Deletion in a regulatory region is associated with underexpression of miR-148b-3p in patients with prostate cancer

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Abstract. Prostate cancer (PCa) is the leading cause of cancer-related death in men. This pathology is complex and heterogeneous; therefore, elucidating the molecular mechanisms that lead to its origin and progression is imperative. MicroRNAs (miRNAs or miRs) are part of the epigenetic machinery that regulates the expression of human genes, therefore, mutations in the genes that encode them can lead to a dysregulation in their expression, which directly impacts their target genes, which could be oncogenes or tumor suppressor genes. In PCa several dysregulated expression levels of miRNAs are associated with perturbed cellular processes. A differential expression of miRNAs such as miR-145-5p and miR-148-3p has been observed in PCa, possibly due to mutations in regions near the miRNAs. However, the molecular mechanisms that lead to the dysregulation of these miRNAs still need to be clarified. Therefore, the present study aimed to analyze the expression of miRNAs and their relationship with mutations in patients with and without PCa. In total, 71 patients were analyzed: 41 of whom had PCa (CAP group) and 30 with benign pathology (BPD group). Underexpression was observed in miR-145-5p and miR-148b-3p in PCa patients (P=0.03 and P=0.001, respectively). In miR-145-5p, no mutations related to its expression were identified. For miR-148b-3p, a set of mutations were identified in the chr12:54337042/54337043 region, which were grouped into the mutation named DelsAAG. Although this mutation's abnormal allele is related to PCa (P=0.017), a statistically significant difference was observed in the expression of miR-148b-3p between carriers and non-carriers of the mutated allele, identifying a mechanism likely to be involved in the miR-148b-3p dysregulation.

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Introduction

Several studies have suggested that the microRNAs (miRNAs or miRs) miR-145-5p and miR-148b-3p are linked to the development of prostate cancer (PCa) as they have been observed to be dysregulated during this disease, and their expression levels differ from patients with benign prostate diseases (1-4). Furthermore, mutations in the genes that code for these miRNAs and in nearby regions have been reported, suggesting a possible explanation for their dysregulation. miR-145-5p has been classified as a tumor suppressor, specifically in PCa; this miRNA has been linked to various mechanisms that promote the development of this pathology (5,6). Some factors have been associated with the underexpression of this miRNA; for example, single nucleotide polymorphisms (SNPs) have been suggested as possible causes of miRNA aberrant expression. In PCa, the rs4705342 polymorphism stands out; in the Asian population, it has been observed that men with the TC/CC genotypes have a significantly lower risk of having PCa than those with the TT genotype. Functionality analyses demonstrated that the T allele increases protein-binding affinity and thus reduces promoter activity, resulting in decreased transcription of the miR-145-5p (7). In recent years, this polymorphism has been studied in the European population. It has been observed that, unlike the Asian population, the C allele appears to be a risk factor in Serbian men (8). These findings highlight the importance of analyzing mutations to identify their involvement in regulating this miRNA and PCa susceptibility.

miR-148b-3p has been observed to be overexpressed in PCa, both in early and metastatic stages (9). Regarding the regulation of this miRNA through single nucleotide

polymorphisms, Chen *et al* (10) observed in 2014 that the rs11170877 (A>G) polymorphism, specifically the G allele and the rs12231393 (T>C) allele C, were associated as a protective factor for gastric cancer in Chinese patients. The rs11170877 variant is located in the exon of COPZ1, the gene that contains or hosts miR-148b. These two polymorphisms form a haplotype due to linkage disequilibrium; this haplotype showed a significant association with this cancer (10). Despite the interesting results obtained with the rs11170877 polymorphism in this type of cancer, few studies have linked this SNP to other types of cancer or even to different populations. Therefore, the present study aimed to identify mutations in miR-145-5p and miR-148b-3p and regions near them and relate them to their expression in patients with and without PCa.

Materials and methods

Patient samples. Non-probabilistic convenience sampling was conducted on prostatic tissue samples collected via transrectal ultrasound-guided 18G core needle biopsy. The biopsies were conducted by a single Board-Certified Diagnostic Radiologist with 20 years of experience in the procedure, utilizing the standard technique, including the sampling of ultrasound-suspicious areas (nodules). The samples were obtained from the tumor tissue of patients with a suspicious diagnosis of PCa based on either positive digital rectal examination or rising prostate specific antigen (PSA). The collected samples were stored in Qiazol® (Qiagen GmbH) at 20˚C for later analysis.

The diagnosis and classification of patients with benign prostatic hyperplasia (BPH) and PCa were performed by a single Board-Certified Pathologist with 7 years of experience using histopathology. The inclusion criteria were as follows: Mexican, ≥18 years, histopathological diagnosis of PCa or BPH. The exclusion criteria included patients with other types of cancer and those who had received chemotherapy or radiotherapy for any other medical reason.

