Identification of dysregulated microRNAs in canine malignant melanoma

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Abstract. Inhibiting aberrantly upregulated microRNAs (miR/miRNAs) has emerged as a novel focus for therapeutic intervention in human melanoma. Thus, identifying upregulated miRNAs is essential for identifying additional melanoma-associated therapeutic targets. In the present study, microarray-based miRNA profiling of canine malignant melanoma (CMM) tissue obtained from the oral cavity was performed and differential expression was confirmed by a reverse transcription-quantitative polymerase chain reaction (RT-qPCR). An analysis of the microarray data revealed 17 dysregulated miRNAs; 5 were upregulated and 12 were downregulated. RT-qPCR analysis was performed for 2 upregulated (miR-204 and miR-383), 3 downregulated (miR-122, miR-143 and miR-205) and 6 additional oncogenic miRNAs (oncomiRs; miR-16, miR-21, miR-29b, miR-92a, miR-125b and miR-222). The expression levels of seven of the miRNAs, miR-16, miR-21, miR-29b, miR-122, miR-125b, miR-204 and miR-383 were significantly upregulated; however, the expression of miR-205 was downregulated in CMM tissues compared with normal oral tissues. The microarray and RT-qPCR analyses validated the upregulation of two potential oncomiRs miR-204 and miR-383. The present study additionally constructed a protein interaction network and a miRNA-target regulatory interaction network using STRING and Cytoscape. In the proposed network, cyclin dependent kinase 2 was a target for miR-383, sirtuin 1 and tumor protein p53 were targets for miR-204 and ATR serine/threonine kinase was a target for both. It was concluded that miR-383 and miR-204 were potential oncomiRs that may be involved in regulating melanoma development by evading DNA repair and apoptosis.

Introduction

MicroRNAs (miR/miRNAs) are small endogenous non-coding RNAs that post-transcriptionally regulate the expression of target genes by binding to the 3'-untranslated regions of mRNAs, causing destabilization, degradation, or translation inhibition (1). Because dysregulation of miRNA expression has been identified in a number of cancers, some miRNAs are categorized as oncogenic miRNAs or 'oncomiRs', a term used to describe either tumor suppressors or oncogenes (2-5). Consequently, miRNAs have been investigated as potential therapeutic targets for several malignant cancers including melanoma (6,7). The tumor burden in mice with liver melanoma metastasis was found to be reduced by anti-miR-182 oligonucleotides that inhibited the upregulated miR-182 in the tumor cells (6). Inhibition of miR-383 over-expression suppressed the proliferation, cell cycle progression and invasion of human epithelial ovarian cancer (EOC) and immortal EOC cell lines (8). Over-expression of miR-203 sensitized malignant melanoma cells to temozolomide drug by targeting glutaminase, which opened new opportunities for chemotherapy-resistant malignant melanoma patients (9). Thus, profiling dysregulated miRNA expression in cancers is an important approach for detecting potential therapeutic targets.

Simpson *et al* 2013 (10) suggested significant overlapping may exist in the clinical and histopathological features of canine and human mucosal melanomas. miRNA expression has been investigated in different canine tumors, including

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B and T-cell lymphoma (11), lymphocytic leukemia (12), transitional cell carcinoma (13), mammary cancer (14), prostate cancer (15) and melanoma (16-18). These studies indicated that the expression patterns of specific miRNAs in specific cancers were similar to those in corresponding human cancers. For example, the upregulation of miR-21 and miR-29b in canine mammary cancer is consistent with their upregulation in human breast cancer (14,19,20) and melanoma (21,22) and miR-145, miR-203, and miR-205 were found to be downregulated in both canine malignant melanoma (CMM) and human malignant melanoma (HMM) (16,17). In the Noguchi *et al* (17) studies of HMM, a total of seven downregulated miRNAs were detected by microarray analysis; three of them were confirmed by quantitative reverse transcription PCR (qRT-PCR). In almost all HMM tumors that have been studied, upregulated miRNA expression has been reported, including the miR-17-92 cluster, miR-222/221, miR-21 and miR-155 (23). Therefore, it is likely that some miRNAs will be upregulated in oral CMM, similar to what Starkey *et al* (18) reported in canine uveal melanoma. However, until now, no upregulated miRNAs in oral CMM have been reported. To investigate this hypothesis, we examined the expression of miRNAs in CMM tissues obtained from the oral cavity using microarray and qRT-PCR analyses. Here we report the upregulation of seven miRNAs in CMM tissues. To understand the biological relevance of miRNAs it is necessary to identify the target genes with which they interact. Protein-protein interactions are essential for cells to maintain systemic biological functions such as replication of DNA, transcription, translation and signal transduction (24). Dysregulation of proteins may collapse the homeostasis process leading to complex diseases and miRNAs may act as master regulators by maintaining the stability of protein-protein interaction networks (25). So, determining the interactions between the proteins encoded by targets of dysregulated miRNAs and other proteins is very important. In this study, we drew a miRNA-target regulatory interaction network with tumor suppressor genes, which revealed miR-383 and miR-204 may play roles in the development of melanoma by avoiding DNA repair and apoptosis.

