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The miR-15a-miR-16-1 Locus is Homozygously Deleted in a Subset of Prostate Cancers

Kati P. Porkka,[1] Erinn-Lee Ogg,[1] Outi R. Saramäki,[1] Robert L. Vessella,[2] Heidi Pukkila,[3] Harri Lähdesmäki,[4] Wytske M. van Weerden,[5] Maija Wolf,[6] Olli P. Kallioniemi,[6] Guido Jenster,[5] and Tapio Visakorpi[1]*

[1]Institute of Medical Technology, University of Tampere and Tampere University Hospital, Tampere, Finland
[2]University of Washington Medical Center, N.E. Pacific Seattle, WA
[3]Department of Signal Processing, Tampere University of Technology, Tampere, Finland
[4]Department of Information and Computer Science, Aalto University, Helsinki, Finland
[5]Department of Urology, Josephine Nefkens Institute, Erasmus University Medical Center, Rotterdam, The Netherlands
[6]Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Helsinki, Finland

MicroRNAs (miRNAs) are small, non-coding RNAs that negatively regulate the expression of protein coding genes. In this study, we screened highly informative prostate cancer cell lines and xenografts ($n = 42$) for miRNA gene copy number and expression changes. The expression profiling showed distinction between cell lines and xenografts as well as between androgen sensitive and independent models. Only a few copy number alterations that were associated with expression changes were identified. Most importantly, the miR-15a-miR-16-1 locus was found to be homozygously deleted in two samples leading to the abolishment of miR-15a, but not miR-16, expression. miR-16 is also expressed from another genomic locus. Mutation screening of the miR-15a-miR-16-1 gene in the model systems as well as clinical samples ($n = 50$) revealed no additional mutations. In conclusion, our data indicate that putative tumor suppressors, miR-15a and miR-16-1, are homozygously deleted in a subset of prostate cancers, further suggesting that these miRNAs could be important in the development of prostate cancer. © 2011 Wiley-Liss, Inc.

INTRODUCTION

MicroRNAs (miRNAs) are small, non-coding RNAs that negatively regulate expression of protein coding genes. A growing number of miRNAs has been implicated in cancer, acting either as tumor suppressive or oncogenic miRNAs (Esquela-Kerscher and Slack, 2006). Genomic aberrations, such as amplifications, deletions, translocations, and mutations, can activate oncogenes and inactivate tumor suppressor genes in a cancer cell. So far, genomic alterations of only few miRNAs have been reported. The first miRNAs that were found to be involved in cancer were miR-15a and miR-16, which were shown to be commonly deleted in chronic lymphocytic leukemia (Calin et al., 2002). These miRNAs are located in a genomic cluster miR-15a-miR-16-1, at the chromosomal region 13q14.3, which is commonly deleted in leukemias, lymphomas and several solid cancers, such as prostate cancer. In a recent study, 22 miRNAs were shown to be often amplified or deleted in hepatocellular carcinoma (Ding et al., 2010). In addition, miR-31 has been shown to be homozygously deleted in malignant mesothelioma (Ivanov et al., 2010). Amplification of a well-established oncogenic miRNA cluster, miR-17-92, has been detected in lymphomas and various solid tumors (Hayashita et al., 2005; He et al., 2005; Rinaldi et al., 2007; Diosdado et al., 2009). In addition, a combined gene copy number and expression analysis has revealed correlation of increased gene copy number and expression of eight miRNAs (miR-30d, miR-139, miR-17-3p, miR-17-5p, miR-18, miR-19a, miR-20a, and miR-122a) in mantle cell lymphomas (Navarro et al., 2009). In prostate cancer, the genomic locus of miR-101 has been found to be deleted (Varambally et al., 2008). In addition to gene copy number variations, mutations can activate or inactivate cancer-related genes. However, mutations of miRNAs have rarely been discovered. In a study that cataloged somatic mutations of the colon cancer cell line COLO-829, one somatic substitution was found in mir-518d (Pleasance

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*Correspondence to: Tapio Visakorpi, Institute of Medical Technology, FIN-33014 University of Tampere, Tampere, Finland. E-mail: tapio.visakorpi@uta.fi

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et al., 2010). In clinical cancers, no mutations of miRNA genes have been described.

The role of miRNAs in prostate cancer, as well as in other cancers, has been extensively studied during the past few years. miRNA expression profiles have been analyzed in several studies (Lu et al., 2005; Mattie et al., 2006; Volinia et al., 2006; Porkka et al., 2007; Ambs et al., 2008; Tong et al., 2008; Schaefer et al., 2010). The possible use of miRNAs as biomarkers in prostate cancer has also been investigated (Mattie et al., 2006; Mitchell et al., 2008; Schaefer et al., 2010). Several miRNAs, such as miR-15a and miR16, have been shown to have tumor suppressor function in prostate cancer (Bonci et al., 2008). Also, miRNAs showing oncogenic potential in prostate cancer, such as miR-21 and miR-125b, have been reported (Ribas et al., 2009; Shi et al., 2010).

We have previously studied miRNA expression (Porkka et al., 2007) as well as epigenetic regulation and androgen regulation of miRNAs in prostate cancer (Rauhala et al., 2010; Waltering at al., 2010). In this study, expression and copy number variations of miRNA genes in prostate cancer cell lines and xenografts were analyzed. In addition, mutation screening of miR15a-16-1 was carried out.

MATERIALS AND METHODS

Cell Line, Xenograft, and Tissue Samples

Prostate cancer cell lines PC-3, LNCaP, DU145, and 22Rv1 were obtained from the American Type Culture Collection (Manassas, VA). LAPC4 and VCaP cell lines were provided by Dr. Charles Sawyers (University of California at Los Angeles, Los Angeles, CA) and Dr. Jack Schalken (Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands), respectively. All cell lines were cultured under recommended conditions.

Freshly frozen LuCaP prostate cancer xenograft samples (LuCaPs 23.1, 23.8, 23.12, 35, 35AI, 41, 49, 58, 69, 70, 73, 77, 78, 81, 86.2, 92.1, 93, 96, 96AI, 105, and 115) were made available for the analyses by one of the co-authors (R.L.V.). Genetic characterization of these xenografts has been reported previously (Laitinen et al., 2002; Saramäki et al., 2006). Freshly frozen PC prostate cancer xenograft samples (PC82, 133, 135, 295, 310, 324, 329, 339, 346, 346I, 346B, 346BI, 347, and 374F) as well as a cell line (PC346c) were also made available for the analyses by one of the co-authors (WvW.). The characteristics of these xenografts have been reported previously (van Weerden et al., 2000; Hendriksen et al., 2006; Marques et al., 2006).

