(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 May 2014 (15.05.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/074874 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/US2013/069237

(22) International Filing Date:
8 November 2013 (08.11.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/724,509 9 November 2012 (09.11.2012) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

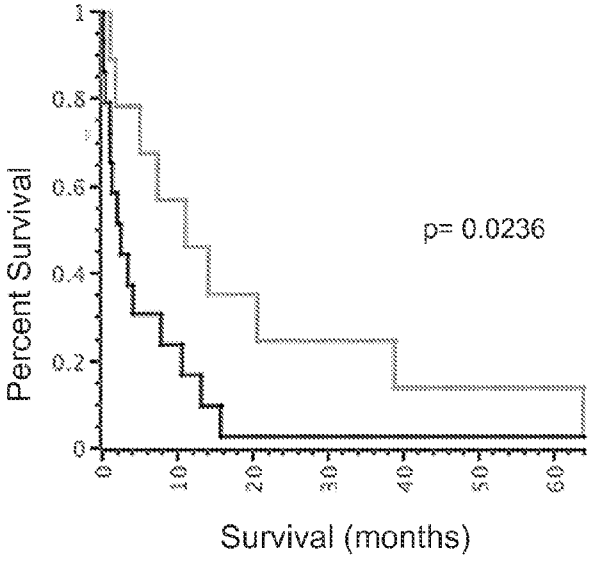
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) **Title**: OSTEOSARCOMA DIAGNOSTIC AND PROGNOSTIC METHODS

Figure 2B



(57) **Abstract**: This disclosure describes, in one aspect, a method that generally includes analyzing a tissue sample from a subject for expression of a 14q32 miRNA, wherein a decrease in expression of the 14q32 miRNA compared to a normal control indicates that the subject has or is at risk of having osteosarcoma. In some embodiments, expression of the 14q32 miRNA of no more than 10% of a normal control further indicates that the subject has or is at risk of having an aggressive osteosarcoma.

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OSTEOSARCOMA DIAGNOSTIC AND PROGNOSTIC METHODS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application Serial No. 61/724,509, filed November 9, 2012, which is incorporated herein by reference.

SUMMARY

This disclosure describes, in one aspect, a method that generally includes analyzing a tissue sample from a subject for expression of a 14q32 miRNA, wherein a decrease in expression of the 14q32 miRNA compared to a normal control indicates that the subject has or is at risk of having osteosarcoma.

In some embodiments, expression of the 14q32 miRNA of no more than 10% of a normal control further indicates that the subject has or is at risk of having an aggressive osteosarcoma.

In some embodiments, the 14q32 miRNA can include miR-382, miR-134, or miR-544. In other embodiments, the 14q32 miRNA can include miR-379, miR-1197, miR-329-1, miR-494, miR-495, miR-376C, miR-1185-1, miR-1185-2, miR-381, miR-655, miR-487A, miR-382, miR-496, miR-154, miR-369, miR-127, miR-299, miR-665, miR-770, miR-432, miR-668, miR-539, miR-433, miR-323, miR-496, miR-654, miR-409, miR-380, miR-485, miR-136, miR-376, miR-431, miR-370, miR-889, miR-381, miR-299, miR-379, miR-411, miR-655, miR-432, miR-487, miR-410, miR-493, miR-656, miR-485, miR-377 or miR-541.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. 14q32 miRNA expression levels and metastasis in human osteosarcoma. (A) Correlation network of 14q32 miRNAs expression in human osteosarcoma samples. Correlation network was constructed with Pearson correlation R >0.9 as the edges. The resulting correlation sub-network contained thirty-six miRNAs and 33 of them mapped to the 14q32 locus. (B) Microarray based transcript levels of highly correlated 14q32 miRNAs in osteosarcoma tumors shown relative to the level observed in FT-14 (human osteosarcoma primary tumor). miRNA profiling data from human osteosarcoma tumors where metastasis was observed show stronger downregulation of 14q32 miRNAs (highlighted) than primary osteosarcoma tumors where metastasis was not observed. (C) Microarray based transcript levels for miR-382 and miR-154. miR-382 and miR-154 show a high level of correlation (R^2= 0.95). (D) qRT-PCR validation for miR-382 and miR-154 expression levels obtained in primary osteosarcoma tumors (FT-14, -7) where metastasis was not observed and three primary osteosarcoma tumors with low levels of (FT-18, -13, -12) where metastasis was observed. miR-382 and miR-154 show a high level of correlation (R^2= 0.99). Measurements were carried out in triplicate and were normalized to the expression levels in two independent normal bone tissues that were also carried out in triplicate.

FIG. 2. Prognostic significance of genes correlated with 14q32 miRNAs in canine osteosarcoma. (A) Unsupervised clustering of human mRNAs (n=385) that shows high-level direct correlations to miR-382 for two normal bone tissues and 11 osteosarcoma tumors, for which we have both mRNA profiles and miRNA profiles. A positive correlation to miR-382 is shown in lighter grey (n=288), and a negative correlation to miR-382 is shown in darker grey (n=97), at the right of the heatmap. Directional functional enrichment analyses (Ingenuity Pathways Analyses) shows that correlating transcripts are enriched in transcripts involved in metastasis, and direction of change is consistent with increased activity of metastatic function in tumors with decreased levels of 14q32 miRNA member, miR-382. Identities of positive and negatively correlating mRNA are provided in Table 6 and 7. (B) Unsupervised hierarchical clustering heatmap of canine mRNA found in 26 canine OS-derived samples that correspond to human mRNAs correlating to miR-382. The bars to at the right side of each heatmap represent correlations to miR-382 (positive = lighter grey or negative= darker grey) observed in the human data indicating that the direction of change

shows similar trends between human and canine data. (C) Kaplan-Meier survival curves generated using the groups shown in FIG. 2B. Survival times are significantly different between the two groups of tumors (p-value = 0.02).

FIG. 3. 14q32 miRNA expression level, metastasis, and outcome in human OS. (A) miR-382 expression levels determined by qRT-PCR in osteosarcoma primary tumor samples with clinical follow-up information. The miRNA expression levels were normalized relative to normal bone tissues. Osteosarcoma samples were ranked based on expression levels of miR-382 from highest to lowest. Primary osteosarcoma patient samples that later developed metastases are highlighted and samples from patients who died due to disease are shown in black. Samples were grouped based on whether they were above or below the median expression level. (B) Kaplan-Meier analysis of metastasis in osteosarcoma patients based on miR-382 expression. Group 1 and Group 2 are significantly different (p-value = 0.01). Patients with lowest levels of miR-382 expression showed increased likelihood of metastasis. (C) Kaplan-Meier analysis of survival in osteosarcoma patients based on miR-382 expression. Patients with lowest levels of miR-382 expression showed decreased likelihood of survival.

FIG. 4. 14q32 orthologous region transcript levels and outcome in canine osteosarcoma. (A) miR-134 and (B) miR-544 expression levels determined by qRT-PCR in canine osteosarcoma primary tumor samples with clinical follow-up information. The miRNA expression levels were normalized relative to reactive osteoblasts. Osteosarcoma samples were ranked based on expression levels of miR-134 from highest to lowest. Samples were grouped based on whether they were above or below the median expression levels. (C and D) Kaplan-Meier analysis of survival in osteosarcoma patients based on miR-134 and miR-544 expression. Survival of dogs with osteosarcoma in Group 1 and Group 2 are significantly different (miR-134 p-value = 0.004, miR-544 p-value = 0.01). Dogs with lowest levels of miR-134 or miR-544 expression showed decreased likelihood of survival.

FIG. 5. Homology between human 14q32 miRNAs and CFA 8:72.3 Mb miRNAs locus. Data was obtained from Entrez gene database. (A-B) Conservation at the gene level. (C-D) Conservation at the miRNA level. While the order is highly maintained only certain miRNAs are shown. The position of miR-379 was used to orient the orthologous canine region of the genome to the human 14q32 locus.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Deregulation of microRNA (miRNA) transcript levels has been observed in many types of tumors including, for example, osteosarcoma. Molecular pathways regulated by differentially expressed miRNAs may contribute to the heterogeneous tumor behaviors observed in naturally occurring cancers. Thus, tumor-associated miRNA expression may provide informative biomarkers for disease outcome and metastatic potential in osteosarcoma patients.

We have discovered an inverse correlation between aggressive tumor behavior—e.g., increased metastatic potential and accelerated time to death—and the residual expression of 14q32 miRNAs (using miR-382 as a representative miRNA of the 14q32 miRNA family) in a series of clinically annotated samples from human osteosarcoma patients. We also have discovered a comparable decrease in expression of orthologous 14q32 miRNAs in canine osteosarcoma samples, with conservation of the inverse correlation between aggressive behavior and expression of orthologous miRNA miR-134 and miR-544. Thus, downregulation of 14q32 miRNA expression is an evolutionarily conserved mechanism that contributes to the biological behavior of osteosarcoma. Consequently, quantifying representative transcripts of the 14q32 miRNA family such as, for example, miR-382, miR-134, and/or miR-544, provide prognostic and/or predictive markers that can assist in the management of patients with this disease.

As used herein, the term "14q32 miRNA" refers to a microRNA transcribed from any portion of the 14q32 locus of the human genome and/or a microRNA transcribed from an orthologous locus of a chromosome of another species such as, for example, the CFA 8 locus of the canine genome. For example, a particular miRNA that is transcribed from the 14q32 locus of the human genome may be characterized as a human 14q32 miRNA, while a particular miRNA that is transcribed from the CFA 8 locus of the canine genome may be characterized as a canine 14q32 miRNA.

Osteosarcoma is a common primary bone tumor, often affecting children and adolescents (Mirabello et al., Cancer 2009, 115:1531-1543; Kansara et al., DNA Cell Biol 2007, 26:1-18). The molecular understanding of osteosarcoma has progressed slowly compared to other cancer types due, at least in part, to its infrequent occurrence and/or the

heterogeneous nature of osteosarcoma in humans. The occurrence of an orthologous disease in dogs, however, provides an opportunity to surmount some of these challenges.

Osteosarcoma is more common in dogs (estimated at >10,000 cases per year), with exquisite breed predilection. Osteosarcoma arises spontaneously in dogs and its biology is similar to the human disease both anatomically and functionally (Khanna et al., Nat Biotechnol 2006, 24:1065-1066). The chronology of osteosarcoma is adapted to lifetime, so progression of osteosarcoma in dogs takes place in a time frame (~2 years) that allows rapid assessment of risk, prediction, and outcome (Khanna et al., Nat Biotechnol 2006, 24:1065-1066). The availability of detailed genome maps for humans and dogs, and the extensive homology in sequence and gene order between both species makes integration and extrapolation of genomic data feasible (Lindblad-Toh et al., Nature 2005, 438:803-819; Shearin et al., Dis Model Mech 2010, 3:27-34).

We have previously identified sets of differentially expressed genes that are associated with tumor behavior in both canine and human osteosarcoma (Scott et al., Bone 2011, 49:356-367). Specifically, we characterized gene signatures that can stratify canine and human osteosarcoma according to the predicted biological behavior of the tumors (Scott et al., Bone 2011, 49:356-367). The signatures in dogs with the worst outcomes are characterized by increased cell cycle gene expression that regulates G2/M transition and DNA damage genes. In contrast, the signatures that are typified in dogs with better outcomes include increased expression of "microenvironment interactions" genes. These patterns also were observed in human osteosarcoma tumor datasets, but the mechanisms responsible for generating these signatures have not yet been fully defined.

Over 40 miRNAs are present between the imprinted genes *DLK1* and *DIO3* at the human 14q32 locus (Seitz et al., Genome Res 2004, 14:1741-1748). We generated a correlation-based network using miRNA expression levels determined by array analyses and observed decreases in expression levels of 14q32 miRNA that are highly correlated in each of the individual tumor tissue samples. The analysis further revealed that expression levels between various of the miRNAs found at the 14q32 locus were highly correlated (FIG. 1A; Table 8). The high correlation in expression levels between 14q32 miRNAs suggests that an individual miRNA from this locus could represent the expression levels of most of 14q32 miRNAs in osteosarcoma.

To demonstrate that the high level of correlation observed for 14q32 miRNAs was derived from meaningful decreases in expression levels, we plotted the expression levels of the 14q32 miRNA members in human osteosarcoma relative to the human osteosarcoma primary tumor sample FT-14. Several of the osteosarcoma tumor samples showed 14q32 miRNA levels similar to those observed in FT-14 while several of the other osteosarcoma tumors showed much more sizable decreases in 14q32 miRNAs. The magnitude of miRNA expression decrease ranged from approximately 3-fold to approximately 20-fold relative to the levels observed in normal bone tissue by miRNA microarray analyses (Sarver et al., Laboratory Invest 2010, 90:753-761; Thayanithy et al., Bone 2012, 50:171-181). The heterogeneity in expression levels was seen in 14q32 locus miRNAs, and is specifically shown for two members at this locus: miR-382 and miR-154 (FIG. 1C). The expression levels observed for miR-382 and miR-154 showed a high level of correlation (R^2= 0.95), exemplary of the high correlations observed across the 14q32 miRNAs. In order to confirm that the results obtained by miRNA microarray for the magnitude of the 14q32 miRNA decreases were meaningful, we carried out qRT-PCR for miR-382 and miR-154. The results show that, relative to normal bone, osteosarcoma samples FT-14 and FT-7 exhibited an approximately 3-fold decrease while osteosarcoma samples FT-13, FT-18, and FT-12 showed an approximately 10-fold to approximately 20-fold decrease in the expression levels of these two 14q32 miRNAs (FIG. 1D). The qRT-PCR results for 14q32 miRNA correlation also showed high correlation (R^2= 0.99) similar to the correlation observed by microarray (R^2=0.95).

