Hsa-miR-1-2/miR-133a-1 Cluster Expression Silencing by DNA Hypermethylation Triggers Oncogenes Contributing to Prostate Cancer Aggressiveness

Cintia Massillo
IBYME: Instituto de Biologia y Medicina Experimental

Rocío Belén Duca
IBYME: Instituto de Biologia y Medicina Experimental

Paula Lucía Farré
IBYME: Instituto de Biologia y Medicina Experimental

Kevin Gardner
Columbia University

Ezequiel Lacunza
Universidad Nacional de la Plata Facultad de Ciencias Medicas

Adriana De Siervi (✉ adesiervi@dna.uba.ar)
IBYME: Instituto de Biologia y Medicina Experimental

Research

Keywords: Prostate Cancer, microRNA, methylation

Posted Date: August 31st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-800913/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

**Background:** Prostate cancer (PCa) is currently the most commonly diagnosed type of cancer. The incidence and mortality of PCa worldwide correlate with increasing age and bad dietary habits. Previously, we investigated the mRNA/miRNA role on PCa development and progression using high fat diet (HFD) chronically fed mice models. Here our main goal was to investigate the effect of HFD on the expression of prostate cancer-related miRNAs and their relevance in PCa patients.

**Methods:** Using microarray data from mice prostate tumors generated by TRAMP-C1 cell inoculation, we focused on the role of three candidate miRNAs (miR-133a-3p/133b/1-3p) and their target genes. Based on data from public databases, we examined the expression levels of hsa-miR-133a-3p/133b/1-3p and their correlation with clinicopathological features in PCa patients. The biological roles of hsa-miR-133a-3p/133b/1-3p and their relevant target genes were investigated by bioinformatics approaches. The promoter methylation of hsa-miR-133a-3p/133b/1-3p host genes and their correlation with mature miRNA expression was further evaluated.

**Results:** We identified 6 up- and 18 down-regulated miRNAs in TRAMP-C1 mice prostate tumors under HFD conditions using miRNA microarrays. Target genes (1,278) of down-regulated miRNAs involved in cancer-related biological processes were identified using DIANA-TarBase and STRING databases. Three down-regulated miRNAs: hsa-miR-133a-3p, 133b and 1a-3p showed nine common target genes that negatively correlated with miRNA expression in prostate tumors from patients. Hsa-miR-133a-3p/133b/1-3p expression levels were significantly decreased in PCa compared to normal tissues and their low expression correlated with bad clinicopathological features in patients. We also examined the promoter region of hsa-miR-133a-3p/133b/1-3p encoding genes in PCa patients and then compared methylation at these loci with mature miRNA expression. This analysis revealed that hsa-miR-1-2/miR-133a-1 cluster promoter hypermethylation decreased hsa-miR-133a-3p/1-3p expression in prostate tumors. Furthermore, CENPF and WHSC1, two common hsa-miR-133a-3p/133b/1-3p target genes, were found significantly up-regulated in PCa and positively correlated with advanced clinicopathological characteristics in PCa patients.

**Conclusion:** Our results provide an explanation for the aggressiveness of PCa and link it mechanistically to the attenuation of hsa-miR-133a-3p/133b/1-3p expression by promoter hypermethylation. Hsa-miR-133a-3p/133b/1-3p downregulation may enhance PCa aggressiveness in part by targeting CENPF and WHSC1. Therefore, hsa-miR-133a-3p/133b/1-3p might be potential therapeutic targets for lethal PCa.

**Background**

Prostate cancer (PCa) is currently the most commonly diagnosed type of cancer and the fifth leading cause of cancer deaths among men over the age of 50 years worldwide (https://gco.iarc.fr/). Although PCa is a multifactorial disease, different epidemiological studies suggested that lifestyle and environmental factors influence the development and progression of this disease [1]. Dietary fats and
obesity have the potential to cause PCa initiation, promotion and progression [2]. The proposed mechanisms for PCa induced by dietary fats are divided into growth factor signaling, lipid metabolism, inflammation and hormonal modulation among others [2]. However, the underlying molecular mechanisms responsible for the effect of high fat diet (HFD) on PCa development and progression remained unknown.

Previously, we generated several preclinical mice models to study the influence of HFD on PCa development and progression. We reported that C-terminal binding protein 1 (CTBP1) depletion in androgen-insensitive PCa xenografts from HFD-fed mice modulated the expression of mRNAs and microRNAs (miRNAs) involved in cancer related pathways which impacts on PCa proliferation and invasion [3–6]. Additionally, recent androgen-sensitive PCa allografts and HFD mice model demonstrated that high fat intake significantly increased tumor growth. Tumors developed in HFD fed mice showed overexpression of oncogenes and oncomiRs compared to control diet (CD) [4, 7, 8].

MiRNAs are endogenous small non-coding RNAs (18–22 nucleotides) that regulate gene expression. Compelling evidence have demonstrated that miRNA expression is deregulated in several human cancer types through numerous mechanisms, including amplification or deletion of miRNA genes, abnormal miRNAs transcription, deregulated epigenetic changes and defects in the miRNA biogenesis machinery [9]. MiRNAs can function either as oncogenes (oncomiRs) or tumor suppressors (tsmiRs) under certain conditions. The deregulated miRNAs have been shown to affect several hallmarks of cancer, including sustaining proliferative signaling, evading growth suppressors, resisting cell death, activating invasion and metastasis, and inducing angiogenesis [9]. In PCa, several miRNAs have been proposed to regulate cell proliferation, cell cycle, apoptosis, as well as invasion and adhesion processes [10]. In addition, diet and lifestyle factors are involved in the regulation of miRNA expression in different tissues and pathologies, including cancer [11–13]. Finally, an increasing number of studies have identified miRNAs as potential biomarkers for PCa diagnosis, prognosis and therapy.