Freshly frozen clinical tumor samples from 36 untreated prostate carcinomas and 14 castration-resistant prostate carcinomas were obtained from Tampere University Hospital (Tampere, Finland). The use of clinical specimens had been approved by the Ethical Committee of the Tampere University Hospital, and written informed consent has been obtained from the patients.

miRNA Microarray Expression Analysis

Total-RNA was extracted from the cell line and xenograft samples by using the *mir*Vana^{TM} miRNA Isolation Kit (Ambion). Before labeling and hybridization of the samples, the integrity of the extracted RNAs was analyzed with the Agilent 2100 Bioanalyzer by using the RNA 6000 Nano Kit (Agilent Technologies). Hybridizations were carried out by using the Agilent Human miRNA Microarrays (Version2, Agilent Technologies), with the miRNA labeling reagent and hybridization kit (Agilent Technologies). The hybridization signals were detected by using the Agilent G2565BA Microarray Scanner System with the Agilent G2567AA Feature Extraction Software (Agilent Technologies). Further data analysis was carried out by using the GeneSpring (v7.3.1) Expression Analysis software (Agilent Technologies). The signal values were normalized by 75th percentile. In clustering analysis of the samples, the "Distance" algorithm was used. All microarray data have been submitted using MIAMExpress to the ArrayExpress database (accession number E-MEXP-2966.)

miRNA Gene Copy Number Analysis

The aCGH profiles of the cell lines and the LuCaP xenografts were obtained from the hybridization of the 244K oligo arrays (Agilent Technologies). The miRNA gene copy number analysis of the data was carried out by using the DNA Analytics 4.0 software (Agilent Technologies). A miRNA gene was considered to be homozygously deleted or highly amplified if the average of three successive oligos surrounding the miRNA locus showed Log2 ratio value lower than −2.5 or higher than +2.5, respectively. The miRNA gene copy number data from the PC xenografts, and from four (LNCaP, 22Rv1, VCaP, and LAPC4) of the cell lines, were obtained from 1M SNP Illumina (Illumina) hybridization analyses. The data was filtered and screened for

miRNA gene copy number alterations. A miRNA gene was considered homozygously deleted or highly amplified if the median value of ten SNPs surrounding the miRNA locus showed the copy number value (CNV) lower than −2 or higher than +2, respectively.

Quantitative Real-time RT-PCR

The miRNA microarray expression results were validated by using the TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA). Briefly, 50 ng of total RNA from each sample was reverse transcribed with miR-15a and miR-16 specific RT primer using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). As a normalization control, miR-103 was used, because it showed non-differential expression across all the samples. qPCR detection was done with miRNA specific fluorescent probes (Applied Biosystems) using the LightCycler FastStart DNA Master HybProbe Kit (Roche Applied Science, F. Hoffman –La Roche, Basel, Switzerland) with the LightCycler qPCR instrument.

FISH Analysis

Human genomic BAC clone RPCI-11-153K13 HS (Invitrogen), containing the miR-15a-miR-16-1 gene cluster, at 13q14.3, was used for the gene copy number analysis by FISH. The probe was labeled with Alexa-fluor-dUTP by nick translation. A PAC 84A6 clone containing the *ETB* gene at 13q22 was used as a reference probe. The probe was labeled with digoxigenin-dUTP. The specificity of the probes was first confirmed by hybridization of metaphase chromosome preparations from the DU145 cell line prepared using routine techniques. After that, 5 μm sections from freshly frozen xenograft tumor blocks were fixed on objective slides and dual-color hybridized essentially as described previously (Hyytinen et al., 1994). After hybridization and washes, the slides were stained with anti-digoxigenin-FITC and counterstained with an antifade solution (Vectashield; Vector Laboratories, Burlingane, CA) containing 4,6-diamidino 2-phenylindole. The FISH signals were detected using an Olympus BX50 epifluorescence microscope (Tokyo, Japan).

Mutation Analysis

Mutation analysis of the miR-15a-miR-16-1 locus at chromosome band 13q14.3 was carried out by direct sequencing of the DNA extracted from six cell lines (PC-3, LNCaP, DU145, 22Rv1 LAPC4, and VCaP), from 32 freshly frozen xenograft and 50 clinical tumor samples. Whole genomic DNA obtained from clinical prostate carcinoma samples was first amplified to increase the overall yield of DNA using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) and 10 ng of original DNA template. The genomic area containing the precursor sequences of both miR-15a and miR-16-1 was PCR amplified from all samples (primer sequences 5′-TGG TTA AGT GTG ACG TTT TGA CAA CCA-3′and 5′-TGT GCT GGG CAC AGA ATG GAC T-3′). The PCR products were purified with QIAquick PCR purification columns (Qiagen, Valencia, CA) and the sequencing reactions in both directions were carried out with nested primers (sequences 5′-TTC AGT TAA GTT TTT GAT GTA GAA ATG-3′ and 5′-TGA AAA GAC TAT CAA TAA AAC TGA AAA-3′) using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit. The samples were sequenced with the ABI 3130*xl* sequencer.

Statistical Analyses

Association between miRNA expression and copy number variations (CNV) values was analyzed using the Pearson correlation. Correlations were calculated for each miRNA expression and CNV value of the corresponding genomic locus. miRNA expression values were 75th percentile normalized using the GeneSpring (v7.3.1) Expression Analysis software (Agilent Technologies). For the PC xenograft samples, a CNV value for each miRNA locus was obtained by calculating the median value of 10 closest probe sets. For the LuCaP xenograft samples, CNV values were quantile normalized, and for each miRNA locus the CNV value was obtained by calculating the median value of three closest probe sets. PC and LuCaP xenograft samples were analyzed separately and combined P values were computed with the Fisher's method. The P values were corrected for multiple testing with the Benjamini and Hochberg method.