To further understand the role of the 14q32 miRNAs in human osteosarcoma, we profiled the mRNA transcript levels of tumor samples for which we also had miRNA profiles. We then identified mRNA that positively and negatively correlated to miR-382. Since all of the tested 14q32 miRNAs expression levels were highly correlated on a sample-by-sample basis, we selected miR-382 as a representative of 14q32 miRNAs. The results of this analysis in human osteosarcoma samples uncovered 288 genes that were positively (see Table 6) correlated to miR-382 expression and 97 genes that were negatively (see Table 7) correlated to miR-382 expression ($R^2 > 0.7$). All of these 385 genes and their direction of change in relation to miR-382 are shown as a heatmap in FIG. 2A. Ingenuity Pathway Analysis (IPA) (accessed April 14, 2012) showed that genes involved in the functional category "metastasis" were enriched in this signature (Enrichment p-value < 0.001 after Benjamini Hochberg

multiple testing correction). Furthermore, genes of this signature in the functional category "metastasis" were predicted to have increased activity (Regulation Z-score 2.123) in osteosarcoma tumor samples that showed the lowest levels of 14q32 miRNAs on the basis of the direction of change of the correlated genes. This provides a potential molecular rationale for the observation of metastasis being present in the samples with the largest decreases in 14q32 miRNA transcript levels.

As a next step in comparative assessment, we mapped the human osteosarcoma genes that positively and negatively correlated with miR-382 (FIG. 2A, direction of change marked in lighter and darker greys) to orthologous canine genes that were included in the Affymetrix canine_2.0 platform (FIG. 2B). Mapping of the human to canine genes provided 219 canine genes, which we then used to perform unsupervised hierarchical clustering of the 26 canine osteosarcoma samples. These 219 genes segregated the canine osteosarcoma samples into two distinct branches. Kaplan-Meier analysis using death as an outcome revealed a statistically significant ($p < 0.05$) difference in survival between dogs in these two distinct branches (FIG. 2C). Dogs in which miR-382-positively correlated mRNAs were expressed at lower levels and in which miR-382-negatively correlated mRNAs were expressed at higher levels had significantly shorter survival times than the other group of dogs. This result shows that genes that correlate with miR-382 are capable of showing survival trends in dogs with osteosarcoma that are consistent with our analysis in humans, thus independently supporting a prognostic role for the 14q32 miRNA transcript level in osteosarcoma.

Because metastasis genes are enriched in miR-382-correlated gene signatures in canine osteosarcoma and miR-382 expression levels showed potential prognostic significance (e.g., ability to predict poor outcome) in the canine osteosarcoma cohort, we tested whether expression levels of 14q32 miRNAs in human showed similar association with outcomes. We analyzed the miRNA profiles obtained from the initial cohort of human osteosarcoma tumor tissue samples. Analysis revealed an inverse relationship: the osteosarcoma samples with the lowest levels of 14q32 miRNAs were from patients that developed metastatic tumors (FIG. 1B-D and 2A). This human osteosarcoma sample cohort was incompletely annotated (no survival data available), but a Mann Whitney test showed that a potential association was present between levels of 14q32 expression levels and metastasis ($p = 0.1357$).

To further examine the association between low 14q32 miRNA levels in osteosarcoma tumors and clinical outcomes, we tested a representative 14q32 miRNA, miR-382, in an independent set of primary tumor samples from 16 human osteosarcoma patients that had more robust clinical follow-up annotation (see Table 4). As determined by qRT-PCR, all of the osteosarcoma tumor tissue samples in this independent set showed lower levels of miR-382 compared to normal bone tissues (FIG. 3A). As we noticed in the first human osteosarcoma dataset, the lowest levels of miR-382 were found in primary osteosarcoma tumors from patients where metastases and/or death were later observed as follow-up. We further noticed that there was a negative correlation between the levels of miR-382 expression and patient survival (FIG. 3A; Table 4). To test whether lower levels of 14q32 were significantly associated with metastasis, we used the Mann Whitney test. This test allowed us to reject the null hypothesis that there was no association between 14q32 level and metastasis ($p < 0.01$).

Further, to examine the prognostic utility of the 14q32 miRNA level, we split the cohort into two groups based on whether the miR-382 level was above the median value (Group 1) or below the median value (Group 2). We compared the two groups using Kaplan-Meier analyses using both metastasis and death as outcomes (FIG. 3B and 3C). Human osteosarcoma tumor samples that had the lowest levels of miR-382 were at higher risk for metastasis ($p = 0.01$) and death ($p = 0.08$) than patients with moderate miR-382 expression independently confirming the observation that 14q32 miRNAs are a potential prognostic tool for osteosarcoma.

We examined the orthologous 14q32 locus miRNAs and genes within the canine genome using the Entrez gene database and were able to find them on CFA 8 (FIG. 5). Similar to human 14q32 locus, canine orthologous miRNAs were also found in the syntenic region between genes *DLK1* and *DIO3*. The order of the miRNA is retained in the canine genome although differences in spacing were observed between human and canine versions. The orthologous canine miRNAs on CFA 8 can, in a manner similar to that observed for 14q32 miRNAs in humans, show decreases in transcript level in canine osteosarcoma tumors. Moreover, the magnitude of these decrease in expression can provide prognostic utility in canines. For example,, we determined the levels of miR-544 and miR-134 (based on identical mature miRNA sequences) in 16 canine osteosarcoma tumors from which RNA was available

by qRT-PCR (FIG. 4A). In all 16 cases, expression decreases were observed in the miRNA relative to reactive canine osteoblasts. As observed in human osteosarcoma, the levels of miR-544 and miR-134 were directly correlated (R^2= 0.88)

The canine osteosarcoma samples that were analyzed by qRT-PCR were derived from dogs with overall survival times ranging from two to 606 days with a median survival of 131.5 days (Table 5). The canine cohort was separated into two groups using the same method used for the human tumor samples. Group 1 was composed of tumor samples with miR-134 (FIG. 4A) and miR-544 (FIG. 4B) expression levels above the median survival days and Group 2 was composed of tumor samples below the median, and these two groups were interrogated via Kaplan-Meier survival analysis (FIG. 4D). Dogs with tumors with more severely reduced levels of miR-134 had higher risk for death (p= 0.004) than dogs with tumors with more moderate decreases in miR-134 expression (FIG. 4C). Similar results were obtained using miR-544 expression levels (p= 0.012, FIG. 4D). Together, these results indicate that the 14q32 miRNA expression levels have prognostic utility in osteosarcoma, a disease that shows conserved features between humans and dogs.

Molecular markers to identify osteosarcoma patients at risk for metastatic progression are important for guiding decisions regarding suitable treatment options. Recently, several molecular markers based on gene expression in tumor tissues have been identified in osteosarcoma. Higher levels of oncogene YY1 expression in primary sites of osteosarcoma are associated with metastasis and poor clinical outcomes (de Nigris et al., BMC Cancer 2011, 11:472). Similarly, overexpression of markers such as cancer-testis antigens, Cystein Rich Protein (CYR61) and Melanoma Antigen Family A (MAGEA) are implicated in predicting tumor progression and prognosis in osteosarcoma (Zou et al., Cancer 2012, 118:1845–1855, Sabile et al., J Bone Mineral Res 2012, 27:58-67). Additionally, the presence of tumor-infiltrating macrophages is associated with metastasis suppression in high-grade osteosarcoma (Buddingh et al., Clinical Cancer Res 2011, 17:2110-2119). These studies show that the development of biomarkers for tumor progression and outcomes have clinical significance and may help develop patient specific treatment strategies.

The cluster of miRNAs at the 14q32 locus show decreased levels of expression in human osteosarcoma (Thayanithy et al., Bone 2012, 50:171-181). Here, we report decreases in miRNA in the orthologous region of CFA 8 cluster members in canine osteosarcoma

tumors. This is consistent with the presence of a commonly conserved mechanism of tumorigenesis involving the orthologous 14q32 locus associated genes and miRNAs between these species. Thus, the cross species comparison of human and canine expression profiles provides an opportunity to define the existence of evolutionarily conserved molecular signatures and to determine whether those signatures influence the outcome of our three patient cohorts (two human and one canine).

In both the human and canine osteosarcoma cases, the human 14q32 miRNAs and its canine orthologs appear to be regulated by a common mechanism, based on the high levels of expression correlation observed between the transcript levels of each member. Analyses of the SMED data reveal that 14q32 miRNA, as a group, are lost in a subset of GIST (3/14) and MFH (5/29). We conclude from this that the level of 14q32 miRNAs in general can be estimated from the measurement of a single member of the family and that measurement of the majority of the 14q32 miRNAs would provide highly similar data. Polycistronic regulation of these miRNAs has previously been proposed as a regulatory mechanism active at this locus and our data supports this concept (Seitz et al., Genome Res 2004, 14:1741-1748).

In an initial set of human osteosarcoma tumors miRNA microarray profiling data we observed that lower levels of 14q32 miRNA were associated with metastases. We further validated this by qRT-PCR for both miR-382 and miR-154 for osteosarcoma tumors from this initial dataset. We also showed that the lowest levels of miR-382 were significantly associated with metastasis and significantly worse survival in a second independent set of human primary osteosarcoma tumors where full clinical follow-up information was available. Taken together, these results suggest that the level of miR-382 may have prognostic utility for predicting the likelihood of developing metastatic tumors and disease outcomes. This observation is supported by three independent dataset from two different species. The association between reduced expression of 14q32 miRNAs and overall survival in humans represented a trend. In dogs, however, the magnitudes of miR-134 and miR-154 reduction were negative predictors of survival. This may be due, at least in part, to companion animal patients being treated with quality of life as the guiding principle. Therefore, metastatic disease is seldom treated aggressively and recurrence often leads to a decision of humane

euthanasia, creating little difference between the disease-free interval and overall survival (Modiano et al., Molecular Therapy 2012, 20:2234-43).

Decreases in 14q32 miRNA levels stabilize cMYC expression in osteosarcoma and subsequently increase the expression of oncogenic miR-17-92 miRNAs (Thayanithy et al., Bone 2012, 50:171-181). Deregulations in 14q32 miRNA-cMYC network are also associated with increased proliferation and apoptotic escape in osteosarcoma (Thayanithy et al., Bone 2012, 50:171-181). The prognostic relevance of decreasing 14q32 miRNA levels may be explained 1) by increased activation of the cMYC/miR-17-92 network leading to poor outcomes 2) via regulation of an alternative cancer signaling pathway(s) either directly or indirectly or 3) as a biomarker whereby decreased levels of 14q32 miRNAs are observed in osteosarcoma cases with poor outcomes.

Genes involved in metastasis are significantly enriched in transcripts correlated to 14q32 miRNAs and the direction of change indicates that metastasis function is also significantly increased (see Tables 6 and 7). CDK5 and TWIST1 can increase metastasis (Feldmann et al., Cancer Res 2010, 70:4460-4469; Reis et al., Oncogene 2004, 23:6684-6692) and are upregulated in osteosarcoma tumors with low levels of 14q32 miRNAs. IFNB1 (Kornbluth et al., J Leukocyte Biol 2006, 80:1084-1102), TEK (Lin et al., Proc Natl Acad Sci USA 1998, 95:8829-8834) and COL18A1 (Brideau et al., Cancer Res 2007, 67:11528-11535; Mendoza et al., Cancer Res 2004, 64:304-310) can decrease metastasis and are downregulated in tumors with low levels of 14q32 miRNAs. *TK1* can be negatively correlated to 14q32 miRNA levels, is involved in the catalysis of phosphorylation of thymidine to deoxythymidine monophosphate, expressed at high levels in proliferating cells, and appears to correlate with high risk in multiple cancer types (Konoplev et al., Am J Clin Pathol 2010, 134:472-477; Xu et al., Tumour Biol 2012, 33:475-483; He et al., Nucleosides, Nucleotides & Nucleic Acids 2010, 29:352-358). This suggests that genes correlated with 14q32 miRNA expression patterns may provide a molecular rational for the observed association between low levels of 14q32 and metastasis and poor survival outcomes and can be further investigated as markers of prognosis.

We have previously demonstrated that comparative studies of human and canine osteosarcoma patients allow study of evolutionary conserved mechanisms of tumorigenesis associated with outcomes (Scott et al., Bone 2011, 49:356-367). A majority of the dogs

evaluated in this study (21/25 in the correlating miRNA analysis and 12/16 in the qRT-PCR analysis) were treated with standard of care, which consists of amputation of the limb to remove the primary tumor followed by with adjuvant chemotherapy. Predictably, treatment generally was associated with increased survival, but the negative correlation with miR-134 and miR-154 was not driven by the type of treatment that the dogs received, and in fact, the data suggest that the dogs that received palliative care and amputation (2, 8 and 58 days survival) would have been reasonable candidates for more prolonged survival with standard of care treatment.

We have therefore identified and confirmed an association between metastasis and 14q32 miRNA expression levels in human osteosarcoma. Further, we have shown that the transcript reductions and prognostic significance for 14q32 miRNAs are conserved in canine osteosarcoma. Based on these integrative, comparative, multi-species analyses, we conclude that larger decreases in 14q32 miRNA expression levels are associated with an increased likelihood of metastases and poor outcomes in osteosarcoma patients.

Thus, this disclosure provides a method that generally includes analyzing a tissue sample from a subject for expression of a 14q32 miRNA. The method can be used as a diagnostic and/or prognostic tool. As a diagnostic tool, the method can inform one whether the subject from which the analyzed sample is obtained has or is at risk of having osteosarcoma. Generally, if the tissue exhibits reduced expression of the 14q32 miRNA compared to a normal control, then the subject has or is at risk of having osteosarcoma.

As a prognostic tool, the method can be used to distinguish, to a clinically relevant extent, whether the osteosarcoma is an aggressive osteosarcoma—i.e., one that exhibits one or more aggressive behaviors such as, for example, metastasis, which can result in shorter survival times—or a nonaggressive osteosarcoma. Generally, if the expression of the 14q32 miRNA is no more than a predetermined percentage of 14q32 miRNA expression compared to a normal control, then the subject has or is at risk of having an aggressive osteosarcoma. With such a prognosis, an appropriate course of treatment can be implemented. Thus, for example, the method can further include treating a subject with treatment appropriate for an aggressive osteosarcoma if the subject exhibits expression of an 14q32 miRNA that is no more than the predetermined percentage of 14q32 miRNA expression indicative of being at risk for aggressive osteosarcoma. As another example, the method can further include treating

a subject with treatment appropriate for a nonaggressive osteosarcoma if the subject exhibits expression of an 14q32 miRNA that is greater than the predetermined percentage of 14q32 miRNA expression indicative of an aggressive osteosarcoma and is less than the predetermined 14q32 miRNA expression level of a normal control.

