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Altered microRNA Expression Profiles and Regulation of INK4A/CDKN2A Tumor Suppressor Genes in Canine Breast Cancer Models

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ABSTRACT

microRNA (miRNA) expression profiling of cancer versus normal cells may reveal the characteristic regulatory features that can be correlated to altered gene expression in both human and animal models of cancers. In this study, the comprehensive expression profiles of the 277 highly characterized miRNAs from the canine genome were evaluated in spontaneous canine mammary tumor (CMT) models harboring defects in a group of cell cycle regulatory and potent tumor suppressor genes of INK4/CDKN2 family including p16/INK4A, p14ARF, and p15/INK4B. A large number of differentially expressed miRNAs were identified in three CMT cell lines to potentially target oncogenes, tumor suppressor genes and cancer biomarkers. A group of the altered miRNAs were identified by miRNA target prediction tools for regulation of the INK4/CDKN2 family tumor suppressor genes. miRNA-141 was experimentally validated for INK4A 3′-UTR target binding in the CMT cell lines providing an essential mechanism for the post-transcriptional regulation of the INK4A tumor suppressor gene in CMT models. A well-recognized group of miRNAs including miR-21, miR-155, miR-9, miR-34a, miR-143/145, and miR-31 were found to be altered in both CMTs and human breast cancer. These altered miRNAs might serve as potential targets for advancing the development of future therapeutic reagents. These findings further strengthen the validity and use of canine breast cancers as appropriate models for the study of human breast cancers. J. Cell. Biochem. 116: 2956–2969, 2015.

KEY WORDS: miRNA; BREAST CANCER; TUMOR SUPPRESSOR; CYCLIN-DEPENDENT KINASE INHIBITOR; INK4A; CANINE CANCER MODEL

miRNAs are evolutionarily conserved, endogenous small structural RNA molecules of 20–22 nucleotides that post-transcriptionally suppress gene expression in a sequence-specific manner [Lagos-Quintana et al., 2001]. The discovery of miRNAs established a new era in translational research and for understanding post-transcriptional regulation of genes as well as their critical regulatory roles in diverse and vital cellular processes including cell cycle, cell proliferation, differentiation, development and apoptosis as well as in disease pathogenesis [Croce and Calin, 2005; Schickel et al., 2008; Stefani and Slack, 2008]. Experimental evidence and the latest miRNA target predictions suggest that more than 60% of protein-coding genes in the human genome are subject to regulation by miRNAs, making them the most abundant single class of regulatory biomolecules known [Lewis et al., 2005; Friedman et al., 2009; Fabian et al., 2010]. An increasing body of experimental data, online-based bioinformatics and prediction tools underscore the property of miRNA-target binding by the 2–7 nucleotide seed region of the 5′-end of mature miRNAs. Since this short complementary site is highly conserved across mammals, thousands of genes appear to be under the post-transcriptional regulation of miRNAs [Lewis et al., 2005]. To date, 2588 mature miRNAs have been annotated in the human genome while mouse and canine genomes account for 1915 and 453 miRNAs, respectively (miRBase Release 21, July 2014) [Kozomara and Griffiths-Jones, 2014].

There is an inherent relationship between miRNAs and cancer because more than 50% of miRNA genes have been found to be located at cancer-associated genomic regions or fragile sites that are also preferential sites for translocation, deletion, amplification, and integration of exogenous genome fragments [Calin et al., 2004; Garzon et al., 2009]. Since miRNAs are encoded by highly conserved naturally occurring genes across mammalian species, evaluation of their expression profiles in cancer models would greatly advance our understanding of regulatory mechanisms involving many critical cancer-associated genes. Global miRNA expression profiling of cancer versus normal cells may provide the characteristic regulatory features of up- and down-regulated miRNAs that can be correlated to

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altered gene expression in cancers. Overexpression of specific oncogenic miRNAs causes down-regulation of target tumor suppressors which contributes to malignant transformation [Rovira et al., 2010]. miRNA profiling studies have helped re-classify human cancers, such as leukemias, based on the developmental lineage and differentiation state of tumors and identified gene expression patterns that correlated with distinct mechanisms of cellular transformation [Lu et al., 2005].

A large number of studies have characterized the comprehensive miRNA expression profiles in human solid and blood-related cancers. But little has been accomplished in non-human cancer models and therefore, miRNA expression and deregulation profiles and regulatory mechanisms in animal cancer models remain largely unknown. Studies with canine breast cancer models have shown a strong genetic correlation with their human counterparts particularly in terms of altered gene expression profiles and frequent loss of cell cycle regulatory genes or cyclin-dependent kinase inhibitors (CKIs) including members of the CDKN2/INK4 tumor suppressor gene family (p16/INK4A, p14ARF and p15/INK4B) [Vail and MacEwen, 2000; DeInnocentes et al., 2009; Klopfleisch and Gruber, 2009; Uva et al., 2009; Klopfleisch et al., 2010; Lutful Kabir et al., 2013]. p16/INK4A is the founder member of the INK4-CKIs and was the first classified as a major tumor suppressor gene (only preceded by p53 for many human malignancies) because the mutations in the INK4A/ARF locus and loss of heterozygosity of the chromosomal region encoding this gene have been reported in a wide range of cancers including melanomas, leukemias, gliomas and lung, breast and bladder cancers [Kamb et al., 1994a; Ruas and Peters, 1998]. Therefore, it is important to study the regulatory mechanisms of this tumor suppressor gene locus in cancers. In this study, the comprehensive expression profile of the 277 most abundantly expressed and highly characterized miRNAs from the canine genome have been evaluated in spontaneous canine mammary tumor (CMT) models. Employing quantitative PCR (qPCR) arrays, this study has identified groups of miRNAs that can serve as potential regulators of the INK4 tumor suppressor genes in CMT cells providing a critical regulatory mechanism and thereby contributing to their altered expression induced by post-transcriptional silencing. Moreover, we have experimentally validated one of the altered miRNAs, miR-141, that binds target INK4A mRNA sequences in CMT cell lines. The deregulated miRNAs could be predicted to target critical biomarker genes and other cell cycle regulators in CMT cells. Additionally, altered regulation of miRNAs in CMT models has been shown to be highly conserved in their relationship with orthologous miRNA expression profiles in human breast cancer.

MATERIALS AND METHODS

CELL LINES AND DEVELOPMENT OF NORMAL CANINE MAMMARY EPITHELIAL CELLS

Four stable and highly transformed CMT cell lines (CMT9, CMT12, CMT27, and CMT28) used in this study were derived from female dogs of different breeds with spontaneous mammary carcinomas or adenocarcinomas [Wolfe et al., 1987]. Normal canine mammary epithelial cells (CMECs) were cultured and developed from mammary biopsy samples collected from dogs and beagles with no history of mammary carcinomas and were verified by histopathological examination. The normal CMEC population was isolated and grown according to previously described methods. Briefly, normal biopsy samples excised surgically were placed immediately in chilled transport medium consisting of tissue culture medium with 10% heat inactivated serum and 2% antibiotics [Wolfe et al., 1986]. Tissues were carefully processed to make single cell suspensions which were grown in appropriate cell culture medium (Leibovitz′s L-15 medium enriched with 10% fetal bovine serum and 2-3X antibiotics) maintaining sterile cell culture conditions [You and Bird, 1995]. Cultured cells had to be free from mycoplasma and fungal contamination based on ultra-structural observation before they were ready for subculture.

All use of animal biopsies for the purpose of establishing cell lines and cultures in this investigation was under the guidance and review of the Auburn University IACUC (Institutional Animal Care and Use Committee) protocol numbers PRN 2009-1633, PRN 2007-1155, and PRN 2005-0826.

EXTRACTION OF SMALL RNAS INCLUDING MIRNAS FROM CMTS AND CMECS

Small RNA populations including miRNAs, were extracted from all three CMT cell lines and CMECs according to the manufacturer′s instructions (QIAGEN, miRNeasy mini-extraction method). 3×10^6 to 1×10^7 cells were grown and trypsinized to be used for RNA extraction. Total RNA extraction and enrichment of small RNAs, including miRNAs, were carried out following phenol-guanidine based lysis and isolation and silica membrane based purification methods. The integrity of small RNA populations was confirmed by formaldehyde denaturing gel electrophoresis (Fig. S1). Denaturing 2% agarose gels were prepared using formaldehyde and N-morpholino propanesulfonic acid buffer (MOPS) in deionized water. The gel was prepared according to manufacturer instructions (MetaPhor agarose, Rockland, Maine) and RNA samples were run under denaturing conditions.