Materials and methods

Sample collection. The CMM tissues used in this study were obtained from dogs (n=10) that had undergone biopsy or surgical resection for diagnosis or treatment at the Veterinary Teaching Hospital, Kagoshima University (Kagoshima, Japan). All melanoma samples were obtained from the oral cavity and were histopathologically diagnosed by two pathologists. Normal oral tissues were obtained from healthy laboratory beagle dogs (n=12). In addition to the CMM and normal oral tissues, we obtained a total of 21 canine tumors and normal tissues to use as microarray reference samples as follows: Mammary tubulopapillary carcinoma (n=4), mammary benign mixed tumor (n=4), hepatic cell carcinoma (n=1), squamous cell carcinoma (n=1), lymphoma (n=1), adenosquamous carcinoma (n=1), mast cell tumor (n=1), malignant peripheral nerve sheath tumor (n=1), normal mammary gland tissue (n=4) and normal hepatic tissue (n=3). The animal experiments were approved by the Kagoshima University's Laboratory Animal Committee (A10031).

Isolation of total RNA. All the tissues were preserved in RNA*later* (Thermo Fisher Scientific Inc., Waltham, MA, USA) immediately after biopsy or surgical resection until used for RNA isolation. Total RNA was isolated from the stored tissues using a mirVana™ miRNA Isolation kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. RNA quantity was measured using either an ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) or a NanoPhotometer™ Pearl (Implen GmbH, München, Germany). RNA quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and RNA integrity numbers were determined (26).

Microarray analysis. Three assays were performed (n=3) using the miRCURY™ LNA microRNA Array, version 11.0 (Exiqon Inc., Woburn, MA, USA). In each assay, Hy3 labeled miRNAs from different CMM tissues but the same references Hy5 labeled miRNAs were used. The reference miRNAs comprised equal amounts of RNA from 21 reference samples from 10 different tissues (listed in the *Sample collection* section), all of which were pooled. Two-color miRNA-microarrays with 264 identical canine miRNA probes were used. Signal extraction was performed using Feature Extraction 10.7.3.1 software (Agilent Technologies). To minimize error, each miRNA was spotted at four different locations on the array and the average signal intensity value of the four spots was used and variable coefficients were calculated [standard deviation (SD) of signal intensity of four spots/average values]. miRNAs with signal intensity variable coefficients >0.5 or with low signal intensity (<100) in both the CMM and reference tissues were excluded from further analysis. The average values of the Hy3/Hy5 (fold change; FC) ratio between the CMM and reference tissues were compared using the Lowess normalization method (27). miRNAs that had FC ratios >2.0 or <0.5 were considered to be dysregulated.

qRT-PCR assays. CMM tissues (n=10) and normal oral tissues (n=12) were used in the qRT-PCRs, which were performed in duplicate using TaqMan microRNA Assays (Thermo Fisher Scientific Inc.; see Table I for assay details) with 2 ng/μl total RNA, according to the optimal reagent concentrations and reaction conditions described in the manufacturer's instructions. The canine miRNA sequences used for the PCRs were identical to the corresponding human miRNA sequences (Table I). The qRT-PCRs were carried out using an Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific Inc.). RNU6B, U6 small nuclear RNA, was used as a quantitative normalization control (13,14). Relative expression levels were calculated using the comparative delta Cq method ($2^{-\Delta\Delta Cq}$) (28). Cq values >36.0 were considered as absence of miRNA expression. The relative expression levels of miRNAs in the CMM tissues were calculated relative to the average values in the normal oral tissues, which were assigned a value of 1.0.