The standard of care aggressive and nonaggressive osteosarcoma are presently similar because practitioners are not currently able to distinguish between aggressive and nonaggressive osteosarcomas. In humans, the standard of care typically includes 10 weeks of pre-operative chemotherapy followed by surgery that preserves the limb and 20 weeks of post-operative chemotherapy. Conventional chemotherapeutic agents including, for example, doxorubicin and cisplatin are currently being used in this treatment. These drugs, along with methotrexate, ifosfamide, or in combination with leucovorin, are often used to treat osteosarcoma in young adults. Percent necrosis in the tumor is used as a predictor for response, but the intensity of therapy is not adjusted based on this biomarker. Moreover, the predictive value of the tumor necrosis response can be as low as 50%. Consequently, practitioners do not have a sufficiently reliable indicator that a particular osteosarcoma is nonaggressive that may allow the practitioner to confidently moderate treatment. Instead, practitioners are reluctant to moderate treatment and will, instead, prescribe treatment that is consistent with the osteosarcoma being aggressive.

In dogs, the standard of practice is amputation of the limb, or in those cases where it is indicated, limb sparing surgery to remove the primary tumor followed by adjuvant chemotherapy. Pre-operative chemotherapy is not generally used in dogs. Post-operative chemotherapy generally is restricted to use of a single drug, with the most common agents used being doxorubicin and carboplatin. Recent studies also have investigated the use of these two drugs in combination with mixed results. There are no tests currently available to predict response to therapy in canine osteosarcoma, so all dogs receive the same intensity of chemotherapy irrespective of potential differences in tumor behavior.

As a prognostic tool, therefore, methods provided herein can allow one to better tune a patient's treatment for osteosarcoma based on the type of osteosarcoma affecting the patient. For example, the methods provided herein can allow a practitioner to distinguish between aggressive and nonaggressive osteosarcomas with greater confidence that is possible using existing methods. This can permit a practitioner to moderate treatment for patients with

nonaggressive osteosarcomas. The moderated treatment may involve prescribing smaller doses of chemotherapeutic drug and/or less frequent chemotherapy, each of which can reduce undesirable side effects of the chemotherapy. Alternatively, the moderated treatment can involve the use of alternative chemotherapeutic agents that may be effective for reducing nonaggressive osteosarcomas but are considered ineffective for reducing aggressive osteosarcomas.

As used herein, the term "expression" and variations thereof refer to the transcription of a RNA (e.g., a microRNA (imRNA) from DNA. Also as used herein, the term "at risk" refers to a subject that may or may not actually possess the described risk. Thus, for example, a subject "at risk" of a neoplastic condition is a subject that exhibits one or more risk factors associated with the condition—e.g., miRNA expression, genetic predisposition, ancestry, age, sex, geographical location, lifestyle, or medical history—even if the subject does not exhibit any clinical sign or symptom of the neoplastic condition.

In some cases, the decrease in 14q32 miRNA expression compared to a normal control can be expressed in terms of a predetermined percentage of 14q32 miRNA expression compared to the normal control. In certain embodiments, 14q32 miRNA expression that indicates a subject that has or is at risk of having osteosarcoma can include 14q32 miRNA expression that is a maximum of no more than 90% of a normal control such as, for example, no more than 80%, no more than 70%, no more than 60%, no more than 50%, no more than 40%, no more than 35%, no more than 30%, no more than 25%, no more than 20%, no more than 15%, no more than 14%, no more than 13%, no more than 12%, no more than 11%, no more than 10%, no more than 9%, no more than 8%, no more than 7%, no more than 6%, no more than 5%, no more than 4%, no more than 3%, or no more than 2% of a normal control. In certain embodiments, 14q32 miRNA expression that indicates a subject that has or is at risk of having osteosarcoma can include, while still exhibiting a decrease compared to a normal control, 14q32 miRNA expression that is a minimum of at least 1% of 14q32 miRNA expression in the normal control such as, for example, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 16%, at least 17%, at least 18%, at least 19%, or at least 20% of the normal control. The 14q32 miRNA expression that indicates that the subject has or is at risk of having an osteosarcoma also can be characterized by any range

that includes, as endpoints, any combination of a minimum 14q32 miRNA expression identified above and any maximum 14q32 miRNA expression identified that is greater than the minimum 14q32 miRNA expression.

In one embodiment, therefore, 14q32 miRNA expression indicative of a subject having or at risk of having an osteosarcoma can be, for example, no more than 40% of a normal control sample.

In another embodiment, 14q32 miRNA expression indicative of a subject having or at risk of having an aggressive osteosarcoma can be, for example, no more than 10% of a normal control sample.

As used herein, "aggressive osteosarcoma" refers to a form of osteosarcoma that exhibits one or more characteristics that are customarily associated with shorter survival times compared to other forms of osteosarcoma. Exemplary characteristics include, for example, metastasis, elevated expression of pro-survival and/or metastatic genes (e.g., *TWIST1*, *cMYC*, and *RUNX2*), and/or decreased expression of tumor suppression genes (e.g., *PTEN*, *FasR*, *ST5*, *ST7*, *ST14*, and *YPEL3*).

As used herein, "nonaggressive osteosarcoma" refers to a form of osteosarcoma that does not exhibit aggressive behaviors (e.g., indolent).

In some embodiments, the 14q32 miRNA can include one or more of miR-382, miR-134, miR-544 miR-379, miR-1197, miR-329-1, miR-494, miR-495, miR-376C, miR-1185-1, miR-1185-2, miR-381, miR-655, miR-487A, miR-496, miR-154, miR-369, miR-127, miR-299, miR-665, miR-770, miR-432, miR-668, miR-539, miR-433, miR-323, miR-496, miR-654, miR-409, miR-380, miR-485, miR-136, miR-376, miR-431, miR-370, miR-889, miR-381, miR-299, miR-379, miR-411, miR-655, miR-432, miR-487, miR-410, miR-493, miR-656, miR-485, miR-377 or miR-541.

Expression of the 14q32 miRNA may be determined using any suitable methods. Conventional methods for measuring miRNA expression include, for example, qRT-PCR, microarrays, and RNA sequencing. In one particular embodiment, the miRNA expression is measured using qRT-PCR. In another particular embodiment, the miRNA expression is measured using a microarray. In another particular embodiment, the miRNA expression is measured using RNA sequencing.

In the preceding description, particular embodiments may be described in isolation for clarity. Unless otherwise expressly specified that the features of a particular embodiment are incompatible with the features of another embodiment, certain embodiments can include a combination of compatible features described herein in connection with one or more embodiments.

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

The term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements; the terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims; unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one; and the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Human osteosarcoma tissue samples.

Human tissues were obtained from two sources. Frozen osteosarcoma tissue samples and normal bone samples (femur/ tibia) of individuals from similar age groups were obtained through the tissue procurement facility at the University of Minnesota (Bionet). Demographic and clinical information is provided in Tables 2 and 3. The excised primary osteosarcoma tumors were obtained prior to the initiation of chemotherapy or radiotherapy with informed consent and following institutional review board-approved protocols. Surgical resections were subjected to standard clinical and histopathological evaluation of hematoxylin and eosin (H&E) stained sections. An additional 16 human osteosarcoma samples with complete clinical follow-up information, including outcomes, were obtained from Pancras C.W. Hogendoorn with approval from the institutional review board in compliance with the

declaration of Helsinki. Demographic and clinical information is provided for these samples in Table 4.

Table 3. Demographic and clinical information of normal bone samples used in the study.

FT	Age	Race	Gender	Source
FT-401	20	Caucasian	F	Femur
FT-402	18	Caucasian	F	Femur
FT-403	15	Caucasian	M	Tibia
FT-404	6	Caucasian	F	Femur
FT-405	11	Caucasian	F	Tibia
FT-406	21	Caucasian	M	Femur

Table 2. Demographic and clinical information of human OS patient samples.

FT #	Diagnosis	Age	Sex	Race	Tumor location	Metastatic status	Additional comments
FT-1	osteosarcoma	16	M	white	right tibia	no	conventional type, high grade
FT-2	osteosarcoma	16	F	white	Mediastinum (bronchus)	Yes	aortic dissenction, margins negative
FT-3	osteosarcoma	10	M	hispanic	right dital femur	no	conventional type, high grade
FT-4	osteosarcoma	64	M	white	soft tissue of neck	no	patient diseased due to metastasis in brain
FT-5	osteosarcoma	40	M	white	tibia	no	high grade OS in the proximal tibia
FT-6	osteosarcoma	17	M	white	tibia	no	conventional type, high grade
FT-7	osteosarcoma	16	M	black	femur	no	conventional type in distal femur
FT-8	osteosarcoma	12	F	white	left distal femur	no	conventional type, high grade
FT-9	osteosarcoma	19	M	white	left lung	yes	conventional type, high grade
FT-10	osteosarcoma	19	M	white	left lung	yes	conventional type, high grade
FT-11	osteosarcoma	25	F	white	right knee	no	soft tissue of the knee, recurrent
FT-12	osteosarcoma	57	M	white	right lung, lower lobe	yes	NA
FT-13	osteosarcoma	24	M	white	right chest wall	yes	NA
FT-14	osteosarcoma	14	M	white	left distal femur	no	NA
FT-15	osteosarcoma	17	M	white	left distal femur	no	high grade OS
FT-16	osteosarcoma	22	M	white	left lung, lower lobe	Yes	metastatic OS extending to pleural surface
FT-17	osteosarcoma	31	F	white	left lung, upper lobe	yes	vimentin+, actin+
FT-18	osteosarcoma	42	M	white	right forearm	no	extraskeletal OS with focal telangiectatic feautures
FT-19	osteosarcoma	31	F	American Indian	right lung, upper lobe	yes	osteoclasts present
FT-336	osteosarcoma	20	M	white	Right pericardial mediastinal mass	yes	NA
FT-337	osteosarcoma	24	M	white	Left distal femur	no	NA
FT-338	osteosarcoma	20	M	white	Right chest wall	yes	NA
FT-339	osteosarcoma	24	M	white	Tumor, left femur	no	high grade, left femur
FT-340	osteosarcoma	20	M	white	Right middle lobe lung	yes	NA

Table 4. Human OS patient sample information and clinical follow-up.

PA-number	Age*	Age years	Sex	Subtype	Tumour Location	Huvos grade	Metastasis	ToM	Deceased	ToD	ToF	pre-operative chemotherapy
H90-02350	200	16.7	M	Telangiectatic	distal femur right	1	yes	0	yes	25	25	PIA
H92-10246	37	3.1	F	Osteoblastic	proximal tibia left	4	no		no		194	PIA
H96-04177	205	17.1	F	Osteoblastic	distal femur left	1	no		no		147	AP
H96-10281	304	25.3	M	Osteoblastic	proximal tibia left	2	yes	27	yes	47	47	AP
H98-13491	200	16.7	M	Osteoblastic	proximal humerus left	1	yes	0	yes	11	11	unknown
H00-03732	198	16.5	M	Osteoblastic	proximal tibia left	3	yes	10	yes	33	33	AP
H01-04259	244	20.3	F	Anaplastic	distal femur right	3	no		no		94	AP
H02-00070	223	18.6	M	Chondroblastic	proximal tibia left	3	yes	21	yes	39	39	AP
H02-01830	137	11.4	F	Osteoblastic	proximal tibia left	2	no		no		77	AP
H05-09073	181	15.1	M	Osteoblastic	proximal tibia right	3	no		no		40	MAP
H06-06362	181	15.1	F	Osteoblastic	proximal tibia left	4	no		no		32	MAP
H06-07381	96	8.0	M	Osteoblastic	distal femur left	2	yes	0	no		31	MAP
H07-01741	103	8.6	M	Unknown	distal femur right	3	yes	2	no		31	MAP
H07-04382	164	13.7	M	Osteoblastic, some giant cells	distal femur right	3	no		no		24	MAP
H07-08899	165	13.8	F	Osteoblastic	distal femur right	3	no		no		24	MAP
H08-02814	114	9.5	F	Osteoblastic, some giant cells	proximal humerus left	3	no		no		17	MAP

*Age in months

ToM	time of metastasis (months)
ToD	time of death (months)
ToF	time of follow up (months)
PIA	Platinum, Ifosfamide, and Adriamycin
AP	Adriamycin and Platinum
MAP	Methotrexate, Adriamycin, and Platinum

Canine osteosarcoma tissue samples and cell lines.