RESULTS

Clustering Analysis

The cell line and xenograft samples were first clustered based on their miRNA expression profiles using the hierarchical clustering method as implemented in GeneSpring's clustering algorithm "Distance." In the resulting hierarchical tree, different sample types were clustered separately from

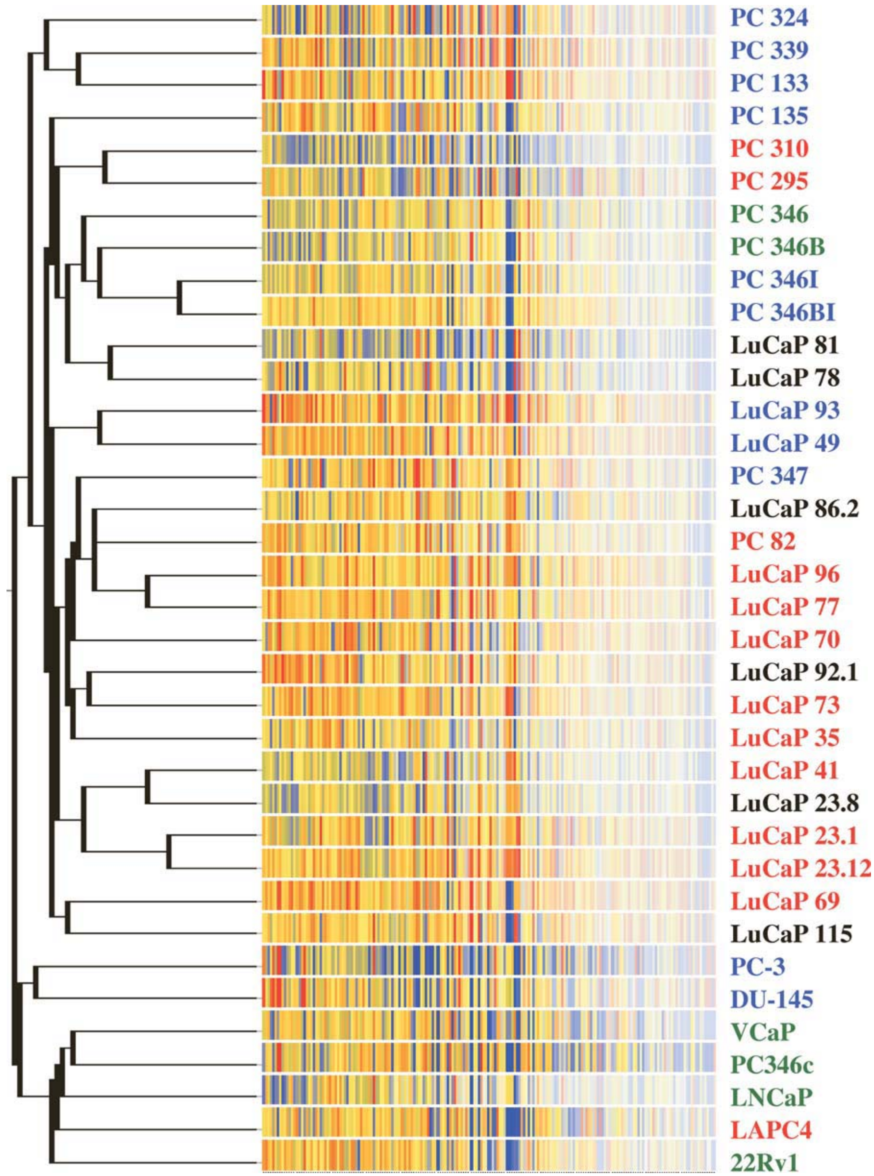

Figure 1. Clustering analysis of prostate cancer cell lines, LuCaP xenograft, and PC xenograft samples. The cell lines form a cluster of their own, separated from the xenograft samples. The two xenograft series (LuCaP and PC) are also mostly clustered in separate subclusters. Androgen status of the samples is indicated by color-coding: blue = androgen-independent, green = androgen-sensitive, red = androgen-dependent, and black = unknown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

each other. The cell lines (22Rv1, LAPC4, LNCaP, PC346c, VCaP, DU145, and PC-3) formed a subcluster of their own that was separated from the xenograft samples, and the samples of the two xenograft series (the LuCaP and the PC series) were mostly, although not completely, clustered in separate subclusters (Fig. 1). In addition, the androgen-independent samples were separated from the androgen-sensitive and androgen-dependent ones. For example, the two androgen receptor (AR) negative, and therefore completely androgen-independent cell lines, PC-3 and DU145, formed a pair that is separated from the AR-positive cell lines (22Rv1, LAPC4, LNCaP, PC346c, and VCaP). Also, the androgen-independent xenografts formed nodes of their own (e.g., LuCaP49 and 93; PC346BI and PC346I; PC133, PC135, PC339, and PC324), separately from the androgen-dependent and androgen-sensitive xenografts within the PC and LuCaP xenograft subclusters (Fig. 1).

miRNA CNV

First, the aCGH data from six prostate cancer cell lines and the 18 LuCaP prostate cancer

TABLE 1. miRNA Genes Showing Homozygous Deletions or High-Level Amplifications in the LuCaP and PC Xenografts

miRNA name	Chromosomal location	Samples
Homozygous deletions		
miR-15a	13q14.3	LuCaP 86.2; PC324
miR-16–1	13q14.3	LuCaP 86.2; PC324
miR-562	2q37.1	PC-329
miR-626	15q15.1	PC-135
miR-744	17p12	PC-324
High-level amplifications		
miR-223	Xq11.1-q12	LuCaP 35
miR-339	7p22.3	LuCaP 105
miR-599	8q22.2	LuCaP 35AI
miR-661	8q24.3	LuCaP 105
miR-875	8q22.2	LuCaP 35AI

xenografts (LuCaP23.1, 23.8, 23.12, 35, 35AI, 41, 69, 70, 73, 77, 78, 81, 86.2, 92.1, 96, 96AI, 105, and 115) obtained with Agilent-platform was analyzed for detection of homozygous deletions (HD) or high-level amplifications of miRNA loci. The alterations detected are summarized in Table 1. HD of miRNA loci was detected in only one LuCaP xenograft (LuCaP86.2), which showed deletion of the miR-15a-miR16-1 locus. High-level amplifications were detected in three LuCaP xenografts, showing the amplifications of miR-223 (LuCaP35), miR-599 and miR-875 (LuCaP35AI), as well as miR-339 and miR-661 (LuCaP105). No HD or high-level amplifications of miRNA genes were detected in the cell lines.

The 1M SNP Illumina data from 12 PC xenografts (PC82, 133, 135, 295, 310, 324, 329, 339, 346, 346B, 374, and 374F) was screened for HD and high-level amplifications of miRNA loci. The alterations detected are summarized in Table 1. Interestingly, the miR-15a-miR-16-1 gene that showed HD in LuCaP 86.2 was detected to be homozygously deleted also in PC324. In addition, HDs of three other miRNAs were detected: miR-562 (PC329), miR-626 (PC135), and miR-744 (PC324). No high-level amplifications of the miRNA genes were detected in the PC xenografts. As with the aCGH analysis, the cell lines studied by the 1M SNP Illumina analysis (LNCaP, 22Rv1, VCaP, and LAPC4) did not show HD or high-level amplifications of miRNA genes.