Statistics. In the microarray experiments, P-values and false discovery rates (FDRs) were analyzed using Welch's test and the Benjamini-Hochberg correction for multiple hypotheses testing using R software (29). For the qRT-PCRs, the miRNA expression levels between CMM and normal oral tissues were

Table I. miRs used in the reverse transcription-quantitative polymerase chain reaction assays.

A, miRNA sequences

Assay name	Assay ID	Mature miRNA sequence	miRBase accession number
hsa-miR-16	000391	UAGCAGCACGUAAAUAUUGGCG	MI0000070
hsa-miR-21	000397	UAGCUUAUCAGACUGAUGUUGA	MI0000077
hsa-miR-29b	000413	UAGCACCAUUUGAAAUCAGUGUU	MI0000105
hsa-miR-92a	000431	UAUUGCACUUGUCCCGGCCUGU	MI0000093
hsa-miR-122	002245	UGGAGUGUGACAAUGGUGUUUG	MI0000442
hsa-miR-125b	000449	UCCCUGAGACCCUAACUUGUGA	MI0000446
hsa-miR-143	002249	UGAGAUGAAGCACUGUAGCUC	MI0000459
hsa-miR-204	000508	UUCCCUUUGUCAUCCUAUGCCU	MI0000284
hsa-miR-205	000509	UCCUUCAUUCCACCGGAGUCUG	MI0000285
hsa-miR-222	002276	AGCUACAUCUGGCUACUGGGU	MI0000299
hsa-miR-383	000573	AGAUCAGAAGGUGAUUGUGGCU	MI0000791

B, Control sequences

Assay name	Assay ID	Control sequence	NCBI accession number
RNU6B	001093	CGCAAGGATGACACGCAAATTCG TGAAGCGTTCCATATTTTT	NR_002752

RNU6B, RNA; U6 small nuclear 6, pseudogene. miR/miRNA, microRNA.

analyzed using the Mann Whitney U-test. Statistical analyses were performed with JMP 10.0 (SAS Institute, Cary, USA). P<0.05 was considered to indicate a statistically significant difference.

Network construction. miRNA targets were predicted using TargetScan 7.1 (30) and 1021 human tumor suppressor genes (with basic annotations) from the Tumor Suppressor Gene Database (TSGene; https://bioinfo.uth.edu/TSGene/). A miRNA-target interaction network was drawn using Cytoscape v3.5 (http://www.cytoscape.org/) (31) and a protein-protein interaction network of tumor suppressor genes was constructed using STRING (confidence score, 0.9) (http://string-db.org/) (32). The two networks were merged within Cytoscape and interconnected nodes were separated to obtain a co-ordinate network. Analysis of basic network parameters (degree, betweenness, centroid value and Eigenvector) was done using Centiscape 2.2 (33). In the network, a node represents a protein (encoded by a target mRNA) or a miRNA and a line represents an interaction between a protein and a miRNA.

Results

Screening of differentially expressed miRNAs by microarray analysis. The microarray analysis revealed 17 dysregulated miRNAs in the CMM tissues based on the FC ratios (Table II). Of the 17 miRNAs, 5 were upregulated (FC ratios >2.0) with no significant FDRs and 12 were downregulated (FC ratios <0.5) and 4 of them had significant FDRs (P<0.05) (Table II).

Confirmation of differentially expressed miRNAs by qRT-PCR. qRT-PCRs were performed to validate some of the dysregulated miRNAs from the microarray analysis (Table II). Because none of the upregulated miRNAs had significant FDRs, we selected the two most highly upregulated miRNAs, miR-204 and miR-383, for validation. From among the downregulated miRNAs, we selected three miRNAs (miR-122, miR-143 and miR-205) that had the most significant FDRs. We also selected six other miRNAs (miR-16, miR-21, miR-29b, miR-92a, miR-125b and miR-222) for validation because they were reported to be dysregulated in cancers other than CMM (13,14,34-36).

We found that seven miRNAs were significantly upregulated [P-values from 0.0001 (miR-21) to 0.025 (miR-29b)], but miR-205 was the only significantly downregulated miRNA (P<0.0001) in the CMM tissues compared with normal oral tissues (Fig. 1). No significant differences were detected in the expression of miR-92a, miR-143 and miR-222 between the CMM and normal oral tissues (Fig. 1).

