

Serum microRNAs in childhood tumors

Solid tumors of childhood display specific serum microRNA profiles

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Abstract

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

Methods: We undertook global quantitative RT-PCR microRNA profiling (n=741) on RNA extracted 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 that the ‘*global-mean*’ approach was optimal. We generated a panel of six microRNAs that were most stable in pediatric serum samples and therefore most suitable for normalization of targeted microRNA qRT-PCR data. Tumor-specific serum microRNA profiles were identified for each tumor type and selected microRNAs underwent confirmatory testing. We identified a panel of microRNAs (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 over-expressed in *MYCN*-amplified high-risk cases (*MYCN*-NB). We also derived candidate microRNA panels for non-invasive 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 microRNA profiling in solid tumors of childhood, and has identified candidate microRNA profiles for prospective testing. *Impact:* We propose a new non-invasive method to diagnose childhood solid tumors.

Introduction

Many solid tumors affecting children rely solely on biopsy for diagnosis. Even where diagnostic body fluid markers exist (e.g. urinary catecholamines in neuroblastoma), biopsy is needed to estimate prognosis, including assessment of *MYCN*-amplification and segmental chromosomal imbalances (1). The identification of novel body fluid tumor markers that may allow non-invasive diagnosis or risk stratification in solid tumors of childhood would be an important advance. One promising approach is measurement of serum microRNA levels.

MicroRNAs are short, non-protein-coding RNAs that post-transcriptionally regulate gene expression (2). Importantly, global microRNA profiles have been shown to classify human cancer tissues, including pediatric solid tumors (2,3). In pediatric malignant germ cell tumors, for example, the miR-371~373 and miR-302/367 clusters are over-expressed in all cases, regardless of patient age, histologic subtype or anatomic site (2) and thus may represent a universal biomarker of this disease. Packaging of specific microRNAs in membrane-bound exosome particles by tumor cells before release into the bloodstream allows for their detection in the serum (4). Indeed, we showed that microRNAs 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 (4-6), findings recently replicated by others (7,8). Importantly, these profiles can detect disease at exceptionally low tumor volumes, demonstrating the sensitivity of this approach (6). MicroRNAs have many qualities that make them suitable tumor markers for translation into clinical practice, including inherent stability and resistance to degradation, even if samples are left at room temperature or subjected to multiple freeze-thaw cycles (9). We therefore hypothesized that other childhood tumors would be characterized by the release

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of specific microRNAs 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. We addressed three important questions: whether it was feasible to extract RNA from the serum of children in sufficient amounts to undertake global microRNA profiling; whether serum housekeeping microRNAs in children would be similar to those described for adults and thus suitable for normalization purposes, and whether lists of candidate biomarkers could be generated for validation in larger, prospective studies. We undertook global quantitative reverse transcription polymerase chain reaction (qRT-PCR) microRNA profiling in serum samples taken from patients at the time of diagnosis of common childhood cancers (n=33) and compared the results with profiles from age- and gender-matched pediatric control samples (n=20). We describe in detail the detection and analysis pipeline used to make these observations, including highly robust quality control and novel normalization steps, essential for translation of these findings into clinical practice. We identify profiles specific for each of the 11 types of pediatric solid tumor investigated. In particular, we report a panel of serum microRNAs that segregate *MYCN*-amplified International Neuroblastoma Risk Group (INRG) (1) 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 (MIQE) (10) (see Supplementary Materials and Methods).

Patient demographics and tumor types analyzed

For the main discovery-phase of the project, we initially recruited 34 patients aged 0-16 years (y) at the time of malignant tumor diagnosis (between April 2010 and June 2013), together with a further 20 anonymized samples from a control group of patients without malignant disease. Clinico-pathological details are listed in Table 1. The tumor group included 18 (53%) male patients and 16 (47%) females with a median age \pm standard deviation of 63 ± 59 months. There were no significant differences in age between the male and female patients within the tumor group ($p=0.35$; two-tailed unpaired *t*-test). The control group included 11 (55%) male patients and 9 (45%) females with a median age of 49 ± 53 months. There were no significant differences in age between the male and female patients within the control group ($p=0.92$). There were also no significant differences by age between the tumor and control groups ($p=0.74$). 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 hepatoblastomas (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's lymphoma (B-NHL)]; v) six sarcomas [three rhabdomyosarcoma (RMS), two Ewings sarcoma (ES), one osteosarcoma (OS)]; vi) one *DICER1*-mutated pleuropulmonary

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blastoma (PPB) (11) 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 ganglio-neuroblastoma (Table 1).