The present study consisted of 71 samples: 41 from patients with PCa diagnosis (PCa group) and 30 from patients diagnosed with benign prostatic disease (BPD group). The BPD group included patients with BPH (n=7), prostatitis (n=18), both hyperplasia and prostatitis (n=4), and intraepithelial neoplasia (n=1). All patients were recruited from the Alvarez & Arrazola Radiologists Clinic in Sinaloa, Mexico, from August 2016 to December 2021. Clinicopathological characteristics including age (applicable to individuals aged ≥18 years, with an upper limit of 90 years), weight, height, body mass index, PSA and family history of cancer were collected through direct questionnaires and the clinical database. An expert pathologist provided the diagnosis and Gleason score. All patients signed informed consent forms, which were reviewed and approved by the Alvarez and Arrazola Radiologists Clinic's Ethics and Research Committee (study approval number P-3103).

RNA and DNA extraction. Tissue samples were used for RNA extraction. The prostate tissue in Qiazol was macerated using sterile pistils, until leaving a homogeneous solution. Total RNA, including miRNAs, was isolated using the miRNEasy kit (Qiagen) according to the manufacturer's protocol. DNA extraction was performed using organic solvents and salt precipitation from the remnants of Qizol® obtained from RNA extraction using the previously described methodology of Chomczynski (1993) (11). The concentration of isolated RNA and DNA was measured with a GENESYS 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc.) at 260 nm. It also allows for the evaluation of RNA and DNA integrity, detection of contaminants, the analysis of low or atypical concentrations, and warning of invalid results. The concentration and integrity analysis were performed in triplicate.

Analysis of polymorphisms in miRNAs. To design primers for miRNA amplification and sequencing, the accession numbers NC_00005 and NC_000012 (https://www.ncbi.nlm.nih.gov/nuccore/NC_000005.10 and https://www.ncbi.nlm.nih.gov/nuccore/NC_000012, respectively) were used for miR-145 and miR-148b, respectively. CLC Sequence Viewer program (https://resources.qiagenbioinformatics.com/manuals/clcsequenceviewer/current/index.php?manual=Introduction_CLC_Sequence_Viewer.html) was used to select a region of ~1,000 base pairs spanning the gene sequence of each selected miRNA to cover the gene and possible regulatory sequences.

Primer-BLAST Tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was employed to design primers used the sequence selected, considering the following characteristics: 40-60% G + C, non-self-complementary and no more than 3˚C (melting temperature difference) between both primers. The primers selected for miR-148b-3p were forward 5'-CAGGCTTTAGAAGCCCCTGA-3' and reverse, 5'-GCGCTTAAATGCCGCTTCA-3' with a 986 bp amplicon. And the primers used for miR-145-5p were forward, 5'-TCTCCAGTAGGTCGTGGACT-3' and reverse, 5'-CACAAGAGGGCGTTCTGAGT-3', with a 1,000 bp amplicon. Sealed 10 μl aliquots of DNA were subsequently sent to the Korean company MACROGEN for capillary electrophoresis sequencing. Once the data was obtained, Chromas V2.6.6 (https://technelysium.com.au/wp/chromas/) was used to clean the sequence, eliminating the ends in which the sequence was not reliable using the default parameters. The reference sequence GRCh38.p14 was obtained using the CLC sequence viewer 8.0 program. Subsequently, the SnackVar v2.4.3 (12) and NovoSNP v.3.0.1 (13) tools were used to identify mutations. Both programs are automatically using the default parameters detect SNVs and indel type mutations by comparing them with a reference sequence. Once the mutations were identified, only those that coincided in both programs and those that had a quality score >40 were included in this analysis.

Reverse transcription (RT) PCR and relative miRNA expression. RT was performed from 10 ng of total RNA with the TaqMan Advanced miRNA cDNA Synthesis kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc.). To obtain cDNA, the TaqMan Advanced miRNA cDNA Synthesis kit was used, which consists of four steps as follows: i) Polyadenylation reaction: First, a reaction mix was prepared where, for a final volume of 5 μl, 0.5 μl of 10x Poly(A) buffer, 0.5 μl of ATP, 0.3 μl of Poly A enzyme and 2 μl of miRNAs were added. Vortexing was briefly performed. The T100 Thermal Cycler (Bio-Rad Laboratories, Inc.) was programmed as follows: 45 min at 37˚C and 10 min at 65˚C. The next

reaction was immediately carried out. ii) Ligation Reaction: The following reaction mixture was prepared for a final volume of 15 μl: 3 μl of 5X DNA ligase buffer, 4.5 μl of 50% PEG 8000, 0.6 μl of 25X ligation adapter, 1.5 μl RNA ligase and 0.4 μl of RNase-free water; and then, the 5 μl reaction mixture obtained in the polyadenylation was added and vortexed briefly. It was subsequently incubated at 16˚C for 60 min in the same thermal cycler. The reverse transcription reaction was carried out immediately afterwards. iii) Reverse transcription (RT) reaction: The following reaction mixture was prepared for a final volume of 30 μl: 6 μl of 5X RT buffer, 1.2 μl of dNTP mix (25 mM), 1.5 μl of 20X Universal RT primer, 3 μl of 10X RT enzyme mix and 3.3 μl of RNase-free water. It was mixed by pipetting, then this reaction mixture was added to the 15 μl of reaction obtained from the ligation reaction and vortexed briefly. The thermal cycler was programmed as follows: 15 min at 42˚C and 5 min at 85˚C. For each of the miRNAs, standard curves of five points of serial dilutions 1:10 were made to obtain the efficiencies from the slope. Quantitative estimations for miR-145-5p (assay ID: 477916_mir), miR-148b-3p (assay ID: 477824_mir), and miR-191-5p (assay ID: 477952_mir) were performed using TaqMan Advance miRNA Assays by Thermo Fisher Scientific, Inc. using a StepOnePlusÔ thermal cycler (Applied Biosystems). To select the normalizing miRNA, the RefFinder tool (https://blooge.cn/RefFinder/) was used, which integrates different algorithms to select normalizers, identifying miR-191-5p as the most appropriate miRNA for the present study.