Of the 17 dysregulated miRNAs identified by microarray analysis (Table II), only miR-204, miR-383 and miR-205 were found to be highly differentially expressed by qRT-PCR. The average FCs for miR-204 and miR-383 were 15.3 and 152.7, respectively, but for miR-205 the average FC was 0.01 (Fig. 1).

The relative expression patterns of miR-204, miR-383 and miR-205 were consistent between the qRT-PCR and microarray results, but there were discrepancies for some of the other miRNAs. For example, miR-122 was downregulated (FC<0.5) in the microarray analysis but significantly

upregulated in the qRT-PCR analysis and miR-143 was downregulated (FC of 0.244) in the microarray analysis but was not found to be significantly differentially expressed by qRT-PCR (Fig. 1).

miRNA-target regulatory interaction network. As indicated in Fig. 2A, the STRING protein interaction network revealed that miR-383 and miR-204 interacted with several common genes (proteins), as was reported previously (37,38). When we separated the connected network and calculated the basic parameters (degree, betweenness, centroid value and eigen-vector) by Centiscape 2.2 through Cytoscape (Fig. 2B), we found all the basic parameters of TP53 (Fig. 3A) had higher value than any of the others. Further, the basic parameters of miR-383, miR-204, SIRT1, CDK2 and ATR (Fig. 3B-F) were higher than the average values, implying these miRNAs and proteins were the hub nodes of this biological network. In the separated miRNA-target interaction network we found that ATR and CDK2 were targets of miR-383 and miR-204 (Fig. 2B). Moreover, miR-204 could regulate the network through TP53 mediated by SIRT1. RBBP7, SMARCB1, and CREBBP were also connected with several nodes and may be related to the regulation of a small cluster network.

Discussion

Some of the dysregulated miRNAs identified in the CMM tissues by microarray analysis were validated by qRT-PCR. The upregulation of seven miRNAs in CMM, namely miR-16, miR-21, miR-29b, miR-122, miR-125b, miR-204, and miR-383 was demonstrated here for the first time. In particular, miR-204 and miR-383 showed extra ordinarily high expression levels in the microarray and qRT-PCR analyses.

Downregulation of miR-145, miR-205 and miR-203 was detected in the microarray analysis, which is consistent with previous studies on CMM (16,17). However, we did not detect dysregulation of other miRNAs that have been reported previously to be downregulated (17). These inconsistencies might be because different microarray platforms and/or samples were used in the two studies. Noguchi *et al* (17) used a CombiMatrix array, whereas we used a miRCURY™ LNA microRNA Array. Thus, there were differences in the miRNAs that were spotted on the arrays. We used CMM tissues from three different dogs and Noguchi *et al* (17) used CMM tissue from only one dog. Finally, in the previous study, miRNA expression was compared between CMM tissue and normal oral mucosal tissue (17), whereas we compared CMM tissues with reference miRNAs from several cancers and normal tissues. We used mixed miRNA reference samples to avoid biases from low signal intensities in the microarray data. Using miRNAs from several different origins means different miRNAs will be included because miRNA expression is highly dependent on the tissue origin and status. Our approach should cover a broad range of miRNAs, thus avoiding misleading FC ratios as a result of weak signals (39). However, because our reference tissues were mostly tumor samples (70.8%), using this kind of miRNA reference samples may have caused miRNAs that are commonly dysregulated in tumors to be overlooked but, importantly, may have revealed miRNAs that are specifically dysregulated in melanoma.

Table II. Dysregulated miRNAs identified in canine malignant melanoma tissues by microarray analysis.

A, Upregulated miRNAs

Upregulated (FC>2.0)

miRNA	FC	FDR
miR-9	2.420	>0.05
miR-149	2.022	>0.05
miR-204	2.781	>0.05
miR-326	2.056	>0.05
miR-383	3.581	>0.05

B, Downregulated miRNAs

Downregulated (FC<0.5)

miRNA	FC	FDR
miR-10	0.486	<0.05
miR-101	0.446	>0.05
miR-122	0.060	<0.05
miR-142	0.385	>0.05
miR-143	0.244	<0.05
miR-195	0.391	>0.05
miR-200c	0.382	>0.05
miR-205	0.100	<0.05
miR-328	0.299	>0.05
miR-487b	0.430	>0.05
miR-652	0.457	>0.05

FC, fold change; FDR, false discovery ratio; miR/miRNA, microRNA.