Data normalization

For details of sample processing, microRNA qRT-PCR profiling and the extensive quality control (QC) steps performed, see Supplementary Materials and Methods. Only a single sample (WT-4) failed initial QC; 53 samples (98%) therefore underwent full qRT-PCR profiling. In initial work, we compared a number of different data normalization methods (12) to define the optimal approach to our global serum microRNA profiling study. We assessed:

- a) the *global mean method*, which used the average Ct value of microRNAs expressed in at least one of the 53 samples analyzed, as described (12);
- b) 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 (12);

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- c) the *modified global mean method of common microRNAs* (12), i.e. those microRNAs expressed in all 53 samples analyzed;
- d) the *ten top-ranking microRNAs* identified as most stably expressed in the study by *geNorm* (13);
- e) the *ten top-ranking microRNAs* identified as most stably expressed in the study by *NormFinder* (14);
- f) the *six top-ranking overlapping housekeeping microRNAs* identified in both d) and e) above;
- g) the *four housekeeping microRNAs* used for initial QC purposes (hkgsQC), namely miR-23a-3p, miR-30c-5p, miR-103a-3p and miR-191-5p;
- h) the *two small nucleolar RNAs* (snoRNAs), RNU38B and RNU49A, present on the Exiqon platform;
- i) the *single small nuclear RNA* (snRNA) RNU6, present on the platform. Both snoRNAs and snRNAs are commonly used for microRNA normalization purposes in tissue samples (12).

Statistical analyses for discovery-phase serum microRNA quantification

Following normalization, we removed miRPlus sequences and microRNAs listed as obsolete according to miRBase v20 (June 2013), the most recent version of the online microRNA registry (www.mirbase.org/). MicroRNA levels were then quantified using the delta Ct method, with fold-change = $2^{(\text{Tumor Ct} - \text{mean 'other tumor' samples Ct})}$ and $2^{(\text{Tumor Ct} - \text{mean control samples Ct})}$. MicroRNAs that had a ≥ 2.0 fold-change in expression in the tumor type under consideration compared with the mean expression value from a) the control group and b) the 'other tumor' group (comprising all other tumor samples except those under consideration) were called as over-expressed and ranked according to fold-change, as described (11).

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Additional analyses were also performed to increase the stringency of our findings further. Firstly, it was necessary for a microRNA to be detected at least 2 Ct values lower in the test group compared with the ‘no template control’ (NTC) sample to be included in the subsequent data analysis. Secondly, a co-efficient of variation (CV) was calculated for each over-expressed microRNA, using the formula $CV = \text{standard deviation for that microRNA} / n$, where n was the mean expression value of all microRNAs within that tumor type. This step identified the level of variation in microRNA expression within the specific tumor type and between the control and ‘other tumor’ group. Thirdly, the Robust Rank Aggregate (RRA) method was employed, as described (15). For this analysis, samples were processed using the BioConductor package ‘*RobustRankAggreg*’ in the statistical software environment *R*. This algorithm detected microRNAs that were ranked consistently higher than expected under the null hypothesis. This was performed by studying the position of each microRNA in the over-expressed list (ranked by fold-change) for each specific tumor type, compared with the ‘other tumor’ and control group. A significance (p -value) score was subsequently assigned for each gene, which calculated how much higher it was positioned in the ranked list than expected by chance, as described (15). This p -value was adjusted for multiple testing using a Bonferroni correction and was used both for re-ranking the genes and as an indicator of the significance of each gene. Adjusted scores of $p < 0.01$ were considered significant. Importantly, the RRA method has been used by others in meta-analysis of cancer datasets (16). Differences in serum microRNA expression levels between experimental groups were assessed using a two-tailed t -test ($p < 0.05$ significant).

Serum microRNA qRT-PCR for technical confirmation

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To maximise sensitivity in the technical confirmation study, cDNA was diluted 1:7.5, rather than the standard 1:50 dilution. To ensure robustness for this phase of the study, previously extracted RNA from each serum sample was assessed in technical triplicate. Each RT product was analyzed by PCR, using the cycling conditions adopted in the discovery-phase full profiling study. The means of the three triplicate values for each microRNA for each sample were calculated. For this targeted work, following QC analysis (Supplementary Materials and Methods), data were normalized using the six top-ranking overlapping housekeeping microRNAs identified in the overlap between the *geNorm* and *NormFinder* methods in the full discovery-phase qRT-PCR profiling study. Differences in serum microRNA expression levels between experimental groups were assessed using a two-tailed *t*-test ($p < 0.05$ significant).

Results

QC and normalization

For full details of the QC steps and the assay pipeline developed, see Supplementary Results and Supplementary Figures S1 to S5. In the discovery-phase global qRT-PCR profiling study, expression of the four housekeeping microRNAs (17) and their mean raw Ct values for all samples were very similar to the levels obtained in our initial QC qRT-PCR (Figure 1A), and were confirmed by linear regression analysis ($p < 0.0001$). The *global mean method*, using the average Ct value of microRNAs expressed in at least one of the 53 samples analyzed ($n=568$), was the optimal normalization approach (Figure 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 microRNAs* (Figure 1B). Use of the *single snRNA* RNU6 (U6), or the *two snoRNAs* RNU38B and RNU49A (snoRNAs), whilst often used for normalizing microRNA expression in tissue samples (12), were not appropriate methods for normalizing serum data, as they resulted in increased technical variation of the data compared with other normalization methods (Figure 1B). Assessment of the *ten top-ranking microRNAs* 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 microRNAs* out of those that were common to the top-ten lists generated using *NormFinder* and *geNorm* [namely miR-140-3p (chromosomal locus 16q22.1), miR-30b-5p (8q24.22), miR-26a-5p (3p22.2), miR-15b-5p (3q25.33), miR-30c-5p (6q13) and miR-191-5p(3p21.31)] (Supplementary Figure S6) also performed well in our analysis (Figure 1B). Consequently, these six microRNAs were used for normalization purposes for the technical confirmation phase of the study, where only selected findings were being explored. All six of these top-ranking housekeeping