The procedures to obtain canine osteosarcoma samples and establish corresponding explants and canine cell lines have been described previously (Scott et al., Bone 2011, 49:356-367; Thomas et al., Genome Res 2005, 15:1831-1837). Briefly, naïve (prior to treatment) samples were obtained from family-owned pet dogs with stage I or stage II appendicular osteosarcoma. Twenty-three of the 37 canine osteosarcoma samples used in this study were derived from the primary tumor in the leg. Samples OSCA-6 (Osteosarcoma Cell ACCR-6), OSCA-11, and OSCA-16 were derived from pulmonary metastases. Participation in the study required the dogs' owners to sign an informed consent form indicating they understood the goals and procedures for this study. Protocols and procedures were reviewed by the appropriate Institutional Animal Care and Use Committee. Samples were collected by attending veterinarians under sterile conditions as part of a diagnostic biopsy procedure, immediately after limb amputation, or at necropsy. Grossly visible tumor was dissected from adjacent normal tissue, rinsed in sterile phosphate buffered saline solution and divided into three portions that were (1) fixed in 10% neutral buffered formalin, (2) snap frozen, and (3) disaggregated into single cell suspensions. Histologically, the cellular composition of the resulting trimmed samples consisted of >90% tumor cells. For the single cell suspensions, debris was removed by passing sequentially through 500- and 200-μm mesh filters. Cells were then placed in a 4-cm^2 culture dish at a density of 1 x 10^6/ml in 1 ml of DMEM supplemented with 10% fetal bovine serum and cultured at 37°C in a 5% CO_2 atmosphere. Cells were allowed to reach ~80% confluence, at which time they were passed to a 10-cm^2 culture dish, and then again to a 25-cm^2 culture flask. By the second passage, the cultures were homogeneous and consisted of large polygonal to plump spindloyd or slightly rounded cells. Histologic diagnosis of osteosarcoma was verified for all tumors from formalin-fixed tissues. Osteoblastic origin of the cultured cells was confirmed at the third passage by expression of alkaline phosphatase and osteocalcin. As needed, cells were maintained in culture using the same conditions described above. Selected cell lines were tested after 30 passages in culture and had no noticeable changes in morphology or loss of alkaline phosphatase expression. These cell lines are tumorigenic in vivo, and, when injected orthotopically into nude mice, they have the capacity to metastasize spontaneously to the

lungs (Wolfe et al., Clinical Exp Metastasis 2011, 28:377-389). Demographic and clinical information for the canine samples is provided in Table 5.

Table 5. Clinical information of canine OS cases.

Case ID	Breed	Gender	Age at Diagnosis	Morphology	Treatment Type	Survival (mo)	Experiment
OSCA-1	Rottweiler	F	9	Osteoblastic	PALL	0.99	GEP
OSCA-04	Rottweiler	F	9.91	Osteoblastic	PALL	0.26	Q
OSCA-6	Rhodesian Ridgeback	F	2.4	Osteoblastic	PALL	0.36	GEP
OSCA-8	Rottweiler	M	2	Osteoblastic	NA	LTF	GEP
OSCA-11	Rottweiler	M	5	Chondroblastic	NA	LTF	GEP
OSCA-12	Rottweiler	MC	7	Osteoblastic	NA	1	GEP
OSCA-16	Rottweiler	M	6.3	Osteoblastic	SOC	1.97	GEP
OSCA-17	Rottweiler	M	8.9	Fibroblastic	SOC	64.01	GEP
OSCA-19	Rottweiler	MC	8	Chondroblastic	SOC	10.52	GEP
OSCA-20	Golden Retriever	MC	7	Osteoblastic	SOC	1.71	GEP
OSCA-21	Rottweiler	MC	11.2	Fibroblastic	SOC	13.12	GEP
OSCA-22	Rottweiler	MC	9.1	Osteoblastic	AMP	0.23	GEP
OSCA-23	Golden Retriever	MC	8.9	Osteoblastic	SOC	13.97	GEP
OSCA-25	Rottweiler	FS	7.1	Fibroblastic	SOC	4.02	GEP
OSCA-29	Rottweiler	F	6.8	Osteoblastic	SOC	2.33	GEP/Q
OSCA-30	German Shepherd	MC	8	Osteoblastic	SOC	20.61	GEP/Q
OSCA-32	Great Pyrenees	F	8.7	Fibroblastic	SOC	11.01	GEP
OSCA-33	Rottweiler	FS	12.08	Osteoblastic	SOC	13.55	Q
OSCA-40	Saint Bernard	FS	5.8	Osteoblastic	SOC	1.18	GEP
OSCA-41*	Alaskan Malamute	M		Osteomyelitis (no tumor)	AMP	>36	GEP/Q
OSCA-45	Irish Setter	FS	11	Osteoblastic	AMP	5.16	Q
OSCA-50	Rottweiler	M	3.7	Osteoblastic	PALL	0	GEP
OSCA-55	Borzoi	F	11.91	Osteoblastic	NA	1.87	Q
OSCA-56	Rottweiler	M	Unknown	Osteoblastic	AMP	LTF	GEP
OSCA-57	Golden Retriever	M	7.8	Osteoblastic	SOC	7.3	GEP/Q
OSCA-59	Golden Retriever	FS	9.4	Chondroblastic	SOC	38.96	GEP
OSCA-60	Mastiff	F	6	Osteoblastic	SOC	4.96	GEP
OSCA-67	Mastiff	FS	12.27	Mixed	PALL	0.07	Q
OSCA-71**	Golden Retriever	FS	6.7	Osteoblastic	SOC	16	GEP
OSCA-73	Golden Retriever	FS	2.5	Osteoblastic	PALL	1.48	GEP
OSCA-75	Golden Retriever	FS	9.8	Osteoblastic	SOC	7.66	GEP/Q
OSCA-77	Belgian	MC	6.1	Osteoblastic	SOC	4	Q
OSCA-78	German Shepherd	M	9.5	Mixed Osteoblastic-fibroblastic	SOC	2.5	GEP/Q
OSCA- 84	Rottweiler	FS	10	NA	SOC	6.48	Q
OSCA- 96	Mastiff	FS	5.17	NA	SOC	11	Q

OSCA- 98	Labrador Retriever	MC	11	Osteoblastic	SOC	4.38	Q
OSCA- 99	Labrador Retriever Mix	FS	10	Osteoblastic	SOC	4.35	Q
OSCA- 109	Irish Setter	M	10.08	Osteoblastic	SOC	1.58	Q

OSCA = Osteosarcoma Cell line Animal Cancer Care and Research Program
NA = information not available in the record
LTF = lost to follow-up
*Final diagnosis was osteomyelitis
**Dog was alive at end of study
canine OS samples used for qRT-PCR and survival analysis are highlighted

RNA isolation.

Total RNA was isolated from 75-100 mg of frozen human osteosarcoma tissue using the miRvana total RNA isolation kit (Ambion Inc, Austin TX, USA) following the manufacturer's protocol. RNA was quantified using the Nanodrop 8000 (NanoDrop Technologies LLC, Wilmington, DE, USA). Samples with RNA Index Number (RIN) values of >6 were included in this study. Total RNA was isolated from canine osteosarcoma tissue using TriPure Isolation Reagent (Roche, Mannheim, Germany). The concentration of RNA from canine osteosarcoma tissue was determined using NanoDrop 1000 UV-Vis spectrophotometer (NanoDrop Technologies LLC, Wilmington, DE, USA) and quality was assessed on a 1.2% formaldehyde agarose gel with ethidium bromide staining. RNA from canine osteosarcoma cryopreserved cells was isolated, quantified, and assessed for quality as previously described (Scott et al., Bone 2011, 49:356-367).

Analysis of osteosarcoma mRNA and miRNA expression.

Messenger RNA expression profiles from human tumors were generated using the human HT-12 Beadchip (Illumina Inc., San Diego, CA, USA) (Fan et al., Genome Res 2004, 14:878-885). Human miRNA expression profile datasets described previously (S-MED; Sarver et al., Laboratory Invest 2010, 90:753-761) were used in this study. Additional clinical information relating to these samples is described in Table 2 (human). Canine osteosarcoma mRNA gene expression data generated on Affymetrix canine_2.0 arrays were also used as previously described (Scott et al., Bone 2011, 49:356-367). Complete clinical and breed information for canine osteosarcoma data is provided in Table 5. miRNAs from the 14q32 cluster were mapped to the canine genome. Two miRNAs (miR-134 and miR-544) that showed 100% conservation and mapped to the predicted region of synteny in canine

chromosome (CFA) 8 were used to examine expression of the 14q32 cluster in dog samples (FIG. 5).

Table 6. Differentially expressed mRNA in osteosarcoma positively correlated to 14q32 encoded miR-382.

Gene Name	Correlation to MiR-382 expression level	Gene Name	Correlation to MiR-382 expression level
MRGPRF	0.96	LOC653598	0.773
RGMA	0.928	CLEC1A	0.773
RGS11	0.928	DKFZP434K191	0.773
TNFRSF25	0.925	KLHL3	0.772
MEG3	0.924	LOC196752	0.772
LIMS2	0.924	RARRES2	0.771
HOXA6	0.917	SPON1	0.77
TAGLN	0.909	COBLL1	0.77
CXCL12	0.901	LOC644634	0.768
ADCY4	0.894	SYT11	0.768
LRRC4C	0.894	PRICKLE2	0.767
RASL12	0.892	OR10T2	0.766
NR2F2	0.89	COL8A1	0.766
FLJ35258	0.889	SETBP1	0.765
MEOX1	0.887	MYOZ3	0.765
PRICKLE1	0.886	PPP1R13B	0.765
CLEC3B	0.885	LOC646897	0.765
GJC2	0.883	RAXL1	0.764
IL1RAPL1	0.877	NET1	0.764
LRRC32	0.876	RNF165	0.764
PHLDB1	0.873	CNN1	0.764
TFIP11	0.87	SOX17	0.763
RAMP3	0.868	TMEM30B	0.763
DARC	0.867	PPP2R3A	0.762
LOC647654	0.867	LOC390876	0.761
FLJ45337	0.866	LOC390414	0.76
COX6BP1	0.866	KLF2	0.76
PEG3	0.866	GVIN1	0.76
TEK	0.865	IGFBP5	0.76
LOC649088	0.864	OTX1	0.76
LOC647873	0.862	MYOM1	0.759
COMP	0.86	LOC643272	0.759
ZNF385D	0.86	NOSTRIN	0.759
RPH3A	0.858	KIT	0.758
LOC643700	0.855	DLG4	0.758
LOC727948	0.855	MALL	0.758
FAM124B	0.854	PODN	0.757
LOC642321	0.853	S100A16	0.756
LOC643423	0.852	PTPRK	0.755
CD79B	0.851	FOXD4L1	0.755
DKFZP686K1684	0.85	PCDHB9	0.753

LOC645241	0.85	LOC441773	0.752
LOC651686	0.849	FLJ21438	0.751
NOL10	0.849	LOC643453	0.75
DEFB109	0.849	PROM1	0.749
LOC649181	0.849	LOC653524	0.749
NCALD	0.848	LOC728411	0.748
IGFL3	0.847	LOC199800	0.748
LOC152024	0.846	SULF1	0.748
LOC643605	0.845	ZNF521	0.747
MYH11	0.844	NISCH	0.747
CPXM1	0.844	PPA2	0.746
CCDC149	0.844	CCDC140	0.746
PTPRB	0.843	LOC642707	0.746
LOC651821	0.843	GPR116	0.745
LOC642118	0.841	ALS2CR14	0.744
IFNB1	0.84	SELP	0.743
KCNMB1	0.839	ESAM	0.742
SGIP1	0.838	LOC644642	0.742
FOXO1	0.838	ZNF160	0.742
FGF9	0.837	LOC644334	0.74
LOC648879	0.837	ARID5B	0.739
PDLIM3	0.836	IGFBP7	0.739
TSHZ2	0.835	DCLRE1C	0.737
LPPR4	0.834	AHI1	0.737
RDH5	0.834	RGS16	0.736
LOC647786	0.832	SLC4A5	0.735
A4GALT	0.831	ABLIM1	0.735
LOC646491	0.828	AHR	0.734
CPT1C	0.827	C8ORF37	0.733
GRK5	0.826	C10ORF10	0.732
LOC642017	0.826	ABI3BP	0.732
LOC649456	0.826	ANKRD30B	0.731
STMN2	0.825	COX19	0.731
KRTAP21-2	0.824	C21ORF55	0.731
CFB	0.824	EVI5	0.731
CARD10	0.823	AGTRL1	0.731
NFIB	0.823	EVI1	0.73
ADCY2	0.821	PDSS2	0.73
FEZ1	0.818	CLEC14A	0.73
DLC1	0.815	SOX18	0.73
SLCO2A1	0.815	TGFBR3	0.73
PGM5	0.815	LOC651268	0.729
NLGN2	0.815	SDHALP1	0.729
SPARCL1	0.814	APCDD1L	0.729
C9ORF106	0.814	KCNJ8	0.729
CACNA1H	0.814	C1S	0.728
RAPGEF3	0.814	PDCD4	0.728
GJA4	0.812	PRSS23	0.728
MGC61598	0.812	CAMK2N1	0.727
DLL1	0.811	COL15A1	0.727
LOC390033	0.81	TM4SF1	0.727

C10ORF108	0.809	LOC648059	0.727
PPP1R14A	0.809	LOC441019	0.726
LDB2	0.809	FBLN2	0.726
C20ORF46	0.809	C1ORF110	0.725
LOC649661	0.806	C14ORF78	0.725
PIK3C2B	0.806	THBS4	0.725
LOC645362	0.805	CPLX1	0.725
COL18A1	0.804	PDZRN4	0.724
TESC	0.803	LOC653489	0.724
FOXD4	0.803	LOC440157	0.723
FGD5	0.803	C10ORF116	0.722
TTN	0.802	GIMAP5	0.72
GABRB1	0.802	LOC653829	0.719
PALM	0.802	RASIP1	0.719
CD34	0.801	RUNDC2C	0.719
LOC648907	0.801	C1ORF115	0.719
MGC10997	0.801	LOC389517	0.719
GAS6	0.801	CLDN5	0.718
CAV1	0.799	ITPR3	0.718
NUAK1	0.798	PNPLA7	0.717
LOC643466	0.798	ZNF223	0.717
LOC643817	0.797	FAM153B	0.717
NOX4	0.797	CRIP2	0.716
LOC647295	0.796	ETS2	0.714
ACACB	0.795	ICA1	0.714
ACTG2	0.794	CEP27	0.714
LOC651309	0.794	TMEM16A	0.713
PPP2R2B	0.793	C8A	0.712
SPRYD5	0.792	PECAM1	0.712
DNHL1	0.792	ACTN4	0.711
ANGPTL2	0.791	VCL	0.711
MFAP5	0.791	FMO3	0.71
CLDN14	0.791	SALL3	0.708
SMOC2	0.791	ARHGAP22	0.708
LOC389634	0.791	EIF2AK4	0.707
LOC653800	0.79	LOC653086	0.707
JAG1	0.79	PLA2G2D	0.707
KIAA0355	0.788	LOC728014	0.706
LOC644113	0.788	SH3BGRL2	0.706
SHANK3	0.786	RAB3IP	0.705
C6ORF189	0.785	CDAN1	0.704
LOC653629	0.785	TIE1	0.703
C11ORF38	0.784	NUBPL	0.703
TLE2	0.784	LOC123688	0.702
LOC645489	0.784	C9ORF80	0.702
MGC24103	0.782		
SEMA6A	0.782		
AKR1C3	0.781		
EPHA4	0.781		
LOC731895	0.779		
ARHGEF3	0.778		