Here our main goal was to investigate the effect of HFD on the expression of cancer-related miRNAs in prostate tumors and their relevance in PCa patients. Starting from microarray data from mice prostate tumors we focused on the role of three candidate miRNAs (miR-133a-3p/133b/1-3p) and their target genes. Using PCa patient samples from public databases, we examined the expression levels of hsa-miR-133a-3p/133b/1-3p and their correlation with clinicopathological features in PCa patients. The biological roles of hsa-miR-133a-3p/133b/1-3p and their relevant target genes were investigated by bioinformatics approaches. Finally, the promoter methylation of hsa-miR-133a-3p/133b/1-3p host genes and their correlation with mature miRNA expression was evaluated.

**Methods**

**Cell culture**
Murine TRAMP-C1 cell line was obtained from the American Type Cell Culture Collection (Manassas, VA) (ATCC: CRL-2730). Cells were cultured in DMEM medium (GIBCO, Thermo Scientific, Massachusetts, USA) supplemented with 10% of fetal bovine serum, antibiotics and 0.25 IU/µl of human recombinant insulin in a 5% CO2 humidified incubator at 37°C.

**PCa allograft and HFD murine model**

We previously reported that HFD significantly increased TRAMP-C1 tumor growth, oncogene and oncomiRs expression [4, 7]. Here, we used the same model to assess miRNA expression in these tumors. Briefly, four-weeks-old C57BL/6J male mice (N = 16) were housed under pathogen-free conditions following the IBYME’s animal care guidelines. Mice were randomized into two dietary groups and fed *ad libitum* during 27 weeks with CD (3,120 kcal/kg, 5% fat) or HFD (4,520 kcal/kg, 37% fat). After 15 weeks of diet, $3 \times 10^6$ TRAMP-C1 cells were subcutaneously injected. Animals were sacrificed in the 27th week and tumor samples were collected. The metabolic state of the animals and tumor volume were previously reported [4].

**RNA isolation and microarrays analysis**

For microarray analysis, total RNA from CD or HFD allografts was isolated using TriReagent (Molecular Research Center) and hybridized with GeneChip® miRNA 4.0 Array (Affymetrix) (N = 3 per group). For miRNA expression analysis, we employed the *Limma* and *pd.mirna.4.0* packages in the R/Bioconductor environment. For differential expression analysis we used the Rank Product Method for two class unpaired data and a fold discovery rate (FDR) < 0.05 [14].

**Functional enrichment analysis**

To investigate the functional role of differentially expressed miRNAs between CD and HFD tumors we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using DIANA-miRPath v3 tool (http://snf-515788.vm.okeanos.grnet.gr/) and STRING database (https://string-db.org/). Briefly, we obtained a list of experimentally validated target genes derived from the differentially expressed mice miRNAs and their human orthologous from DIANA-TarBase v7 that were entered into the STRING database for KEGG analysis.

**Principal component analysis (PCA)**

Publicly available log2(norm_count + 1) gene expression values for TCGA Prostate Cancer (PRAD) and the GTEx project patient samples were downloaded from UCSC’s Xena Browser (http://xena.ucsc.edu/) [15]. PCA was performed to determine samples distribution based on the expression of the target genes identified from the downregulated miRNAs. We included in the analysis normal prostates (n = 100) (GTEx), normal adjacent tissue (NAT) (n = 52) and prostate tumors (n = 497) (TCGA-PRAD) samples. For PCA plots, the R function "prcomp" from stats package (version 4.0.2) was used.

**Correlation matrix**
Expression levels of down-regulated miRNAs in HFD tumors and their respective target genes were obtained from TCGA-PRAD patient cohort data available in UCSC Xena. Expression data of miRNA mature strand [miRNA-Seq (IlluminaHiSeq_miRNASeq)] and genes [RNAseq (IlluminaHiSeq)] were downloaded as log2 (RPM + 1) values. PCa samples from 497 patients were included in the present analysis. We generated a correlation matrix for each miRNA and their target genes applying the Spearman correlation coefficient using the Hmisc R package. For all down-regulated miRNAs, target genes with a negative correlation coefficient rho <= -0.2 plus p-Value < 0.05 were selected for further analysis.

**Single-sample gene-set enrichment analysis (ssGSEA)**

A ssGSEA was performed to analyze the coordinated regulation of a defined gene signature and the activation of specific biological processes in PCa samples. Log2 (RPM + 1) gene expression values of 497 PCa samples were obtained from TCGA-PRAD cohort and loaded into GenePattern web-tool (https://www.genepattern.org). A gene set enrichment profile was obtained for each sample using a nine-gene signature obtained from the correlation matrix described above. Then, Spearman correlation analysis was performed between the expression of three down-miRNAs and the enrichment score obtained from the ssGSEA (GraphPad Prism 8.0.1). Afterwards, to analyze the contribution of the 9 genes in specific biological processes a compute overlaps was performed against C1, C2, C3, C4, C5, C6, C7, C8 and H collections in Molecular Signatures Database (MSigDB) (http://www.gsea-msigdb.org/gsea/msigdb/). The top 10 genesets with FDR q-value less than 0.05 were selected to graphed the gene/geneset overlap matrix.

**Bioinformatics analysis databases**

MiRNA and gene expression levels in addition to clinical-pathological data (e.g. Gleason score, pathological state N or T, etc.) were obtained from two independent PCa patient datasets. The RNA-seq and miRNA-seq data from TCGA-PRAD cohort was downloaded using UCSC Xena resource. PCa samples (n = 497) and NAT (n = 52) with expression data of miRNA mature strand [miRNA-Seq (IlluminaHiSeq_miRNASeq)] and genes [RNAseq (IlluminaHiSeq)] as log2 (RPM + 1) values were included in the present study. The miRNA microarray and exon/whole-transcript microarray information from Taylor cohort was downloaded from GEO dataset (GSE21032) [16]. PCa samples (n = 111) and normal prostates (n = 28) with expression data of miRNA mature strand [Agilent-019118 Human miRNA Microarray 2.0 G4470B (miRNA ID version)] and genes [{[HuEx-1_0-st}] Affymetrix Human Exon 1.0 ST Array [probe set (exon) version]} as generalized log-transformed (glog2) and exon (core + extended) normalized signal intensity values respectively were included in the present study.