Relative expression analysis. To obtain relative miRNA expression of miR-145-5p and miR-148b-3p, the previously described method by Taylor *et al* (2019) (14) was used based on the ΔΔCq method ($2^{-\Delta\Delta Cq}$ algorithm) (15) and the miR-191-5p was used as reference gene. First, the average of the Cq of the control samples was calculated; subsequently, the average Cq obtained was subtracted individually for each test sample, obtaining a ΔCq, and then, the RQ was calculated using the formula $2^{\Delta\Delta Cq}$. Subsequently, the normalized expression was obtained for each sample using the formula RQ problem gene/RQ normalizing gene; and finally, the normalization of the expression per sample was carried out using the Log2 of each result obtained in the previous formula.

Statistical analysis. Once the information was collected, the data was organized into tables and figures. Measures of central tendency (mean, median, mode), measures of dispersion (standard deviation), and confidence intervals were estimated for the quantitative variables. For the qualitative variables, the frequencies and percentages were estimated, as well as their respective confidence intervals. To identify differences between means of the different quantitative variables in patients with and without PCa, the unpaired Student's t-test and Mann-Whitney U statistics were performed for parametric and non-parametric variables, respectively. To find a relationship between qualitative variables, the Chi-square test was used. For the correlation analyses, the Pearson's correlation coefficient and the Spearman's test were used, for parametric and non-parametric variables, respectively. All the analyses were carried out using the statistical package SPSS v.20 (IBM Corp.). To determine if the distribution of the genotypes of each mutation was in Hardy-Weinberg (EHW) equilibrium, the SNPStats software (http://www.snpstats.net/start.htm) was used. Similarly, the haplotype frequencies and linkage analysis were calculated using the same software. P<0.05 was considered to indicate a statistically significant difference.

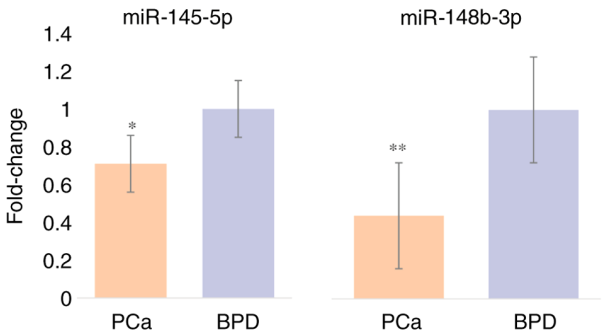

Figure 1. Relative expression of miR145-5p and miR-148b-3p. Analyzing the expression of both miRNAs, a decreased expression was observed in patients with PCa. miR, microRNA; PCa, prostate cancer; BPD, benign prostatic diseases. *P<0.05 and **P<0.01.

Results

miRNAs expression analysis. Relative expression of miR-145-5p and miR-148b-3p was quantified in all samples of BPD and PCa patients (Fig. 1). When analyzing miR-145-5p expression, an expression factor of 0.71±0.15 was identified in PCa patients compared with BPD patients, showing a significant difference (P=0.033), and miR-148b-3p was underexpressed in PCa patients with an expression factor of 0.44±0.28 with a statistically significant difference between groups (P=0.001).

Identification of mutations. A total of 18 mutations were identified in the analyzed sequence for miR-148b-3p in 71 study samples (Table I). Different types of mutations were observed, which were single nucleotide variations (SNVs), deletions (Del), an insertion (Ins), and a duplication (Dup). Among the mutations obtained, 12 had already been registered, while the remaining six had not. In position chr12:54337042/54337043, there were four different deletions, two of which were previously registered (rs1465330647 and rs1187801243), and two which have not been reported in any database (Fig. 2). The rs1465330647 mutation is the deletion of AAAGAA, the rs1187801243 mutation is the loss of AAGAA, and the other two unrecorded mutations comprise the deletion of AAAGA in one case and AAGAAG in the other. As they are very similar to each other and have a low frequency separately, they were grouped into a single variable called DelsAAG. To analyze the relationship of these mutations with the expression of miR-148b-3p, those with the highest frequency of the mutated allele were selected: DupCT (rs59761210), SNV G/T (rs1381668656), SNV A/C (NA), DelG (rs368666376), SNV G/C (rs56818309) and DelsAAG (Table I). Mutation analysis for miR-145-5p was only performed on 44 of the 71 study samples since

Table I. Mutations identified for miR-148b-3p.