LOC143666	0.777
FLJ41423	0.776
THY1	0.776
LOC642299	0.775
CASQ2	0.774
LOC641989	0.774
RHOJ	0.774
C20ORF160	0.773

For human samples, mRNA data were assayed for quality control as described (Sarver et al., J Cardiovasc Transl Res 2010, 3:204-211). Fluorescence values were obtained from the Illumina detection system without background subtraction and were quantile normalized using GeneData Expressionist Software (GeneData Inc, San Francisco, CA, USA). Multiple probes to the same gene product were then averaged to obtain a value for each gene. The dataset was filtered to identify genes with a variance >1 across the human osteosarcoma tumor dataset and two normal bone samples. The mRNA expression data were composed of human tumors and normal bone samples which had been previously profiled for miRNA expression levels, allowing us to directly calculate Pearson correlation coefficients between miR-382 and mRNA transcript levels. mRNAs that showed positive correlation >0.7 are listed in Table 6, and mRNAs that showed negative correlation are listed < -0.7 in Table 7. These mRNAs and their direction of change (increased for negative correlation and decreased for positive correlation) were submitted to Ingenuity Pathway Analyses to determine enriched functions where the direction of change was consistent with increased or decreased activity of enriched functions. The quantile normalized human miRNA array data were first filtered to determine miRNAs that showed variance >0.1 across the 15 human osteosarcoma tumors. Pearson correlations were then calculated between all pairs of miRNA. All pairs of miRNA with R^2 >0.9 were then used to generate a correlation network visualization of the osteosarcoma miRNAome. Pairwise Pearson correlations used to generate FIG. 1A are provided in Table 8.

Table 7. Differentially expressed mRNA in osteosarcoma negatively correlated to 14q32 encoded miR-382.

Gene Name	Correlation to MiR-382 expression level	Gene Name	Correlation to MiR-382 expression level
C14ORF142	-0.892	UBE2C	-0.741
PRKCA	-0.884	EXOSC4	-0.741

PTRH2	-0.88	C9ORF105	-0.74
PIGP	-0.873	STRAP	-0.738
CALU	-0.87	LOC134997	-0.737
POLR2J3	-0.863	G3BP2	-0.736
GPC4	-0.861	CLDN12	-0.733
C7ORF49	-0.86	HIRIP3	-0.733
CCDC86	-0.849	KDELR2	-0.733
P4HA2	-0.844	BYSL	-0.73
PSMD14	-0.84	TWIST1	-0.729
MRPS18C	-0.839	HMGN2	-0.729
MRPS33	-0.835	IARS	-0.729
LASS2	-0.827	TYMS	-0.725
TRIB3	-0.823	C13ORF34	-0.725
ATP1B1	-0.816	SDHB	-0.725
SPR	-0.808	PFN2	-0.724
NEU1	-0.803	METTL1	-0.722
B4GALNT1	-0.803	MRPL40	-0.721
SDC2	-0.799	FEN1	-0.719
PPIH	-0.799	RPN2	-0.719
RAD51AP1	-0.795	GGH	-0.717
NOP5/NOP58	-0.794	CMTM6	-0.717
POLQ	-0.794	C19ORF10	-0.717
COQ2	-0.792	UBL5	-0.716
SDC4	-0.791	AARS2	-0.716
MRC2	-0.79	CKS2	-0.715
NOMO1	-0.786	PCOLCE2	-0.714
PIP4K2C	-0.782	EBP	-0.714
HIST1H1C	-0.781	C3ORF58	-0.714
COMMD9	-0.778	UBE2T	-0.713
MRPL16	-0.777	C14ORF2	-0.711
PRDX4	-0.776	LOC654121	-0.709
UQCRQ	-0.773	DCXR	-0.709
PRICKLE4	-0.77	GCUD2	-0.708
HNRPC	-0.767	CDK4	-0.707
C6ORF125	-0.766	STX8	-0.707
MARS	-0.766	M160	-0.706
TFRC	-0.766	C2ORF7	-0.705
EPDR1	-0.765	LOC646197	-0.702
ALDOA	-0.763	CCDC99	-0.701
RUNX2	-0.762	HIST2H2BE	-0.701
DCI	-0.76	CDCA5	-0.701
PSMA3	-0.757		
MRPL13	-0.757		

TK1	-0.753
NDUFA1	-0.753
PET112L	-0.751
P4HA1	-0.75
HIST1H2BK	-0.749
PCNA	-0.749
SNRPC	-0.747
CDK5	-0.747
FAM96A	-0.742

Table 8. 14q32 subnetwork pairwise correlations.

Row	genomic position	HS_212	HS_214.1	hsa-miR-127	hsa-miR-134	hsa-miR-154	hsa-miR-154*	hsa-miR-299-3p	hsa-miR-299-5p	hsa-miR-323	hsa-miR-329	hsa-miR-368	hsa-miR-369-3p	hsa-miR-370	hsa-miR-376a	hsa-miR-376a*	hsa-miR-376b	hsa-miR-377	hsa-miR-379	hsa-miR-381	hsa-miR-382	hsa-miR-409-3p	hsa-miR-410	hsa-miR-411	hsa-miR-431	hsa-miR-432	hsa-miR-485-3p	hsa-miR-487a	hsa-miR-487b	hsa-miR-493-3p	hsa-miR-493-5p	hsa-miR-495	hsa-miR-496	hsa-miR-654	hsa-miR-655	hsa-miR-656
HS_212			0.84	0.77	0.74	0.77	0.73	0.92	0.79	0.87	0.85	0.64	0.67	0.74	0.71	0.71	0.70	0.77	0.73	0.84	0.88	0.78	0.73	0.76	0.55	0.81	0.79	0.80	0.77	0.85	0.81	0.83	0.93	0.88	0.77	0.82
HS_214.1	100584039	0.84		0.88	0.92	0.91	0.85	0.71	0.93	0.96	0.94	0.85	0.88	0.93	0.92	0.92	0.84	0.91	0.92	0.94	0.94	0.95	0.93	0.93	0.79	0.91	0.92	0.89	0.95	0.89	0.95	0.90	0.88	0.92	0.95	0.93
hsa-miR-127	100419125	0.77	0.88		0.97	0.97	0.94	0.72	0.95	0.90	0.94	0.93	0.93	0.92	0.96	0.94	0.96	0.94	0.94	0.93	0.90	0.94	0.94	0.98	0.78	0.96	0.94	0.91	0.95	0.94	0.93	0.91	0.88	0.90	0.97	0.91
hsa-miR-134	100590784	0.74	0.92	0.97		0.98	0.94	0.64	0.97	0.91	0.96	0.97	0.98	0.91	0.99	0.94	0.96	0.95	0.97	0.94	0.91	0.97	0.97	0.98	0.85	0.97	0.95	0.92	0.97	0.93	0.96	0.92	0.85	0.87	0.99	0.94
hsa-miR-154	100595859	0.77	0.91	0.97	0.98		0.94	0.67	0.99	0.92	0.96	0.92	0.94	0.93	0.96	0.94	0.95	0.96	0.97	0.92	0.95	0.98	0.96	0.99	0.83	0.98	0.96	0.95	0.97	0.97	0.98	0.94	0.87	0.90	0.98	0.97
hsa-miR-154*	100595895	0.73	0.85	0.94	0.94	0.94		0.66	0.92	0.84	0.91	0.89	0.93	0.84	0.90	0.86	0.95	0.88	0.96	0.85	0.87	0.90	0.88	0.93	0.85	0.92	0.87	0.95	0.87	0.94	0.90	0.90	0.83	0.81	0.93	0.91
hsa-miR-299-3p	100559922	0.92	0.71	0.72	0.64	0.67	0.66		0.68	0.76	0.77	0.57	0.60	0.66	0.61	0.59	0.62	0.61	0.64	0.76	0.74	0.65	0.60	0.67	0.49	0.73	0.67	0.66	0.66	0.74	0.67	0.72	0.82	0.81	0.67	0.65
hsa-miR-299-5p	100559890	0.79	0.93	0.95	0.97	0.99	0.92	0.68		0.93	0.97	0.89	0.92	0.93	0.94	0.91	0.90	0.95	0.98	0.92	0.96	0.98	0.94	0.98	0.87	0.96	0.94	0.93	0.95	0.97	0.98	0.93	0.87	0.91	0.97	0.97
hsa-miR-323	100561871	0.87	0.96	0.90	0.91	0.92	0.84	0.76	0.93		0.94	0.86	0.87	0.91	0.91	0.90	0.86	0.94	0.89	0.96	0.95	0.94	0.93	0.93	0.77	0.95	0.93	0.90	0.95	0.91	0.95	0.92	0.95	0.92	0.93	0.93
hsa-miR-329	100562924	0.85	0.94	0.94	0.96	0.96	0.91	0.77	0.97	0.94		0.89	0.92	0.91	0.93	0.88	0.89	0.91	0.96	0.92	0.95	0.95	0.92	0.95	0.84	0.95	0.92	0.92	0.94	0.96	0.96	0.90	0.89	0.90	0.97	0.95
hsa-miR-368	100575823	0.64	0.85	0.93	0.97	0.92	0.89	0.57	0.89	0.86	0.89		0.98	0.83	0.97	0.89	0.95	0.89	0.91	0.91	0.81	0.90	0.95	0.94	0.81	0.94	0.88	0.84	0.93	0.83	0.88	0.87	0.78	0.77	0.94	0.84
hsa-miR-369-3p	100601731	0.67	0.88	0.93	0.98	0.94	0.93	0.60	0.92	0.87	0.92	0.98		0.86	0.97	0.89	0.95	0.89	0.95	0.90	0.84	0.93	0.94	0.95	0.88	0.94	0.88	0.89	0.93	0.87	0.90	0.88	0.79	0.79	0.95	0.87
hsa-miR-370	100447276	0.74	0.93	0.92	0.91	0.93	0.84	0.66	0.93	0.91	0.91	0.83	0.86		0.92	0.95	0.86	0.91	0.92	0.90	0.91	0.96	0.91	0.95	0.76	0.90	0.91	0.87	0.94	0.89	0.92	0.85	0.83	0.90	0.95	0.90
hsa-miR-376a	100576208	0.71	0.92	0.96	0.99	0.96	0.90	0.61	0.94	0.91	0.93	0.97	0.97	0.92		0.95	0.96	0.95	0.95	0.95	0.89	0.96	0.99	0.98	0.81	0.96	0.93	0.90	0.98	0.89	0.94	0.92	0.85	0.86	0.98	0.91
hsa-miR-376a*	100576877	0.71	0.92	0.94	0.94	0.94	0.86	0.59	0.91	0.90	0.88	0.89	0.89	0.95	0.95		0.93	0.96	0.89	0.92	0.91	0.94	0.96	0.95	0.71	0.93	0.96	0.88	0.97	0.87	0.94	0.90	0.84	0.90	0.97	0.91
hsa-miR-376b	100576587	0.70	0.84	0.96	0.96	0.95	0.95	0.62	0.90	0.86	0.89	0.95	0.95	0.86	0.96	0.93		0.93	0.91	0.89	0.86	0.90	0.94	0.94	0.76	0.95	0.92	0.91	0.92	0.89	0.90	0.91	0.84	0.82	0.95	0.89
hsa-miR-377	100598184	0.77	0.91	0.94	0.95	0.96	0.88	0.61	0.95	0.94	0.91	0.89	0.89	0.91	0.95	0.96	0.93		0.91	0.94	0.95	0.95	0.97	0.96	0.74	0.96	0.98	0.91	0.96	0.92	0.97	0.94	0.90	0.90	0.96	0.96
hsa-miR-379		0.73	0.92	0.94	0.97	0.97	0.96	0.64	0.98	0.89	0.96	0.91	0.95	0.92	0.95	0.89	0.91	0.91		0.89	0.90	0.97	0.93	0.97	0.91	0.93	0.89	0.94	0.94	0.95	0.94	0.90	0.83	0.84	0.96	0.94
hsa-miR-381	100582058	0.84	0.94	0.93	0.94	0.92	0.85	0.76	0.92	0.96	0.92	0.91	0.90	0.90	0.95	0.92	0.89	0.94	0.89		0.91	0.94	0.95	0.95	0.72	0.95	0.92	0.85	0.96	0.88	0.92	0.93	0.93	0.91	0.95	0.89
hsa-miR-382	100590406	0.88	0.94	0.90	0.91	0.95	0.87	0.74	0.96	0.95	0.95	0.81	0.84	0.91	0.89	0.91	0.86	0.95	0.90	0.91		0.95	0.92	0.93	0.76	0.94	0.95	0.93	0.93	0.95	0.98	0.94	0.92	0.95	0.94	0.98