For statistical analysis normality of data was assessed using Shapiro-Wilk test and homogeneity of variances was analyzed by boxplot. We used a paired sample t-test or a Sign Median test to compare the expression between PCa and NAT samples (N = 52 per group) and a Mann Whitney test to compare the expression between PCa and normal prostate samples (normal N = 28, PCa N = 111). Also, one-way
ANOVA followed by Tukey or Kruskal-Wallis test followed by Dunn were used to analyze the statistical differences between PCa samples according to Gleason grade (N = 497 TCGA-PRAD, N = 111 Taylor).

Progression free interval curves were plotted using the Kaplan-Meier method and compared by log-rank test using Xena resource from TCGA-PRAD cohort.

**Integrated mature miRNA expression and promoter methylation analysis**

Hsa-miR-1-2/miR-133a-1, miR-1-1/miR-133a-2 and miR-133b promoter methylation was evaluated in 52 paired PCa and NAT samples from the TCGA-PRAD cohort. DNA methylation data (Illumina Infinium HumanMethylation450) was downloaded as beta value using UCSC Xena resource. The Ensembl browser (http://www.ensembl.org) was employed to identify the coding genes of the mentioned miRNAs: MIR1-2: chr18:19,408,965 – 19,409,049, MIR133A1: chr18:19,405,659 – 19,405,746, MIR1-1: chr20:62,554,306 – 62,554,376, MIR133A2: chr20:62,564,912 – 62,565,013, MIR133B: chr6:52,148,923 – 52,149,041.

Methylation probes targeting miRNA promoter regions were identified by mapping 5000 base pairs upstream of miRNA TSS (hsa-miR-1-2/miR-133a-1 promoter: chr18:19,409,049 – 19,414,049, hsa-miR-1-1/miR-133a-2 promoter: chr20:62,554,306-5000-62,544,306, hsa-miR-133b promoter: chr6:52,008,721 – 52,013,721). The correlation between promoter methylation and mature miRNA expression was assessed by a Spearman correlation analysis in 497 PCa samples using GraphPad Prism 8.0.1.

**Results**

**HFD modulate the expression of multiple miRNAs in murine prostate tumors**

Previously, we reported that HFD significantly increased TRAMP-C1 tumor growth, oncogenes and oncomiRs expression in C57BL/6J male mice [4,7]. In this work, we investigated the impact of HFD on miRNA expression profile from these tumors using a high-throughput platform. GeneChiP miRNA 4.0 Affymetrix was hybridized with total RNA isolated from CD or HFD TRAMP-C1 tumors. After data normalization, we selected differentially expressed miRNAs with a FDR<0.05 and identified 18 down- and 6 up-regulated miRNAs in HFD tumors compared to CD group (Figure 1, Table 1). Particularly, mmu-miR-133a-3p, 133b and 1a-3p showed the most robust expression changes between the two groups. As miR-133a-3p and 1a-3p are expressed from the same miR-1/133a genomic clusters (located on chromosomes 2 and 18 in mouse and 18 and 20 in human and mmu-miR-133a-3p and 133b are part of the same miRNA gene family they were selected for further analyses.

**Down-regulated miRNAs by HFD modulate cancer related pathways**

To explore the functional role of the differentially expressed miRNAs and pathways, we used DIANA-miRPath v3 tool (http://snf-515788.vm.okeanos.grnet.gr/) and STRING database (https://string-db.org/). Since each miRNA can control several genes, we selected only the deregulated miRNAs that had orthologous in human to obtain a more robust result. We employed DIANA-TarBase v7 of the miRPath
tool, which is a reference database of experimentally supported miRNA targets, and followed the Figure 2A workflow. Eight of the down-modulated mouse miRNAs (8 out of 18) and 10 of its orthologous human miRNAs (10 out of 18) showed validated target genes (Figure 2AI). Additionally, two (2 out of 6) of the up-regulated mouse miRNAs and 2 of its human orthologous presented validated target genes. Next, we excluded common target genes among down- and up-regulated miRNAs from the same species and selected common target genes between species (human and mice). This analysis revealed that down-regulated miRNAs showed 1,278 target genes common to human and mice, while up-regulated miRNAs did not present common target genes (Table S1). We continue working with down-modulated miRNAs and their target genes. For this group of genes, KEGG signaling pathways were identified with a FDR<0.05 using STRING database (Figure 2B, Table S2). Target genes of the down-modulated miRNAs showed enriched processes mainly associated with cancer-related and signal transduction pathways (Figure 2B).

To determine the relevance of down-regulated miRNAs-target genes in human samples, we performed a PCA of the 1,278 target genes using the normalized expression data from normal prostate (GTEX), NAT and prostate tumors samples (TCGA-PRAD). The 2-dimensional scatterplot of the first two principal components revealed marked differences in overall gene expression between normal prostate and PCA samples (Figure 2C).

In order to found relevant target genes for the down-modulated miRNAs, within the 1,278 genes, we performed a correlation matrix, using the expression data of the down-modulated miRNAs and their target genes of prostate tumors from patients available in TCGA-PRAD (UCSC Xena). From this analysis, we selected the target genes that showed a negative Spearman correlation coefficient rho <-0.2 and a p-Value <0.05 (Figure 2AII) with its regulatory miRNA. Next, to shorten the number of relevant miRNAs and target genes, we choose common target genes that negatively correlate with the expression of the previously selected miRNAs hsa-miR-133a-3p, 133b and 1-3p. We found nine common target genes (BCL7A, CENPF, CPSF6, ELAVL1, NR1P1, PAICS, RALA, RBM15B and WHSC1) with a significant negative correlation with the mentioned miRNAs (Figure 3A, Table S3). Also, hsa-miR-133a-3p, 133b and 1-3p showed a strong positive correlation between them with a significant p-value (Figure 3A). Additionally, we performed a ssGSEA to explore the degree to which the 9 common target genes were coordinately up- or down-regulated within the prostate tumor samples in two different PCa datasets: TCGA-PRAD and Taylor (GSE21032). As shown in Figure 3B-C we found that the 9 target genes showed a positive ssGSEA-enrichment in most of PCa samples in both datasets. Moreover, a strong negative correlation between the ssGSEA-enrichment and the expression of miRs 133a-3p, miR-133b and miR-1-3p were found in the TCGA-PRAD dataset. A similar result was found for 133a-3p, miR-133b in Taylor dataset. Finally, we analyzed the contribution of the 9 target genes to specific gene sets annotated in MSigDB collections. As shown in Figure 3D, three of the nine target genes (WHSC1, CENPF and PAICS) were found to be significantly enriched in cancer-related gene sets.