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microRNAs were transcribed from different genomic loci, adding strength to the suitability of this panel of microRNAs for normalizing all serum expression data, including from adult and pediatric patients. Indeed, five of the six microRNAs were previously reported as being abundant and stably expressed in serum and plasma in adult patients (17). Furthermore, two of the six microRNAs (miR-30c-5p and miR-191-5p) overlapped with the four housekeeping microRNAs used for the initial qRT-PCR QC analyses. It should also be noted that the *four housekeeping microRNAs* used in the initial QC checks (hkgsQC) also performed well for normalization purposes in the full discovery-phase profiling study (Figure 1B), indicating their suitability for screening serum samples.

Serum microRNAs in different tumor types

No consistent profile of microRNA over-expression common to all tumor groups was identified when compared with the control group. However, for each of the 11 tumor types studied, we identified microRNAs that were over-expressed compared with both the cohort of other childhood tumors and the control group. The number of such microRNAs ranged from 22 to 49 (mean 34), depending on tumor type (Supplementary Tables S1 and S2). Using the RRA method, the lists were refined to 16 to 26 (mean 21) serum microRNAs in each tumor group (Supplementary Table S1). As the MYCN-NB group had the largest coefficient of variance for the delta Ct (miR-23a-3p - miR451a) hemolysis levels (Supplementary Figure S1E), we only included microRNAs that were ≥ 2 fold greater than the ‘other tumor’ group and control group in both of the MYCN-NB samples being interrogated. This reduced the number of microRNAs called as over-expressed 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.

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The lists of over-expressed serum microRNAs 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 (Figure 1C). MicroRNAs for the heatmap were chosen based on their overall abundance and potential value in differential diagnosis. For two microRNAs (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 (18) (Supplementary Figure S7). Ten microRNAs from the heatmap were chosen for subsequent validation in the technical confirmation study (shown in italics in Figure 1C and listed in Supplementary Table S2), along with the six top-ranking housekeeping microRNAs 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 microRNAs were detected at approximately 3 Ct values lower in the 1:7.5 dilutions compared with the 1:50 dilutions (Supplementary Figure S8). The suitability of all 25 samples for the technical confirmation study was verified by assessment of triplicate UniSp6 values (Supplementary Figure S9A). Expression levels of the six housekeeping microRNAs were assessed in technical triplicate and showed high consistency (Supplementary Figure S9B).

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

***MYCN*-amplified high-risk neuroblastoma (MYCN-NB) vs. non-*MYCN*-amplified low-risk neuroblastoma (NB).**

Treatment schedules for neuroblastoma rely on distinguishing tumors by the presence or absence of INRG high-risk molecular abnormalities, such as *MYCN*-amplification (1). Interestingly, our most striking findings were found in the *MYCN*-NB group. The five

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over-expressed serum microRNAs (miR-124-3p, miR-9-3p, miR-218-5p, miR-490-5p and 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 (Figure 1C, panel 1). Levels of these five microRNAs in individual tumor types in the discovery-phase test set are highlighted in boxplot analysis (Figure 2A). Significant over-expression of all five microRNAs in the MYCN-NB samples versus the ‘other tumor’ group was shown in the subsequent confirmatory qRT-PCR experiments, performed in technical triplicate ($p < 0.05$ for all comparisons) (Figure 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 ($p < 0.05$) (Figure 2C). In addition, in the technical confirmation set, the panel of all five microRNAs successfully distinguished MYCN-NB from NB samples ($p = 0.031$) (Figure 2C).

Hepatoblastoma (HB) vs. 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 e.g. neuroblastoma. For HB, of the 49 over-expressed serum microRNAs, miR-483-3p, miR-122-3p and miR-205-5p were ranked in the top ten by fold-change (Supplementary Table S5), with similar fold-changes versus the controls and ‘other tumor’ group. Due to its greater abundance (Supplementary Figure S7) (18), miR-122-5p, rather than miR-122-3p, was selected for confirmatory testing along with miR-483-3p and miR-205-5p (Figure 1C, panel 2). The findings for these three microRNAs in individual tumor types in the discovery-phase test set are highlighted by boxplot analysis (Figure 3A). Significant over-expression of each of the three microRNAs in the HB group was verified in the technical confirmation study, versus both the ‘other tumor’ group (which included all eight MYCN-

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NB/NB samples, two of which showed liver involvement) and the control group ($p < 0.05$ for all comparisons) (Figure 3B). For the neuroblastomas, there was no association between liver involvement and greater abundance of the HB-associated microRNAs. It was however noted that levels of the liver-specific miR-122-5p (19) were occasionally increased in non-HB samples, for example, in a case of pancreatic RMS presenting with obstructive jaundice (RMS-2) (Figure 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 microRNAs were individually significantly elevated in HB ($p < 0.05$ for all comparisons) (Figure 3C). Furthermore, the three microRNA panel also distinguished HB from MYCN-NB/NB ($p = 0.0001$) (Figure 3C).

