozygous C/C as well as the heterozygous A/C rs4245739 genotypes were associated with a decreased risk compared with the A/A genotype, supporting our findings for prostatic cancer regarding the significance of even the heterozygous genotype (Zhou *et al.* 2013).

Furthermore, our results indicated that not only are miR-191-5p and miR-887 expressed in the normal prostate, but are also elevated in some tumours compared with local benign tissue. The MDM4 protein changes induced by both miR-191-5p and miR-887 indicate that the effects induced by miR-887 may be stronger than those induced by miR-191-5p. The effects of any given miRNA on a specific target gene's expression levels will not simply be a function of binding affinity, but will also be related to the expression levels of the given miRNA and its target genes, and the number and transcription levels of any additional genes targeted by the same miRNA. Thus, although expressed at relatively low levels, miR-887 may still significantly affect MDM4 function *in vivo*. Interestingly, the miRNA target prediction algorithm Targetscan (Human release 6.2, www.targetscan.org) predicts 54 putative target genes for miR-191-5p, but only ten for miR-887, indicating that the latter may have fewer genes 'competing' for binding. Hence, both miRNAs probably possess the capacity to significantly affect MDM4 activity in tumours. As putative tumour suppressor miRNAs in prostate cancer, both miR-191-5p and miR-887 may be assessed in future studies as potential candidate biomarkers or therapies.

In this study, we found only one cell line (PC3) to have the 'C' allele for the *MDM4* genotype and we demonstrated

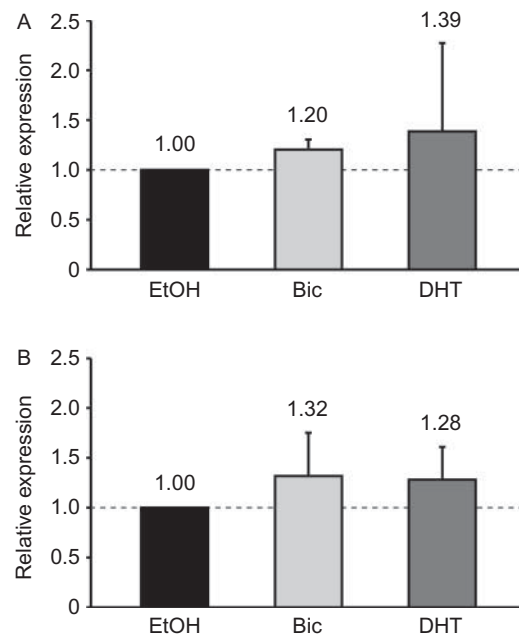
**Figure 5**

miR-191-5p and miR-887 induce reductions in PC3 cell viability. With reference to the negative control miRNA mimic, over-expression of miR-191-5p and miR-887 induced a reduction in cell viability by more than a third ($P=0.0002$, $P=0.001$ respectively), which was also similar to the effect of treatment with the C-allele perfect match positive control miRNA mimic. Mean \pm s.d., $n=3$ (** $P<0.01$).

that both miRNAs are able to induce a decrease in PC3 cell viability, further highlighting their potential as tumour suppressor miRNAs. However, to verify the MDM4-mediated effects of miR-191-5p/miR-887 miRNAs on cell viability, these results need to be replicated in an additional rs4245739 SNP 'C' allele-containing cell line. MDM4 has been shown to negatively regulate the tumour suppressor p53 in prostate cancer and several other cancers (Ramos et al. 2001, Wade et al. 2013, Wang et al. 2013). However, the reduction in cell viability following miR-191-5p/miR-887 over-expression cannot be attributed to enhanced p53 activity as PC3 cells are p53-null (Simone et al. 2013). miR-191-5p and miR-887 may therefore be modulating these effects through additional direct gene targets independent of MDM4. Alternatively, other MDM4-associated pathways may be involved. Given that the cell viability changes for both miRNAs were similar to the MDM4 siRNA-positive control, we believe the latter scenario is likely. Besides p53, MDM4 interacts with several other proteins including p21, a cyclin-dependent kinase inhibitor whose expression is associated with reduced proliferation in prostate cancer (Jin et al. 2008, Lee & Lu 2011, Peng et al. 2013), the transcription factor E2F1, which is known to play a role in cell-cycle regulation in prostate cancer (Strachan et al. 2003, Lee et al. 2013), the E3 ubiquitin ligase MDM2 that also harbours polymorphisms associated with the risk of prostate cancer (Badciong & Haas 2002, Yang et al. 2012) and both Smad3 and Smad4, which are major activating components of the transforming growth factor- β (TGF- β)