**Hsa-miR-133a-3p, miR-133b and miR-1-3p expression are decreased in PCa**

In order to further understand the pattern expression of the three selected miRNAs in prostate tumors from patients, we performed a bioinformatic analysis using the TCGA-PRAD, and Taylor (GSE21032)
datasets. First, we evaluated the expression of the selected miRNAs in prostate primary tumors in comparison to NAT and normal prostates. As shown in Figure 4A, we found hsa-miR-133a-3p, 133b and 1-3p significantly downregulated in PCa compared to NAT (TCGA-PRAD cohort). Additionally, hsa-miR-133a-3p, 133b and 1-3p expression were significantly decreased in PCa compared to normal prostate (Taylor cohort) (Figure 4B). These results proposed that hsa-miR-133a-3p, miR-133b and miR-1-3p might act as tumor suppressors in PCa.

**Hsa-miR-133a-3p, miR-133b and miR-1-3p expression negatively correlated with Gleason grade**

To correlate hsa-miR-133a-3p, 133b and 1-3p with PCa stage, we used TCGA-PRAD and Taylor (GSE21032) patient cohorts, which were divided into groups according to Gleason's score on histopathology (6, 7 and 8-9). As shown in Figure 5, hsa-miR-133a-3p, 133b and 1-3p were found to be significantly down-regulated according to Gleason score increased in both datasets. Although the expression of the selected miRNAs did not affect overall survival in PCa patients (data not shown), a low expression of hsa-miR-133a-3p, 133b and 1-3p correlated with worst progression-free interval (Figure 5 A-C) in the TCGA-PRAD cohort. Thus, hsa-miR-133a-3p, 133b and 1-3p low expression correlated with high gleason score and worst progression free interval.

**Hsa-miR-1-2/miR-133a-1 promoter hypermethylation is increased in PCa and negatively correlated with miRNA expression**

The hsa-miR-1/133 family is located at three different *loci* (as clustered miRNAs) at chromosomes 18q11.2 (miR-1-2/miR-133a-1), 20q13.33 (miR-1-1/miR-133a-2), and 6p12.2 (miR-206/miR-133b) [17]. Here, we analyzed the methylation status at miR-1-2/miR-133a-1, miR-1-1/miR-133a-2 and miR-206/miR-133b promoters. Based upon the Ensembl and UCSC Xena resources, two, six and thirteen methylation probes were identified targeting the hsa-miR-1-2/miR-133a-1, miR-133b and miR-1-1/miR-133a-2 promoters, respectively (Figure 6, Table 2). We compared the methylation status at the three cluster promoters in 52 paired PCa and NAT samples. As shown in Figure 6, one probe (cg17106157) targeting miR-1-2/133a-1 (Figure 6A) and two probes (cg14487577 and cg22720139) targeting miR-133b promoters (Figure 6B) showed significantly higher beta values in PCa compared to NAT. Also, ten and one probes targeting miR-1-1/133a-2 promoter showed lower and higher beta values in PCa vs NAT respectively (Figure 6C). Then, a correlation analysis between promoter methylation and mature miRNA expression levels was performed in 497 PCa samples. As presented in Table 2, Spearman correlation analysis showed that hsa-miR-133a-3p and hsa-miR-1-3p expression negatively correlated with cg17106157 (chromosome 18) probe and hsa-miR-133a-3p and hsa-miR-1-3p expression negatively correlated with cg05898333 (chromosome 20) probe. Moreover, hsa-miR-133a-3p and hsa-miR-1-3p expression positively correlated with cg15580304, cg14523475, cg08148458 and cg22617703 probes targeting the miR-1-1/miR-133a-2 promoter. Therefore, the decreased expression observed in hsa-miR-1-3p/miR-133a-3p in prostate tumors might be due to a hsa-miR-1-2/miR-133a-1 cluster promoter hypermethylation.
**CENPF and WHSC1 are increased in primary prostate tumors from patients and positively correlated with Gleason grade**

We found that hsa-miR-133a-3p, 133b and 1-3p showed nine common target genes with negative correlation. Then, we analyzed the expression pattern of the nine target genes in prostate tumors and normal samples from TCGA-PRAD and Taylor (GSE21032) datasets. As shown in Figure 7A, we found that CENPF, WHSC1, CPSF6, RBM15B, PAICS and ELAVL1 expression was significantly increased in primary prostate tumors compared to NAT (TCGA-PRAD dataset). Moreover, CENPF, WHSC1 and PAICS expression were significantly increased in prostate tumors compared to normal prostates (Taylor dataset, Figure 7B). With these results we analyzed the correlation of CENPF, WHSC1 and PAICS with PCa stage. CENPF and WHSC1 were found to be significantly up-regulated according as Gleason score increases in TCGA-PRAD and Taylor datasets (Figure 8A-B). Moreover, a high expression of these genes correlates with a worst progression free interval (Figure 8A-B). These results collectively suggest that common target genes of hsa-miR-133a-3p, 133b and 1-3p act as oncogenes favoring PCa aggressiveness.

**Discussion**

Here we demonstrated that HFD markedly reduces the expression of potential tsmiRs in TRAMP-C1 tumors developed as allograft in C57BL/6J mice. Target genes modulated by these tsmiRs regulate processes mainly associated to cancer related pathways. Among these, mmu-miR-133a-3p, miR-133b and miR-1-3p were dramatically diminished in tumors by HFD. Additionally, human orthologous miRNAs were significantly downregulated in human prostate tumors compared to NAT and normal prostates. Besides, a low expression of hsa-miR-133a-3p, 133b and 1-3p correlated with more aggressive pathological features of prostate tumors, such as Gleason score and progression free-interval in PCa patients.