REVERSE TRANSCRIPTION OF MIRNAS AND SMALL RNAS ISOLATED FROM CMECS AND CMTS

The qPCR array protocol begins with the reverse transcription (RT) of miRNAs. Approximately 100–200 ng small RNAs or ~500 ng total RNAs isolated from CMECs and CMT cells were used as starting material. The RT step was performed according to manufacturer′s instructions (QIAGEN, miScript miRNA PCR Array). Briefly, 4 μl 5X HiSpec buffer, 2 μl 10X nucleics mix, 2 μl reverse transcriptase mix, RNase-free water ~11.5 μl (adjusted depending on RNA concentration) and template miRNA were added to make a 20 μl reaction and gently mixed on ice. Reactions were incubated for 60 min at 37°C and finally for 5 min at 95°C to inactivate reverse transcriptase. The special RT buffer mix (HiSpec buffer) allowed selective conversion of mature miRNAs and certain small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) to cDNA and locked long RNAs in a conformation that rendered small RNAs to be selectively reverse transcribed. Mature miRNAs were polyadenylated

by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers.

QPCR ARRAY FOR MIRNA EXPRESSION PROFILE

miRNA qPCR arrays were assayed according to the manufacturer's instruction (SABiosciences, QIAGEN). cDNA prepared in the reverse transcription reaction served as the template for qPCR analysis using the miRNA PCR arrays containing miRNA-specific forward primers arrayed in the plate (for entire miRNA panels), universal primer (reverse primer) and SYBR Green PCR master mix. The oligo-dT primer (~65 nt) contains a 3′ degenerate anchor and a universal tag sequence on the 5′ end where the reverse primer binds promoting amplification of mature miRNAs in the qPCR step. The qPCR arrays were designed in 96-well plate formats (4×96-well plate) containing 277 canine miRNAs to be profiled. A complete list of these miRNAs from the CanFam genome assembly has been provided (supplemental Table S1). Cycling conditions for the qPCR array were initial activation of hotstart taq DNA polymerase at 95°C for 15 min and then 40 cycles of three step PCR amplification including denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 70°C for 30 s. The qPCR arrays were performed in triplicates for each cell line.

QPCR ARRAY CONTROLS

The assays were optimized based on sample RNA input and the detection system and each assay was validated using the quality control (QC) report to determine reverse transcription efficiency and that positive PCR control (PPC) values passed or were within the correct range calculated for all samples analyzed. Particularly, miRNA reverse transcription control (miRTC) assessed the performance of a reverse transcription reaction by detecting template cDNA synthesized from the kit's built-in control RNA. This control monitored for any variables that may inhibit the reverse transcription reaction. The PPC contained a pre-dispensed artificial DNA sequence and the assay that detected it. If the RNA sample was of high quality, the cycling program was correctly run and the thresholds were correctly defined. A set of endogenous control RNAs, including small nucleolar and small nuclear (snoRNAs/snRNAs) that are highly conserved and widely expressed in different tissues, were used in the normalization of miRNA expression. To avoid any variability in results and analysis, a panel of six snoRNAs/snRNAs (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6-2) were used as reference endogenous controls giving an average measure of relatively stable expression in cells.

DATA ANALYSIS

A web-based miRNA PCR Array data analysis tool (miScript miRNA PCR Array Data Analysis, SABiosciences, QIAGEN) was used to analyze the qPCR data. An important feature of miRNA qPCR array data analysis is called 'fold regulation'. Fold-regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change. A two fold-change threshold was arbitrarily defined by the data analysis tool. Calculation of fold-change in expression was defined by the $\Delta\Delta$CT method for relative quantification (Fig. S2). Statistical significance (P-value <0.05) was determined by volcano plots (see supplemental methods).

3′-UTR AMPLIFICATION AND SEQUENCING

The canine p16 3′-UTR sequence, including exons 2 and 3 derived from CMT28, was aligned with p16 sequences from other mammals. Primers (forward: 5′ TTCCTGGACACGCTGGTGGTGCTGC 3′, and reverse: 5′ ATACAAATGGAAATTT AAGGGAAAGGGAAGGC 3′) were designed using primer design software (Vector NTI, Invitrogen). The canine p16 3′-UTR sequence was amplified by touch-down (TD) RT-PCR [Korbie and Mattick, 2008]. The TD-PCR protocol was RT (48°C, 45 min), denaturation (94°C, 2 min), and 10 cycles of denaturation (94°C, 1 min), annealing 1 min (primer annealing temperature plus 10°C decreasing 1°C/cycle) and elongation (68°C, 1 min) followed by 25 cycles of PCR amplification. The TD-annealing temperature range for p16 3′-UTR was 67–77°C. PCR products were analyzed semi-quantitatively on 2% agarose electrophoresis gels and gel-purified 3′-UTR amplicons were authenticated by DNA sequencing (MGH DNA Core, Cambridge, MA) and analyzed by alignments of published sequences. Expression of p16, p14, and p15 INK4 mRNAs in CMEC and CMT cell lines were carried out according to previously described methods [Lutful Kabir et al., 2013].

CUSTOM CONSTRUCTS OF CANINE CDKN2A 3′-UTR VECTOR AND MIRNA 3′-UTR REPORTER ASSAYS

miRNA 3′-UTR target clones were used for miRNA target identification and functional validation of predicted targets and to study the regulatory effects of miRNAs on target genes. Canine p16/CDKN2A 3′-UTR reporter clone constructs were designed and customized for miRNA 3′-UTR reporter assays. The canine p16/CDKN2A 3′-UTR sequence (441 bp spanning from downstream of exon 2 to 3′-UTR) was inserted downstream of the secreted *firefly* luciferase (fluc) reporter gene in the pEZX-MT01 vector system (GeneCopoeia Inc. Rockville, MD) (Fig. S3).

Normal CMEC and CMT cells were grown in 24-well plates for 18–24 h. Cells were ≥80% confluent prior to transfection at a density of $1.5-6 \times 10^4$ cells/well. On the second day, cells were co-transfected with 0.1 μg of CDKN2A 3′-UTR miRNA target clone expression vector (or target control vector) and 0.2 μg of miR-141 precursor clone expression vector (or miRNA scramble control vector). These cloning vectors were incubated with 20–25 μl serum-free Minimum Essential Medium (MEM, Gibco, NY) and 1.0–1.5 μl transfection reagent (TransIT-LT1, Mirus Bio, WI) for 15–30 min at room temperature before adding to the cells. Cells were then transferred to 96-well plates ($0.3–1.2 \times 10^4$ cells/well) at 24–48 h post-transfection and incubated for 24 h. The *firefly* and *renilla* luciferase activities were measured in all transfected cells according to manufacturer instructions (GeneCopoeia Inc. Rockville, MD).

PREDICTION OF MIRNA TARGETS

TargetScan (Release 6.2) tool was used to search for predicted canine miRNA targets. Predicted miRNA targets were identified based on multiple algorithms that essentially searched for the presence of conserved 8mer (an exact match to positions 2–8 of the mature

miRNA followed by an 'A') or 7mer (an exact match to positions 2–7 of the mature miRNA followed by an 'A') sites at the 3′-UTR sequence that matched the seed region (position 2 to 7) of each miRNA [Lewis et al., 2005; Grimson et al., 2007; Reczko et al., 2011]. This online prediction tool considers matches to annotated human orthologs as defined by UCSC whole genome alignments [Karolchik et al., 2014]. The predicted binding of miR-141 seed sequence with target INK4A/CDKN2A was further validated *in silico* by a bioinformatics software (Sfold 2.2, a software for statistical folding of nucleic acids and studies of regulatory RNAs) (http://sfold.wadsworth.org/cgi-bin/starmirtest2.pl).

RESULTS

COMPREHENSIVE EXPRESSION PROFILE OF THE 277 MIRNAS FROM THE CANINE GENOME

A comprehensive expression profile of miRNAs from the canine genome was evaluated in CMTs by miRNA qPCR arrays. Three CMT cell lines (CMT12, CMT27, and CMT28) were used to profile the 277 best characterized and most abundantly expressed mature miRNAs from the canine genome. Each of these cell lines has been previously investigated for INK4 tumor suppressors that were differentially expressed and frequently deleted in these cell lines [Lutful Kabir et al., 2013]. First, small RNAs including miRNAs were isolated from CMECs and CMT cells. The integrity of small RNA populations was validated by performing denaturing gel electrophoresis (Fig. S1).