In this study, the microarray and qRT-PCR results were consistent for the relative expressions of miR-204, miR-383 and miR-205. However, the discrepant expressions of miR-122 and miR-143 between the microarray and qRT-PCR results may be explained by differences in the control samples that were used in the two experiments; that is, a mixed sample reference in the microarray analysis and normal oral tissues in the qRT-PCRs. For the same reason, differential expression of miR-16, miR-21, miR-29b and miR-125b was not detected in the microarray analysis but was detected by qRT-PCR. miR-21 and miR-29b are known to be upregulated in several tumors; for example, miR-21 in mouse BL/6 melanoma cells (40), miR-29b in human breast cancer (20) and both miRNAs in canine mammary cancer (14). These findings indicate that miR-21 and miR-29b are common oncomiRs in several species. Thus, the microarray screening method that we used may have masked the differential expression of these miRNAs because they are not specific to melanoma but commonly shared among several kinds of tumors.

While the significant downregulation of miR-205 can be explained, upregulation of miR-204 and miR-383 expression has not been reported in CMM until now. Indeed, miR-204
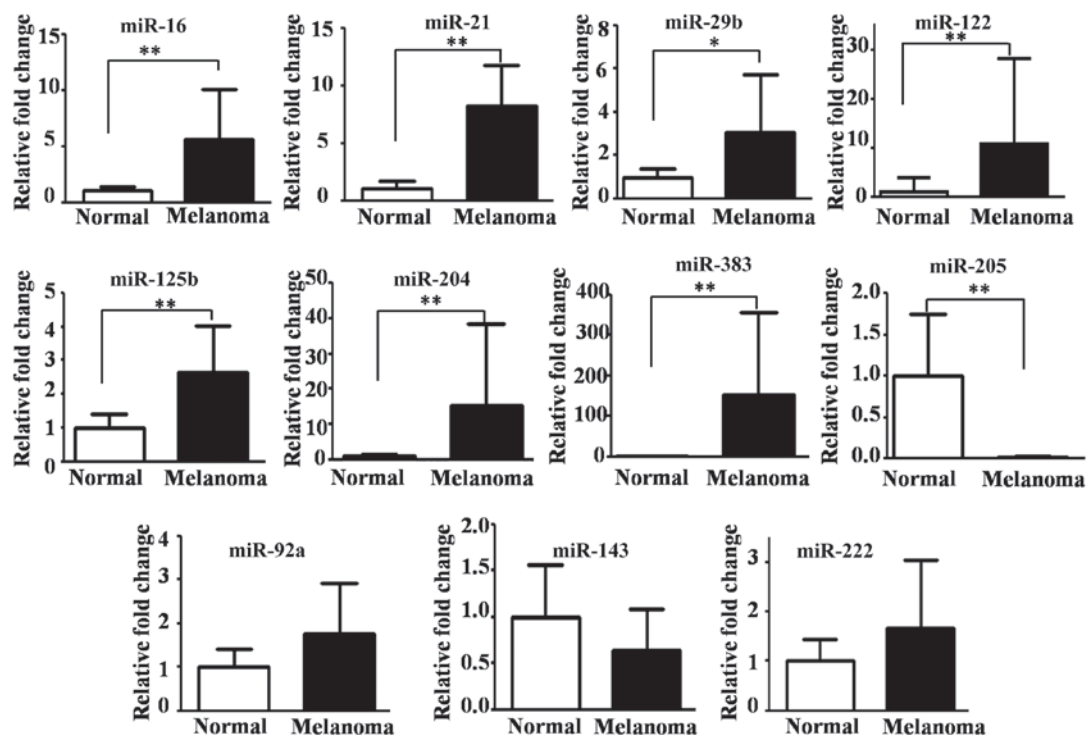
Figure 1. Reverse transcription-quantitative polymerase chain reaction validation of five dysregulated miRNAs from the microarray assays and six other cancer-associated miRNAs. Relative expression levels in CMM tissues (Melanoma) and normal oral tissues (Normal) are shown. The mean expression levels of the Normal samples were set to 1.0. P-values were determined by the Mann Whitney U-test (*P<0.05, **P<0.01). The bars indicate standard error. miR/miRNA, microRNA; CMM, canine malignant melanoma.

was reported to be upregulated in old HMM patients compared with young HMM patients (41); however, no comparison between melanoma and normal tissue was performed and the target mRNA was not defined. In another study, miR-204 was found to be downregulated in malignant melanoma compared with benign nevi (42), but the age of the patients was not considered and the comparisons were between malignant melanoma and benign nevi tissues. In prostate cancer and breast cancer studies, miR-204 was reported to be both up- and downregulated (43-47), maybe because of different experimental designs and individual identity.