Mutation	Position	ID	Mutated allele frequency
SNV G/T	54336876	NA	0.01
SNV G/A	54337007	NA	0.01
DupCT	54337015	rs59761210	0.15[a]
SNV C/G	54337017	rs555306172	0.01
SNV G/T	54337018	rs1381668656	0.11[a]
SNV A/C	54337021	NA	0.10[a]
SNV A/C	54337042	rs1953883052	0.02
SNV A/C	54337043	rs918405335	0.01
DelAAAGA	54337042	NA	0.01
DelAAAGAA	54337042	rs1465330647	0.08
DelAAGAA	54337043	rs1187801243	0.02
DelAAGAAG	54337043	NA	0.09
DelG	54337045	rs368666376	0.12[a]
InsA	54337045	NA	0.01
SNV G/A	54337045	rs879146327	0.01
SNV A/C	54337046	rs1048873442	0.01
SNV A/C	54337047	rs1481050082	0.02
SNV G/C	54336970	rs56818309	0.27[a]
DelsAAG[b]	54337042/54337043	NA	0.21[a]

[a]Mutations selected by the frequency of the mutated allele; [b]Clustering of deletions (DelAAAGA, DelAAAAGAA, DelAAGAA, DelAAGAAG) (n=71). MicroRNA, miR; SNV, single nucleotide variations; dup, duplication; Del, deletion; Ins, insertion.

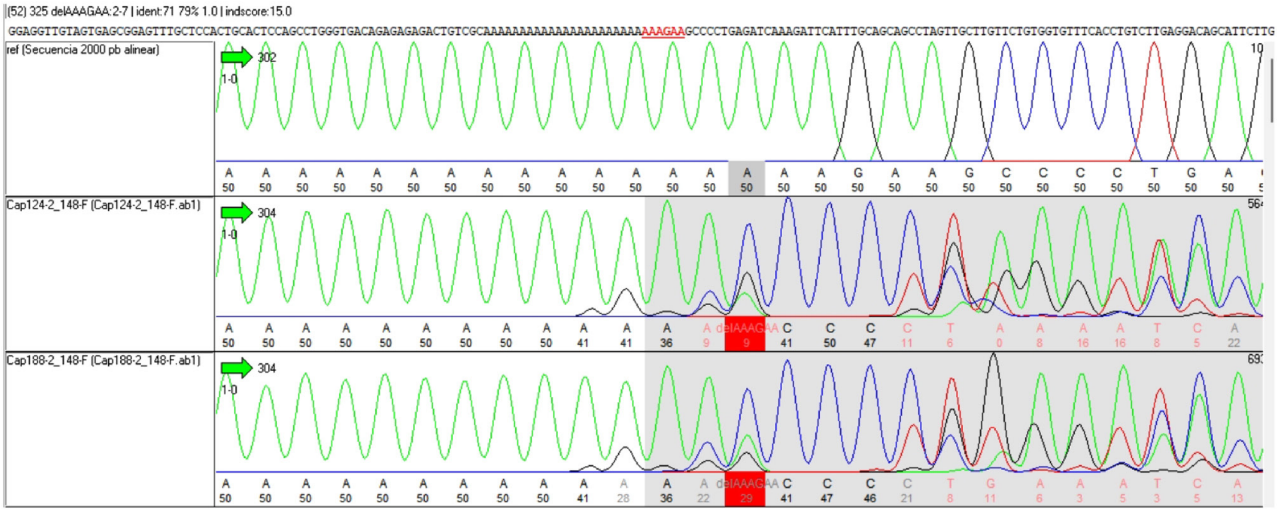

Figure 2. DelsAAG mutations analysis. Identification of DelsAAG deletions using the NovoSNP program in two samples of prostate cancer patients.

the remaining samples could not be sequenced. Of the 44 samples, 21 were from the PCa and 23 from the BPD groups. Mutation analysis for miR-145-5p was performed, where 62 mutations were identified, of which 24 had previously been reported. Of the 62 mutations, 59 were single nucleotide variations (SNV), two deletions (Del), and one duplication (Dup). Most of the mutations had a low frequency of the mutated allele; for this reason, only the rs353291 mutation, consisting of a T/C SNV with a frequency of 0.18 of the mutated alleles in the total population, was chosen.

Analysis by inheritance model. Subsequently, an analysis of the possible inheritance models of these six mutations of miR-148-3p, was carried out according to the response variable that groups patients in PCa and BPD using the SNPStat.net software. Of the six mutations, only DelsAAG was significant for the codominant (P=0.03, AIC=95.4), dominant (P=0.01, AIC=94.7), and over dominant (P=0.02, AIC=94.9) models. To conduct subsequent analyses using genotypes and alleles, the inheritance model was selected according to the lowest value of the Akaike Information Criterion (AIC) obtained.