The miRNA qPCR arrays for the miRNAs from the canine genome revealed that nearly 80% ($151 + 45 + 24 = 220$ out of 277 total miRNAs) were deregulated in CMT cell lines compared to normal CMEC and the expression of nearly 20% ($30 + 27 = 57$ out of 277) of the miRNAs remained nearly unchanged or were not detected in both CMEC and CMT cells (Fig. 1). In this study, the majority of the miRNAs whose expression was determined to be altered in the CMT cell lines could be divided into three groups including, up-/down-regulated, up-regulated and down-regulated miRNAs, in order to characterize the biological importance of altered miRNAs from each group in regulating critical genes in canine breast cancer models. The up-/down-regulated group comprises a large number of miRNAs (151 out of 277) in which each miRNA was up- and/or down-regulated differentially in CMT cell lines rather than only up- or only down-regulated in all three CMT cell lines. A second group of miRNAs (45 out of 277) were up-regulated in all three CMT cell lines showing that each of these miRNAs was positively regulated (>2 fold change in expression) in all CMT cell lines suggesting that they may have an important contribution in cancer progression. A third category was the down-regulated miRNAs (24 out of 277) in which each miRNA was negatively regulated (<−2 fold change in expression) in all three CMT cell lines (Fig. 1A–B). These altered miRNAs were further investigated for their potential roles in targeting INK4 tumor suppressors and other gene expression defects which have been evaluated in CMT models.

FOLD-CHANGE IN EXPRESSION OF MIRNAS IN CMT CELLS

Calculation of relative fold-change in miRNA expression was determined using the ΔΔCT method of relative quantification (described in Materials and Methods). The fold-difference in expression from the qPCR array data was determined following two layers of normalization, one performed using the reference genes and then by comparing levels in normal CMECs. The results from miRNA expression profiles in terms of fold-change or fold-regulation were represented by graphical analyses. As described in Materials and Methods, a two fold change threshold has been defined by the data analysis program and is an arbitrary threshold commonly applied in data analysis of microarrays and qPCR assays. An important graphical representation of fold-change in expression is the scatter plot that compares expression level ($2^{-\Delta Ct}$) of each gene in the test sample (CMT cells) versus the control sample (CMEC) (Fig. 2A–C). The scatter plot represents miRNA expression in CMT cells in a biologically significant way by defining relative expression in terms of either more than two fold up-regulation or down-regulation compared to normal CMEC. The scatter plot from each cell line distinguishes the three altered groups of miRNAs as shown in Figure 1. For example, miR-203, miR-9, miR-429, or miR-200a/b were highly up-regulated in all three CMT cell lines (Fig. 2A–C, marked by triangles and in red) whereas miR-1, miR-133a/b/c, or miR-214 were prominently down-regulated in all CMT cell lines (indicated by blue arrows). In contrast, miR-141 and miR-200c were shown to be differentially expressed in these cells as they were highly up-regulated in CMT12 and CMT27 cell lines but were identified as down-regulated in the CMT28 cell line. The miRNAs that are plotted in between the boundaries of two fold change (black circles in between the two diagonal boundary lines) indicate small or no change in miRNA expression levels as these values are close or equal to 1 (Fig. 2A–C). A majority of the altered miRNAs were examined to be both biologically (>2 fold-change in expression) and statistically significant (P-value <0.05). However, a fraction of them were found to be statistically insignificant although their fold-change values were higher or relevant to biologically significant (Fig. S4).

Figure 3A–B shows only up-regulated and down-regulated miRNAs in all three CMT cell lines. Another group of differentially expressed miRNAs (25 out of 151 up-/down-regulated miRNAs) were taken into consideration from all three CMT cell lines as their fold regulation range was from 10 to several hundred suggesting that they may have important gene regulatory functions in cancer development (Fig. 3C). The remainder of the differentially expressed miRNAs were also analyzed to predict potential miRNA targets.

MIRNA TARGET PREDICTION AND RECOVERY OF TARGET SITE IN THE NEWLY CHARACTERIZED P16/P14 3′-UTR

Only a few of the recently developed online resources are available for miRNA target prediction based on the canine genome. We used TargetScan 6.2 which is one of the most widely used miRNA target prediction programs that predicts miRNA binding sites through the identification of complementary seed sequence matches in the 3′-UTR of mRNAs and the assessment of their evolutionary conservation [Reczko et al., 2011]. We first identified a number of miRNAs from the altered expression panel that could potentially target INK4 tumor suppressor genes in CMT models. These INK4 tumor suppressor genes, including p16/INK4A, p14ARF, and p15/INK4B, are frequently mutated in a wide range of cancers in both humans and dogs and particularly inactivation of the p16/INK4A

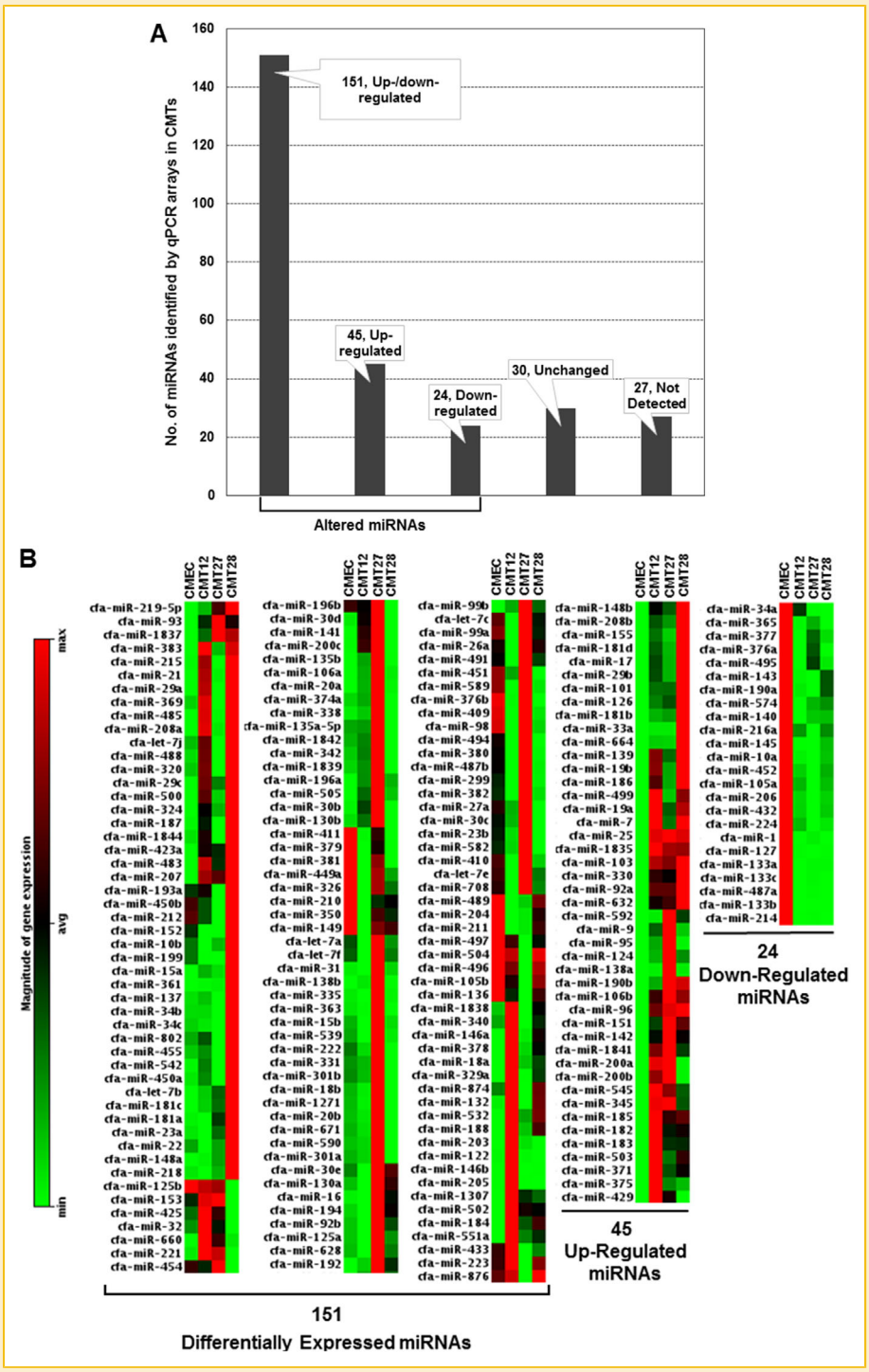

Fig. 1. Comprehensive expression profile of the dog miRNome. (A) miRNAs identified by qPCR arrays were divided into altered, unchanged, or not detected groups. 'Up-/down-regulated' = expression of each miRNA was up- and/or down-regulated differentially in CMT cells rather than only up- or only down-regulated in all three CMT cell lines; 'up-regulated' = expression of each miRNA was up-regulated (by >2 fold change) in all three CMT cell lines; 'down-regulated' = expression of each miRNA was down-regulated (by <−2 fold change) in all three CMT cell lines; 'unchanged' = expression of each miRNA was not changed comparing normal CMEC and CMT cell lines or their normalized fold regulation value is close or equal to 1; 'not detected' = expression was undetectable or miRNAs were not found to be expressed in both CMEC and CMT cells. (B) Heat maps for relative expression level of miRNAs in CMT cell lines in comparison to CMEC. The brighter red color in the heat maps indicates higher level of miRNA expression. Conversely, the brighter green color indicates least expression or down-regulation of miRNA in CMT cells. The numbers in the heat maps (151, 45, and 24) indicate the corresponding groups of altered miRNAs specified in the bar chart (A).