hsa-miR-409-3p	100601435	0.78	0.95	0.94	0.97	0.98	0.90	0.65	0.98	0.94	0.95	0.90	0.93	0.96	0.96	0.94	0.90	0.95	0.97	0.94	0.95		0.97	0.98	0.85	0.95	0.94	0.94	0.98	0.94	0.97	0.93	0.88	0.91	0.97	0.96
hsa-miR-410	100602051	0.73	0.93	0.94	0.97	0.96	0.88	0.60	0.94	0.93	0.92	0.95	0.94	0.91	0.99	0.96	0.94	0.97	0.93	0.95	0.92	0.97		0.97	0.81	0.97	0.95	0.91	0.99	0.89	0.96	0.94	0.87	0.88	0.97	0.94
hsa-miR-411	100559430	0.76	0.93	0.98	0.98	0.99	0.93	0.67	0.98	0.93	0.95	0.94	0.95	0.95	0.98	0.95	0.94	0.96	0.97	0.95	0.93	0.98	0.97		0.83	0.97	0.95	0.92	0.98	0.94	0.96	0.92	0.88	0.90	0.99	0.94
hsa-miR-431	100417116	0.55	0.79	0.78	0.85	0.83	0.85	0.49	0.87	0.77	0.84	0.81	0.88	0.76	0.81	0.71	0.76	0.74	0.91	0.72	0.76	0.85	0.81	0.83		0.81	0.72	0.84	0.79	0.81	0.82	0.77	0.65	0.69	0.81	0.81
hsa-miR-432	100420586	0.81	0.91	0.96	0.97	0.98	0.92	0.73	0.96	0.95	0.95	0.94	0.94	0.90	0.96	0.93	0.95	0.96	0.93	0.95	0.94	0.95	0.97	0.97	0.81		0.96	0.94	0.97	0.94	0.97	0.96	0.91	0.91	0.97	0.94
hsa-miR-485-3p	100591554	0.79	0.92	0.94	0.95	0.96	0.87	0.67	0.94	0.93	0.92	0.88	0.88	0.91	0.93	0.96	0.92	0.98	0.89	0.92	0.95	0.94	0.95	0.95	0.72	0.96		0.90	0.95	0.92	0.97	0.91	0.89	0.94	0.97	0.95
hsa-miR-487a	100588584	0.80	0.89	0.91	0.92	0.95	0.95	0.66	0.93	0.90	0.92	0.84	0.89	0.87	0.90	0.88	0.91	0.91	0.94	0.85	0.93	0.94	0.91	0.92	0.84	0.94	0.90		0.91	0.97	0.95	0.94	0.88	0.86	0.92	0.96
hsa-miR-487b	100582595	0.77	0.95	0.95	0.97	0.97	0.87	0.66	0.95	0.95	0.94	0.93	0.93	0.94	0.98	0.97	0.92	0.96	0.94	0.96	0.93	0.98	0.99	0.98	0.79	0.97	0.95	0.91		0.91	0.96	0.94	0.88	0.92	0.98	0.93
hsa-miR-493-3p	100405206	0.85	0.89	0.94	0.93	0.97	0.94	0.74	0.97	0.91	0.96	0.83	0.87	0.89	0.89	0.87	0.89	0.92	0.95	0.88	0.95	0.94	0.89	0.94	0.81	0.94	0.92	0.97	0.91		0.96	0.91	0.91	0.89	0.94	0.97
hsa-miR-493-5p	100405165	0.81	0.95	0.93	0.96	0.98	0.90	0.67	0.98	0.95	0.96	0.88	0.90	0.92	0.94	0.94	0.90	0.97	0.94	0.92	0.98	0.97	0.96	0.96	0.82	0.97	0.97	0.95	0.96	0.96		0.94	0.90	0.92	0.97	0.99
hsa-miR-495	100569894	0.83	0.90	0.91	0.92	0.94	0.90	0.72	0.93	0.92	0.90	0.87	0.88	0.85	0.92	0.90	0.91	0.94	0.90	0.93	0.94	0.93	0.94	0.92	0.77	0.96	0.91	0.94	0.94	0.91	0.94		0.92	0.91	0.92	0.93
hsa-miR-496		0.93	0.88	0.88	0.85	0.87	0.83	0.82	0.87	0.95	0.89	0.78	0.79	0.83	0.85	0.84	0.84	0.90	0.83	0.93	0.92	0.88	0.87	0.88	0.65	0.91	0.89	0.88	0.88	0.91	0.90	0.92		0.91	0.87	0.91
hsa-miR-654	100576324	0.88	0.92	0.90	0.87	0.90	0.81	0.81	0.91	0.92	0.90	0.77	0.79	0.90	0.86	0.90	0.82	0.90	0.84	0.91	0.95	0.91	0.88	0.90	0.69	0.91	0.94	0.86	0.92	0.89	0.92	0.91	0.91		0.91	0.90
hsa-miR-655	100585700	0.77	0.95	0.97	0.99	0.98	0.93	0.67	0.97	0.93	0.97	0.94	0.95	0.95	0.98	0.97	0.95	0.96	0.96	0.95	0.94	0.97	0.97	0.99	0.81	0.97	0.97	0.92	0.98	0.94	0.97	0.92	0.87	0.91		0.95
hsa-miR-656	100602856	0.82	0.93	0.91	0.94	0.97	0.91	0.65	0.97	0.93	0.95	0.84	0.87	0.90	0.91	0.91	0.89	0.96	0.94	0.89	0.98	0.96	0.94	0.94	0.81	0.94	0.95	0.96	0.93	0.97	0.99	0.93	0.91	0.90	0.95	

Integrative analysis of human and canine gene and miRNA expression data.

Human genes that showed positive and negative correlation to miR-382 were mapped to canine genes using gene symbols. The direction of change was tracked using dark grey (negative correlation) and light grey (positive correlation) tags for each gene so that the direction of change in the human data could be observed in analogous fashion as previously described (Scott et al., Bone 2011, 49:356-367). Unsupervised hierarchical clustering was carried out with the Pearson correlation as the metric using average linkage following log base 2 transformation of mean-centered data using Cluster3.0. Heatmaps were generated from the CDT files generated by Cluster3.0 using Java Treeview (Saldanha, Bioinformatics 2004, 20:3246-3248).

qRT-PCR.

qRT-PCR of RNA from both human and canine osteosarcoma samples was carried out using normalization to U6 snRNA. First strand cDNA was synthesized from total RNA using a miScript reverse transcription kit (Qiagen, Valencia, CA, USA). miRNA was quantified with an miRscript SYBR Green PCR kit (Qiagen, Valencia, CA, USA) using cDNA equivalent of 50 ng total RNA per reaction. Mature miRNA-specific forward primers provided in Table 1 were purchased from Integrated DNA Technologies (Coralville, IA, USA) and the universal reverse primer provided by the manufacturer.

Table 1. Oligonucleotides used for qRT-PCR analysis.

Name and miRbase number of the microRNA	Oligo sequence	Seq ID No:
miR-154 MIMAT0000452	GGAGGTTATCCGTGTTGCCTTCG	1
miR-382 MIMAT0000737	GGGGAAGTTGTTCGTGGTGGATTCG	2
miR-544 MIMAT0003164	GGGGGATTCTGCATTTTTAGCAAGTTC	3
miR-134 MIMAT0000447	TGTGACTGGTTGACCAGAGGGG	4

Real-time PCR was performed at 55°C annealing following standard protocol of the manufacturer in 7500 Real Time PCR system (Applied Biosystems/Life Technologies, Inc., Carlsbad, CA USA) and threshold cycles (CT) were calculated using Sequence Detection Software (SDS v1.2.1, Applied Biosystems/Life Technologies, Inc., Carlsbad, CA USA). Relative gene expression (expressed as fold difference relative to normal bone tissues (human

OS) or relative to reactive osteoblasts (canine OS)) was calculated from the average of triplicate CT value measurements using the 2-ΔΔCt method (Schmittgen et al., Nat Protoc 2008, 3:1101-1108) except for situations where the RNA available was limited allowing only duplicate reactions to be carried out. The standard error is represented as $2^{-(\Delta\Delta CT \pm SD)}$.

Statistical analyses

Kaplan-Meier survival curves were generated with the GeneData Analyst statistical package using the clinical data provided in Tables 4 and 5. Whitney Mann U tests were conducted using an online tool available at vassarstats.net/utest.html.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference in their entirety. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. A method comprising:

analyzing a tissue sample from a subject for expression of a 14q32 miRNA, wherein a decrease in expression of the 14q32 miRNA compared to a normal control indicates that the subject has or is at risk of having osteosarcoma.

2. The method of claim 1 wherein the expression of the 14q32 miRNA is no more than 60% of the 14q32 miRNA expression in a normal sample.

3. The method of claim 2 wherein the expression of the 14q32 miRNA is no less than 20% of the 14q32 miRNA expression in a normal sample.

4. The method of claim 3, further comprising treating the subject for a non-aggressive osteosarcoma.

5. The method of claim 1 wherein expression of the 14q32 miRNA of no more than 10% of a normal control further indicates that the subject has or is at risk of having an aggressive osteosarcoma.

6, The method of claim 5 further comprising treating the subject for an aggressive osteosarcoma.

7. The method of any preceding claim wherein the 14q32 miRNA comprises miR-382, miR-134, or miR-544.

8. The method of any preceding claim wherein the 14q32 miRNA comprises miR-379, miR-1197, miR-329-1, miR-494, miR-495, miR-376C, miR-1185-1, miR-1185-2, miR-381, miR-655, miR-487A, miR-382, miR-496, miR-154, miR-369, miR-127, miR-299, miR-665, miR-770, miR-432, miR-668, miR-539, miR-433, miR-323, miR-496, miR-654, miR-409, miR-380, miR-485, miR-136, miR-376, miR-431, miR-370, miR-889, miR-381, miR-299,

miR-379, miR-411, miR-655, miR-432, miR-487, miR-410, miR-493, miR-656, miR-485, miR-377 or miR-541.

9. The method of any preceding claim wherein the subject is a human.

10. The method of any preceding claim wherein the subject is a canine.

11. The method of any preceding claim wherein the analyzing a tissue sample from a subject for expression of a 14q32 miRNA comprises qRT-PCR, microarrays, or RNA sequencing.