Table II. Allele and genotypic frequencies of miR-148b-3p mutations.

Mutation	Inheritance model	Genotype	Group BPD	Group PCa	RM (95% CI)	P-value
dupCT rs59761210	Dominant	CT/CT	21 (70%)	32 (78%)	0.66 (0.22-1.92)	0.44
		CT/CTCT	7 (23%)	7 (17%)		
		CTCT/CTCT	2 (7%)	2 (5%)		
		CTCT	0.18	0.13	0.66 (0.22-1.92)	0.44
		G/G	22 (73%)	33 (81%)		
SNP G/T rs1381668656	Codominant	G/T	8 (27%)	8 (19%)	0.67 (0.22-2.04)	0.48
		T/T	-	-		
		T	0.13	0.1	0.72 (0.33-1.56)	0.41
		G/G	22 (74%)	33 (80%)		
DelG rs368666376	Recessive	G/G^{-}	7 (23%)	8 (20%)	0.00 (0.00-NA)	0.19
		G^{-}/G^{-}	1 (3%)	0 (0%)		
		G^{-}	0.13	0.1	0.41 (0.31-0.55)	0.24
		G/G	13 (44%)	23 (56%)		
SNV G/C rs56818309	Overdominant	G/C	16 (53%)	16 (39%)	0.56 (0.22-1.45)	0.23
		C/C	1 (3%)	2 (5%)		
		C	0.3	0.24	0.56 (0.22-1.45)	0.23
		A/A	23 (77%)	36 (88%)		
SNV A/C 54337021	Codominant	A/C	7 (23%)	5 (12%)	0.46 (0.13-1.61)	0.22
		C/C	-	-		
		C	0.12	0.06	0.49 (0.15-1.63)	0.24
		AAG/AAG	26 (87%)	25 (61%)		
DelsAAG	Dominant	AAG/AAG^{-}	1 (3%)	9 (22%)	4.16 (1.22-14.17)	0.014
		AAG^{-}/AAG^{-}	3 (10%)	7 (17%)		
		AAG^{-}	0.12	0.28	4.16 (1.22-14.17)	0.017

MicroRNA, miR; BPD, benign prostatic disease; PCa, prostate cancer; RM, odds ratio.

For the rs59761210 mutation, the dominant model was selected; for the rs368666376 mutation, the recessive model, for the rs56818309 mutation, the over dominant model; and for the DelsAAG mutation, the dominant model. In the case of the rs1381668656 and SNV A/C mutations, the analysis by different inheritance models was not obtained because the mutated homozygous genotype was not observed in the study groups; hence the codominant inheritance model was selected. In the case of miR-145-5p for the rs353291 mutation, none of the inheritance models were statistically significant; therefore, the model with the lowest AIC value was chosen (over dominant model, AIC=62.2).

Genotypic and allele frequencies and their association with PCa. The genotype and allele frequencies were calculated for each of the selected mutations, and both genotypes and alleles were associated with PCa. The allele and genotypic frequencies of the rs59761210, rs1381668656, rs368666376, rs56818309 and SNV A/C mutations are in Hardy-Weinberg equilibrium (P=0.052, P=0.59, P=0.98, P=0.36 and P=0.44, respectively); in the case of the DelsAAG mutation, this was not observed in Hardy-Weinberg equilibrium (P=0.001). The DelsAAG mutation was significantly related to the presence of PCa (P=0.014), wherein a dominant model, both mutated homozygotes and heterozygotes, were related to the pathology, unlike normal homozygotes. Likewise, the analysis by alleles revealed that patients who carry the DelsAAG mutation are 4.16 (P=0.017) times more likely to develop PCa; the other mutations were not observed to be related to the pathology (Table II). The rs353291 mutation demonstrated a frequency of the heterozygous genotype of 33.3% in patients with PCa, and 13% in the BPD group. The genotype analysis found no statistically significant relationship between the genotypes and the presence of PCa. The mutated allele presents a frequency of 0.21 in the PCa group and 0.15 in the BPD group; no significant relationship between the mutated allele and the pathology was observed (P=0.11) (Table III).

Linkage disequilibrium and analysis by haplotypes. Linkage disequilibrium analysis was performed to identify possible haplotypes, where the set of mutations of rs368666376, rs56818309, and DelsAAG was found to be in linkage disequilibrium. In total, three haplotypes (haplotypes I, II and III) were obtained from the set of mutations, with 'haplotype I' having the normal alleles. An analysis was performed in which each haplotype was linked to the presence of PCa. However, no statistically significant association was found. In the case of miR-145-5p, since only one mutation was studied, this analysis was not performed.

Table III. Allele and genotypic frequencies of the rs353291 mutation.

Mutation	Inheritance model	Genotype	Group BPD	Group PCa	RM (95% CI)	P-value
SNV T/C rs353291		T/T	18 (78.3%)	13 (61.9%)		
					3.33 (0.73-15.17)	0.11
	Sobredominant	T/C	3 (13%)	7 (33.3%)		
		C/C	2 (8.7%)	1 (4.8%)	3.33 (0.73-15.17)	0.11
		C	0.15	0.21		

BPD, benign prostatic disease; PCa, prostate cancer; RM, odds ratio.