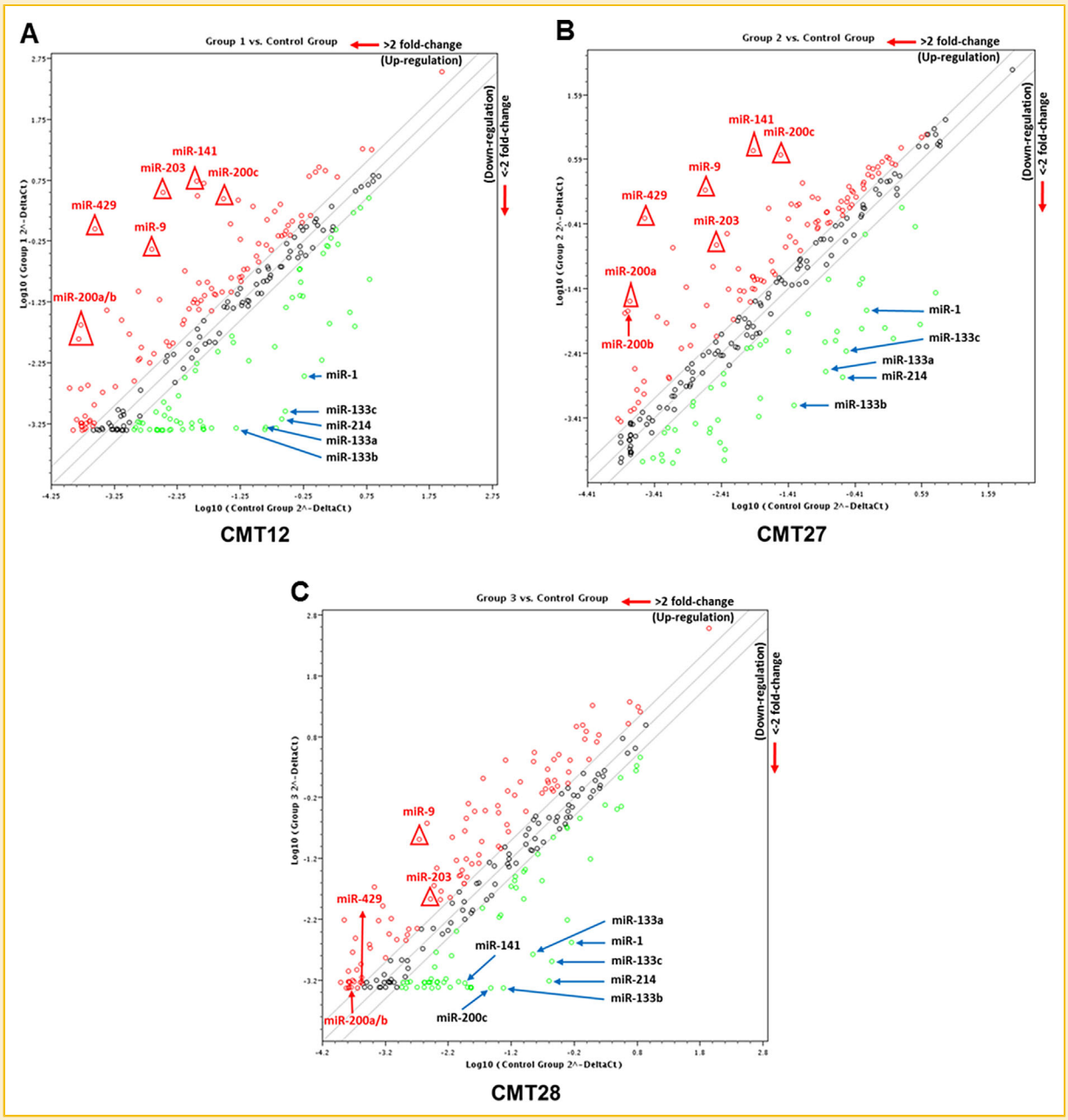

Fig. 2. Fold-regulation of miRNAs in CMT cells. (A–C) Scatter plots of CMT vs CMEC log ($2^{-\Delta Ct}$) showing fold-change in miRNA expression. The diagonal line in the center indicates a fold change value ($2^{-\Delta\Delta Ct}$) of 1 or no change in expression. The two diagonal boundary lines drawn above and below the center line indicate the fold-change in gene expression threshold (defined as 2). Each circle is plotted by a pair of coordinates (x,y) defined by a point on the X-axis, denoted by log ($2^{-\Delta Ct}$) or relative normalized expression of a miRNA in CMEC, and a point on the Y-axis, obtained from the log ($2^{-\Delta Ct}$) or relative normalized expression of a miRNA in a respective CMT cell line. Therefore, each circle indicates a miRNA's relative fold-regulation change in CMT cells compared to CMEC. All miRNAs indicated by red circles are up-regulated and those by green circles are down-regulated in CMT cell lines. The miRNAs that fall in between the two diagonal boundary lines (above and below the middle line, respectively) are nearly unchanged in expression (fold-change is 1 or close to 1 and less than 2) and are designated by black circles. Several common and highly up-regulated miRNAs are indicated by either red triangles or red arrows whereas common downregulated miRNAs are indicated by blue arrows. Fig. A, B, and C indicate the individual scatter plots for CMT12, CMT27, and CMT28 cells, respectively.

was observed in nearly one-third of human malignancies [Kamb et al., 1994a,b; Ruas and Peters, 1998; Sharpless, 2005; DeInnocentes et al., 2009; Lutful Kabir et al., 2013]. Prediction of experimentally identified miRNA targets would establish post-transcriptional regulatory mechanisms pertinent to the regulation of individual INK4 tumor suppressor genes. The TargetScan tool identified several miRNA target sites from putative 3′-UTR sequences generated from mammalian sequence alignments. We found that miR-141, miR-300, miR-514 and miR-653 potentially target p16/INK4A and p14ARF mRNAs that share common exons 2 and 3 including 3′-UTRs [Mao et al., 1995; Stone et al., 1995] while miR-375 could target p15/INK4B mRNA. However, all these miRNAs