Wilms tumor (WT) vs. all neuroblastomas (MYCN-NB/NB).

Due to their anatomical proximity, it may be difficult both clinically and radiologically to distinguish WT from neuroblastoma; indeed, some neuroblastomas may be intra-renal (20, 21). Due to the different management and prognosis of these two tumor types, it is important to differentiate them diagnostically (21). We screened our profiling data from the discovery-phase test set for microRNAs that might be informative in this differential diagnosis setting. We identified miR-129-5p (over-expressed 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 (Figure 1C, panel 4 and Figure 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 over-expressed in the majority of MYCN-NB/NB cases (Figure 4B). In ‘differential diagnosis’ plots, miR-143-3p

Serum microRNAs in childhood tumors distinguished WT from MYCN-NB/NB ($p=0.0005$), with miR-129-5p being over-expressed in six of eight MYCN-NB/NB cases versus WT (Figure 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 microRNAs that were over-expressed for RMS, ES and OS are listed in Supplementary Tables S7-S9, respectively. Overall levels of representative microRNAs specific for RMS, ES and OS versus the other sarcoma subtypes are illustrated in Figure 1C, (panels 5-7, respectively). In particular, overall analysis (Figure 5A) and ‘differential diagnosis’ plots of the global profiling data (i.e. discovery-phase test set) (Figure 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 microRNA panel ($p=0.0098$) (Figure 5B). For OS, miR-500a-5p, miR-512-5p and miR-519a-3p showed much higher serum expression than other tumors, including ES (Supplementary Figure S10).

Discussion

We report a robust, quality controlled pipeline suitable for quantifying serum microRNA 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 biological variation between samples, to allow identification of lists of over-expressed serum microRNAs 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 microRNA qRT-PCR data, the ‘*global mean*’ method (12) was optimal. In addition, we generated a panel of six housekeeping microRNAs 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 microRNAs showed substantial overlap with findings from adult samples (17).

The most striking tumor findings were for *MYCN*-amplified high-risk neuroblastoma (*MYCN*-NB). The blood-based microRNA panel identified here has clinical relevance, due to the current requirement for surgical biopsy to confirm the diagnosis and look for INRG high-risk genomic changes, such as *MYCN*-amplification (1). Very recently, qRT-PCR detection of neuroblastoma-specific messenger RNAs (mRNAs) in peripheral blood from children at diagnosis of advanced stage neuroblastoma have been reported, with high levels of *TH* and *PHOXB2* representing clinically useful biomarkers of risk (22). However, potential blood-based mRNA biomarkers can be subject to considerable variation in levels for technical as well as biological reasons (23,24). In particular, mRNAs are inherently unstable at room temperature and rapidly degrade in blood samples that are not stored correctly (23, 24). In contrast, serum microRNAs offer particular advantages as blood-based biomarkers as they are not prone to such technical variations (9).

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The MYCN-NB-specific serum microRNAs identified here are of biological relevance. It has been known for some time that *MYCN* amplification status of neuroblastoma tissue samples determines global microRNA profiles (25) and our findings are in keeping with this observation. MiR-124-3p is the most abundant neuronal-specific microRNA and silencing of miR-124 in neuroblastoma cells *in vitro* resulted in their differentiation (26). In addition, expression of miR-9 is activated by MYCN protein, which directly binds to the promoter region of this microRNA (27). In neuroblastoma tissues, high miR-9 levels correlated with *MYCN*-amplification, tumor grade and metastatic status (27).

Other tumor-specific serum microRNAs are of biological relevance. 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 (28). Here, we found elevated serum levels of miR-122-5p in HB samples compared with other tumors and controls. Down-regulation of miR-122 in HB (compared with normal liver tissue) (28), but detection at elevated levels in the serum of HB patients is consistent with other observations (11). Passive leakage of microRNAs from tumor cells into the bloodstream, due to abnormal cellular function, may account for these findings. Alternatively, there may be active and selective microRNA release mechanisms from tumor cells, which may subsequently promote an environment suitable for tumor cell growth and metastasis (11). It should be noted that miR-122 is a general non-specific marker of liver injury, and is increased in the serum in jaundiced patients, for example (29). Therefore, a wider panel of serum microRNAs, as identified in this study, is likely to offer increased specificity for HB compared with other tumors and disease states. Of note, one of the additional microRNAs, in the panel that we identified, miR-205, has been shown contribute to hepatocyte differentiation (30).