Figure 1A

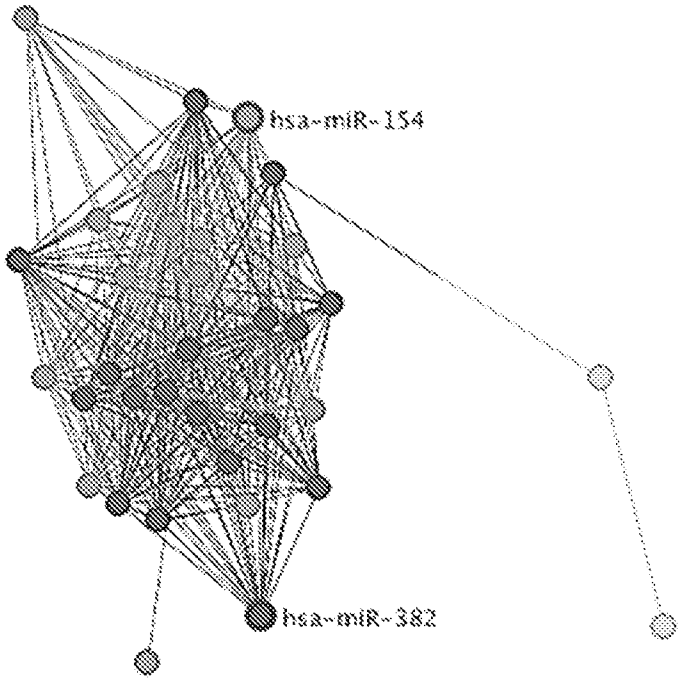


Figure 1B

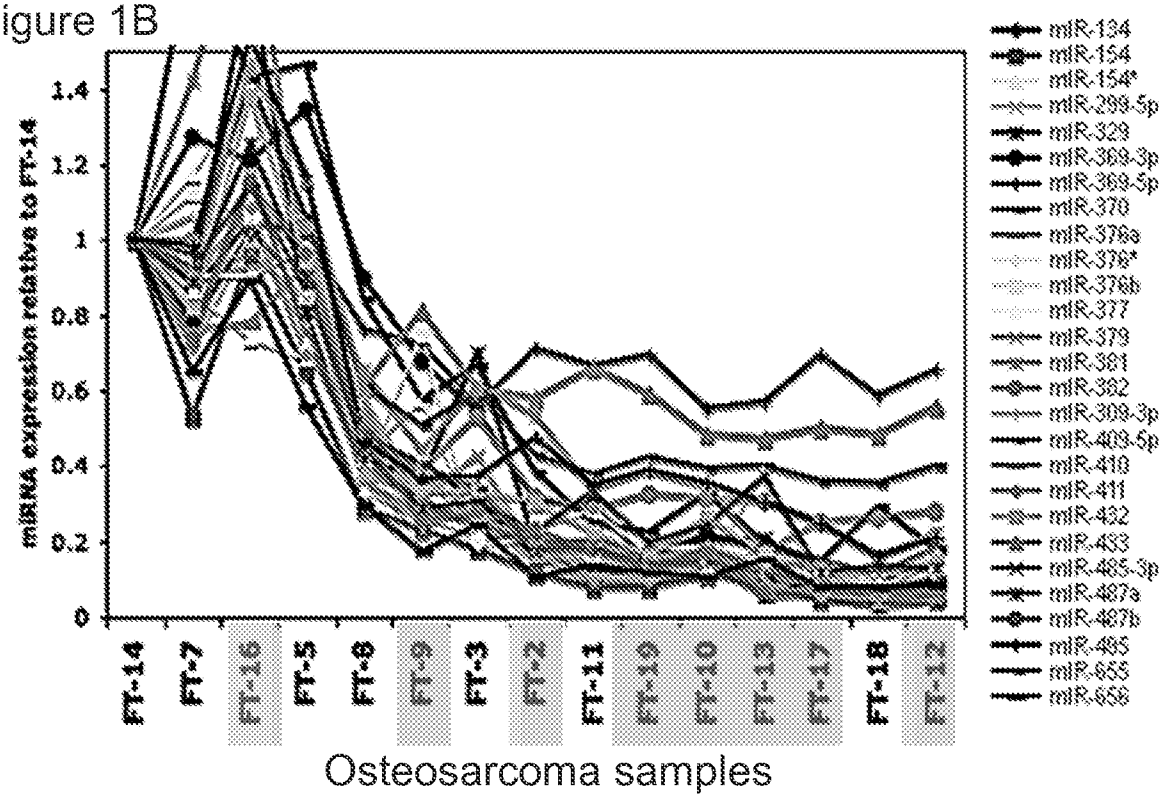


Figure 1C

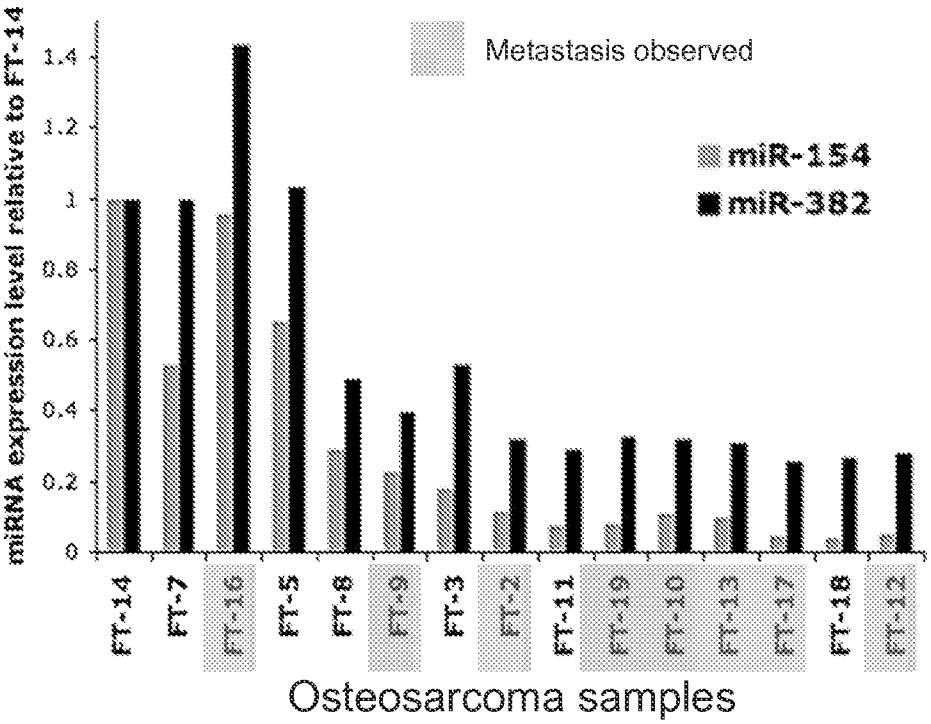


Figure 1D

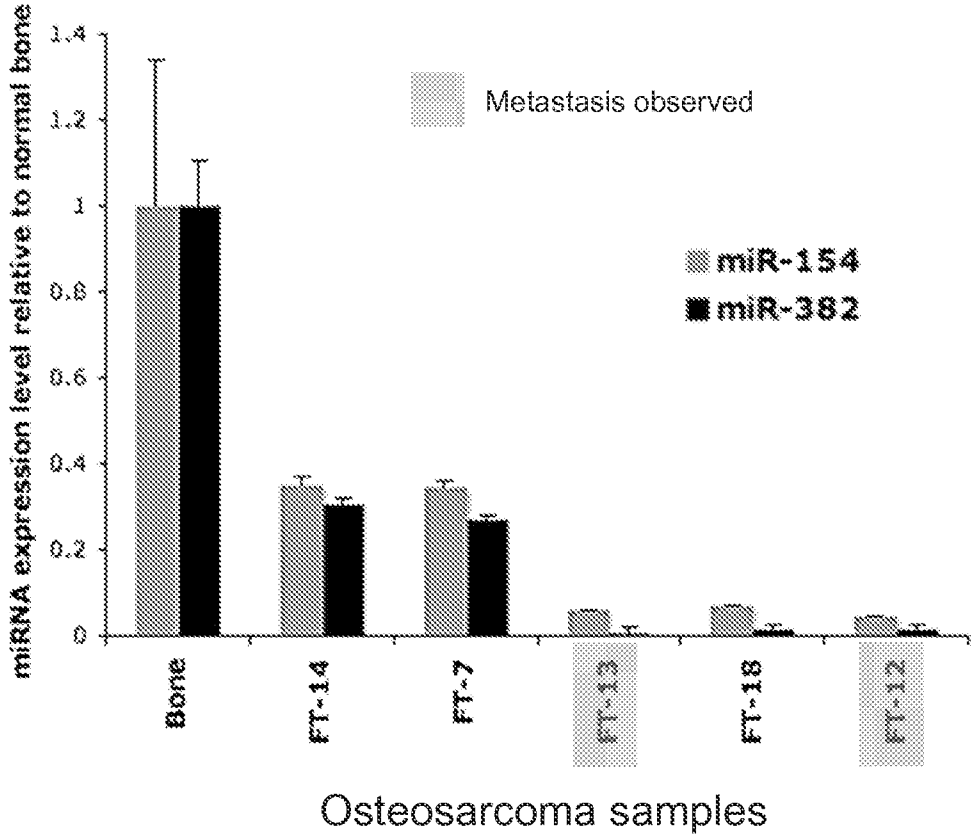


Figure 2A

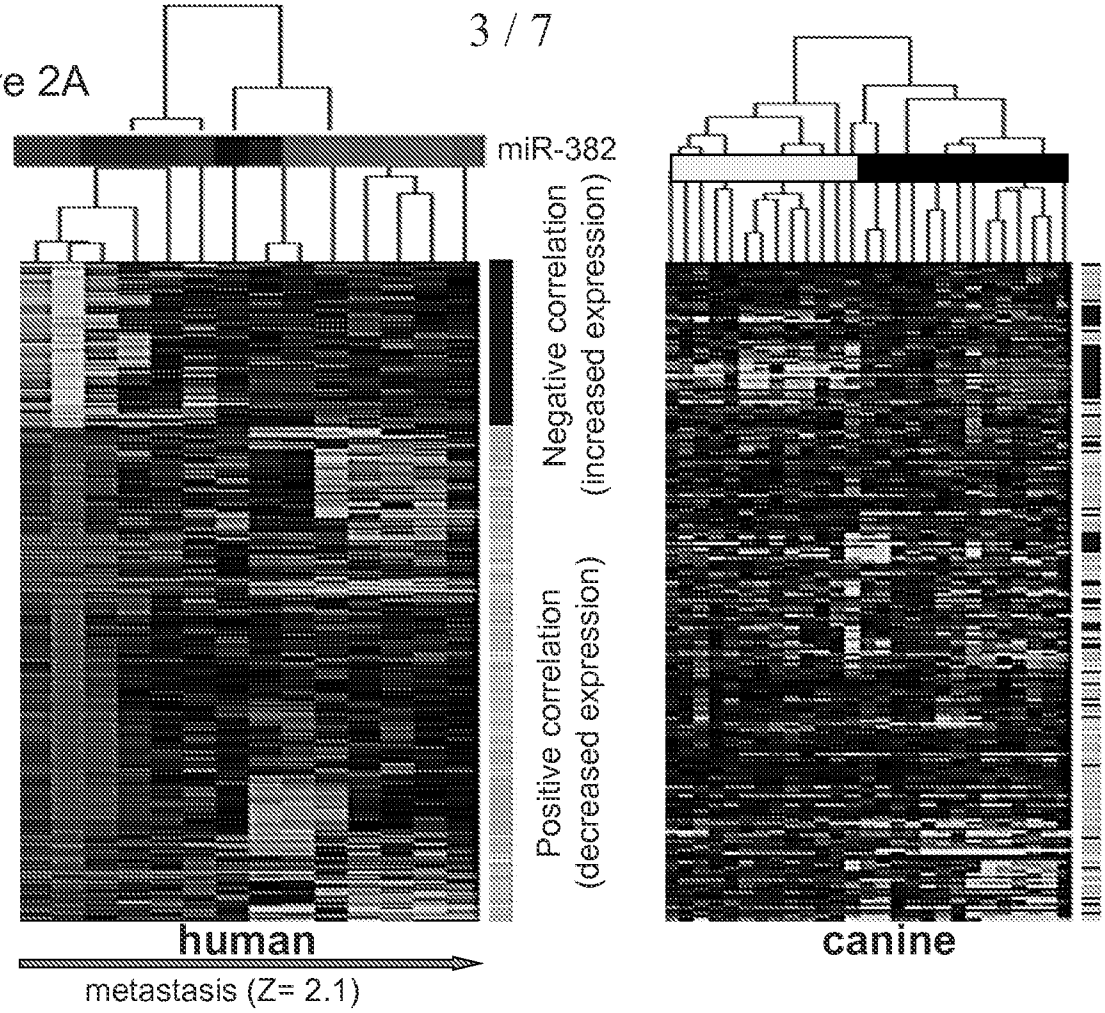


Figure 2B

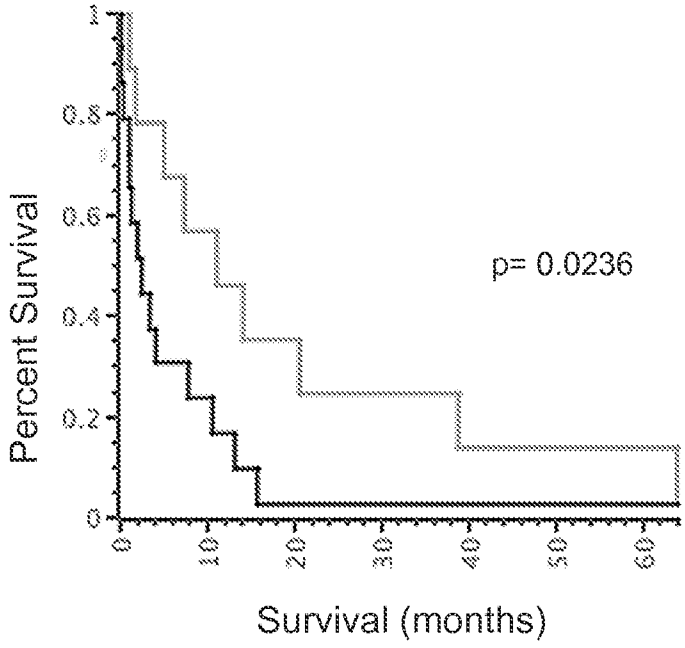


Figure 3A

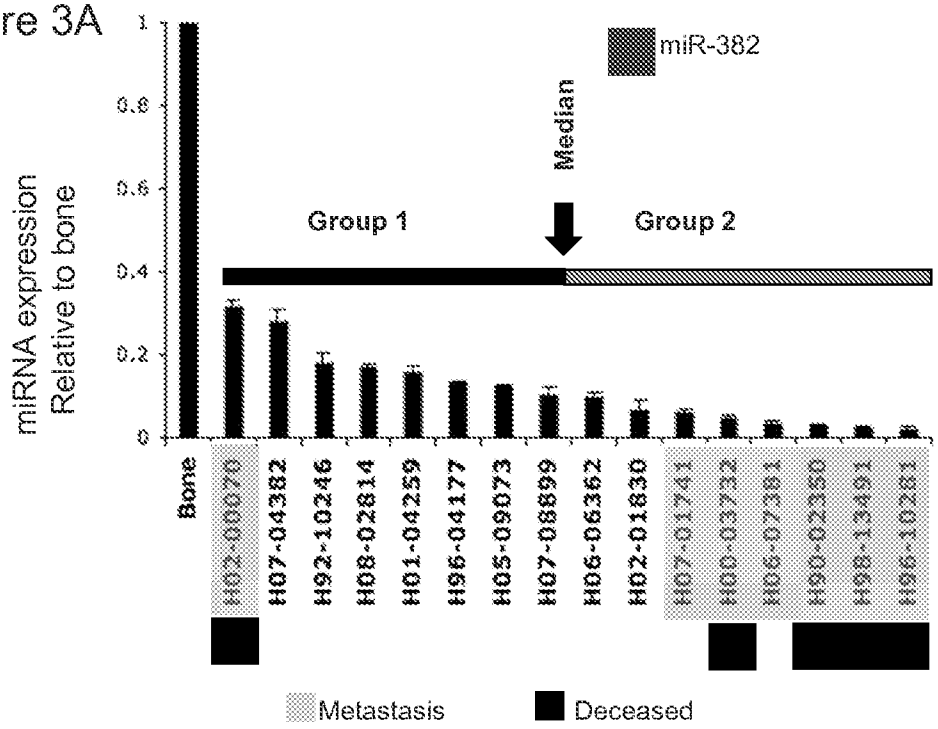


Figure 3B

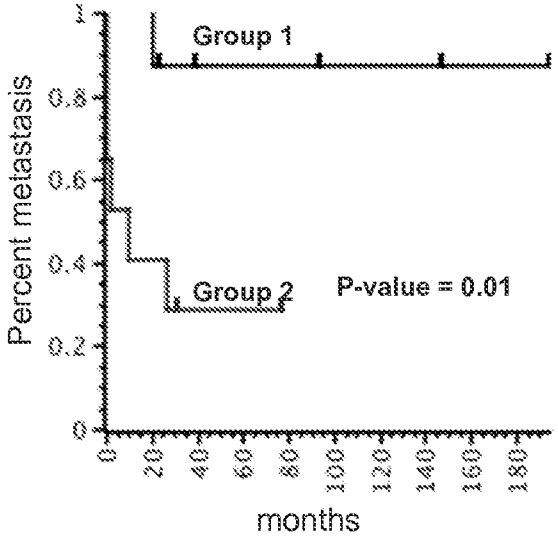


Figure 3C

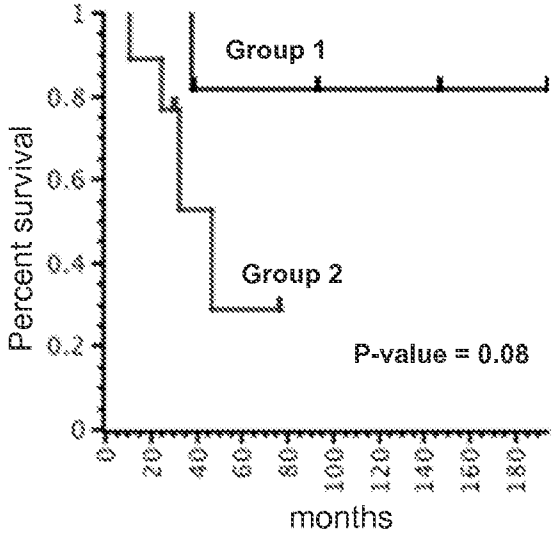


Figure 4A

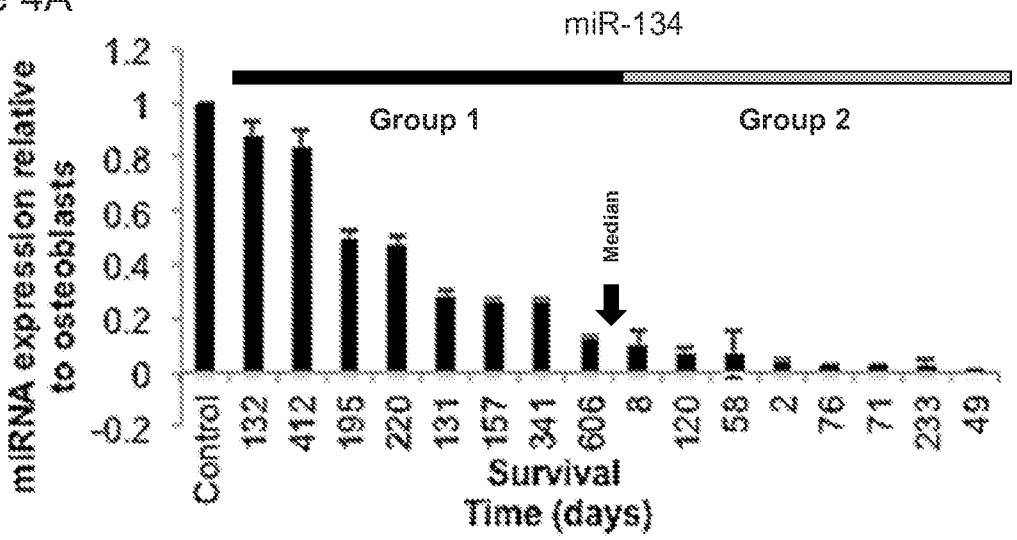


Figure 4B

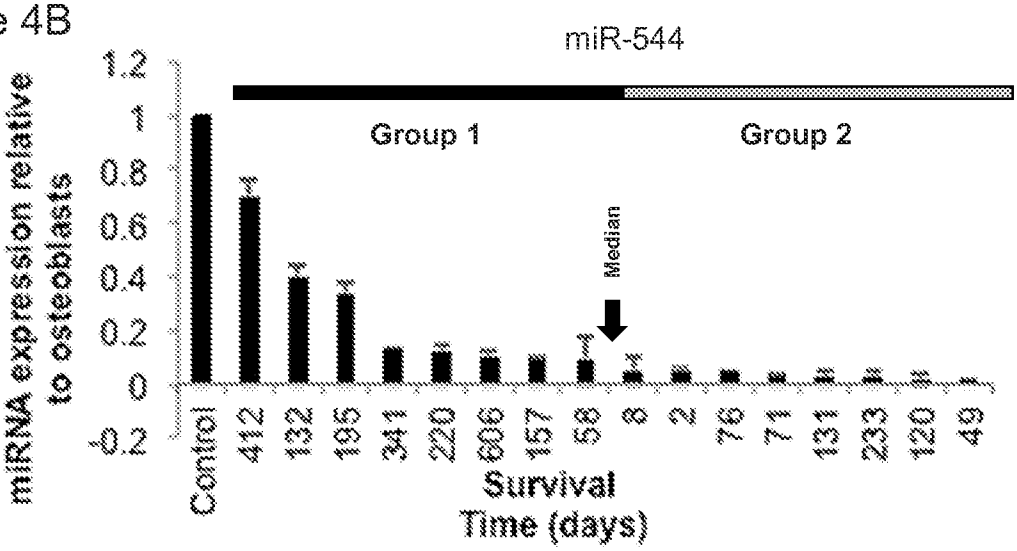


Figure 4C

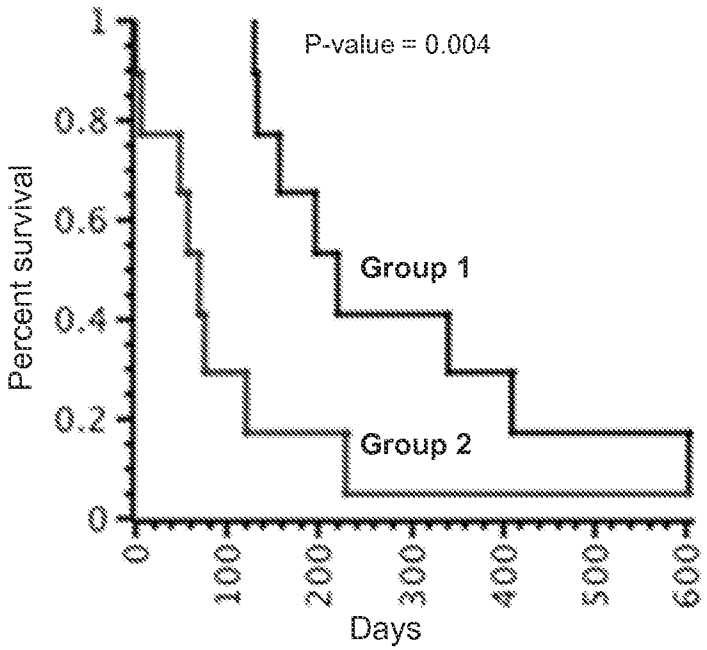
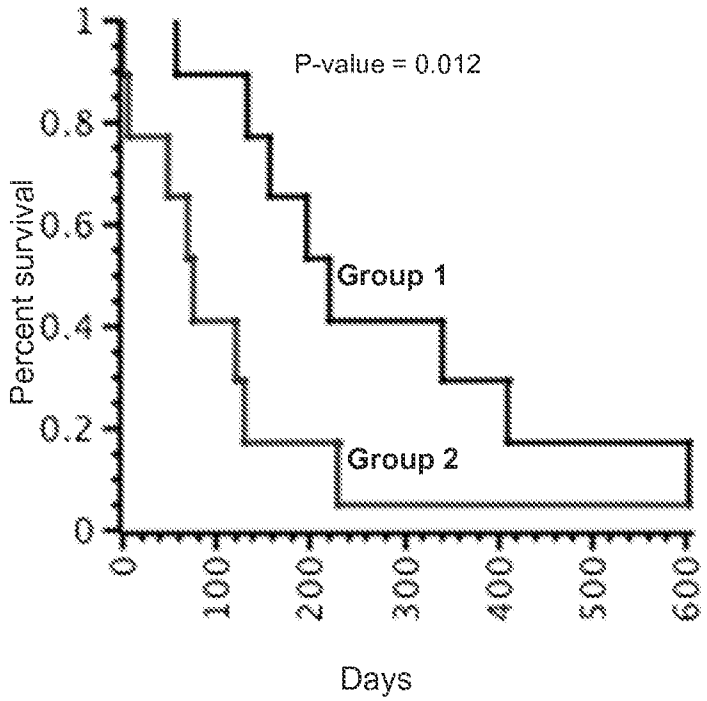


Figure 4D



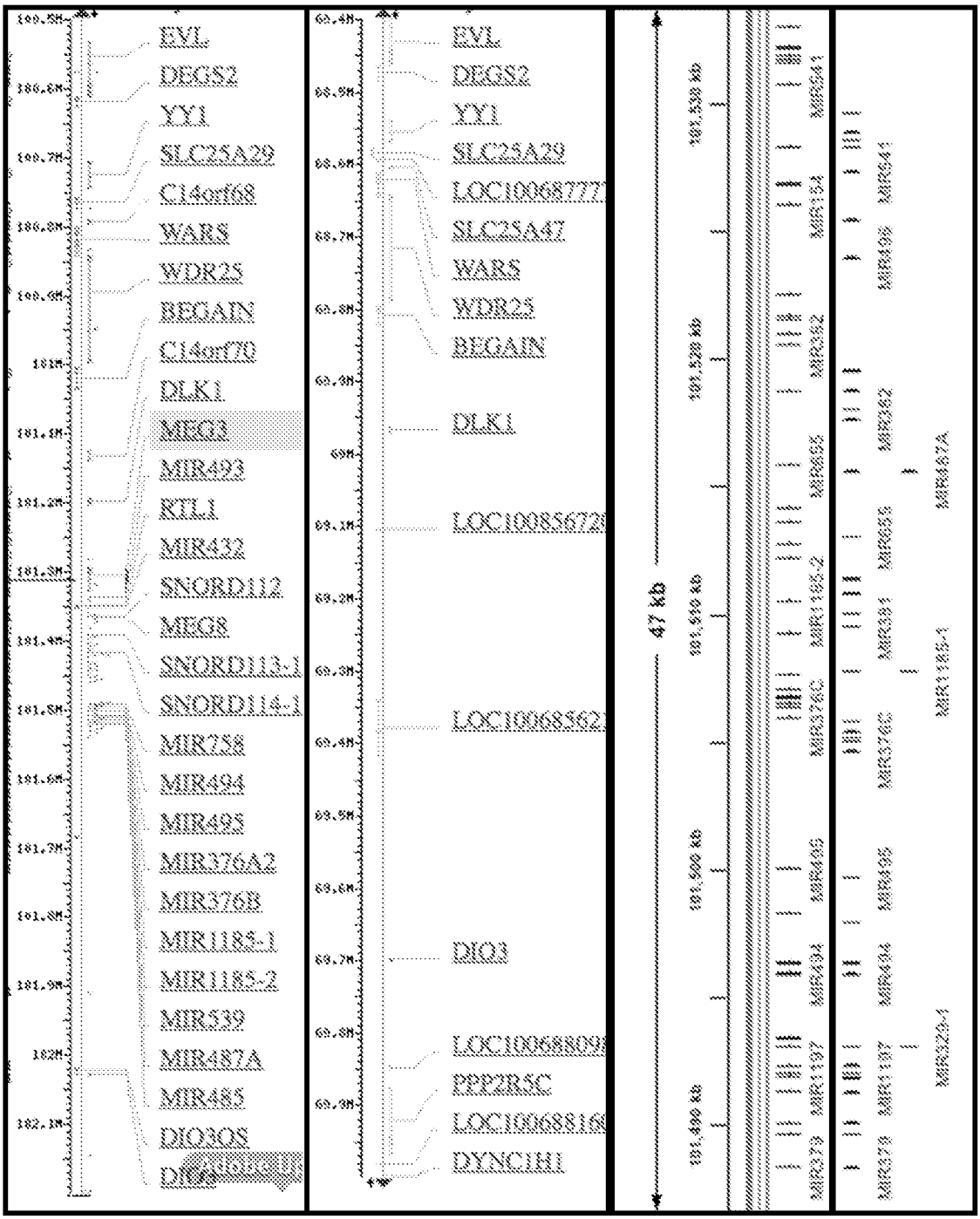


Figure 5A Figure 5B Figure 5C Figure 4D

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/069237

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	AARON L SARVER ET AL: "MicroRNAs at the human 14q32 locus have prognostic significance in osteosarcoma", ORPHANET JOURNAL OF RARE DISEASES, BIOMED CENTRAL LTD, LO, vol. 8, no. 1, 11 January 2013 (2013-01-11), page 7, XP021140112, ISSN: 1750-1172, DOI: 10.1186/1750-1172-8-7 the whole document ----- -/--	1-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 December 2013	20/12/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bruma, Anja

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VENUGOPAL THAYANITHY ET AL: "Perturbation of 14q32 miRNAs-cMYC gene network in osteosarcoma", BONE, PERGAMON PRESS., OXFORD, GB, vol. 50, no. 1, 10 October 2011 (2011-10-10), pages 171-181, XP028350833, ISSN: 8756-3282, DOI: 10.1016/J.BONE.2011.10.012 [retrieved on 2011-10-18] the whole document page 177, column 1 - page 179, column 1 figures 1-3,6-7	1-11