TP53 is a well-known tumor suppressor gene located in the center of the network with a high centroid value (Fig. 3A). SIRT1, an indirect regulator of TP53, is a direct target of miR-204 in the network and has been reported to be downregulated in canine melanoma (48). SIRT1 acts as a tumor suppressor via β-catenin and has reminiscent effects on TP53 in colon cancer (49). Abnormal expression of β-catenin was reported in melanoma (50,51), so the miR-204-mediated downregulation of SIRT1 revealed in the network may cause β-catenin-mediated cell survival by evading TP53 in melanoma.

Up-regulation of miR-383 expression has been observed in primary HMM tumor cell lines compared with normal human epidermal melanocytes (52). In their study, Mueller et al (52) found that miR-383 was downregulated in snail stable knockdown melanoma cells by transfection of an antisense snail plasmid construct, named as-snail, compared with the parental melanoma cell line. Snail belongs to the snail superfamily of zinc finger transcription factors and is involved in the development of malignant melanoma through direct repression of E-cadherin expression (53). Indeed, the

transcriptional profile of the as-snail cells was reported to be more similar to normal melanocytes than malignant melanoma cells (52). However, the detailed biological functions of miR-383 have not been reported so far. In our study, miR-383 was upregulated in CMM tissues. Liao et al (54) showed that ATR was the direct target of miR-383 and ATR was found to play a central role in the ATM/ATR pathway involved in DNA damage recognition and initial phosphorylation (55). Liao et al (54) also showed that GADD45γ, MDC1, and H2AX were all negatively correlated with miR-383 expression. Moreover, a recent study showed that loss of function or mutations of ATR lead to the development of melanoma (56). In testicular embryonal carcinoma miR-383 overexpression was found to reduce CDK2 expression at the protein level, which was also found to be necessary for proper DNA repair (57). Furthermore, CREB binding protein, a known co-activator of TP53, was found to be a direct target of miR-383 (58). There is also a possibility that miR-383 has indirect control over apoptosis via TP53 inhibition through CDK2. So, our network analysis and the above discussion suggest that miR-383 may be involved in DNA damage repair and apoptosis phenomena in melanoma. In this study, we demonstrated the dysregulation of 17 miRNAs in CMM and investigated the probable biological functions of these miRNAs based on their target genes. Our study is valid not only for dog but also for human because dog has been considered as a good preclinical model for human melanoma (10). Further studies are required to clarify the functions of the dysregulated miRNAs by for example, detecting the actual target genes and their pathways and analyzing their differential expression patterns in established canine melanoma cell lines (59,60) to determine the roles of
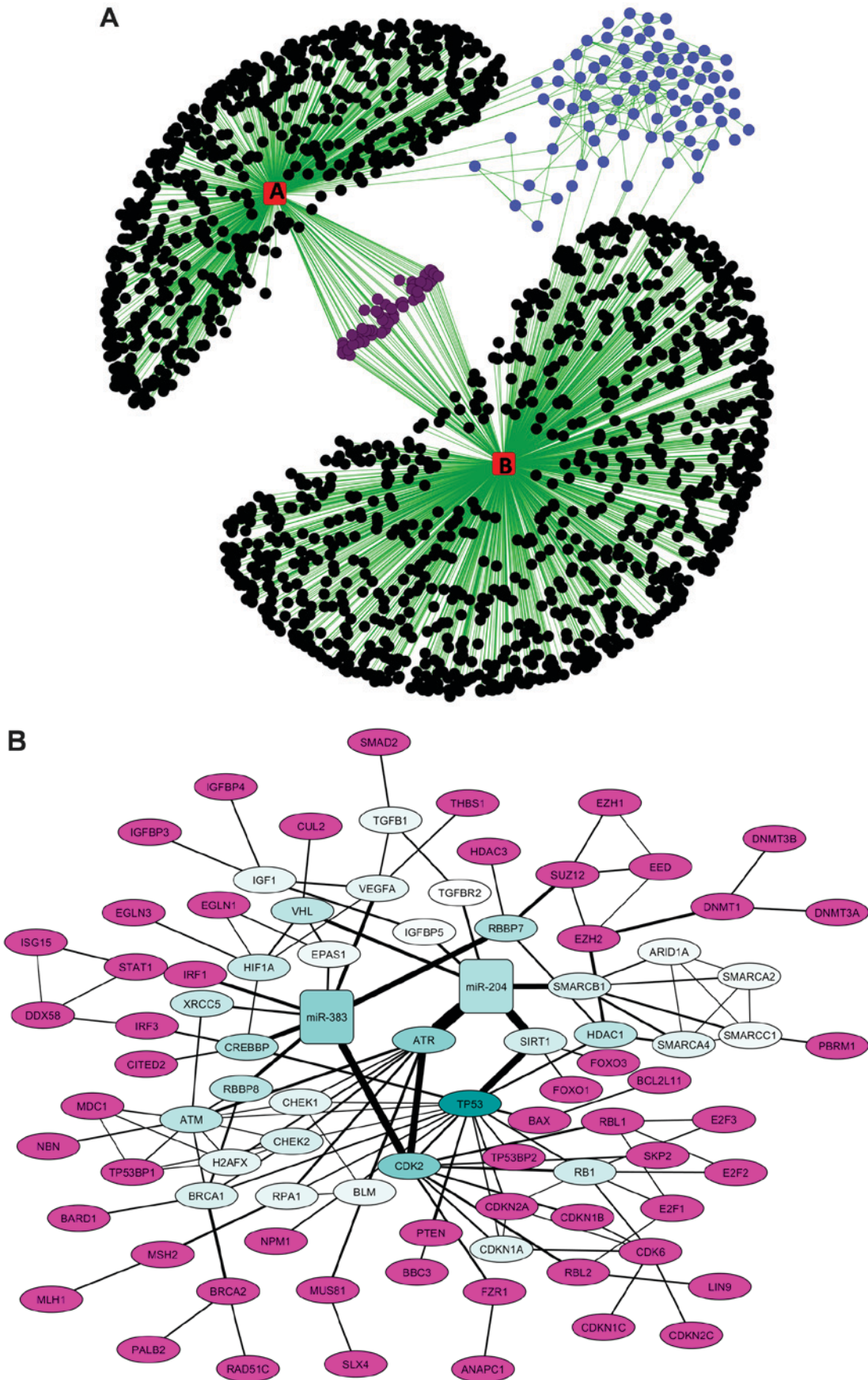
Figure 2. miRNA-target regulatory interaction network. (A) miRNA-target regulatory network merged with the tumor suppressor genes protein interaction network. The red squares indicate miRNA nodes [(A) miR-383; (B) miR-204]. Black circles indicate targets (mRNAs) of single miRNAs, purple circles indicate targets shared by miRNAs and blue circles indicate tumor suppressor genes predicted to be targeted by one or both of the miRNA. The edges (lines) connecting two nodes are indicative of regulation (interaction). (B) Separated co-ordinate network showing the interactions between microRNAs and tumor suppressor genes. The node colors indicate the CV; pink gradient indicates CVs lower than average, blue gradient indicates CVs higher than average. Edge width indicates the betweenness measurement. miR/miRNA, microRNA; CV, centroid value.
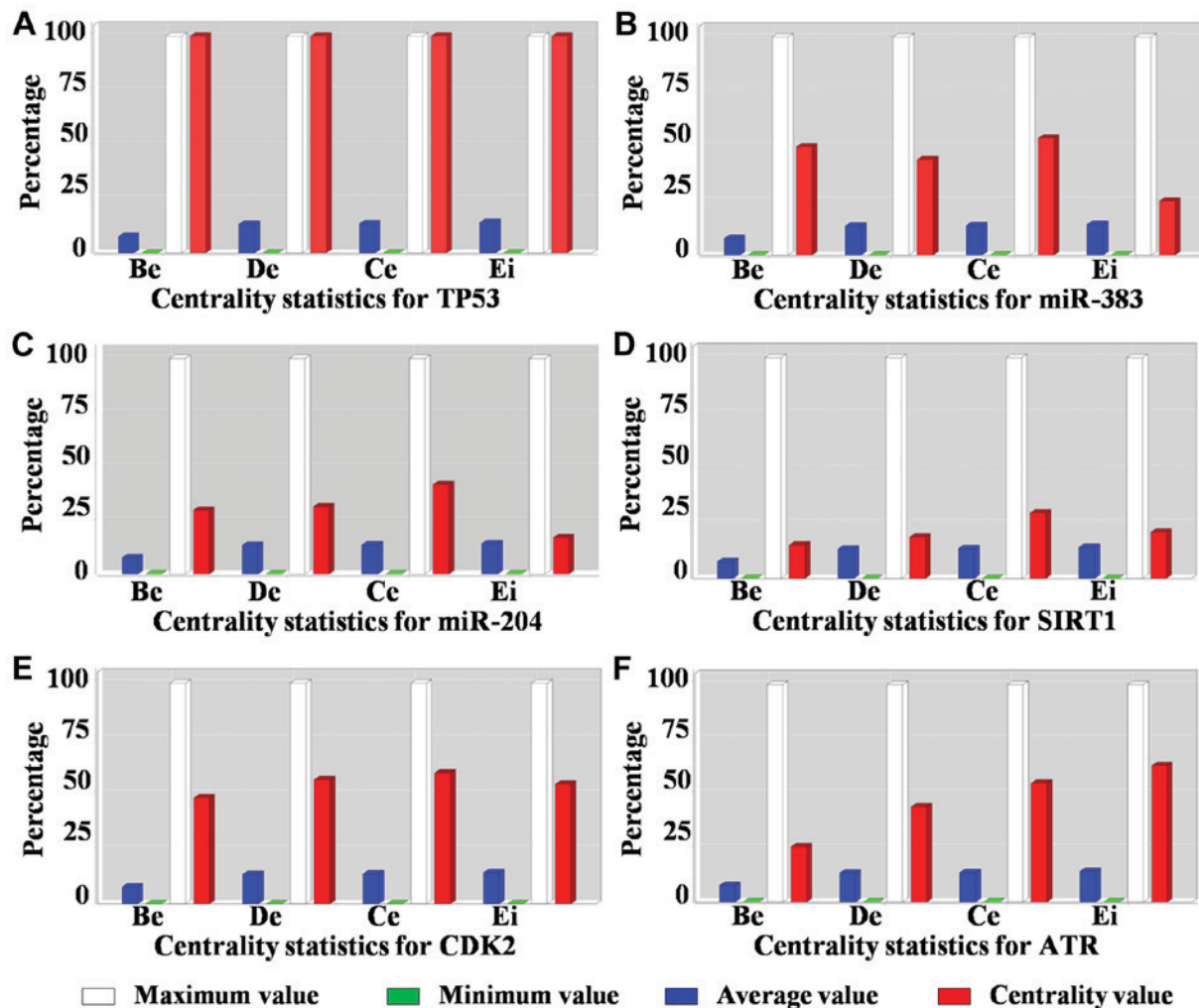
Figure 3. Centrality measures of the hub nodes in the miRNA-target regulatory network. Betweenness (Be), degree (De), centroid value (Ce) and eigenvector (Ei) plots for (A) TP53, (B) miR-383, (C) miR-204, (D) SIRT1, (E) CDK2 and (F) ATR respectively. miR/miRNA, microRNA; TP53, tumor protein p53; SIRT1, sirtuin 1; CDK2, cyclin dependent kinase 2; ATR, ATR serine/threonine kinase.