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The serum panel for Wilms tumor (WT) included miR-141-5p, which has been reported to be abundant in normal mature renal tissue and down-regulated in WT (31). The elevated serum miR-141-5p levels at diagnosis in WT patients are likely to be due to the same cellular microRNA release mechanisms suggested for miR-122-5p in HB above. For RMS, serum levels of miR-183-5p were elevated at diagnosis. This is consistent with over-expression of miR-183-5p in RMS tissues, leading to down-regulation of the tumor suppressor transcription factor *EGR1* and increased cell migration (32). For osteosarcoma (OS), seven of the 44 (16%) over-expressed serum microRNAs were from the large microRNA cluster on chromosome 19, at 19q13, termed C19MC, including miR-512-5p and miR-519a-3p. Serum levels of these microRNAs were very low indeed in other tumor groups and control samples. Interestingly, genomic amplification at 19q13 occurs frequently in OS and may account for these findings (33).

Our detection of a panel of differentially expressed serum microRNAs in central nervous system gliomas (GL) (Supplementary Table S13) is likely to reflect disturbance of the blood brain barrier, allowing transfer of microRNAs into the bloodstream. Indeed, similar findings have been reported recently (34). The serum GL panel included miR-155-5p and miR-124-3p. In GL tissues, miR-155 levels were noted to be higher than in normal brain, associated with higher pathological grade and poor clinical outcomes, revealing miR-155 expression as a potential independent prognostic factor and therapeutic target for human glioma (35). Furthermore, in a study investigating altered microRNA expression during GL malignant transformation, both miR-155-5p (increased) and miR-124-3p (decreased) showed altered expression between GL stem cells and neural stem cells (36). Interestingly, apart from MYCN-NB samples, the GL group was the only other in which we could detect miR-124-3p in the serum (Figure 2, panel 1). MiR-124-3p is under-expressed in pediatric

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GL (pilocytic astrocytoma) compared with non-neoplastic brain tissue (37), and again detection in the serum is likely due to active release or passive leak of miR-124-3p from the GL tumor cells across the disrupted blood brain barrier.

As the number of samples assessed for each of the tumor subtypes is small in the present proof-of-principle study, it will be important to confirm our findings in larger, prospective investigations. The fact that the panel of serum microRNAs 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 microRNAs identified here represent useful candidate biomarkers for improving the accuracy of pediatric cancer diagnosis. Such markers may reduce or obviate the need for diagnostic histological 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.

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References

1. Ambros PF, Ambros IM, Brodeur GM, Haber M, Khan J, Nakagawara A, et al. International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee. *Br J Cancer*. 2009;100:1471-82.
2. Palmer RD, Murray MJ, Saini HK, van Dongen S, Abreu-Goodger C, Muralidhar B, et al. Malignant germ cell tumors display common microRNA profiles resulting in global changes in expression of messenger RNA targets. *Cancer Res*. 2010;70:2911-23.
3. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435:834-8.
4. Murray MJ, Halsall DJ, Hook CE, Williams DM, Nicholson JC, Coleman N. Identification of MicroRNAs From the miR-371~373 and miR-302 Clusters as Potential Serum Biomarkers of Malignant Germ Cell Tumors. *Am J Clin Pathol*. 2011;135:119-25.
5. Gillis AJ, Rijlaarsdam MA, Eini R, Dorssers LC, Biermann K, Murray MJ, et al. Targeted serum miRNA (TSmiR) test for diagnosis and follow-up of (testicular) germ cell cancer patients: A proof of principle. *Mol Oncol*. 2013;7:1083-92.
6. Murray MJ, Coleman N. Testicular cancer: a new generation of biomarkers for malignant germ cell tumours. *Nat Rev Urol*. 2012;9:298-300.
7. Belge G, Dieckmann KP, Spiekermann M, Balks T, Bullerdiek J. Serum levels of microRNAs miR-371-3: a novel class of serum biomarkers for testicular germ cell tumors? *Eur Urol*. 2012;61:1068-9.
8. Dieckmann KP, Spiekermann M, Balks T, Flor I, Loning T, Bullerdiek J, et al. MicroRNAs miR-371-3 in serum as diagnostic tools in the management of testicular germ cell tumours. *Br J Cancer*. 2012;107:1754-60.

Serum microRNAs in childhood tumors

9. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008;105:10513-8.
10. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009;55:611-22.
11. Murray MJ, Bailey S, Raby KL, Saini HK, de Kock L, Burke GA, et al. Serum levels of mature microRNAs in DICER1-mutated pleuropulmonary blastoma. *Oncogenesis*. 2014;3:e87.
12. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, et al. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol*. 2009;10:R64.
13. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3:RESEARCH0034.
14. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*. 2004;64:5245-50.
15. Kolde R, Laur S, Adler P, Vilo J. Robust rank aggregation for gene list integration and meta-analysis. *Bioinformatics*. 2012;28:573-80.
16. Vosa U, Vooder T, Kolde R, Vilo J, Metspalu A, Annilo T. Meta-analysis of microRNA expression in lung cancer. *Int J Cancer*. 2013;132:2884-93.
17. Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, Wrang Teilum M, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. *Methods*. 2013;59:S1-6.