The hsa-miR-1, -133 and − 206 family are located at three different loci at chromosomes 20q13.33 (miR-1-1/miR-133a-2), 18q11.2 (miR-1-2/miR-133a-1) and 6p12.2 (miR-206/miR-133b) [17]. The mature miR-133 isomers (A and B) are highly similar, differing only at the 3′-terminal base, with miR-133a-1/2 terminating G-3′ and miR-133b with A-3′, respectively [18]. Due to their close locations at distinct loci, miR-1/133a, miR-206/133b are constituted as clustered miRNAs [17]. Recent studies showed miR-1, -133 and − 206 family deregulation in cancer, in which typically they function as tumor suppressors [19, 20].

Hsa-miR-133a-3p is probably the most studied miRNA of this family and has been extensively reported as down-regulated in several types of cancer and predicted a poor prognosis [21–27]. In PCa, a low expression of hsa-miR-133a-3p has been associated with the recurrence and distant metastasis of PCa [28–32]. Likewise, a recent study from Tang et al demonstrated that hsa-miR-133a-3p expression is reduced in PCa tissues compared with the NAT and benign prostate lesion tissues, particularly in bone metastatic PCa tissues. Also, low expression of miR-133a-3p is significantly correlated with advanced clinicopathological characteristics and shorter bone metastasis-free survival in PCa patients [33].
As referred to hsa-miR-133b and miR-1, different studies demonstrated that both miRNAs are among the most down-regulated miRNAs in PCa compared non-cancerous prostate tissues and significantly decreased in recurrent PCa specimens in comparison to non-recurrent PCa samples [34–36]. MiR-1 is further down-regulated in cancer progression and alone can predict disease recurrence [35]. Also, miR-133b and miR-1 have sufficient power to distinguish recurrent PCa specimens from non-recurrent [34, 35, 37]. Lastly, both miRNAs have been reported to target the oncogenic function of purine nucleoside phosphorylase (PNP) in PCa [31]. Thus, both miR-133b and miR-1 may exert similar tumor suppressor activities and coordinately regulate the expression of oncogenes controlling PCa initiation and progression.

Based on our and other groups’ findings, we propose hsa-miR-133a-3p, 133b and 1-3p as a promising miRNA to be studied as potential biomarkers for PCa diagnosis and prognosis.

In this work, we also aim to find relevant common target genes for hsa-miR-133a-3p, miR-133b and miR-1-3p. We performed a correlation analysis using data from PCa patients available in public algorithms. This analysis allowed us to find nine relevant common target genes for the three miRNAs with a significant negative correlation. Among the nine target genes, CENPF, WHSC1 and PAICS were significantly increased in primary prostate tumors compared to NAT and normal prostates, and positively correlated with more aggressive pathological features of PCa as Gleason grade and a worst progression-free interval. Also, the three target genes were significantly enriched in several cancer-related biological processes, suggesting that may act as oncogenes in PCa.

MiRNAs may be epigenetically silenced by DNA methylation of their encoding genes [9]. DNA hypermethylation was found to downregulate several tsmiRNAs, whereas DNA hypomethylation was reported to upregulate oncomiRs [38]. In this study, we analyzed the methylation status at miR-1-2/miR-133a-1, miR-1-1/miR-133a-2 and miR-206/miR-133b promoters in PCa and normal samples and examined the correlation between promoter methylation and mature miRNA expression. This bioinformatics analysis suggests that hypermethylation of hsa-miR-1-2/miR-133a-1 cluster promoter might decrease hsa-miR-1-3p/miR-133a-3p expression in prostate tumors. Therefore, epigenetic repression of the hsa-miR-1-2/miR-133a-1 cluster may play a critical role in PCa aggressiveness by activating CENPF, WHSC1 and PAICS.

Centromere protein F (CENPF) is a key component of the kinetochore complex and plays a crucial role in chromosome segregation and cell cycle progression. Different studies have investigated the role of CENPF in PCa. Two independent reports demonstrated that CENPF overexpression in PCa tissues is linked to higher Gleason grade, advanced pathological tumor stage, the presence of metastasis, worst overall survival and prostate-specific antigen (PSA) failure [39, 40]. CENPF is also a critical regulator of cancer metabolism potentially through its role in the mitochondria. Shahid et al. reported that CENPF silencing increased inactive forms of pyruvate kinase M2, a rate limiting enzyme needed for an irreversible reaction in glycolysis, and reduced global bio-energetic capacity, Acetyl-CoA production,
histone acetylation, and lipid metabolism [41]. Further untargeted metabolomics analysis revealed that silencing of CENPF alters the global metabolic profile of PCa cells and inhibits cell proliferation [42].

Meanwhile, Wolf-Hirschhorn syndrome candidate 1 (WHSC1/NSD2) is a histone methyltransferase enzyme that targets dimethyl and trimethyl H3K36. WHSC1 has been shown to be up-regulated in prostate tumors and promote malignant growth and progression [43, 44]. WHSC1 silencing in DU145 and PC-3 tumor cells decreased cell proliferation, colony formation and strikingly diminished cell migration and invasion [45]. Additionally, pharmacologically inhibition of WHSC1 with MCTP-39 inhibited DU145 prostate tumor growth in vivo [46]. WHSC1 levels positively correlate with the presence of an immunosuppressive microenvironment. Genetic and pharmacological inhibition of WHSC1 restores antigen presentation via an epigenetic regulation of gene expression at the chromatin and DNA methylation levels [44].

Therefore, based on our and previous studies, CENPF and WHSC1 have a critical role in PCa pathogenesis and progression.

