Research Article

Solid Tumors of Childhood Display Specific Serum microRNA Profiles

Matthew J. Murray1,2,3, Katie L. Raby2, Harpreet K. Saini4, Shivani Bailey1,2, Sophie V. Wool1, Jane M. Tunnacliffe1, Anton J. Enright4, James C. Nicholson1, and Nicholas Coleman2,5

Abstract

Background: Serum biomarkers for diagnosis and risk stratification of childhood solid tumors would improve the accuracy/timeliness of diagnosis and reduce the need for invasive biopsies. We hypothesized that differential expression and/or release of microRNAs (miRNAs) by such tumors may be detected as altered serum miRNA profiles.

Methods: We undertook global quantitative reverse transcription PCR (qRT-PCR) miRNA profiling (n = 741) on RNA from 53 serum samples, representing 33 diagnostic cases of common childhood cancers plus 20 controls. Technical confirmation was performed in a subset of 21 cases, plus four independent samples.

Results: We incorporated robust quality control steps for RNA extraction, qRT-PCR efficiency and hemolysis quantification. We evaluated multiple methods to normalize global profiling data and identified the `global mean’ approach as optimal. We generated a panel of six miRNAs that were most stable in pediatric serum samples and therefore most suitable for normalization of targeted miRNA qRT-PCR data. Tumor-specific serum miRNA profiles were identified for each tumor type and selected miRNAs underwent confirmatory testing. We identified a panel of miRNAs (miR-124-3p/miR-9-3p/miR-218-5p/miR-490-5p/miR-1538) of potential importance in the clinical management of neuroblastoma, as they were consistently highly overexpressed in MYCN-amplified high-risk cases (MYCN-NB). We also derived candidate miRNA panels for noninvasive differential diagnosis of a liver mass (hepatoblastoma vs. combined MYCN-NB/NB), an abdominal mass (Wilms tumor vs. combined MYCN-NB/NB), and sarcoma subtypes.

Conclusions: This study describes a pipeline for robust diagnostic serum miRNA profiling in childhood solid tumors, and has identified candidate miRNA profiles for prospective testing.

Impact: We propose a new noninvasive method with the potential to diagnose childhood solid tumors. Cancer Epidemiol Biomarkers Prev; 24(2); 350–60. ©2014 AACR.

Introduction

Current challenges in the clinical management of pediatric solid tumors include a requirement for tissue biopsy at initial diagnosis and difficulties in detecting minimal residual disease. The identification of novel body fluid tumor markers that may allow noninvasive diagnosis, risk stratification, or follow-up in solid tumors of childhood would be an important advance. One promising approach is measurement of serum microRNA (miRNA) levels.

miRNAs are short, non–protein-coding RNAs that posttranscriptionally regulate gene expression (1). Importantly, global miRNA profiles have been shown to classify human cancer tissues, including pediatric solid tumors (1, 2). In pediatric malignant germ cell tumors (GCT), for example, the miR-371–373 and miR-302/367 clusters are overexpressed in all cases, regardless of patient age, histologic subtype, or anatomic site (1), thus representing universal biomarkers. Packaging of specific miRNAs in membrane-bound exosome particles by tumor cells before release into the bloodstream allows for their detection in the serum (3). Indeed, we showed that miRNAs from the miR-371–373 and miR-302/367 clusters are detectable at high levels in the serum of patients at the time of malignant GCT diagnosis, and that levels fall with treatment (3–5), findings recently replicated by others (6–9). Importantly, these profiles can detect disease at low tumor volumes, demonstrating the sensitivity of this approach (5). miRNAs have many qualities that make them suitable tumor markers for translation into clinical practice, including inherent stability and resistance to degradation, even if the samples are left at room temperature or subjected to multiple freeze-thaw cycles (10). We therefore hypothesized that other childhood tumors would be characterized by the release of specific miRNAs into the bloodstream, which would be detectable as an altered serum profile compared with control serum samples.