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18. Anglesio MS, Wang Y, Yang W, Senz J, Wan A, Heravi-Moussavi A, et al. Cancer-associated somatic DICER1 hotspot mutations cause defective miRNA processing and reverse-strand expression bias to predominantly mature 3p strands through loss of 5p strand cleavage. *J Pathol.* 2013;229:400-9.
19. Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, et al. LNA-mediated microRNA silencing in non-human primates. *Nature.* 2008;452:896-9.
20. Kessler OJ, Siegel JF, Brock WA. Intrarenal neuroblastoma masquerading as Wilms' tumor. *Urology.* 1998;51:313-6.
21. Sellaturay SV, Arya M, Banisadr S, Murthi GV, Sebire NJ, Duffy PG. Primary intrarenal neuroblastoma: a rare, aggressive tumour of childhood mimicking Wilms' tumour. *J Pediatr Urol.* 2006;2:522-4.
22. Viprey VF, Gregory WM, Corrias MV, Tchirkov A, Swerts K, Vicha A, et al. Neuroblastoma mRNAs predict outcome in children with stage 4 neuroblastoma: a European HR-NBL1/SIOPEN study. *J Clin Oncol.* 2014;32:1074-83.
23. Rainen L, Oelmueller U, Jurgensen S, Wyrich R, Ballas C, Schram J, et al. Stabilization of mRNA expression in whole blood samples. *Clin Chem.* 2002;48:1883-90.
24. Viprey VF, Corrias MV, Kagedal B, Oltra S, Swerts K, Vicha A, et al. Standardisation of operating procedures for the detection of minimal disease by QRT-PCR in children with neuroblastoma: quality assurance on behalf of SIOPEN-R-NET. *Eur J Cancer.* 2007;43:341-50.
25. Chen Y, Stallings RL. Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. *Cancer Res.* 2007;67:976-83.

Serum microRNAs in childhood tumors

26. Huang TC, Chang HY, Chen CY, Wu PY, Lee H, Liao YF, et al. Silencing of miR-124 induces neuroblastoma SK-N-SH cell differentiation, cell cycle arrest and apoptosis through promoting AHR. *FEBS Lett.* 2011;585:3582-6.
27. Ma L, Young J, Prabhala H, Pan E, Mestdagh P, Muth D, et al. miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol.* 2010;12:247-56.
28. Gyugos M, Lendvai G, Kenessey I, Schlachter K, Halasz J, Nagy P, et al. MicroRNA expression might predict prognosis of epithelial hepatoblastoma. *Virchows Arch.* 2014;464:419-27.
29. Shifeng H, Danni W, Pu C, Ping Y, Ju C, Liping Z. Circulating liver-specific miR-122 as a novel potential biomarker for diagnosis of cholestatic liver injury. *PLoS One.* 2013;8:e73133.
30. Kim N, Kim H, Jung I, Kim Y, Kim D, Han YM. Expression profiles of miRNAs in human embryonic stem cells during hepatocyte differentiation. *Hepatol Res.* 2011;41:170-83.
31. Senanayake U, Das S, Vesely P, Alzoughbi W, Frohlich LF, Chowdhury P, et al. miR-192, miR-194, miR-215, miR-200c and miR-141 are downregulated and their common target ACVR2B is strongly expressed in renal childhood neoplasms. *Carcinogenesis.* 2012;33:1014-21.
32. Sarver AL, Li L, Subramanian S. MicroRNA miR-183 functions as an oncogene by targeting the transcription factor EGR1 and promoting tumor cell migration. *Cancer Res.* 2010;70:9570-80.
33. Atiye J, Wolf M, Kaur S, Monni O, Bohling T, Kivioja A, et al. Gene amplifications in osteosarcoma-CGH microarray analysis. *Genes Chromosomes Cancer.* 2005;42:158-63.

Serum microRNAs in childhood tumors

34. Yang C, Wang C, Chen X, Chen S, Zhang Y, Zhi F, et al. Identification of seven serum microRNAs from a genome-wide serum microRNA expression profile as potential noninvasive biomarkers for malignant astrocytomas. *Int J Cancer*. 2013;132:116-27.
35. Sun J, Shi H, Lai N, Liao K, Zhang S, Lu X. Overexpression of microRNA-155 predicts poor prognosis in glioma patients. *Med Oncol*. 2014;31:911.
36. Liu S, Yin F, Zhang J, Wicha MS, Chang AE, Fan W, et al. Regulatory Roles of miRNA in the Human Neural Stem Cell Transformation to Glioma Stem Cells. *J Cell Biochem*. 2014;[Epub ahead of print].
37. Ho CY, Bar E, Giannini C, Marchionni L, Karajannis MA, Zagzag D, et al. MicroRNA profiling in pediatric pilocytic astrocytoma reveals biologically relevant targets, including PBX3, NFIB, and METAP2. *Neuro Oncol*. 2013;15:69-82.