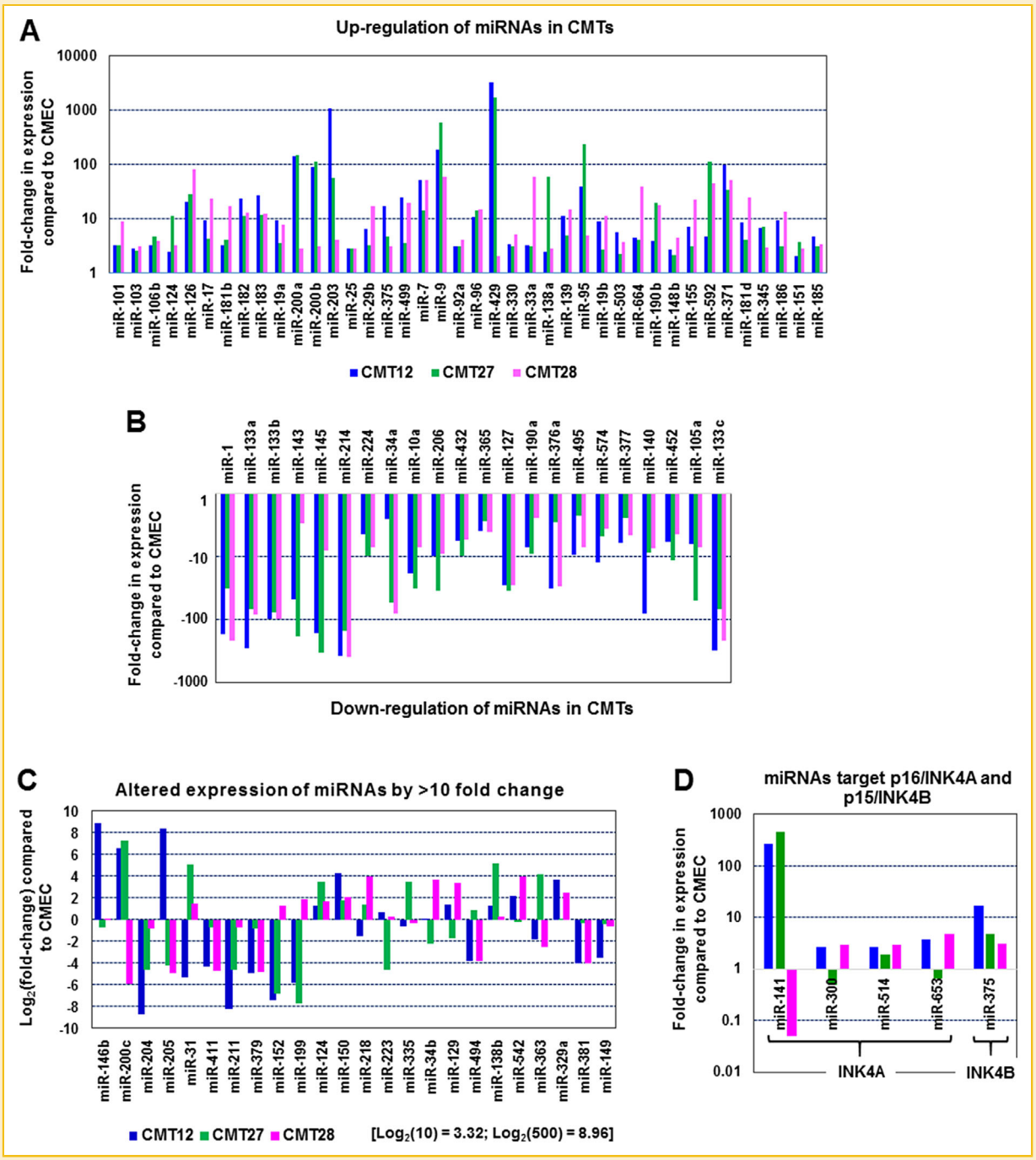

Fig. 3. Altered miRNA expression profiles in CMT cell lines. Among experimentally validated miRNAs devoid of technical errors, (A) 40 miRNAs were up-regulated and (B) 22 were down-regulated in all three CMT cell lines. (C) Another group of differentially expressed miRNAs were identified based on their altered expression by more than 10-fold in CMT cell lines compared to CMECs. (D) Five differentially expressed miRNAs (identified in CMTs) potentially target p16/INK4A and p15/INK4B genes. In Figure 3A–D, logarithmic scales (on the X-axis) were drawn to show the trends in fold-regulation. In Figure 3C, values were $\log_2$ transformed.

were determined as differentially expressed or altered in three CMT cell lines (Fig. 3D). The potential binding of miR-141 seed sequence with p16/INK4A 3′-UTR target was verified by another prediction tool (Sfold, Fig. 4A).

Comparison of canine p16 sequence alignments revealed that these miRNA binding homologies were located in the 3′-UTR of the p16/INK4A mRNA in CMT cells. Surprisingly, p16 from at least one canine cell line (CMT28) was found to have a shorter length 3′-UTR sequence thereby omitting the target site for miR-141 (Fig. 4). Although p16 mRNA from CMT28 has definite 3′-UTR length followed by a poly (A) tail as determined by 3′ RACE-PCR [Lutful Kabir et al., 2013], these were found to have shorter lengths

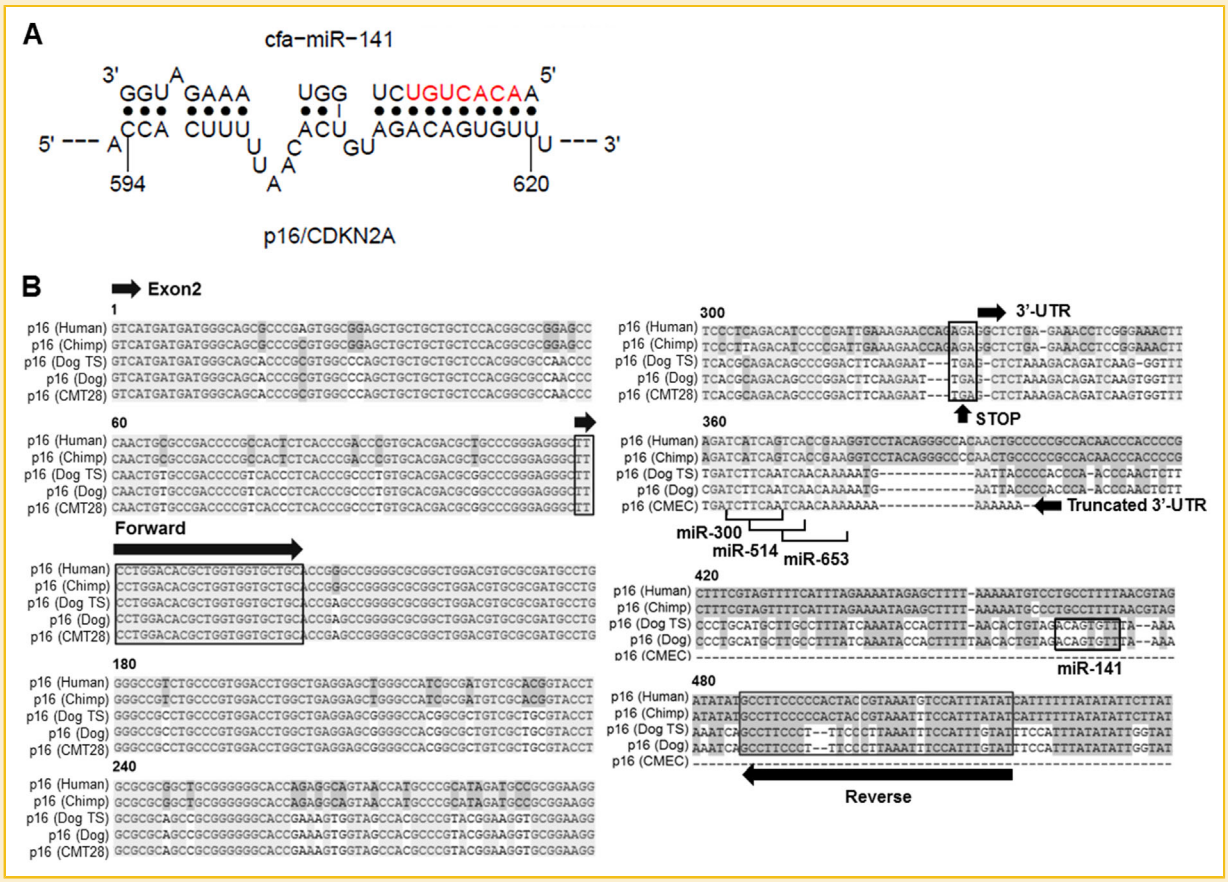

Fig. 4. Prediction of miRNA target binding sites in the p16/INK4A mRNA sequence. (A) Hybrid conformation of miR-141 seed (bases in red) binding to p16/CDKN2A (GenBank: AB675384) 3′-UTR target analyzed by miRNA target prediction tool (Sfold 2.2). (B) Alignments of the p16 mRNA sequence spanning exon 2 to the 3′-UTR from different species were analyzed. Canine p16 exon 2 to exon 3 including 3′-UTR sequences, including putatively generated canine p16 sequences from genome alignments obtained from TargetScan (as indicated by Dog TS), published p16 sequences from thymic lymphoma indicated by 'p16 (Dog)' (GenBank: AB675384) and from CMT28 (GenBank: JQ796920) show variable lengths of 3′-UTR. The sequence alignments indicated a highly conserved exon 2 including start and stop codons for all dog sequences and 3′-UTR regions. The forward and reverse primers are shown by the forward and reverse arrow, respectively. Several predicted miRNA binding sites are indicated in the 3′-UTR. The binding site for miR-141 is missing in p16 3′-UTR from CMT28.

compared to the published canine p16 3′-UTR (GenBank: AB675384). Since p16 and p14 are alternatively spliced and the canine p14ARF 3′-UTR has never been defined, amplification of the missing 3′-UTR could allow identification of miRNA target sites as well as additional information characterizing these alternatively spliced transcripts. For this purpose, primers were designed to amplify the region of the p16/p14 mRNA that could span the highly conserved exons 2 and 3 including 3′-UTR with ~100 bp of additional downstream sequence (Fig. 4B).