Table IV. Analysis of the mean difference in the expression of miR-148b-3p between the genotypes of the mutations in the study population.

Mutation	Genotype	N	Average	Standard error	P-value
dupCT rs59761210	CT/CT	53	-0.88	0.23	
	CT/CTCT	14	-0.88	0.30	0.17
	CTCT/CTCT	4	0.41	0.71	
SNP G/T rs1381668656	G/G	55	-0.54	0.23	
	G/T	16	-0.73	0.33	0.69
	T/T	0	-	-	
DelG rs368666376	G/G	56	-0.71	0.21	
	G/G^{-}	14	-0.66	0.49	0.9
	G^{-}/G^{-}	1	-	-	
SNV G/C rs56818309	G/G	36	-1.03	0.31	
	G/C	32	-0.34	0.23	0.18
	C/C	3	-0.27	0.29	
SNV A/C 54337021	A/A	59	-0.69	0.21	
	A/C	12	-0.66	0.44	0.95
	C/C	0	-	-	
DelsAAG	AAG/AAG	51	-0.36	0.19	
	AAG/AAG^{-}	10	-2.10	0.75	0.004 (0.03[a])
	AAG^{-}/AAG^{-}	10	-0.95	0.33	

[a]Multiple comparisons using Tukey's test, difference between normal homozygous and heterozygous genotypes.

Relationship of mutations with miRNAs expression. The gene expression of miR-148b-3p between the genotypes and alleles of each mutation was compared and was observed that there is a statistically significant difference between the expression of the DelsAAG mutation genotypes (P=0.004), specifically between the normal homozygous genotypes with a mean expression of -0.35 and heterozygous with a mean of -2.10 (P=0.003) (Table IV). Similarly, this analysis compared alleles, where it was identified that the patients carrying the mutated allele of the DelsAAG mutation had a mean of -1.53. In the case of non-carriers, a mean of -0.36 was found, with a statistically significant difference (P=0.005). The remaining mutations were not observed with significant differences in the analyses by genotypes or by alleles (Table V). To identify a relationship between the rs353291 mutation and the expression of miR-145-5p, mean difference analyses were performed between genotypes and alleles. It was observed that the patients with the heterozygous genotype had a mean miR-145-5p expression of -0.38, however, no statistically significant difference was observed when compared with the other genotypes. The allele analysis revealed that patients carrying the mutated allele had a mean expression of -0.38 and non-carrier patients had a mean expression of -0.17; however, no statistically significant difference was observed between groups (P=0.47).

Discussion

miRNAs are small non-coding RNAs and are part of the epigenetic machinery that can regulate the expression of genes; therefore, increasingly altered mechanisms are identified due to the malfunction of these miRNAs, for example, it has been observed that several miRNAs dysregulated in PCa, are related to the regulatory T cell marker FOXP3, suggesting

Table V. Analysis of the mean difference in the expression of miR-148b-3p between the alleles of the mutations in the study population.

Mutation	Allele	N	Average	Standard error	P-value
dupCT rs59761210	Carrier	18	-0.13	0.28	0.046
	Normal	53	-0.88	0.23	
SNP G/T rs1381668656	Carrier	33	-0.53	0.22	0.53
	Normal	109	-0.73	0.16	
DelG rs368666376	Carrier	1	0.03	-	0.65
	Normal	70	-0.7	0.19	
SNV G/C rs56818309	Carrier	32	-0.34	0.23	0.09
	Normal	39	-0.98	0.28	
SNV A/C 54337021	Carrier	12	-0.66	0.44	0.95
	Normal	130	-0.7	0.14	
DelsAAG	Carrier	20	-1.53	0.42	0.005
	Normal	51	-0.36	0.19	

a relationship between immune cells, miRNAs and PCa (16). Therefore, the identification of dysregulated miRNAs could have implications both in understanding the pathology and in identifying possible targets for the detection, prognosis and treatment of various cancers (17,18).

The dysregulation of the expression of a miRNA can be due to mutations in its coding genes or in their regulatory regions; for example, SNPs in miR-146a and in miR-100 have been identified that act as a factor of bad and an improved prognosis in PCa. In addition, it has been mentioned that these polymorphisms could alter miRNA function (19). Furthermore, it has been observed that mutations can affect the biogenesis of the mature miRNA while it is in the 'seed' region of the passenger strand, thus decreasing its expression (20,21). Finally, mutations in the promoter regions can also cause changes in the expression of miRNAs, which have been observed in the miR-143/145 cluster associated with PCa (22).

In the case of miR-145-5p, several mutations were identified; among them, the one with the highest frequency was the rs353291 mutation. This mutation is located in an intronic region of the CARMN gene, 450 bp downstream of miR-145-5p. According to the NCBI database, this SNV has a mutated allele frequency of C=0.40 and normal allele frequency of T=0.60 in the global population. In the present study, the frequencies in the general population were C=0.18 and T=0.82, which are remarkably similar to those reported in the African and Afro-American population (C=0.23 and T=0.77) and to those reported in Latin Americans (C=0.30 and T=0.70). The Asian population is the most distinct from the frequencies observed in the present study (C=0.41 and T=0.59) (23).