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Sample	Code	Gender	Age (mo)	Cancer Type	Additional Comments	Confirmatory Study
1	MYCN-NB-1	M	98	MYCN-amplified HR neuroblastoma	Undifferentiated	✓
2	MYCN-NB-2	M	6	MYCN-amplified HR neuroblastoma	Undifferentiated	✓
3	NB-1	M	67	LR neuroblastoma	Ganglio-neuroblastoma	✓
4	NB-2	F	21	LR neuroblastoma	Differentiating	✓
5	HB-1	M	14	Hepatoblastoma	Mixed fetal and embryonal type histology	✓
6	HB-2	M	85	Hepatoblastoma	Mixed fetal and embryonal type histology	✓
7	HB-3	F	25	Hepatoblastoma	Fetal type histology	
8	HB-4	F	14	Hepatoblastoma	Fetal type histology	✓
9	WT-1	F	54	Wilms tumor	Biopsy - anaplastic histology	✓
10	WT-2	M	22	Wilms tumor	Biopsy -stromal type histology; WAGR	✓
11	WT-3	F	12	Wilms tumor	Biopsy - mixed type histology	
12	WT-4	F	25	Wilms tumor	Biopsy - triphasic histology. Failed QC.	
13	WT-5	M	41	Wilms tumor	Bilateral tumors	
14	WT-6	F	16	Wilms tumor	Biopsy - stromal type histology	
15	WT-7	F	97	Wilms tumor	Biopsy - triphasic histology	
16	RMS-1	M	121	Rhabdomyosarcoma	Abdominal	✓
17	RMS-2	M	52	Rhabdomyosarcoma	Pancreatic site; jaundiced; embryonal type	
18	RMS-3	F	104	Rhabdomyosarcoma	Nasopharyngeal site; embryonal type	✓
19	ES-1	M	152	Ewings sarcoma	Bone and soft tissue of cervical vertebrae	
20	ES-2	M	34	Ewings sarcoma	Soft tissue of lumbo-sacral region	
21	OS-1	F	103	Osteosarcoma	Previous RMS/Li Fraumeni syndrome	
22	NHL-1	M	176	B-cell non-Hodgkin's lymphoma	Burkitt's lymphoma	✓
23	NHL-2	M	110	B-cell non-Hodgkin's lymphoma	Burkitt's lymphoma	✓
24	HD-1	F	186	Hodgkin's disease	Classical, nodular sclerosing subtype	
25	HD-2	M	156	Hodgkin's disease	Classical, nodular sclerosing subtype	✓
26	HD-3	M	176	Hodgkin's disease	Classical, nodular sclerosing subtype	✓
27	HD-4	F	159	Hodgkin's disease	Classical, nodular sclerosing subtype	
28	HD-5	F	172	Hodgkin's disease	Classical, nodular sclerosing subtype	
29	PPB-1	F	133	Pleuropulmonary blastoma	Germline <i>DICER1</i> mutation	
30	GL-1	M	53	Central nervous system glioma	Pilocytic astrocytoma WHO 2007 grade I	✓
31	GL-2	F	68	Central nervous system glioma	Pilocytic astrocytoma WHO 2007 grade I	
32	GL-3	M	172	Central nervous system glioma	Gliomatosis cerebri, WHO 2007 grade III	
33	GL-4	F	17	Central nervous system glioma	Oligodendroglioma WHO 2007 grade II	
34	GL-5	M	59	Central nervous system glioma	Pilocytic astrocytoma WHO 2007 grade I	✓
35	MYCN-NB-3	F	17	MYCN-amplified HR neuroblastoma	Undifferentiated; abdominal	✓
36	MYCN-NB-4	M	32	MYCN-amplified HR neuroblastoma	Undifferentiated; abdominal	✓
37	NB-3	M	5	LR neuroblastoma	Poorly differentiated	✓
38	NB-4	M	3	LR neuroblastoma	Poorly differentiated	✓
39	C-1	F	38	N/A	N/A	
40	C-2	M	159	N/A	N/A	
41	C-3	F	34	N/A	N/A	✓
42	C-4	F	142	N/A	N/A	✓
43	C-5	F	129	N/A	N/A	
44	C-6	M	23	N/A	N/A	
45	C-7	F	21	N/A	N/A	
46	C-8	M	57	N/A	N/A	
47	C-9	M	34	N/A	N/A	
48	C-10	F	38	N/A	N/A	✓
49	C-11	M	134	N/A	N/A	
50	C-12	M	48	N/A	N/A	
51	C-13	M	74	N/A	N/A	
52	C-14	F	140	N/A	N/A	
53	C-15	M	181	N/A	N/A	
54	C-16	M	29	N/A	N/A	
55	C-17	M	26	N/A	N/A	
56	C-18	F	50	N/A	N/A	
57	C-19	M	36	N/A	N/A	✓
58	C-20	F	86	N/A	N/A	