Here, we report the findings of a proof-of-principle study testing this hypothesis at the time of diagnosis of common childhood cancers, using a quantitative reverse transcription PCR (qRT-PCR)
approach. We addressed three important questions: (i) whether it was feasible to extract RNA from the serum of children in sufficient amounts to undertake global miRNA profiling; (ii) whether serum housekeeping miRNAs in children would be similar to those described for adults; and (iii) whether lists of candidate biomarkers could be generated for validation in larger, prospective studies. We identify serum miRNA profiles specific for each of the 11 types of pediatric solid tumor investigated. In particular, we report

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NOTE: Samples 3 to 38 were independent samples used for the technical confirmation study only.

Abbreviations: MYCN-NB, MYCN-amplified high-risk (HR) neuroblastoma; NB, non-MYCN-amplified low-risk (LR) neuroblastoma; HB, hepatoblastoma; WT, Wilms tumor; RMS, rhabdomyosarcoma; ES, Ewing sarcoma; OS, osteosarcoma; B-NHL, B-cell non-Hodgkin lymphoma; HD, Hodgkin disease; PPB, pleuropulmonary blastoma; GL, central nervous system glioma; C, control samples; WAGR, Wilms tumor, aniridia, genitourinary abnormalities, and retardation syndrome.
Mean of four housekeeping miRNAs in QC and full qRT-PCR run

A

Mean of four housekeeping miRNAs in QC and full qRT-PCR run

B

Cumulative distribution

C

Average QC HK raw C\text{t} value

Average full HK raw C\text{t} value

miR-124-3p
miR-15b-3p
miR-218-5p
miR-490-5p
miR-1838
miR-106-5p
miR-122-5p
miR-205-5p
miR-489-3p
miR-106b-3p
miR-10a-5p
miR-187-5p
miR-141-3p
miR-577
miR-34e-3p
miR-30b-3p
miR-143-3p
miR-129-5p
miR-33b-3p
miR-370
miR-99b-3p
miR-93-3p
miR-193-5p
miR-214-3p
miR-214-5p
miR-224-3p
miR-500a-5p
miR-512-5p
miR-618a-3p
miR-132-3p
miR-120b-5p
miR-506b-5p
miR-125a-3p
miR-125b-3p
miR-181a-3p
miR-181a-5p
miR-193a
miR-185-5p

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a panel of serum miRNAs that segregate MYCN-amplified International Neuroblastoma Risk Group (INRG; ref. 11) high-risk neuroblastoma from non-MYCN-amplified INRG low-risk neuroblastoma and other tumors.

Materials and Methods

The study received approval from the Multicenter Research Ethics Committee (reference 02/4/71) and Local Research Ethics Committee (reference 01/128) and was performed with full informed parental consent. All experimental steps were compliant with the Minimum Information for Publication of Quantitative Real-time PCR Experiments (12).

Patient demographics and tumor types analyzed

For the main discovery-phase of the project, we initially recruited 34 patients ages 0 to 16 years at the time of malignant tumor diagnosis (between April 2010 and June 2013), together with a further 20 age- and gender-matched anonymized samples from a control group of patients without malignant disease. Clinicopathologic details are listed in Table 1. The 34 tumors were from 11 different tumor types. They comprised (i) four neuroblastomas [two MYCN-amplified INRG high-risk tumors (MYCN-NB); two non-MYCN-amplified INRG low-risk tumors (NB)]; (ii) four hepa-toblastomas (HB); (iii) seven Wilms tumors (WT); (iv) seven lymphomas [five cases of classical nodular-sclerosing Hodgkin’s disease (HD) and two cases of B-cell non-Hodgkin lymphoma (B-NHL)]; (v) six sarcomas [three rhabdomyosarcoma (RMS), two Ewing sarcoma (ES), one osteosarcoma (OS)]; (vi) one DICER1-mutated pleuropulmonary blastoma (PPB; ref. 13), and (vii) five central nervous system tumors (gliomas; three WHO 2007 grade I, one grade II, and one grade III) (Table 1).

For the technical confirmation phase of the study, we used 25 samples. These comprised 17 representative tumor and four control serum samples from the discovery-phase test set (Table 1), plus four independent neuroblastoma serum samples from two MYCN-NB and two NB cases. In total, the confirmatory set comprised four MYCN-NB samples, four NB, three HB, two WT, two RMS, two B-NHL, two HD, two gliomas, and four control samples (Table 1). There was no difference in tumor volume between the MYCN-NB and NB cases. While all four of the MYCN-NB cases were undifferentiated, the grade of the NB cases varied from poorly differentiated to differentiating and ganglioneuroblastoma (Table 1).