The present results indicated that the rs353291 mutation is unrelated to CaP or miR-145-5p underexpression. At present, there is only one study that analyzes the relationship of this miR-145-5p SNV with cancer in an Australian population, which observed that the mutated allele was significantly related to breast cancer; additionally, it was suggested that this SNV could be part of the gene regulation mechanisms of miR-145-5p. It should be noticed, however, that they did not analyze miRNA expression (24). The dysregulation of this miRNA in CaP is probably caused by molecular mechanisms other than mutations close to the miRNA, such as DNA methylation. This is consistent with previous research; Zaman *et al* (2010) (25), reported that the promoter of this miRNA was hypermethylated in PCa cell lines. Similarly, previous studies have shown that methylation plays a role in regulating miR-145-5p expression, which contributes to tumor growth and response to treatment (26,27). Furthermore, Xue *et al* (2015) (28), observed that miR-145-5p targets the DNMT3b methyltransferase, one of its mechanisms as a tumor suppressor miRNA. In addition, they observed that DNMT3b leads to hypermethylation of the miRNA promoter, with feedback regulation between the two. To the best of the authors' knowledge, the only study analyzing the regulation of this miRNA by epigenetic mechanisms in tissue from patients with PCa is that of Suh *et al* (2011) (29) in the United States population, who observed a relationship between the underexpression of this miRNA and the hypermethylation in its promoter region. This suggested that epigenetic mechanisms in the regulation of miR-145-5p may be the most common cause of its gene expression dysregulation.

At present, the mechanisms that lead to the dysregulation of miR-148b-3p in PCa have not been studied. The present study identified a set of deletions with a start site at chr12:54337042/54337043, which were unified and called DelsAAG. This mutation was related to the presence of PCa; likewise, it was associated with the underexpression of miR-148b-3p. These deletions are located in a particular region, specifically in the first intron of the COPZ1 gene, in the sequence comprising chr12:54337020–54337044, there is a set of 25 adenines in tandem, behaving as a pure or perfect microsatellite, and as previously reported, this type of repeated sites are hot spots for mutations, as they are susceptible to the generation of deletions and insertions, being highly polymorphic, having mutation rates ranging from 103 and 106 for each cell generation, thus this could explain the existence of the mutations in the analyzed site (30-32). According to the GeneCards database (33), this mutation is