Association of miRNA CNV and Expression

Association of miRNA CNV and expression was studied in PC and LuCaP xenografts by using the Pearson correlation. The two xenograft series were analyzed separately, because the copy number data was obtained with different platforms (for the LuCaP xenografts with 244K oligo arrays and for the PC xenografts with 1M SNP Illumina hybridization), which created high variation between the two data sets. In both PC xenograft series, miR-15a and miR-744 showed statistically positive correlation between gene copy number and expression at the false positive rate (FDR) threshold of 0.05 (corrected P values 0.015 for both miRNAs). In the LuCaP xenograft series, miR-191, and let-7g showed statistically significant positive association between the miRNA gene copy number and the expression (corrected P values 0.00056 and 0.014, respectively). Also, miR-15a was found to be marginally significant with corrected P value of 0.07. If the two xenograft series were combined for the association analysis, statistically significant association was found between gene copy number and expression of miR-15a, miR-191, and miR-744 (corrected P values 0.00016, 0.00016, and 0.0078, respectively).

Next, we concentrated on miRNAs that showed either HD or high-level amplification. Of the 10 miRNAs that showed HD (miR-15a, miR-16, miR-562, miR-626, and miR-744) or high-level amplifications (miR-223, miR-339, miR-599, miR-661, and miR-875) in at least one of the xenografts, six miRNAs (miR-339, miR-599, miR-661, miR-562, miR-626, and miR-875) showed no detectable expression in any of the xenografts. miR-15a and miR-16 were generally expressed at high level in the xenografts studied, whereas in miR-223 at moderate and miR-774 at low level. The expression and copy number values of miR-15a, miR-16, and miR-223 in the LuCaP xenografts and of miR-15a, miR-16, and miR-744 in the PC xenografts are shown in Figure 2. Xenografts with the miR-15a locus HD (LuCaP 86.2 and PC324) showed low expression values, whereas no such association was seen with miR-16, which is located in the same genomic cluster. The high-level amplification (miR-223) did not show association of the copy number and expression (Fig. 2). The expression of miR-744 showing putative HD was generally so low that it was considered unreliable for qRT-PCR analysis and was therefore excluded.

Confirmation of the HD of miR-15a-16-1 Loci

The HDs of miR-15a-miR-16-1 locus detected in LuCaP 86.2 and PC324 by aCGH and 1M

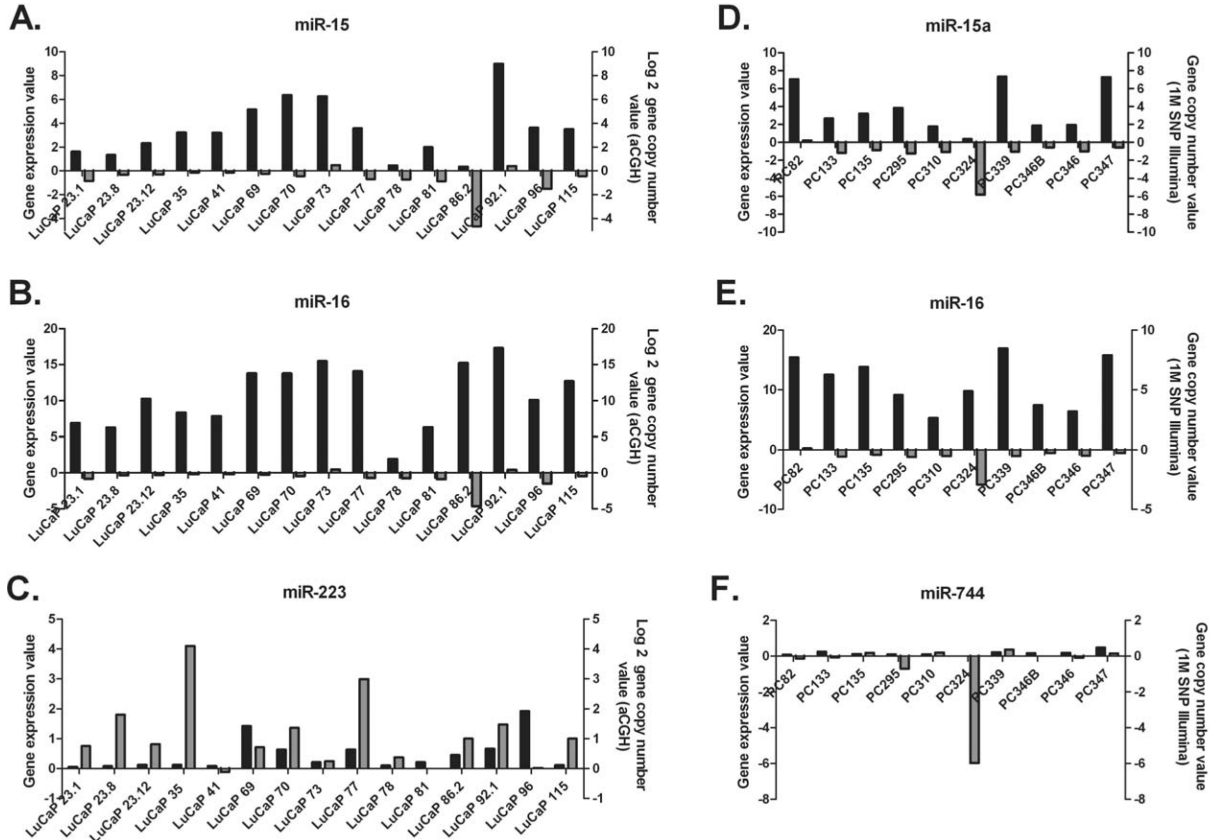

Figure 2. Combined copy number and expression analysis of miRNAs in LuCaP and PC prostate cancer xenograft samples. Copy number and expression values of (A) miR-15-a, (B) miR-16, and (C) miR-223 in LuCaP xenografts as well as (D) miR-15a, (E) miR-16, and (F) miR-744 in PC xenografts. The low copy number value of miR-15a, indicating a homozygous deletion in LuCaP 86.2 and PC324, is associated with low expression value of this miRNA in the same samples. Gray columns indicate the gene copy number values and black columns the expression values.

SNP Illumina analyses, respectively, were validated by FISH. The miR-15a-miR-16-1 cluster is located at 13q14.3. No signals with the genomic probe specific for the miR-15a-miR-16-1 locus were detected in LuCaP 86.2 and PC324, whereas the reference probe specific for the *ETB* gene locus at 13q22 gave two signals (Fig. 3). The control samples without deletions of the miR-15a-miR-16-1 according to arrays (LuCaP 92.1 and PC82, respectively) showed clear signals with both the miR-15a-miR-16-1 probe and the reference probe (Fig. 3). Therefore, the FISH analysis confirmed the HD of miR-15a-miR-16-1 locus in the LuCaP 86.2 and PC324 xenograft samples. In addition, the HDs were also confirmed by genomic PCR (data not shown).