Data normalization

Samples were obtained and processed as described (3). miRNAs were quantified as described (13) using miRNA Ready-to-Use PCR Human Panels I and II (Exiqon). We performed extensive quality control steps to measure hemolysis and the efficiencies of RNA extraction, reverse transcription and qRT-PCR. A detailed account of this assay pipeline is available in the Supplementary Materials and Methods. Only a single sample (WT-4) failed initial quality control; 53 samples (98%) therefore underwent full qRT-PCR profiling. In initial work, we compared a number of different data normalization methods (14) to define the optimal approach to our global serum miRNA profiling study. We assessed:

i the global mean method, which used the average C_t value of miRNAs expressed in at least one of the 53 samples analyzed, as described (14);

ii the modified global mean method, which did not take into account other samples in the dataset and instead used a sample-specific normalization factor plotted on a linear scale (14);

iii the modified global mean method of common miRNAs (14), i.e., those miRNAs expressed in all 53 samples analyzed;

iv the 10 top-ranking miRNAs identified as most stably expressed in the study by geNorm (15);

v the 10 top-ranking miRNAs identified as most stably expressed in the study by NormFinder (16);

vi the six top-ranking overlapping housekeeping miRNAs identified in both (iv) and (v) above;

vii the four housekeeping miRNAs used for initial quality control purposes (hkgQC), namely miR-23a-3p, miR-30c-5p, miR-103a-3p, and miR-191-5p;

viii the two small nuclear RNAs (snRNA), RNU49A and RNU49B, present on the Exiqon platform;

ix the single small nuclear RNA (snRNA) RNU6, present on the platform. Both snRNAs and snRNAs are commonly used for miRNA normalization purposes in tissue samples (14).

Statistical analyses for discovery-phase serum miRNA quantification

Following normalization, we removed miRPlus sequences and miRNAs listed as obsolete according to miRBase (www.mirbase.org/). miRNA levels were then quantified using the delta C_t method, with fold change \( \frac{2^{(C_t \text{WT} - C_t \text{NB})}}{2^{(C_t \text{WT} - C_t \text{NB})}} \) and \( \frac{2^{(C_t \text{WT} - C_t \text{NB})}}{2^{(C_t \text{WT} - C_t \text{NB})}} \), that had a \( \geq 2.0 \) fold change in expression in the tumor type under consideration compared with the mean expression value from (i) the control group and (ii) the “other tumor” group (comprising all other tumor samples except those under consideration) were called as overexpressed and ranked according to fold change, as described previously (13).

Additional analyses were also performed to increase the stringency of our findings further. First, it was necessary for a miRNA to be detected at least 2 C_t values lower in the test group compared with the ‘no template control’ (NTC) sample to be included in the subsequent data analysis. Second, a coefficient of variation (CV) was calculated for each overexpressed miRNA, using the formula \( CV = SD / n \times n \), where \( n \) was the mean expression value of all miRNAs within that tumor type. This step identified
the level of variation in miRNA expression within the specific tumor type and between the control and ‘other tumor’ group. Third, the Robust Rank Aggregate (RRA) method was employed, as described (ref. 17; Supplementary Materials and Methods). Adjusted RRA scores of $P < 0.01$ were considered significant. Importantly, the RRA method has been used by others in meta-analysis of cancer datasets (18). Differences in serum miRNA expression levels between experimental groups were assessed using a two-tailed $t$ test ($P < 0.05$ significant).

**Serum miRNA qRT-PCR for technical confirmation**

To maximise sensitivity in the technical confirmation study, cDNA was diluted 1:7.5, rather than the standard 1:50 dilution. For this targeted work, following quality control analysis (Supplementary Materials and Methods), data were normalized using the six top-ranking overlapping housekeeping miRNAs identified in the overlap between the geNorm and NormFinder methods in the full discovery-phase qRT-PCR profiling study. Differences in serum miRNA expression levels between experimental groups were assessed using a two-tailed $t$ test ($P < 0.05$ significant).