In conclusion, our results demonstrated that HFD dramatically reduces the expression of tsmiRs in androgen-sensitive prostate tumors. Additionally, 3 miRNAs: hsa-miR-133a-3p, -133b and −1-3p are epigenetically silenced by promoter hypermethylation and functions as a tsmiRs in PCa. Moreover, their low expression significantly correlated with advanced clinicopathological characteristics of PCa patients, including Gleason score and progression free interval. Besides, the expression of hsa-miR-133a-3p, -133b and −1-3p negatively correlates with CENPF and WHSC1, two PCa driver oncogenes. Although evaluations of the three miRNAs and their target genes expression in larger populations are still needed, our results indicate that hsa-miR-133a-3p, -133b, -1-3p CENPF and WHSC1 are functional drivers of PCa and may be target for advanced PCa treatment.

Conclusions

HFD modulates the expression of a substantial number of miRNAs in PCa. Attenuation of hsa-miR-133a-3p, -133b and −1-3p expression by promoter methylation in prostate tumors may enhance PCa development, in part by targeting CENPF and WHSC1. Hsa-miR-133a-3p, -133b, -1-3p, CENPF and WHSC1 are functional drivers of PCa and might be potential targets for additional therapeutic opportunities for lethal PCa.

Abbreviations

CD: control diet; CENPF: Centromere Protein F; HFD: high fat diet; MeS: metabolic syndrome; miRNA: microRNA; NAT: normal adjacent tissue; OncomiR: oncogenic miRNA; PAICS: Phosphoribosylaminomidazole carboxylase, phosphoribosylaminimidazole succinocarboxamide synthetase; PCa: prostate cancer; TsmiR: Tumor suppressor miRNA; WHSC1: Wolf-Hirschhorn syndrome candidate 1.
Declarations

Acknowledgements

We would like to express our gratitude to all those who financed the subject.

Funding

This research was supported by the Argentinean Agency of Science and Technology (ANPCyT PICT 2014-324; PICT 2015-1345, PICT 2018-1304, PICT START UP-2019-21) and by Williams Foundation (Argentina).

Availability of data and materials

Microarray data have been deposited in GEO database (accession number GSE181350).

The patient datasets analyzed during the current study are available in UCSC’s Xena Browser (http://xena.ucsc.edu/) and GEO datasets (https://www.ncbi.nlm.nih.gov/gds). ssGSEA analysis was performed using GenePattern (https://www.genepattern.org/).

Author’s contributions

CM and ADS designed the study. CM and RBD carried out the experiments. CM, RBD, PLF and EL performed bioinformatics analyses. CM, RBD, PLF, EL, and ADS interpreted the data. CM and ADS wrote the manuscript with contributions of KG and approval from all authors.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interest

References

1. Ornish D, Weidner G, Fair WR, Marlin R, Pettengill EB, Raisin CJ, et al. Intensive lifestyle changes may affect the progression of prostate cancer. J Urol. 2005;174(3):1065-9; discussion 1069-70.
2. Narita S, Nara T, Sato H, Koizumi A, Huang M, Inoue T, et al. Research Evidence on High-Fat Diet-Induced Prostate Cancer Development and Progression. J Clin Med. 2019;8(5):597.
3. Porretti J, Dalton GN, Massillo C, Scalise GD, Farré PL, Elble R, et al. CLCA2 epigenetic regulation by CTBP1, HDACs, ZEB1, EP300 and miR-196b-5p impacts prostate cancer cell adhesion and EMT in metabolic syndrome disease. Int J Cancer. 2018;143:897–906.
4. Massillo C, Dalton GN, Porretti J, Scalise GD, Farré PLL, Piccioni F, et al. CTBP1/CYP19A1/estradiol axis together with adipose tissue impacts over prostate cancer growth associated to metabolic syndrome. Int J Cancer. 2019;144:1115–27.

5. Dalton GN, Massillo C, Scalise GD, Duca R, Porretti J, Farré PL, et al. CTBP1 depletion on prostate tumors deregulates miRNA/mRNA expression and impairs cancer progression in metabolic syndrome mice. Cell Death Dis. 2019;10:299.

6. Moiola CP, Luca P De, Zalazar F, Cotignola J, Rodríguez-Seguí SA, Gardner K, et al. Prostate tumor growth is impaired by CtBP1 depletion in high-fat diet-fed mice. Clin Cancer Res. 2014;20:4086–95.

7. Massillo C, Duca RB, Lacunza E, Dalton GN, Farré PL, Taha N, et al. Adipose tissue from metabolic syndrome mice induces an aberrant miRNA signature highly relevant in prostate cancer development. Mol Oncol. 2020;14:2868–83.

8. Duca RB, Massillo C, Dalton GN, Farré PL, Graña KD, Gardner K, De Siervi A. MiR-19b-3p and miR-101-3p as potential biomarkers for prostate cancer diagnosis and prognosis. Am J Cancer Res 2021. In press.

9. Peng Y, Croce CM. The role of microRNAs in human cancer. Signal Transduct Target Ther. 2016;1:15004.

10. Massillo C, Dalton GN, Farré PL, De Luca P, De Siervi A. Implications of microRNA dysregulation in the development of prostate cancer. Reproduction 2017;154:R81–97.

11. Palmer JD, Soule BP, Simone BA, Zaorsky NG, Jin L, Simone NL. MicroRNA expression altered by diet: Can food be medicinal? Ageing Res Rev. 2014;17:16-24.

12. Slattery ML, Herrick JS, Mullany LE, Stevens JR, Wolff RK. Diet and lifestyle factors associated with miRNA expression in colorectal tissue. Pharmgenomics Pers Med. 2017;10:1-16.

13. Tarallo S, Pardini B, Mancuso G, Rosa F, Di Gaetano C, Rosina F, et al. MicroRNA expression in relation to different dietary habits: A comparison in stool and plasma samples. Mutagenesis. 2014;29(5):385-91.

14. Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank products: A simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett. 2004;573:83–92.

15. Goldman MJ, Craft B, Hastie M, Repečka K, McDade F, Kamath A, et al. Visualizing and interpreting cancer genomics data via the Xena platform. Nat Biotechnol. 2020;38:675-678.

16. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, et al. Integrative genomic profiling of human prostate cancer. Cancer Cell. 2010;18:11–22.