Expression of miR-15a and miR-16 According to qRT-PCR

The expression data of miR-15a and miR-16 in LuCaP and PC xenograft series obtained with arrays was validated with qRT-PCR using the TaqMan microRNA Assays (Applied Biosystems). The qRT-PCR analysis confirmed the background level expression of miR-15a in samples that showed HD of the miR-15a-miR-16-1 gene cluster (LuCaP 86.2 and PC324). The expression of miR-16, however, was not decreased in these samples by either of the analysis methods (Fig. 4).

Mutation Analysis of the miR-15a-miR-16-1 Gene

In addition to HD of miR-15a and miR-16 locus in LuCaP 86.2 and PC324, most of the other xenografts contained putative heterozygous deletions of the locus according to array analyses (data not shown). To study whether the remaining gene copy could be inactivated by a mutation, thereby showing the "second hit" of inactivation of this gene, six prostate cancer cell lines, 32 prostate cancer xenografts, and 50 clinical prostate carcinoma tumor samples were screened for mutations of the miR-15a-miR-16-1

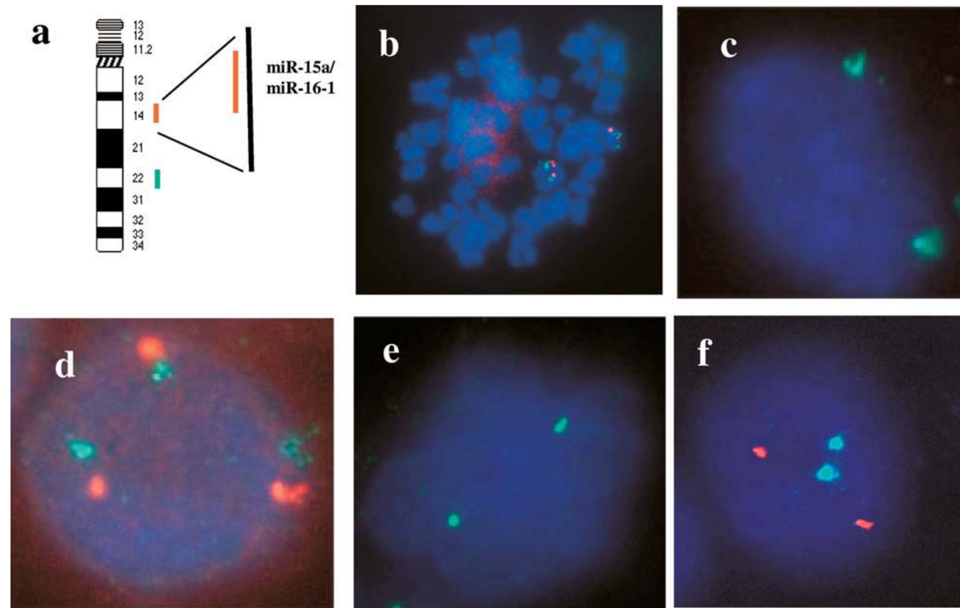

Figure 3. FISH analysis of the miR15a-miR16-1 gene. (a) Schematic presentation of the FISH probes for the chromosome 13: the red line indicates the BAC RPC-11-153K13 probe for the miR15a-miR16-1 genomic locus at 13q14.3, and the green line the PAC probe 84A6 for the reference *ETB* gene at 13q22. The black line indicates the region homozygously deleted in the LuCaP 86.2. (b) DU145 metaphase chromosomes confirming the specificity of the probes. (c) LuCaP 86.2 showing homozygous deletion of the miR15a-miR16-genomic locus (no red signals) and two copies of the *ETB* reference gene (green signals). (d) LuCaP 92.1 showing three copies of the miR15a-miR-16-1 gene (red signals) and three copies of the *ETB* gene (green signals). (e) PC324 showing homozygous deletion of miR15a-miR16-genomic locus (no red signals), and two copies of the ETB gene (green signals). (f) PC82 showing two copies of the miR-15a-16-1 gene (red signals) and two copies of the *ETB* gene (green signals). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

gene cluster. The sequenced areas covered both pre-miR15a and pre-miR-16-1 sequences, as well as 73 bp upstream of the miR-15a precursor and 159 bp downstream of the miR-16-1 precursor. Only one mutation was found in the xenograft sample PC346BI, in which the mutation was located in the region coding for the mature miRNA-15a sequence (Fig. 5b). PC346BI is a subline of the xenograft PC346B, in which there was no mutation of miR-15a-miR-16-1, indicating that the mutation has arisen during growth selection in female mice (Marques et al., 2006). No other mutations were detected in the samples studied, indicating that mutations of the miR-15a-miR-16-1 gene cluster are rare in prostate cancer.

DISCUSSION

In this study, expression of miRNAs as well as their gene copy numbers in prostate cancer were analyzed. First, clustering analysis of the samples based on their miRNAs expression profiles was carried out. One of the main results of the clustering analysis was that the different sample types were clustered separately from each other: the cell lines (22Rv1, LAPC4, LNCaP, PC346c, VCaP, DU145, and PC-3) formed a subcluster of their own that was separated from the xenograft samples, indicating that the miRNA expression profiles in the in vitro growing cell lines and the in vivo growing xenograft samples are different. In addition, samples of the two xenograft series (the LuCaP and the PC series) were mostly, although not completely, clustered in separate subclusters. One reason for this clustering to their "own" series could be the fact that these xenografts have been grown in different mouse background (Balb-c nu/nu and NMRI nu/nu) that may affect miRNA expression. The other reason could be that the PC series is mainly established form primary tumors, whereas the LuCaP series from metastases. The other main result that can be seen from the clustering analysis is that the androgen-independent samples were separated from the androgen-sensitive and androgen-dependent samples. For example, the two androgen receptor (AR) negative, and therefore completely androgen-independent cell lines, PC-3 and DU145, formed a pair that is separated from the other, AR-positive cell lines (22Rv1, LAPC4, LNCaP, PC346c, and VCaP). Also, the androgen-independent xenografts formed nodes of their own (e.g., LuCaP49 and 93; PC346BI and PC346I; PC133, PC135, PC339, and PC324), separately from the androgen-dependent and