Surprisingly, touch-down RT-PCR assays demonstrated 3′-UTR expression (including the extended downstream region) in CMEC and CMT28 cells detecting longer 3′-UTR amplification (Fig. 5A) although this was unexpected given the truncated sequence of the p16 mRNAs from these cell lines [Lutful Kabir et al., 2013]. Similar PCR amplification was then performed in other CMT cell lines that were defective for both p16 and p14 expression (CMT12 and CMT27) or only p16 expression (CMT9). CMT9 (not CMT12 or CMT27) that never expressed p16 but did express p14, was found to amplify this elongated 3′UTR similar to CMEC and CMT28 (Fig. 5A) suggesting that this longer 3′-UTR is expressed as part of the p14 mRNA but not p16 mRNA in CMEC and CMT cells. Sequencing of the amplicons also revealed that the new 3′-UTR for the INK4 message was more likely to be the part of the p14 mRNA. The alignment of the longer 3′-UTR from p14 mRNA recovered the miR-141 target site that was previously absent from the existing p16 3′-UTR from CMEC (Fig. 5B). This experimental evidence demonstrated that one miRNA can regulate two genes encoding a common target site in the 3′-UTR while cells regulate the variable 3′-UTR lengths between two alternatively spliced INK4A transcripts for fine tuning their expression in a tissue-specific manner and possibly for an escape from miRNA-mediated silencing.

FUNCTIONAL VALIDATION OF MIR-141 TARGET-BINDING IN CMT CELLS

As the newly discovered 3′-UTR of the canine CDKN2A (INK4A/p14) mRNA was found to harbor a miR-141 target sequence (Fig. 5), the

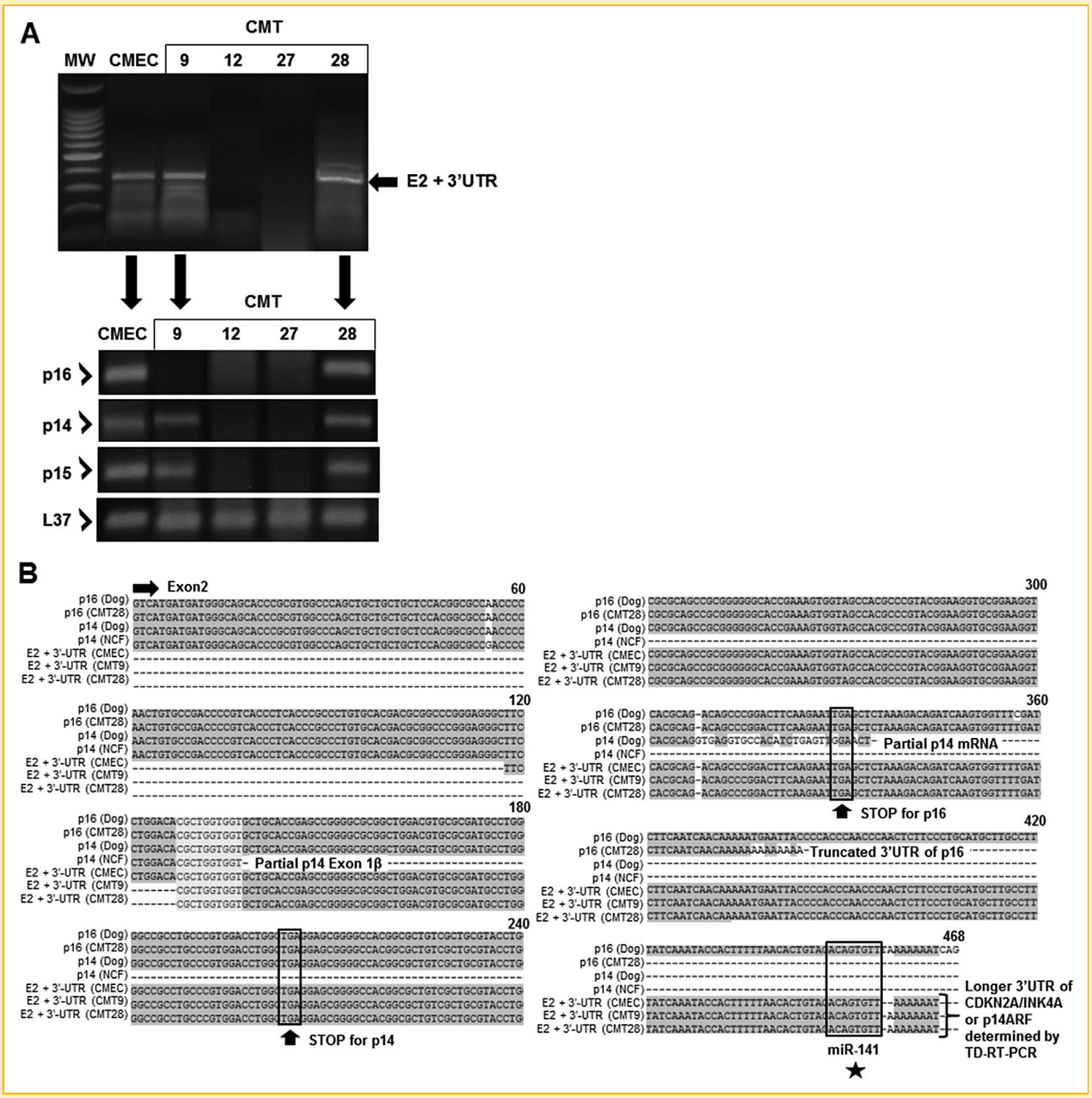

Fig. 5. Amplification of the INK4A exon 2 to exon 3 including 3′-UTR (E2-3′UTR) sequence in CMECs, CMT9, CMT12, CMT27, and CMT28 cell lines. (A) The authenticity of the amplicons was validated by sequencing and NCBI BLAST analysis. In the upper panel, the ~380 bp E2-3′-UTR amplicon bands were observed in CMEC, CMT9, and CMT28. The expression of the longer 3′-UTR in these cell lines correspond to p14 expression in the lower panel (indicated by down-arrows). The lower panel showing the differential expression profile of p16, p14, and p15 in CMT cell lines. L37, a ribosomal protein, was used as an internal control transcript. (B) Sequencing and alignment of the longer 3′-UTR of INK4A or p14ARF mRNA amplified in CMEC, CMT9, and CMT28 (indicated by E2 + 3′-UTR). In addition to published p16 sequences from dog and CMT28, the predicted p14 mRNA from dog (GenBank: FM883643) and the partial p14 mRNA sequence from NCF or normal canine fibroblasts (GenBank: JQ801342) were compared with newly discovered INK4A 3′-UTR sequences. The longer 3′-UTR sequences of the INK4A gene that are likely to be spliced to p14ARF obtained from CMEC, CMT9, and CMT28 are shown to harbor the predicted miR-141 target site. (E2 = Exon 2).

miR-141 target (CDKN2A 3′-UTR) binding needed to be validated by a 3′-UTR reporter assay. CMEC and CMT cell lines including CMT12, CMT27, and CMT28 were co-transfected with a CDKN2A 3′-UTR miRNA target clone expression vector (or target control vector) encoding *firefly* and *renilla* dual luciferase reporters and a miR-141 precursor clone expression vector (or miRNA scrambled control vector) encoding a GFP reporter (described in Materials and Methods). Transfection and expression of these genes in the CMEC and CMT cell lines were assessed by the respective reporter gene expression.

The silencing of the CDKN2A 3′-UTR target by miR-141 (resulting from its seed sequence binding to the matched target sequence) was