17. Xie M, Dart DA, Owen S, Wen X, Ji J, Jiang W. Insights into roles of the miR-1, -133 and -206 family in gastric cancer (Review). Oncol Rep. 2016; 36(3):1191-1198.

18. Mitchelson KR. Roles of the canonical myomiRs miR-1, -133 and -206 in cell development and disease. World J Biol Chem. 2015; 6(3):162-208.
19. Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol. 2004;5(3):R13.

20. Nohata N, Hanazawa T, Enokida H, Seki N. MicroRNA-1/133a and microRNA-206/133b clusters: Dysregulation and functional roles in human cancers. Oncotarget. 2012;3:9-21

21. Kawakami K, Enokida H, Chiyomaru T, Tatarano S, Yoshino H, Kagara I, et al. The functional significance of miR-1 and miR-133a in renal cell carcinoma. Eur J Cancer. 2012;48(6):827-36.

22. Wong TS, Liu XB, Wong BYH, Ng RWM, Yuen APW, Wei WI. Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue. Clin Cancer Res. 2008;CCR-07-0666.

23. Szafrańska AE, Davison TS, John J, Cannon T, Sipos B, Maghnouj A, et al. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. Oncogene. 2007;26:4442-4452.

24. Wang H, An H, Wang B, Liao Q, Li W, Jin X, et al. MiR-133a represses tumour growth and metastasis in colorectal cancer by targeting LIM and SH3 protein 1 and inhibiting the MAPK pathway. Eur J Cancer. 2013; 49(18):3924-35.

25. Ma Y, Zhang P, Yang J, Liu Z, Yang Z, Qin H. Candidate microRNA biomarkers in human colorectal cancer: Systematic review profiling studies and experimental validation. Int J Cancer. 2012;130(9):2077-87.

26. Pignot G, Cizeron-Clairac G, Vacher S, Susini A, Tozlu S, Vieillefond A, et al. MicroRNA expression profile in a large series of bladder tumors: Identification of a 3-miRNA signature associated with aggressiveness of muscle-invasive bladder cancer. Int J Cancer. 2013;132(11):2497-91.

27. Kano M, Seki N, Kikkawa N, Fujimura L, Hoshino I, Akutsu Y, et al. MiR-145, miR-133a and miR-133b: Tumor-suppressive miRNAs target FSCN1 in esophageal squamous cell carcinoma. Int J Cancer. 2010; 127(12):2804-14.

28. Pashaei E, Pashaei E, Ahmady M, Ozen M, Aydin N. Meta-analysis of miRNA expression profiles for prostate cancer recurrence following radical prostatectomy. PLoS One. 2017;12(6):e0179543.

29. Coarfa C, Fiskus W, Eedunuri VK, Rajapakshe K, Foley C, Chew SA, et al. Comprehensive proteomic profiling identifies the androgen receptor axis and other signaling pathways as targets of microRNAs suppressed in metastatic prostate cancer. Oncogene. 2016;35(18):2345-56.

30. Tao J, Wu D, Xu B, Qian W, Li P, Lu Q, et al. microRNA-133 inhibits cell proliferation, migration and invasion in prostate cancer cells by targeting the epidermal growth factor receptor. Oncol Rep. 2012;27(6):1967-75.

31. Kojima S, Chiyomaru T, Kawakami K, Yoshino H, Enokida H, Nohata N, et al. Tumour suppressors miR-1 and miR-133a target the oncogenic function of purine nucleoside phosphorylase (PNP) in prostate cancer. Br J Cancer. 2012;106:405-413.

32. Wang BD, Ceniccola K, Yang Q, Andrawis R, Patel V, Ji Y, et al. Identification and functional validation of reciprocal microRNA-mRNA pairings in African American prostate cancer disparities. Clin Cancer Res. 2015;21(21):4970-84.
33. Tang Y, Pan J, Huang S, Peng X, Zou X, Luo Y, et al. Downregulation of miR-133a-3p promotes prostate cancer bone metastasis via activating PI3K/AKT signaling. J Exp Clin Cancer Res. 2018;37(1):160.

34. Li SM, Wu HL, Yu X, Tang K, Wang SG, Ye ZQ, et al. The putative tumour suppressor miR-1-3p modulates prostate cancer cell aggressiveness by repressing E2F5 and PFTK1. J Exp Clin Cancer Res. 2018;37(1):219.

35. Karatas OF, Guzel E, Suer I, Ekici ID, Caskurlu T, Creighton CJ, et al. miR-1 and miR-133b are differentially expressed in patients with recurrent prostate cancer. PLoS One 2014;9(6):e98675.

36. Martens-Uzunova ES, Jalava SE, Dits NF, Van Leenders GJLHLH, Møller S, Trapman J, et al. Diagnostic and prognostic signatures from the small non-coding RNA transcriptome in prostate cancer. Oncogene. 2012;31:978–91.

37. Hudson RS, Yi M, Esposito D, Watkins SK, Hurwitz AA, Yfantis HG, et al. MicroRNA-1 is a candidate tumor suppressor and prognostic marker in human prostate cancer. Nucleic Acids Res. 2012;40(8):3689-703.

38. Wang S, Wu W, Claret FX. Mutual regulation of microRNAs and DNA methylation in human cancers. Epigenetics. 2017;12(3):187-197.

39. Göbel C, Özden C, Schroeder C, Hube-Magg C, Kluth M, Möller-Koop C, et al. Upregulation of centromere protein F is linked to aggressive prostate cancers. Cancer Manag Res. 2018;10:5491-5504.

40. Zhuo YJ, Xi M, Wan YP, Hua W, Liu YL, Wan S, et al. Enhanced expression of centromere protein F predicts clinical progression and prognosis in patients with prostate cancer. Int J Mol Med. 2015;35(4):966-72

41. Shahid M, Lee MY, Piplani H, Andres AM, Zhou B, Yeon A, et al. Centromere protein F (CENPF), a microtubule binding protein, modulates cancer metabolism by regulating pyruvate kinase M2 phosphorylation signaling. Cell Cycle. 2018;17(24):2802-2818.