**Results**

**Quality control and normalization**

For full details of the quality control steps and the assay pipeline developed, see Supplementary Results and Supplementary Figs. S1–S5. In the discovery-phase global qRT-PCR profiling study, expression of the four housekeeping miRNAs (19) and their mean raw $C_v$ values for all samples were very similar to the levels obtained in our initial quality control qRT-PCR (Fig. 1A), and were confirmed by linear regression analysis ($P < 0.0001$). The global mean method, using the average $C_v$ value of miRNAs expressed in at least one of the 33 samples analyzed ($n = 568$), was the optimal normalization approach (Fig. 1B) and was therefore used in the global profiling study. No additional benefit was identified by using either the modified global mean method or the modified global mean method of common miRNAs (Fig. 1B). Use of the single snRNA RNU6 (U6), or the two snRNAs RNU138B and RNU40A, while often used for normalizing microRNA expression in tissue samples (14), were not appropriate methods for normalizing serum data, as they resulted in increased technical variation of the data compared with other normalization methods (Fig. 1B). Assessment of the 10 top-ranking miRNAs using the geNorm and NormFinder algorithms showed only marginal inferiority to the global mean method as a normalization approach.

Normalization using the six top-ranking housekeeping miRNAs common to the top-10 lists generated using NormFinder and geNorm (namely miR-140-3p (chromosomal locus 16q22.1), miR-30b-5p (6q24.22), miR-26a-5p (3p22.2), miR-15b-5p (3q25.33), miR-30c-5p (6q13), and miR-191-5p (3p21.31); Supplementary Fig. S6) also performed well in our analysis (Fig. 1B). Consequently, these six miRNAs were used for normalization purposes for the technical confirmation phase of the study. Five of these miRNAs are also abundant and stably expressed in serum/plasma in adult patients (19). The four housekeeping miRNAs used in the initial QC checks (hkgCQC) also performed well for normalization purposes in the full discovery-phase profiling study (Fig. 1B), indicating their suitability for screening serum samples.

**Serum miRNAs in different tumor types**

No consistent profile of miRNA overexpression common to all tumor groups was identified when compared with the control group. However, for each of the 11 tumor types studied, we identified miRNAs that were overexpressed compared with both the cohort of other childhood tumors and the control group. The number of such miRNAs ranged from 22 to 49 (mean 34), depending on tumor type (Supplementary Tables S1 and S2). The RRA method refined this to 16 to 26 (mean 21) serum miRNAs in each tumor group (Supplementary Table S1). As the MYCN-NB group had the largest coefficient of variance for the delta $C_v$ (miR-23a-3p-miR-451a) hemolysis levels (Supplementary Fig. S1E), we only included miRNAs that were $> 2$-fold greater than the ‘other tumor’ group and control group in both of the MYCN-NB samples being investigated. This reduced the number of miRNAs called as overexpressed in MYCN-NB from 96 to 35 (Supplementary Table S1), avoiding identification of serum microRNAs that were released from red blood cells rather than being disease associated.

The lists of overexpressed serum miRNAs for each tumor group are shown in Supplementary Tables S3 to S13. Selected findings for the individual tumor groups were used to generate an expression heatmap (Fig. 1C). miRNAs for the heatmap were chosen based on their overall abundance and potential value in differential diagnosis. For two miRNAs (miR-122 and miR-877), the $-5p$ strands were selected rather than the $-3p$ strands identified in the global profiling study, due to the greater abundance of the $-5p$ strands (20) (Supplementary Fig. S7). Ten miRNAs from the heatmap were chosen for subsequent validation in the technical confirmation study (italics in Fig. 1C; Supplementary Table S2), along with the six top-ranking housekeeping miRNAs that overlapped in the NormFinder and geNorm lists. In the technical confirmation study, the 1:7.5 cDNA dilution increased sensitivity and did not inhibit the PCR reaction. As expected, the 16 miRNAs were detected at approximately 3 $C_v$ values lower in the 1:7.5 dilutions compared with the 1:50 dilutions (Supplementary Fig. S8). The suitability of all 25 samples for the technical confirmation study was verified by assessment of triplicate U6snP6 values (Supplementary Fig. S9A). Expression levels of the six housekeeping miRNAs were assessed in technical triplicate and showed high consistency (Supplementary Fig. S9B).