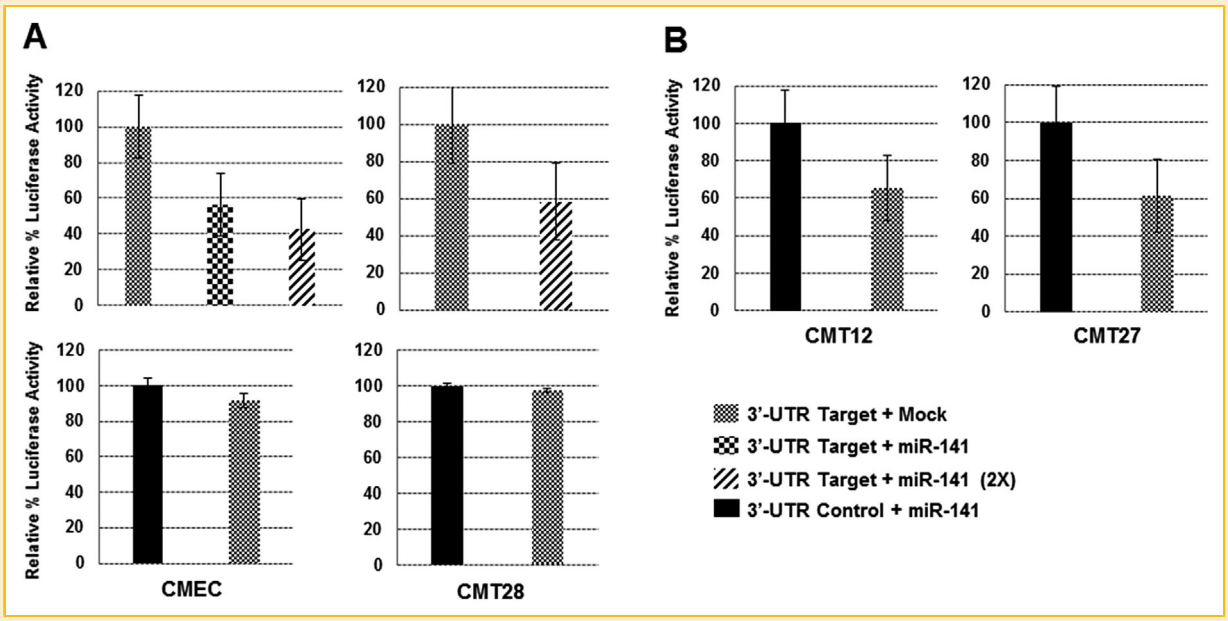

Fig. 6. Validation of miR-141 target binding by 3′-UTR luciferase assay. (A) CMEC and CMT28 cells were co-transfected with either CDKN2A 3′-UTR target cloning vector (or target control vector without the miR-141 binding sequence) encoding *firefly* and *renilla* luciferase reporter expression and a miR-141 precursor cloning vector encoding GFP expression. 90 ng of 3′-UTR target clone and two different concentrations of miR-141 (120 and 240 ng as indicated by 2X) were used to transfect the cells grown in 24-well plates. Cells were also mock transfected (3′-UTR target clone and transfection reagent with serum-free medium) to assess endogenous miR-141 function. In each case, relative percent luciferase activity was calculated by measuring the ratio of *firefly* to *renilla* luciferase activities in the assay. The *renilla* luciferase activity was used to normalize the firefly luciferase signal in the same CMEC or CMT28 cell line. (B) CMT12 and CMT27 cell lines were similarly transfected and assayed for the validation of miR-141 3′-UTR target binding. These cells were also co-transfected with the 3′-UTR target clone and the miRNA scrambled control clone in addition to mock transfection to determine the specific binding of endogenous miR-141 with the 3′-UTR target sequence. Error bars indicate standard error for each experimental value.

analyzed by comparing the diminished activity of *firefly* luciferase which was inserted upstream from the 3′-UTR sequence in the expression vector. Expression of miR-141 caused variable levels of suppression of target 3′-UTRs in CMEC and CMT cell lines. When miR-141 was overexpressed in CMEC and CMT28, by transfecting with a higher concentration, it caused further reduction in luciferase expression (up to 60%) thereby silencing 3′-UTR target and validating miR-141 CDKN2A target binding (Fig. 6A).

Two other cell lines (CMT12 and CMT27) that highly expressed miR-141 (Fig. 3C), were used to investigate the endogenous effects of miR-141. A control target 3′-UTR clone (lacking the miR-141 target binding site) was designed and used to assess the inhibitory effect of endogenously expressed miR-141 on the cells transfected with the CDKN2A 3′-UTR target. Transfecting CMT12 and CMT27 cell lines with the control target 3′-UTR sequence revealed that endogenously expressed miR-141 knocked down CDKN2A 3′-UTR target expression in these cells (Fig. 6B, compare 'control' bars to 'mock' bars) suggesting a dominant competitive function of endogenously expressed miR-141 in silencing the CDKN2A 3′-UTR target sequence in these cells. Similar treatment in the CMT28 cell line demonstrated no endogenous inhibitory effect on the CDKN2A 3′-UTR target (Fig. 6A), reconfirming the down-regulation of miR-141 in this cell line. Additionally, this experimental evidence suggests that lack of miR-141 mediated silencing in the CMT28 cell line was permissive for p14ARF mRNA expression leading to the discovery of the longer 3′-UTR sequence containing the miR-141 target site in these cells.

PREDICTION OF OTHER CANINE MIRNA TARGETS INCLUDING CELL CYCLE REGULATORS

Several other miRNAs whose expression was altered in CMT cells, were investigated for targeting of other genes known to regulate the cell cycle. A group of down-regulated miRNAs were predicted to target the cyclin-CDK genes and E2F transcription factors that function downstream of the p16 in the same regulatory pathway suggesting that these miRNAs may act as potential tumor suppressors in canine breast cancer (Fig. 7). Expression of these potential cell proliferation suppressors was down-regulated in CMT cells. In the target prediction analysis, we identified more miRNA candidates from the deregulated panel (Fig. 3) that could potentially target other genes in CMT cell models. Among these genes were included luminal epithelial specific markers and hormone receptors including estrogen receptor 1 (ESR1), progesterone receptor (PR) and proto-oncogenes such as the epidermal growth factor receptors (EGFR/HER2 family) (Table I). Most of these miRNAs are down-regulated in CMT cells supporting their important roles in silencing oncogenes involved in tumor progression. A very highly upregulated miRNA family including miR-429 (over-expressed in CMT12 and CMT27 by >1000 fold) and miR-200c (over-expressed in CMT12 and CMT27 by 100–150 fold) (Fig. 3) were predicted to target a gene known as the ERBB receptor feedback inhibitor 1 (ERRFI1) in a highly conserved fashion (the target sites are conserved across mammalian species) (Fig. S5). ERRFI1 has been characterized by many studies as a potent tumor suppressor gene that inhibits the

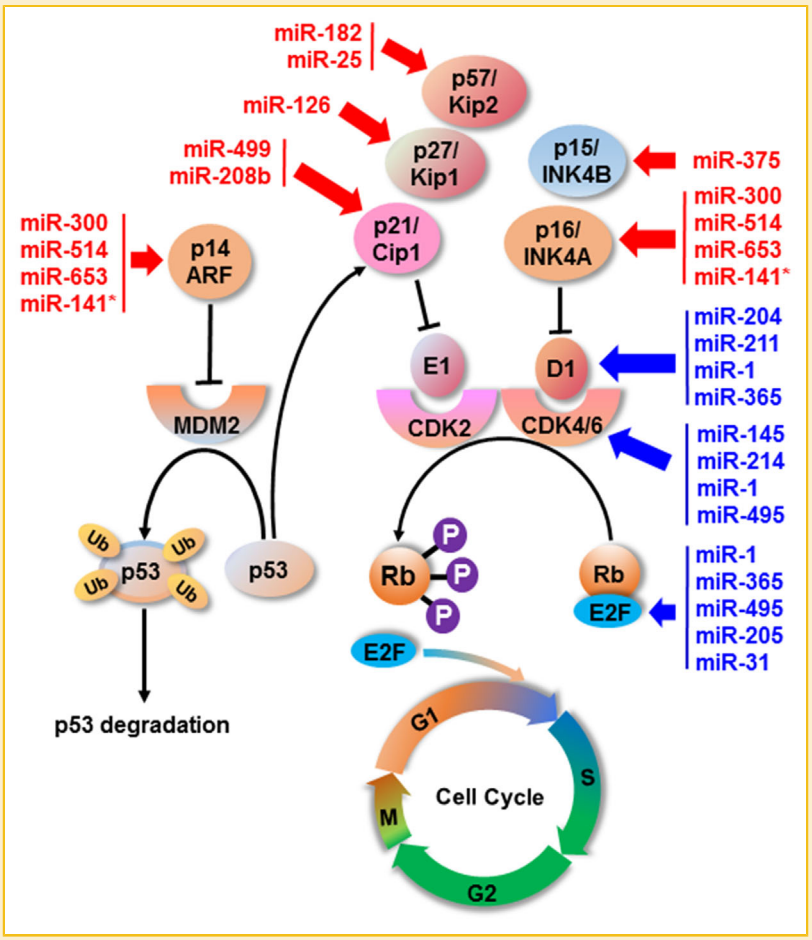

Fig. 7. Prediction of miRNA targets among key cell cycle regulatory genes. A group of up- and down-regulated miRNAs in CMT cells identified by qPCR arrays, are shown to potentially target different genes in the p16-Rb and p14-p53/p21 anti-proliferative pathways in the cell cycle. miRNAs in red were up-regulated and in blue were down-regulated in CMT cell lines. The red and blue arrows indicate the potential post-transcriptional silencing of target genes by miRNAs that were up- and down-regulated in CMT cells, respectively. miR-141 (labeled by red asterisk), a differentially expressed miRNA, was experimentally validated for silencing the INK4A gene expression in CMT cell lines. The inhibition of downstream proteins is indicated by T-bars. Ub = ubiquitin molecule conjugated to p53.

kinase domain of ERBB [Anastasi et al., 2005; Zhang et al., 2007a, b]. Therefore, these highly up-regulated miRNAs in CMT cells also have the potential to promote potent oncogenic functions by targeting and down-regulating key tumor suppressor genes.

miRNA expression profiles from the canine genome are not only associated with CMT cells but are also highly correlated with those found in human breast cancer. A number of miRNAs that are altered in both canine and human breast cancers have been identified in the current study and in several human studies (Table II), suggesting conserved regulation of their potential oncogenic and tumor suppressive functions. Several well-known miRNAs have proven

TABLE I. Altered miRNAs in CMT cells targeting oncogene growth factors and hormone receptors in the context of CMT models

Target genes	*EGFR	*HER2/ERBB2	*ESR1	*PR
miRNAs	miR-1 miR-206	miR-376a miR-432 miR-214 miR-140 miR-199	miR-203	miR-190a

*EGFR/HER2/ERBB2, Epidermal Growth Factor Receptor family; ESR1, Estrogen Receptor 1; PR, Progesterone Receptor.