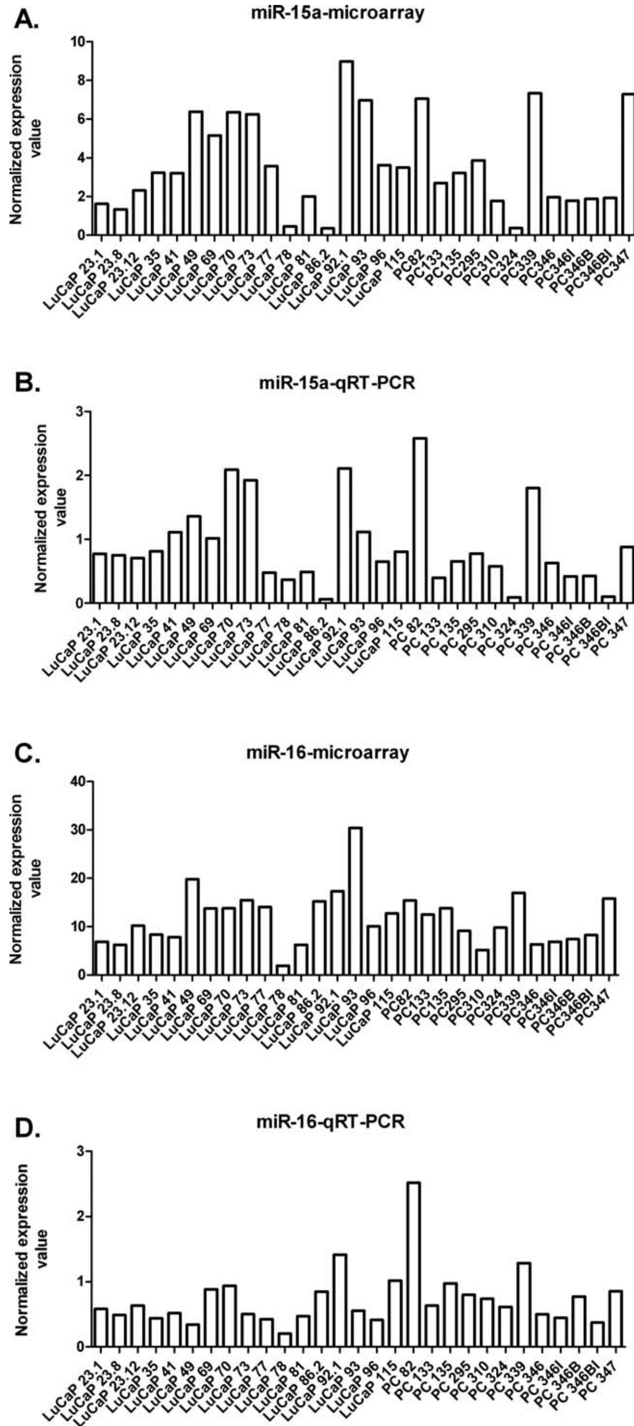

Figure 4. Validation of the microarray expression results by the qRT-PCR. Expression of miR-15a in xenografts according to (A) microarray and (B) qRT-PCR, as well as expression of miR-16 in xenografts according to (C) microarray and (D) qRT-PCR.

androgen-sensitive xenografts within the PC and LuCaP xenograft subclusters. These results confirm our previous findings with smaller sample material (Porkka et al., 2007). In addition, the data suggest that androgens and the androgen receptor play a role in regulating the expression of some miRNAs. Indeed, we have demonstrated that, for example, miR-21, −22, −29a, −29b, −141, and −494, are regulated by androgens (Waltering et al., 2010).

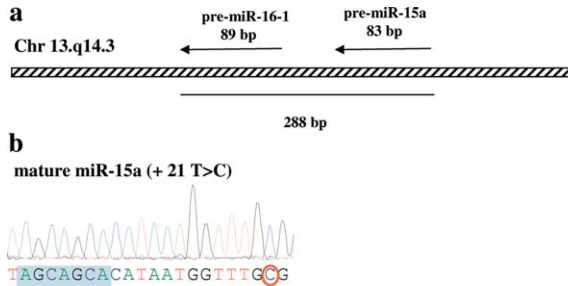

Figure 5. Mutation analysis of the miR-15a-miR-16-1 gene cluster. (a) Schematic presentation of the miR-15a-miR-16-1 gene cluster at chromosome 13q14.3. (b) Mutation at the mature miR-15a coding sequence (+21 T>C) detected in the xenograft sample PC346BI. The seed sequence is highlighted in light blue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Second, by gene copy number analysis we found a homozygous deletion of the miR-15a-miR-16-1 gene cluster in two prostate xenograft samples (LuCaP86.2 and PC324) and heterozygous deletions in most of the other samples studied (data not shown). Heterozygous deletions of the miR-15a-miR-16-1 gene have previously been detected to be common in various cancer types, such as chronic lymphocytic leukemia (Calin et al., 2002), myeloma, mantle cell lymphoma, lung cancer (Bandi et al., 2009), breast cancer, and ovarian cancer (Zhang et al., 2006). HD of miR-15a-miR-16-1 has been reported in chronic lymphocytic leukemia (Calin et al., 2002), but not in other cancer types. In prostate cancer, deletions of this miRNA gene have not been reported before. HD of the miR-15a-miR-16-1 locus was associated with abolished expression of miR-15a but not of miR-16. This must be due to the fact that miR-16 is expressed also from another genomic locus, miR-15b-miR-16-2 at 3q26. This locus was intact in the xenograft samples.

Heterozygous deletion of chromosome band 13q14 region is one of the most common DNA copy number alterations in prostate cancer (Visakorpi et al., 1995). Thus, not surprisingly most of the other samples studied here contained heterozygous deletion of the miR-15a-miR-16-1 locus. However, the heterozygous deletion was not associated with decreased expression of miR15a-miR-16-1. This finding is maybe not surprising since loss of one copy of a putative tumor suppressor gene is not generally associated with decreased expression. Instead, according to the Knudson's "two-hit hypothesis," the remaining allele is often mutated. Thus, we screened 88 samples for mutation in the miR-15a-miR-16-1 genes. We found only one variation indicating that mutations of this miRNA gene are rare in prostate cancer. In addition, the mutation is not in the seed sequence region, which makes it questionable whether it has any significance in target silencing. However, in some studies, mutations outside the miRNA coding regions have been shown to affect mature miRNA expression. For example, a germ-line variation of the primary transcript of miR-16 in chronic lymphocytic leukemia (CLL), leading to diminished expression of the mature miR-16, has been reported in one study (Calin et al., 2005). In addition, mutation in a mouse model of CLL in the 3′ flanking region of miR-16-1 precursor has been described, leading to decreased expression of miR-16 (Raveche et al., 2007). Mutations of the miR-15a-miR-16-1 gene have been screened for also in various other cancer types, but have not been detected (Yazici et al., 2009).