Taken together, our data strongly suggest important clinical applications based on serum miRNA quantification. The most promising are described in the following sections.

**MYCN-amplified high-risk neuroblastoma versus non–MYCN-amplified low-risk neuroblastoma**

Treatment schedules for neuroblastoma rely on distinguishing tumors by the presence or absence of INRG high-risk molecular
abnormalities, such as MYCN amplification (11). Interestingly, our most striking findings were found in the MYCN-NB group. The five overexpressed serum miRNAs (miR-124-3p/miR-9-3p/miR-218-5p/miR-490-5p/miR-1538) that were top-ranking compared with both the ‘other tumor’ group (including NB samples) and the controls (Supplementary Table S3) are shown in the heatmap (Fig. 1C, panel 1). Levels of these five miRNAs in individual tumor types in the discovery-phase test set are highlighted in boxplot analysis (Fig. 2A). Significant overexpression of all five miRNAs in the MYCN-NB samples versus the ‘other tumor’ group was shown in the subsequent confirmatory qRT-PCR experiments, performed in technical triplicates (P<0.05 for all comparisons; Fig. 2B). In a focussed ‘differential diagnosis’ analysis of the MYCN-NB versus the NB group in the confirmatory study, there was a significant difference in expression levels for miR-124-3p and miR-9-3p individually (P < 0.05; Fig. 2C). In addition, in the technical confirmation set, the panel of all five miRNAs successfully distinguished MYCN-NB from NB samples (P = 0.031; Fig. 2C).

Hepatoblastoma versus all neuroblastomas (MYCN-NB/NB)

In children presenting with a liver tumor/enlargement, it may be important to distinguish a primary lesion, such as HB, from involvement by a tumor from elsewhere, for example, neuroblastoma. For HB, of the 49 overexpressed serum miRNAs, miR-483-3p, miR-122-3p, and miR-205-5p were ranked in the top 10 by fold change (Supplementary Table S5), with similar fold changes versus the controls and ‘other tumor’ group. Because of its greater abundance (Supplementary Fig. S7; ref. 20), miR-122-5p, rather than miR-122-3p, was selected for confirmatory testing along with miR-483-3p and miR-205-5p (Fig. 1C, panel 2). The findings for these three miRNAs in individual tumor types in the discovery-phase test set are highlighted by boxplot analysis (Fig. 3A). Significant overexpression of each of the three miRNAs in the HB group was verified in the technical confirmation study, versus both the ‘other tumor’ group (which included all eight MYCN-NB/NB samples, two of which showed liver involvement) and the control group (P < 0.05 for all comparisons; Fig. 3B). For the neuroblastomas, there was no association between liver involvement and greater abundance of the HB-associated miRNAs. It was however noted that levels of the liver-specific miR-122-5p (21) were occasionally increased in non-HB samples, for example, in a case of pancreatic RMS presenting with obstructive jaundice (RMS-2; Fig. 3B). Accordingly, miR-122-5p needed to be a part of a larger panel to ensure sufficient specificity for HB. In focussed ‘differential diagnosis’ plots for the HB group versus the combined MYCN-NB/NB group, all three miRNAs were individually significantly elevated in HB (P < 0.05 for all comparisons; Fig. 3C). Furthermore, the three miRNA panel also distinguished HB from MYCN-NB/NB (P = 0.0001; Fig. 3C).