the miRNA-target interactions in CMM tumor genesis and therapy.

We have demonstrated the upregulation of potential oncomiRs, miR-16, miR-21, miR-29b, miR-122, miR-125b, miR-204 and miR-383 in CMM tissues. In particular, the strong upregulation of miR-383 in CMM tissues compared with normal oral tissues identified by microarray screening was confirmed by qRT-PCR. We conclude that miR-383 and miR-204 may promote melanoma development by regulating both the DNA repair/checkpoint and apoptosis. To identify therapeutic targets in melanoma, further studies are required to verify the biological significance of the miRNA target genes.

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Availability of data and materials

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

Authors' contributions

NU, MR, TM and NaM were involved in designing the study. NU, MR, TM, TI, NoM and HK collaborated in the data analysis. NaM acquired the funding. NU, MR, YCL, TM, TI, HK, YM and NaM performed the experiments. NaM was involved in project administration. NU, TI, NoM and HK acquired the resources. NoM, YM and NaM supervised the study. NaM was involved in data validation. TM wrote the original draft of the manuscript. NU, MR, YM and NaM revised and edited the original draft. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent to use the specimens in this study was obtained from the dog patient's owners. The animal experiments were approved by the Kagoshima University's Laboratory Animal Committee (approval no. A10031).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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