In functional studies, miR-15a-miR-16-1 has been shown to control cell survival, proliferation, and invasion in prostate cancer (Bonci et al., 2008). miR-15a and miR-16 have been shown to target several well-established oncogenes, such as *BCL2*, *CCND1*, *CCND2*, *CCNE1*, and *WNT3A* (Bonci et al., 2008; Bandi et al., 2009), thus indicating the tumor suppressor function of this miRNA gene. In addition, it has been shown that systemic delivery of miR-16 inhibits growth of metastatic prostate tumors via down-regulation of multiple cell-cycle genes (Takeshita et al., 2010). We studied the association of the deletion of miR-15a-miR-16-1 gene and expression of BCL2 by immunohistochemistry stainings of the xenograft samples, but no association of the expression of the BCL2 protein and the miR-15a-miR-16-1 gene copy number was detected (data not shown). However, it has been previously demonstrated that the combined loss of both miR-15a and miR-16 results in increased levels of BCL2 protein (Cimmino et al., 2005). One possible explanation for the contradictory results obtained here could be that miR-15a-miR-16-1 has a homologous gene locus, miR-15b-miR-16-2, at 3q26. miR-16-2 and miR-16-1 genes produce identical mature miRNA sequences, whereas miR-15a and miR-15b have a four base difference in their mature miRNA sequences. However, all these miRNAs (miR-15a, miR-15b, and miR-16) share the same seed sequence, indicating that they all target the same mRNAs. Therefore, even though the expression of miR-15a and miR-16 from the miR-15a-miR-16-1 locus was abolished, the putative expression of miR-15b and miR-16

from the homologous locus, miR-15b-miR-16-2 at 3q26, could be enough for inhibiting the expression of the targets, such as BCL2 in the prostate cancer xenograft samples studied here.

In the association analysis of the miRNA CNV and expression, miR-15a, miR-191, let-7g, and miR-744 showed statistically significant association. Of these, miR-15a showed homozygous deletion in two samples and putative heterozygous deletion in most of the other samples (data not shown). The tumor suppressor function of miR-15a has been discussed in the previous paragraph. Expression of miR-191 was at the background level and of miR-744 at very low level, which is why they probably do not have any functional role in prostate cancer. Let-7g was expressed in all the cell line and xenograft samples at moderate to high level. In our previous studies with clinical prostate tumor samples, let-7g was expressed at relatively low level and did not show differential expression between benign and malignant samples (Porkka et al., 2007; Martens-Uzunova et al., unpublished). The let-7 miRNA family has been proposed to function in tumor suppression, because reduced expression of let-7 family members is common in non-small cell lung cancer (NSCLC), and this reduction is correlated with poor prognosis (Takamizawa et al., 2004; Yanaihara et al., 2006). Ectopic expression of let-7g has been shown to induce cell cycle arrest and cell death, and to reduce NSCLC tumors in human and mice (Kumar et al., 2008).

The expression and gene copy number analyses were done using 42 well-established prostate cancer models, that is, cell lines and two sets of xenografts (the LuCaP series and the PC series). These samples, especially the xenografts, are very good and informative representatives of prostate cancer, because they contain many genetic alterations that have frequently been found also in clinical prostate cancer (Laitinen et al., 2002; Saramäki et al., 2006). Therefore, the genomic changes discovered here are likely to reflect the changes of the miRNA genes also in clinical prostate carcinomas.

In summary, miRNA gene copy number and expression analyses identified only a few miRNAs that are affected by CNV. Most importantly, homozygous deletion of the miR-15a-miR-16-1 locus was found in two samples, which together with previously published data, suggest that they are involved in the development of prostate cancer.

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REFERENCES

Ambs S, Prueitt RL, Yi M, Hudson RS, Howe TM, Petrocca F, Wallace TA, Liu CG, Volinia S, Calin GA, Yfantis HG, Stephens RM, Croce CM. 2008. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. Cancer Res 68:6162–6170.

Bandi N, Zbinden S, Gugger M, Arnold M, Kocher V, Hasan L, Kappeler A, Brunner T, Vassella E. 2009. miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer. Cancer Res 69:5553–5559.

Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, Memeo L, D'Urso L, Pagliuca A, Biffoni M, Labbaye C, Bartucci M, Muto G, Peschle C, De Maria R. 2008. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat Med 14:1271–1277.

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM. 2002. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 99:15524–15529.

Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M, Iuliano R, Palumbo T, Pichiorri F, Roldo C, Garzon R, Sevignani C, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. 2005. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 353:1793–1801.

Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 102:13944–13949.

Ding J, Huang S, Wu S, Zhao Y, Liang L, Yan M, Ge C, Yao J, Chen T, Wan D, Wang H, Gu J, Yao M, Li J, Tu H, He X. 2010. Gain of miR-151 on chromosome 8q24.3 facilitates tumour cell migration and spreading through downregulating RhoGDIA. Nat Cell Biol 12:390–399.

Diosdado B, van de Wiel MA, Terhaar Sive Droste JS, Mongera S, Postma C, Meijerink WJ, Carvalho B, Meijer GA. 2009. MiR-17-92 cluster is associated with 13q gain and c-myc expression during colorectal adenoma to adenocarcinoma progression. Br J Cancer 101:707–714.

Esquela-Kerscher A, Slack FJ. 2006. Oncomirs—microRNAs with a role in cancer. Nat Rev Cancer 6:259–269.

Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y, Takahashi T. 2005. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res 65:9628–9632.

He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM. 2005. A microRNA polycistron as a potential human oncogene. Nature 435:828–833.

Hendriksen PJ, Dits NF, Kokame K, Veldhoven A, van Weerden WM, Bangma CH, Trapman J, Jenster G. 2006. Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res 66:5012–5020.

Hyytinen E, Visakorpi T, Kallioniemi A, Kallioniemi OP, Isola JJ. 1994. Improved technique for analysis of formalin-fixed, paraffin-embedded tumors by fluorescence *in situ* hybridization. Cytometry 16:93–99.

Ivanov SV, Goparaju CM, Lopez P, Zavadil J, Toren-Haritan G, Rosenwald S, Hoshen M, Chajut A, Cohen D, Pass HI. 2010.