Wilms tumor versus all neuroblastomas (MYCN-NB/NB)

Because of their anatomical proximity, it may be clinically and radiologically difficult to distinguish WT from neuroblastoma; indeed, some neuroblastomas may be intrarenal (22, 23). Because of the different management and prognosis of these two tumor types, it is important to differentiate them diagnostically (23). We screened our profiling data from the discovery-phase test set for miRNAs that might be informative in this differential diagnosis setting. We identified miR-129-5p (overexpressed in both the MYCN-NB and NB lists; Supplementary Tables S3 and S4, respectively) and miR-143-3p (10.7 greater fold change in levels in WT compared with the combined MYCN-NB/NB samples; data not shown) for subsequent testing (Fig. 1C, panel 4 and Fig. 4A). The technical confirmation study established that miR-143-3p was increased in WT compared with the ‘other tumor’ group (P = 0.003), with miR-129-5p being overexpressed in the majority of MYCN-NB/NB cases (Fig. 4B). In ‘differential diagnosis’ plots, miR-143-3p distinguished WT from MYCN-NB/NB (P = 0.0005), with miR-129-5p being overexpressed in six of eight MYCN-NB/NB cases versus WT (Fig. 4C).

Sarcoma differential diagnosis

Sarcomas may present with bone lesions (ES/OS), soft tissue masses (RMS/ES), or both (ES). Consequently, discriminating these tumors is important, but may be challenging. Serum miRNAs that were overexpressed for RMS, ES, and OS are listed in Supplementary Tables S7 to S9, respectively. Overall levels of representative miRNAs specific for RMS, ES, and OS versus the other sarcoma subtypes are illustrated in Fig. 1C, (panels 5–7, respectively). In particular, overall analysis (Fig. 5A) and ‘differential diagnosis’ plots of the global profiling data (i.e., discovery-phase test set; Fig. 5B) showed that miR-214-3p, miR-214-5p, and miR-92b-3p individually segregated ES from RMS/OS (P < 0.05 for all comparisons), as did the three miRNA panel (P = 0.0098; Fig. 5B). For OS, miR-500a-5p, miR-512-5p, and miR-519a-3p showed much higher serum expression than other tumors, including ES (Supplementary Fig. S10).

Discussion

We report a robust, quality controlled pipeline suitable for quantifying serum miRNA levels in pediatric patient cohorts (and potentially adult samples), including the assessment of RNA extraction, degree of sample hemolysis and qRT-PCR efficiency. The described approach minimizes technical alterations and maximizes true biologic variation between samples, to allow identification of lists of overexpressed serum miRNAs between study groups, as exemplified here for common solid tumors of childhood. We assessed multiple normalization approaches and identified that for high-throughput global serum miRNA qRT-PCR data, the ‘global mean’ method (14) was optimal. In addition, we generated a panel of six housekeeping miRNAs that were most stable in pediatric serum samples and therefore suitable for normalizing qRT-PCR data in more targeted low-throughput studies. Interestingly, the six miRNAs showed substantial overlap with findings from adult samples (19).

The most striking tumor findings were for MYCN-amplified high-risk neuroblastoma (MYCN-NB). The blood-based miRNA panel identified here has potential clinical value, due to the current requirement for surgical biopsy to confirm the diagnosis of neuroblastoma and test for INRG high-risk genomic changes, such as MYCN amplification (11). Very recently, qRT-PCR detection of neuroblastoma-specific miRNAs in peripheral blood from children at diagnosis of advanced stage neuroblastoma has been reported, with high levels of TH and PHOXB2 representing clinically useful biomarkers of risk (24). However, potential blood-based mRNA biomarkers can be subject to considerable
Figure 3.
Serum miRNA expression in hepatoblastoma (HB) versus all neuroblastomas (MYCN-NB/NB). A, raw Ct values (y-axis) of three highly overexpressed miRNAs in HB (miR-483-3p, miR-205-5p, and miR-122-5p) relative to other tumors and controls in the discovery-phase test set. B, mean normalized relative expression values for the miRNAs listed in A above, in HB samples versus 'other tumors' and control samples, in the confirmatory qRT-PCR study. C, mean normalized relative expression values for the miRNAs listed in A above, in HB samples versus MYCN-NB/NB samples, in the technical confirmation qRT-PCR study. In addition, an equally weighted overall mean-adjusted relative expression value for the specific three miRNA panel was calculated. Error bars, SEM. For tumor abbreviations, see Table 1.
variation in levels, for technical as well as biologic reasons (25, 26). In particular, mRNAs are inherently unstable at room temperature and rapidly degrade in blood samples that are not stored correctly (25, 26). In contrast, serum miRNAs offer particular advantages as blood-based biomarkers as they are not prone to such technical variations (10).