TABLE II. Altered expression of miRNAs that are associated with canine and human breast cancers

	Regulation in breast cancer	
miRNAs	Human	Dog
miR-21	Up-regulated (oncogenic) (35)	Up-regulated/differentially expressed
miR-155	Up-regulated (oncogenic) (36)	Up-regulated
miR-9	Up-regulated (oncogenic) (37)	Up-regulated
miR-34a	Down-regulated (tumor suppressor) (38)	Down-regulated
miR-143/145	Down-regulated (tumor suppressor) (35)	Down-regulated
miR-31	Down-regulated (tumor suppressor) (39)	Down-regulated/ differentially expressed

to be oncogenic and upregulated in human breast cancers including miR-21 [Iorio et al., 2005], miR-155 [Zhang et al., 2013], and miR-9 [Ma et al., 2010]. Whereas miRNAs that function as tumor suppressors and are down-regulated in human breast cancers included miR-34a [Li et al., 2013], miR-143/145 [Iorio et al., 2005] and miR-31 [Laurila and Kallioniemi, 2013]. In Table II, these miRNAs have been shown to reflect a similar altered regulation in canine breast cancer models.

DISCUSSION

Increasing numbers of miRNAs in mammalian genomes clearly indicate that their regulatory functions in gene expression can manipulate diverse areas of cellular processes including disease mechanisms. A large number of miRNAs have been identified as deregulated in human breast cancer compared to normal breast tissue. Overexpression of certain oncogenic miRNAs and the loss of several tumor suppressor miRNAs have been shown to cause loss of regulation of vital cellular functions involved in breast cancer pathogenesis [Harquail et al., 2012]. This is the first evidence of comprehensive expression profiles of the 277 best characterized miRNAs from the canine genome evaluated in CMT models using a quantitative PCR array strategy. The quantitative expression profile estimated that nearly 80% of total miRNAs are differentially expressed in all three CMT cell lines compared to CMEC and a number of altered miRNAs were identified to potentially regulate critical cell cycle and cancer-associated genes.

One of the important functions of miRNAs is to regulate cell cycle progression and arrest by targeting the expression of multiple cell cycle regulatory genes. They can regulate cell proliferation by specifically targeting expression of cyclin-CDK complexes (involved in cellular proliferation) and CDK inhibitors (acting as growth suppressors) [Liu et al., 2008; Wang and Blelloch, 2009]. This study identified groups of up-/down-regulated miRNAs and investigated their roles in promoting canine breast cancer and in targeting INK4 tumor suppressor and other cancer-associated genes. Several altered miRNAs in CMT cells were analyzed *in silico* for their potential roles in silencing critical cell cycle regulator and tumor suppressor genes including INK4 and other CKI family members. miR-141, miR-300, miR-514, and miR-653 were found to potentially target p16/INK4A and p14ARF as these transcripts share a large overlapping region of exons 2–3 including the 3′-UTRs while miR-375 was predicted to target p15/INK4B (Fig. 4A). Additionally, several up-regulated or oncogenic miRNAs in CMTs were identified as potentially targeting Cip/Kip family members of the CKIs, for example, miR-499 and miR-208b targeting p21/Cip1, miR-126 targeting p27/Kip1 and miR-182 and miR-25 targeting p57/Kip2 expression (Fig. 7). Another group of downregulated miRNAs were predicted to post-transcriptionally regulate cyclin D, CDK4/6 and E2F genes encoding proteins that play essential roles in cell cycle progression (Fig 7). All these miRNAs are subject to experimental validation for their target sequence binding and for functional suppression of gene expression. In this study, the highly deregulated miR-141 in CMT cells (Fig. 3D) was experimentally authenticated for post-transcriptional silencing of INK4A genes.

Differentially expressed miRNAs were highly correlated with the expression defects of their predicted target mRNAs in CMTs. For example, miR-141 was differentially expressed in CMT cell lines showing its sharp up-regulation in CMT12 and CMT27 (by ~266 and ~460 fold, respectively) but down-regulation in CMT28 (by ~20 fold). Up-regulated miR-141 was predicted to target p16/INK4A mRNA and expression of this gene was missing in CMT12 and CMT27 [Lutful Kabir et al., 2013]. In contrast, CMT28 cells down-regulated miR-141 and did express p16 mRNA though as a mutant message [Lutful Kabir et al., 2013]. This suggests that expression of p16 in CMT12 and CMT27 cells may be post-transcriptionally regulated by miR-141 promoting neoplastic potential. Functional validation of the miRNA-target binding potential of these differentially expressed miRNAs in canine breast cancer models is necessary to further decipher the complex regulatory mechanisms of miRNAs in breast cancer and many reflect fundamental differences in phenotype and genotype in these CMTs.

Hunting for miRNA target sites in 3′-UTRs of the INK4A mRNAs revealed striking evidence of additional regulation and expression of INK4A 3′-UTRs in CMTs. As miR-141 was predicted to target the p16 mRNA, we intended to identify the target site from the 3′-UTR sequence alignments. The complementary target site in the 3′-UTR for the matched seed sequence of miR-141 was found in the published p16 mRNA sequences but missing in the sequences obtained from CMT28 cells because this message was expressed in these cells with a truncated 3′-UTR [Lutful Kabir et al., 2013]. This could be because the original p16 mRNA 3′-UTR sequence and length had been previously determined by 3′-RACE PCR in CMT28 cell line [Lutful Kabir et al., 2013]. We have investigated that truncated p16 mRNAs that do not include the miR-141 binding site are frequently expressed in CMT cells and may be a common aspect of the breast cancer phenotype. A number of reports have suggested that 3′-UTR length variability may be the result of a mechanism to remove miRNA target sites from key mRNAs in proliferating cells [Sandberg et al., 2008; Shrout, 2013]. Surprisingly, the longer 3′-UTR sequence of the p16/INK4A gene was found to be expressed in CMT28. To verify this unlikely expression of an extended 3′-UTR as part of p16 mRNA in CMT28, PCR amplification was performed in other CMT cell lines that were previously examined for p16 and p14 expression. This gene expression analysis verified that the longer 3′-UTR was also expressed in the CMT9 cell line that does not express p16, but only p14, suggesting that this mRNA sequence is expressed as the 3′UTR of p14 but not of p16 and was derived from the common INK4A gene locus. This evidence demonstrated how cells can shift the 3′-UTR length from longer to shorter forms between two alternatively spliced transcripts and, as a consequence eliminate the miRNA target sites in the dominant tumor suppressor gene, p16. This newly discovered 3′-UTR and miR-141 target site in INK4A/p14 was further validated using a miRNA 3′-UTR reporter assay. This experimental evidence demonstrated that miR-141 endogenously knocked down INK4A/p14 mRNA (containing the 3′-UTR target site) when over-expressed in CMT12 and CMT27 cell lines. In contrast, down-regulation of miR-141 in CMT28 cells could not knock down the 3′-UTR target and to silence the p14 mRNA expression in this cell line.

The very first evidence of a comprehensive expression profile of the 277 canine miRNAs in the canine mammary cancer cells revealed a large number of altered miRNAs (up-/down-regulated) that might serve as potential oncogenic and tumor suppressor targets for advancing the development of future therapeutics. Validation of the regulation of p16/p14 INK4A tumor suppressors by an altered miRNA (miR-141) demonstrated its importance in canine breast cancer. Evidence of cellular management of the miRNA regulatory mechanism for p16 and p14 transcripts emanating from the INK4A locus was also identified by truncation of the 3′-UTR. Additionally, this study reported several oncogenic and tumor suppressor miRNAs that are similarly altered in both human and canine breast cancers. These correlations further strengthen the validity and use of canine mammary cancers as appropriate intermediate models for the study of human breast cancers.

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