Pro-tumorigenic effects of miR-31 loss in mesothelioma. J Biol Chem 285:22809–22817.
Kumar MS, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA, Jacks T. 2008. Suppression of non-small cell lung tumor development by the let-7 microRNA family. Proc Natl Acad Sci USA 105:3903–3908.
Laitinen S, Karhu R, Sawyers CL, Vessella RL, Visakorpi T. 2002. Chromosomal aberrations in prostate cancer xenografts detected by comparative genomic hybridization. Genes Chromosomes Cancer 35:66–73.
Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. 2005. MicroRNA expression profiles classify human cancers. Nature 435:834–838.
Marques RB, van Weerden WM, Erkens-Schulze S, de Ridder CM, Bangma CH, Trapman J, Jenster G. 2006. The human PC346 xenograft and cell line panel: A model system for prostate cancer progression. Eur Urol 49:245–257.
Mattie MD, Benz CC, Bowers J, Sensinger K, Wong L, Scott GK, Fedele V, Ginzinger D, Getts R, Haqq C. 2006. Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. Mol Cancer 5:24.
Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. 2008. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA 105:10513–10518.
Navarro A, Beà S, Fernández V, Prieto M, Salaverria I, Jares P, Hartmann E, Mozos A, López-Guillermo A, Villamor N, Colomer D, Puig X, Ott G, Solé F, Serrano S, Rosenwald A, Campo E, Hernández L. 2009. MicroRNA expression, chromosomal alterations, and immunoglobulin variable heavy chain hypermutations in Mantle cell lymphomas. Cancer Res 69:7071–7078.
Pleasance ED, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, Greenman CD, Varela I, Lin ML, Ordóñez GR, Bignell GR, Ye K, Alipaz J, Bauer MJ, Beare D, Butler A, Carter RJ, Chen L, Cox AJ, Edkins S, Kokko-Gonzales PI, Gormley NA, Grocock RJ, Haudenschild CD, Hims MM, James T, Jia M, Kingsbury Z, Leroy C, Marshall J, Menzies A, Mudie LJ, Ning Z, Royce T, Schulz-Trieglaff OB, Spiridou A, Stebbings LA, Szajkowski L, Teague J, Williamson D, Chin L, Ross MT, Campbell PJ, Bentley DR, Futreal PA, Stratton MR. 2010. A comprehensive catalogue of somatic mutations from a human cancer genome. Nature 463:191–196.
Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL, Visakorpi T. 2007. MicroRNA expression profiling in prostate cancer. Cancer Res 67:6130–135.
Rauhala HE, Jalava SE, Isotalo J, Bracken H, Lehmusvaara S, Tammela TL, Oja H, Visakorpi T. 2010. miR-193b is an epigenetically regulated putative tumor suppressor in prostate cancer. Int J Cancer 127:1363–1372.
Raveche ES, Salerno E, Scaglione BJ, Manohar V, Abbasi F, Lin YC, Fredrickson T, Landgraf P, Ramachandra S, Huppi K, Toro JR, Zenger VE, Metcalf RA, Marti GE. 2007. Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice. Blood 109:5079–5086.
Ribas J, Ni X, Haffner M, Wentzel EA, Salmasi AH, Chowdhury WH, Kudrolli TA, Yegnasubramanian S, Luo J, Rodriguez R, Mendell JT, Lupold SE. 2009. miR-21: An androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. Cancer Res 69:7165–7169.
Rinaldi A, Poretti G, Kwee I, Zucca E, Catapano CV, Tibiletti MG, Bertoni F. 2007. Concomitant MYC and microRNA cluster miR-17-92 (C13orf25) amplification in human mantle cell lymphoma. Leuk Lymphoma 48:410–412.
Rosenwald A, Campo E, Hernández L. 2009. MicroRNA expression, chromosomal alterations, and immunoglobulin variable heavy chain hypermutations in Mantle cell lymphomas. Cancer Res 69:7071–7078.
Saramäki OR, Porkka KP, Vessella RL, Visakorpi T. 2006. Genetic aberrations in prostate cancer by microarray analysis. Int J Cancer 119:1322–1329.
Schaefer A, Jung M, Mollenkopf HJ, Wagner I, Stephan C, Jentzmik F, Miller K, Lein M, Kristiansen G, Jung K. 2010. Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. Int J Cancer 126:1166–1176.
Shi XB, Xue L, Ma AH, Tepper CG, Kung HJ, White RW. 2011. miR-125b promotes growth of prostate cancer xenograft tumor through targeting pro-apoptotic genes. Prostate 71:538–549.
Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T, Takahashi T. 2004. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64:3753–3756.
Takeshita F, Patrawala L, Osaki M, Takahashi RU, Yamamoto Y, Kosaka N, Kawamata M, Kelnar K, Bader AG, Brown D, Ochiya T. 2010. Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes. Mol Ther 18:181–187.
Tong AW, Fulgham P, Jay C, Chen P, Khalil I, Liu S, Senzer N, Eklund AC, Han J, Nemunaitis J. 2008. MicroRNA profile analysis of human prostate cancers. Cancer Gene Ther 16:206–216.
Varambally S, Cao Q, Mani RS, Shankar S, Wang X, Ateeq B, Laxman B, Cao X, Jing X, Ramnarayanan K, Brenner JC, Yu J, Kim JH, Han B, Tan P, Kumar-Sinha C, Lonigro RJ, Palanisamy N, Maher CA, Chinnaiyan AM. 2008. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science 322:1695–1699.
Visakorpi T, Kallioniemi AH, Syvänen AC, Hyytinen ER, Karhu R, Tammela T, Isola JJ, Kallioniemi OP. 1995. Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. Cancer Res 55:342–347.
Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. 2006. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA 103:2257–2261.
Waltering KK, Porkka KP, Jalava SE, Urbanucci A, Kohonen PJ, Latonen LM, Kallioniemi OP, Jenster G, Visakorpi T. Androgen regulation of micro-RNAs in prostate cancer. Prostate (in press). DOI: 10.1002/pros.21276.
van Weerden WM, Romijn JC. 2000. Use of nude mouse xenograft models in prostate cancer research. Prostate 43:263–271.
Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM, Harris CC. 2006. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9:189–198.
Yazici H, Zipprich J, Peng T, Akisik EZ, Tigli H, Isin M, Akisik EE, Terry MB, Senie RT, Li L, Peng M, Liu Z, Dalay N, Santella RM. 2009. Investigation of the miR16–1 ($C > T$) + 7 substitution in seven different types of cancer from three ethnic groups. J Oncol 2009:827532.
Zhang L, Huang J, Yang N, Greshock J, Megraw MS, Giannakakis A, Liang S, Naylor TL, Barchetti A, Ward MR, Yao G, Medina A, O'brien-Jenkins A, Katsaros D, Hatzigeorgiou A, Gimotty PA, Weber BL, Coukos G. 2006. microRNAs exhibit high frequency genomic alterations in human cancer. Proc Natl Acad Sci USA 103:9136–9141.