The MYCN-NB–specific serum miRNAs identified here are of biologic relevance. It has been known for some time that MYCN amplification status of neuroblastoma tissue samples determines global miRNA profiles (27) and our findings are in keeping with this observation. miR-124-3p is the most abundant neuronal-specific miRNA and silencing of miR-124 in neuroblastoma cells in vitro resulted in their differentiation (28). In addition, expression of miR-9 is activated by MYCN protein, which directly binds to the promotor region of this miRNA (29). In neuroblastoma tissues, high miR-9 levels correlated with MYCN amplification, tumor grade, and metastatic status (29).

Other tumor-specific serum miRNAs are also of biologic significance. For example, miR-122 is highly abundant in liver tissues and is considered liver specific. Recently, reduced miR-122 expression has been shown in HB compared with normal liver tissue (30). Here, we found elevated serum levels of miR-122-5p in HB samples compared with other tumors and controls. Downregulation of miR-122 in HB (compared with normal liver tissue; ref. 30), but detection at elevated levels in the serum of HB patients is consistent with observations in other tumors (13), and may reflect passive miRNA leakage from tumor cells. It should be noted that miR-122 is a general nonspecific marker of liver injury, and is increased in the serum in jaundiced patients, for example (31). Therefore, a wider panel of serum miRNAs, as identified in this study, is likely to offer increased specificity for HB compared with other tumors and disease states. Further discussion of the biologic relevance of our findings in individual tumor types is provided in the Supplementary Discussion.

As the number of samples assessed for each of the tumor subtypes is small in the current proof-of-principle study, it will be important to confirm our findings in larger, prospective investigations. The fact that the panel of serum miRNAs that distinguished the initial group (n = 4) of MYCN-NB from NB patients was confirmed in a small independent panel of patient samples (n = 4) highlights that these changes are promising and worthy of further testing. If confirmed in future studies, we propose that the panels of childhood solid tumor-associated serum miRNAs identified here represent useful candidate biomarkers for improving the accuracy of pediatric cancer diagnosis. Such serum markers may reduce or obviate the need for diagnostic histologic biopsy and the associated risks of anaesthesia and surgery. Furthermore, compared with the labor-intensive diagnostic techniques in current clinical use, a qRT-PCR approach, based on the analysis pipeline reported here, is likely to be more cost-effective, thereby offering health economic as well as clinical benefits.

Figure 4.
Serum miRNA expression in Wilms tumor (WT) versus all neuroblastomas (MYCN-NB/NB). A, raw C. values (y-axis) of two miRNAs (miR-129-5p and miR-143-3p) relative to other tumors and controls in the discovery-phase test set. B and C, mean normalized relative expression values in MYCN-NB/NB (miR-129-5p) or WT (miR-143-3p) samples versus (B) ‘other tumors’ and control samples or (C) each other, in the technical confirmation qRT-PCR study. Error bars, SEM. For tumor abbreviations see Table 1.
Figure 5.
Serum microRNA expression in sarcoma subtypes. A, raw Ct values (y-axis) of three selected miRNAs (miR-214-3p/miR-214-5p/miR-92b-3p) in Ewing sarcoma samples compared with other sarcoma samples (OS/RMS) in the discovery-phase test set. B, ‘Differential diagnosis’ plots for ES versus RMS/OS in the discovery-phase test set. In addition, an equally weighted overall mean-adjusted relative expression value for the specific three miRNA panel was calculated. Error bars, SEM. For tumor abbreviations see Table 1.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M.J. Murray, N. Coleman
Development of methodology: M.J. Murray, K.L. Raby, H.K. Saini
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.J. Murray, K.L. Raby, H.K. Saini, S.V. Wool, J.M. Tunnacliffe, J.C. Nicholson
Writing, review, and/or revision of the manuscript: M.J. Murray, K.L. Raby, H.K. Saini, S. Bailey, J.C. Nicholson, N. Coleman
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.J. Murray, K.L. Raby, H.K. Saini, S.V. Wool, J.M. Tunnacliffe, A.J. Enright, N. Coleman
Study supervision: M.J. Murray, N. Coleman

References

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