42. Shahid M, Kim M, Lee MY, Yeon A, You S, Kim HL, et al. Downregulation of CENPF Remodels Prostate Cancer Cells and Alters Cellular Metabolism. Proteomics. 2019;19(11):e1900038.

43. Stangl-Kremser J, Lemberger U, Hassler MR, Garstka N, Grubmüller B, Haitel A, et al. The prognostic impact of tumour NSD2 expression in advanced prostate cancer. Biomarkers. 2020;25(3):268-273.

44. Want MY, Tsuji T, Singh PK, Thorne JL, Matsuzaki J, Karasik E, et al. WHSC1/NSD2 regulates immune infiltration in prostate cancer. J Immunother Cancer. 2021;9(2):e001374.

45. Ezponda T, Popovic R, Shah MY, Martinez-Garcia E, Zheng Y, Min DJ, et al. The histone methyltransferase MMSET/WHSC1 activates TWIST1 to promote an epithelial-mesenchymal transition and invasive properties of prostate cancer. Oncogene. 2013;32(23):2882-90.

46. Aytes A, Giacobbe A, Mitrofanova A, Ruggiero K, Cyrla J, Arriaga J, et al. NSD2 is a conserved driver of metastatic prostate cancer progression. Nat Commun. 2018;9(1):5201.

Tables
Due to technical limitations, tables are only available as a download in the Supplemental Files section.

**Figures**

Figure 1

HFD regulates the expression of miRNAs in TRAMP-C1 tumors from mice. Heat map representation for miRNA expression in CD and HFD allografts. The heat map was generated based on differentially expressed miRNAs with a FDR <0.05 in TRAMP-C1 tumors developed in HFD compared to CD fed mice. CD, control diet; HFD, high-fat diet.
Figure 2

Functional enrichment analysis of validated miRNA targets. (A) Flow chart used for the analysis. (B) Barplot representation of the top 20 significant KEGG pathways associated with the target genes of the down-modulated miRNAs of TRAMP-C1 tumors from HFD-fed mice compared to CD-fed mice. (C) Scatterplot of the two principal components of PCA from the gene expression data. The green, blue and red circles represent normal prostates, NAT and prostate tumors samples respectively.
Figure 3

miRNA-mRNA correlation. (A) Correlation matrix showing the extent of correlation between the expression of miRNAs and mRNAs in PCa from the TCGA-PRAD cohort based on Spearman's linear correlation. (B-C) Spearman correlation between the ssGSEA enrichment scores and mature miRNA expression in prostate tumors from TCGA-PRAD (B) and Taylor (C) datasets. (D) Gene/gene set overlap matrix for nine common target genes with MSigDB collections.
Figure 4

Hsa-miR-133a-3p, miR-133b and miR-1-3p are decreased in human prostate tumors compared with non-tumor tissues. The differential analysis was performed based upon two independent datasets, (A) TCGA-PRAD: PCa tissues compared with NAT (NAT, n = 52; PCa, n = 52). Read per millions values are graphed. Data were analyzed using paired t-test. (B) Taylor (GSE21032): PCa tissues compared with normal prostates (normal prostates, n = 28; PCa, n =113). glog2 values are graphed. Data were analyzed using Mann Whitney test.
Low expression of hsa-miR-133a-3p, miR-133b and miR-1-3p correlates with poor clinicopathological features of PCa patients. (A-C) Hsa-miR-133a-3p, miR-133b and miR-1-3p expression levels in PCa tissues with different Gleason score in two independent datasets (TCGA-PRAD and Taylor [GSE21032]). Data were analyzed using Kruskal-Wallis test with Dunn's multiple comparisons. Kaplan–Meier plots of progression free interval curves according to the relative expression levels of hsa-miR-133a-3p (A), miR-
133b (B) and miR-1-3p (C) in primary PCa tissues from TCGA-PRAD cohort. Patients were categorized into two groups based on the median expression levels of miRNA. Data were analyzed using log-rank Test.

Figure 6

Hsa-miR-1-2/miR-133a-1 promoter hypermethylation is increased in PCa and negatively correlated with miRNA expression. The methylation levels of hsa-miR-1-2/133a1 (A), hsa-miR-133b (B) and hsa-miR-1-1/133a2 (C) promoter regions were estimated from the TCGA-PRAD dataset in 52 paired PCa tissues compared to NAT. Beta values are graphed. Data were analyzed using paired t-test.
CENPF, WHSC1 and PAICS expression are increased in primary prostate tumors from patients. (A) CENPF, WHSC1, CPSF6, RBM15B, NRP1, RALA, PAICS, BCL7A and ELAVL1 and expression levels in paired PCa tissues compared with NAT by analyzing the PCa mRNA sequencing dataset from TCGA-PRAD (NAT, n = 52; PCa, n = 52). Read per millions values are graphed. Data were analyzed using paired t-test. (B) CENPF, WHSC1, CPSF6, RBM15B, NRP1, RALA, PAICS, BCL7A and ELAVL1 expression levels in primary tumors
compared to normal prostates by analyzing the expression profiling by array dataset from Taylor (GSE21032). Normalized signal intensity values are graphed. Data were analyzed using Mann Whitney test.

**Figure 8**

**CENPF** and **WHSC1** expression positively correlated with Gleason grade. (A-C) **CENPF**, **WHSC1** and **PAICS** expression levels in PCa tissues with different Gleason score in two independent datasets (TCGA-PRAD and Taylor (GSE21032)).
and Taylor [GSE21032]). Data were analyzed using Kruskal-Wallis test with Dunn's multiple comparisons. Kaplan–Meier plots of progression free interval curves according to the relative expression levels of CENPF (A), WHSC1 (B) and PAICS (C) in primary PCa tissues from TCGA-PRAD cohort. Patients were categorized into two groups based on the median expression levels of mRNA. Data were analyzed using log-rank Test.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- GraficalAbstractModeloV2.pdf
- Table1.pdf
- Table2.pdf
- SupplementalsMassilloDuca2021.pdf