X	VENUGOPAL THAYANITHY ET AL: "Combinatorial Treatment of DNA and Chromatin-Modifying Drugs Cause Cell Death in Human and Canine Osteosarcoma Cell Lines", PLOS ONE, vol. 7, no. 9, 5 September 2012 (2012-09-05), page e43720, XP055092749, DOI: 10.1371/journal.pone.0043720 the whole document page 1, column 2, lines 10-13 page 5, column 1, paragraph 2 page 7, column 2, paragraph 2 - page 9, column 1, paragraph 1 figures 2,3	1-11
X	AARON L SARVER ET AL: "S-MED: Sarcoma microRNA Expression Database", LABORATORY INVESTIGATION, vol. 90, no. 5, 8 March 2010 (2010-03-08), pages 753-761, XP055092750, ISSN: 0023-6837, DOI: 10.1038/labinvest.2010.53 the whole document	1-11
A	MILCAH C SCOTT ET AL: "Molecular subtypes of osteosarcoma identified by reducing tumor heterogeneity through an interspecies comparative approach", BONE, PERGAMON PRESS., OXFORD, GB, vol. 49, no. 3, 5 May 2011 (2011-05-05), pages 356-367, XP028327705, ISSN: 8756-3282, DOI: 10.1016/J.BONE.2011.05.008 [retrieved on 2011-05-15] the whole document	1-11

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/069237

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEONIDAS BENETATOS ET AL: "The microRNAs within the DLK1-DIO3 genomic region: involvement in disease pathogenesis", CELLULAR AND MOLECULAR LIFE SCIENCES, 1 January 2012 (2012-01-01), XP055037453, ISSN: 1420-682X, DOI: 10.1007/s00018-012-1080-8 the whole document -----	1-11

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