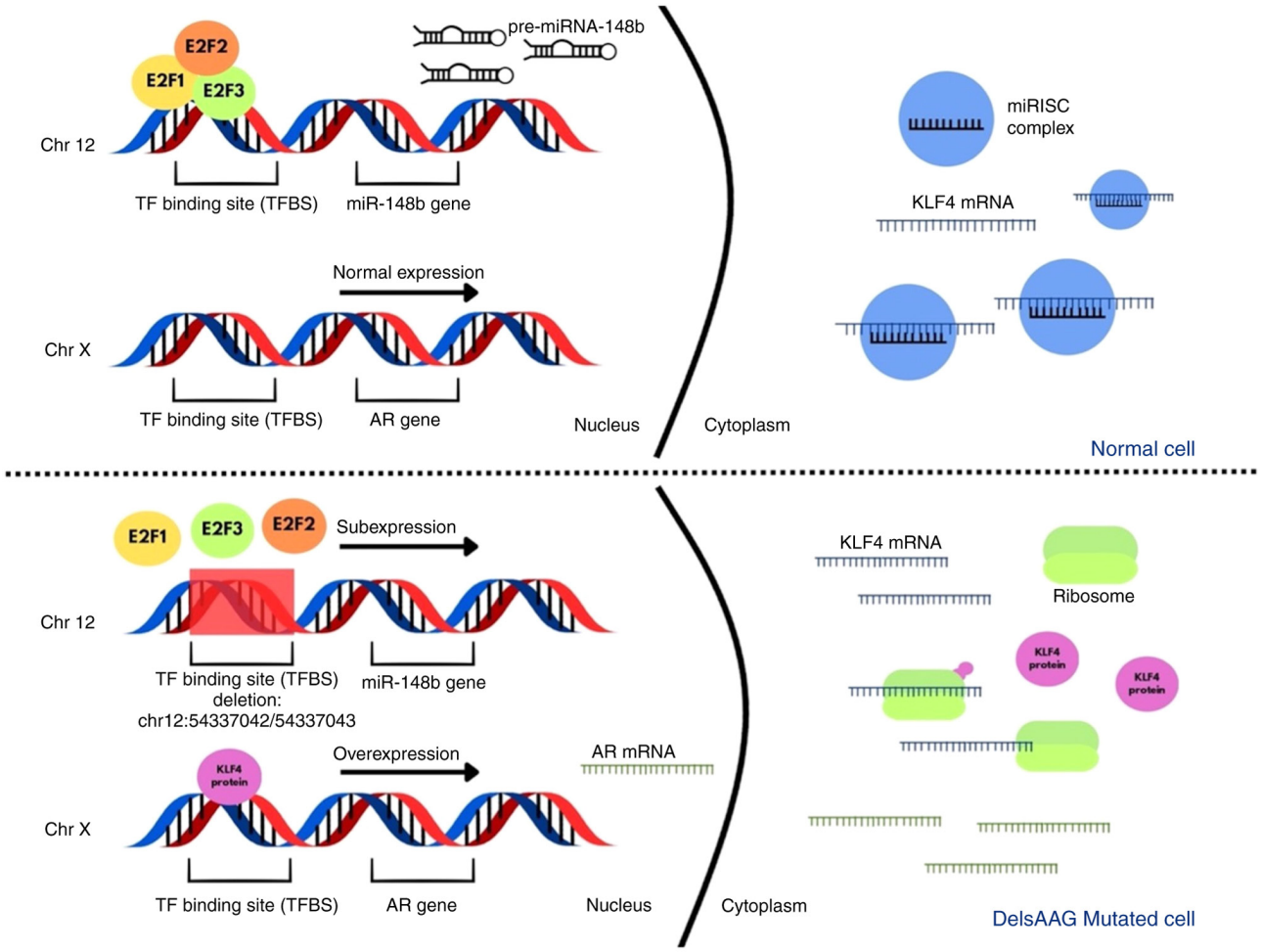

Figure 3. Regulation of miR-148b-3p gene expression. Deletion in a site close to miR148b-3p could decrease the affinity of transcription factors, affecting miRNA transcription and decreasing its expression, culminating in the proliferation and progression of prostate cancer cells through AR. miR, microRNA; AR, androgen receptor.

in a region that functions as a proximal intragenic enhancer (ID: GH12J054336), which could be a key cis-acting regulator promoting the expression of miR-148b-3p (34). Similarly, according to the ENSEMBL database, this mutation is found within a binding site for the transcription factors E2F1, E2F2 and E2F3 (chr:54337040-54337055) (35); previously these transcription factors have been identified to regulate the expression of other miRNAs, such as miRNAs 17 and 20, by binding to their promoter regions and promoting their expression (36,37). This is consistent with previous studies, as ~50% of the variants or mutations in transcription factor binding regions (RUFT) are in intronic regions (38). Previously, some RUFT mutations in Pac have been reported to modify their affinity and be involved in pathology development. For example, Huang *et al* (2014) (39), reported that an SNV increases the binding affinity of HOXB13, resulting in the overexpression of RFX6, leading to proliferation, migration and invasion of prostate tumor cells. Likewise, the rs7077275 polymorphism increases the binding of CTFC, which leads to the overexpression of CTBP2, decreasing apoptosis, and increasing tumor growth in PCa (40). Eickhardt *et al* (41) reported in 2016 that deletions are more disruptive variations than SNVs, leading to a more significant modification in the affinity of the transcription factors for the affected sequence. The aforementioned description indicated that the DelsAAG mutation could affect the binding of transcription factors, resulting in the absence or reduction of miR-148b-3p transcription.

This miRNA is directly involved in molecular mechanisms related to the development of PCa. Tomeva *et al* (2022) (42), observed that miR-148b-3p was underexpressed in tumors that occur at the androgen receptor (AR). Feng *et al* (43), in 2019 demonstrated that this miRNA was underexpressed in tumor tissue of patients with PCa. In addition, they observed that the 3'-untranlated region of the KLF4 gene contains the conserved miR-148b-3p binding site, making it a potential target for this miRNA binding, which was verified through their *in vitro* experiments (43). KLF4 is a transcription factor that can regulate the expression of various genes and that has been previously observed to be overexpressed in PCa (44). This transcription factor has an important implication in the prostate, since Siu *et al* (2016) (45), demonstrated a reciprocal relationship between KLF4 and the AR, where AR can induce the expression of this transcription factor. KLF4 can then bind to the AR promoter generating its transcription. In turn, AR activity is commonly altered in PCa, which promotes the evolution and progression of this cancer (46,47). Therefore, it was proposed by the auhtors that the mutation near miR-148b-3p, which includes a set of deletions in a binding

site for transcription factors, promotes miRNA underexpression, which means that it cannot regulate the expression of KLF4 increasing its expression and, as a result, leading to the overexpression of the AR, enhancing the proliferation and progression of PCa (Fig. 3).

In conclusion, mutations in regions near the gene that codes for a miRNA may be related to its expression and be part of the regulation mechanisms in a pathogenic event such as PCa. Thus, the present study becomes the first to propose a method for regulating the expression of miR-148b-3p in PCa, involving deletions in a transcription factor binding site.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FBH and FLO performed the statistical analysis, critically reviewed literature findings and drafting the manuscript. NGM, MAA and EAM collected the sample, conceived and designed the study and revised the manuscript. FBH and NGM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. P-3103) by the Alvarez and Arrazola Radiologists Clinic's Ethics and Research Committee (Mazatlán, Mexico). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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