signalling pathway that has been shown to repress growth in prostate cancer (Kadakia et al. 2002, Bjerke et al. 2014, Mishra et al. 2014). As MDM4 probably plays a role in cancer susceptibility and progression via multiple pathways, further study is required to assess the prostate-specific effects of miRNA-mediated MDM4 levels on each of these pathways.

Furthermore, we analysed the correlation between MDM4 mRNA and miR-191-5p and miR-887 in the only two publicly available prostate cancer datasets with matched miRNA:mRNA data – one from MSKCC and the other from TCGA. In both of these datasets, there was no correlation between MDM4 mRNA and miR-191-5p/miR-887. This is consistent with the data shown in Fig. 3 and Supplementary Figure 1C, where we observed that these miRNAs reduced the levels of MDM4 protein but not MDM4 mRNA. These results are not surprising given that the miRNA machinery frequently does not affect mRNA levels *in situ* but only inhibits translation of these genes (Bartel 2004). Unfortunately, MDM4 protein expression is not tested in the TCGA or MSKCC datasets; therefore, we were unable to test for negative correlations between the miRNAs and MDM4 protein in large patient cohorts. Nevertheless, using a small panel of prostate and ovarian

**Figure 6**

(Anti)-androgen regulation of miRNAs, (A) miR-191-5p (B) miR-887, in LNCaP prostate cancer cells. LNCaP cells were treated with either ethanol (Mock) or 10 μ M anti-androgen (bicalutamide (Bic)) or 10 nM androgen (DHT) for 24 h. Data are expressed as the mean \pm s.e.m., which is a mean of $n=3$ analysed in triplicates.

cancer cell lines, we assessed endogenous MDM4 protein levels and detected significantly lower MDM4 levels in the heterozygous A/C lines (PC3 and OV90) compared with the lines homozygous A/A for the rs4245739 SNP, possibly at least in part reflecting endogenous miR-191-5p/miR-887 regulation of MDM4 expression via the C-allele in these heterozygous cell lines. A similar but weaker pattern was observed for *MDM4* mRNA genotype-specific expression. Large-scale studies, using larger cell line panels and patient tissues, are now required in order to determine the true significance of correlations between MDM4 protein expression and genotype for prostatic tissues.

We also assessed *MDM4* expression correlation with genotype in familial, multi-tissue and multi-population cohorts, revealing a strong correlation between the rs4245739 C-allele and lower *MDM4* mRNA expression in multiple non-tumour cells. These observations are further emphasised by a recent large-scale study genotyping approximately 50 000 SNPs in approximately 2100 genes, which revealed significant correlations between mRNA expression and the MDM4 SNP rs2169137 which is in linkage disequilibrium with rs4245739 SNP (Ganesh et al. 2013).

Finally, in an effort to identify the mechanism of regulation of the two identified miRNAs, we analysed effects of androgen and anti-androgen on their expression. We did not observe any significant effects of either DHT or bicalutamide on the expression of miR-191-5p or miR-887. Therefore, the mechanism by which these miRNAs are regulated in prostate cancer remains to be elucidated in future studies.

In conclusion, herein, we identified an association between the *MDM4* rs4245739 SNP and two miRNAs, miR-191-5p and miR-887. This not only validates findings from studies of ovarian cancer but also identifies new modes of MDM4 regulation in prostate cancer. Specifically, the down-regulation of MDM4 in C-allele-containing prostate cancer-derived cells induced by miR-191-5p and miR-887 presents a mechanism by which the un-targeted A-allele may be associated with an increased risk of prostate cancer.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0013>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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