Table 1. Clinico-pathological characteristics of the patients in the tumor and control groups. The gender, age, cancer type of the patients and which samples were used in the confirmatory study are listed. Key: MYCN-NB=*MYCN*-amplified high-risk neuroblastoma (n=4 samples), NB=*non-MYCN*-amplified low-risk neuroblastoma (n=4), HB=hepatoblastoma (n=4), WT=Wilms tumor (n=6),

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RMS=rhabdomyosarcoma (n=3), ES=Ewing's sarcoma (n=2), OS=osteosarcoma (n=1), B-NHL=B-cell non-Hodgkin's lymphoma (n=2), HD=Hodgkin's disease (n=5), PPB=pleuropulmonary blastoma (n=1), GL=Central nervous system glioma (n=5), C=control samples (n=20). HR= high-risk; LR= low-risk. Samples 1-34 and 39-58 were used in the discovery-phase global profiling study, while samples 35-38 were used in the technical confirmation study.

Figure Legends

Figure 1. Discovery-phase global qRT-PCR profiling. A) Comparison of serum housekeeping microRNA expression in the initial QC and full qRT-PCR analyses. The graph shows the mean raw Ct values (*y*-axis) of the four housekeeping microRNAs (miR-23a-3p, miR-30c-5p, miR-103a-3p, and miR-191-5p) in the initial QC and full qRT-PCR run for each of the 53 samples (*x*-axis) taken forward for full analysis. Key: QC = quality control; HK = housekeeping. For tumor abbreviations please refer to Table 1. B) Assessment of normalization approaches for global microRNA profiling in serum samples from pediatric cancer patients and controls. The graph shows the cumulative distribution of serum microRNA expression (*y*-axis) plotted against the standard deviation (*x*-axis) for all 53 samples in the cohort for nine different normalization approaches (identified by the color key). Normalization key: hkgsQC = using the four housekeeping microRNAs used in the initial QC screening in A) above; snoRNAs = using the two snoRNAs on the qRT-PCR platform and U6 = using the single small nuclear RNA RNU6 on the platform. C) Heatmap showing overall expression levels of selected serum microRNAs identified by global qRT-PCR profiling. Columns represent each individual tumor type or the control group. Each row represents the mean normalized serum expression level for the listed microRNA (*global mean* method), using \log_2 Ct values across each tumor type or control group. Key: Selected serum microRNA signature for: panel 1=MYCN-NB vs. NB; 2=HB vs. MYCN-NB/NB; 3=WT; 4=MYCN-NB/NB vs. WT; 5/6/7=RMS, ES and OS, respectively, vs. the other two sarcoma subtypes; 8=HD vs. B-NHL; 9=PPB; 10=glioma. For tumor abbreviations see Table 1. Italicized microRNAs were those used for the technical confirmation study. Red=microRNA over-expression; blue=microRNA under-expression.

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Figure 2. Serum microRNA expression in MYCN-amplified high-risk

neuroblastoma (MYCN-NB). A) The boxplots show the median (black bar) and inter-quartile range (dark blue bar) raw Ct values (*y*-axis) of five highly over-expressed microRNAs in MYCN-NB (miR-124-3p, miR-9-3p, miR-218-5p, miR-490-5p and miR-1538) relative to other tumors and controls, across the 53 samples in the global qRT-PCR profiling study (discovery-phase test set). Whiskers represent 1.5x inter-quartile range. Dots represent outlier samples. B) The graphs show the mean normalized relative expression values (horizontal bars) and SEM (vertical bars) for the microRNAs listed in A) above, in MYCN-NB samples versus ‘other tumor’ and control samples, in the confirmatory qRT-PCR study. C) The ‘differential diagnosis’ graphs show the mean normalized relative expression values for miR-124-3p and miR-9-3p in MYCN-NB samples versus NB samples, in the technical confirmation qRT-PCR study. In addition, an overall mean-adjusted relative expression value for the specific five microRNA panel was calculated, where each of the five microRNAs listed in A) above was given an equal weighting. Confirmatory qRT-PCR data were normalized to the six top-ranking housekeeping microRNAs identified using *geNorm* and *NormFinder* (Supplementary Figure S6), and are shown relative to the mean expression levels in the control group. Error bars=standard error of the mean (SEM). For tumor abbreviations see Table 1.

Figure 3. Serum microRNA expression in hepatoblastoma (HB) vs. all

neuroblastomas (MYCN-NB/NB). A) The boxplots show the median (black bar) and inter-quartile range (light green bar) raw Ct values (*y*-axis) of three highly over-expressed microRNAs in HB (miR-483-3p, miR-205-5p and miR-122-5p) relative to other tumors and controls, across the 53 samples in the full qRT-PCR study (discovery-phase test set). Whiskers represent 1.5x inter-quartile range. Dots

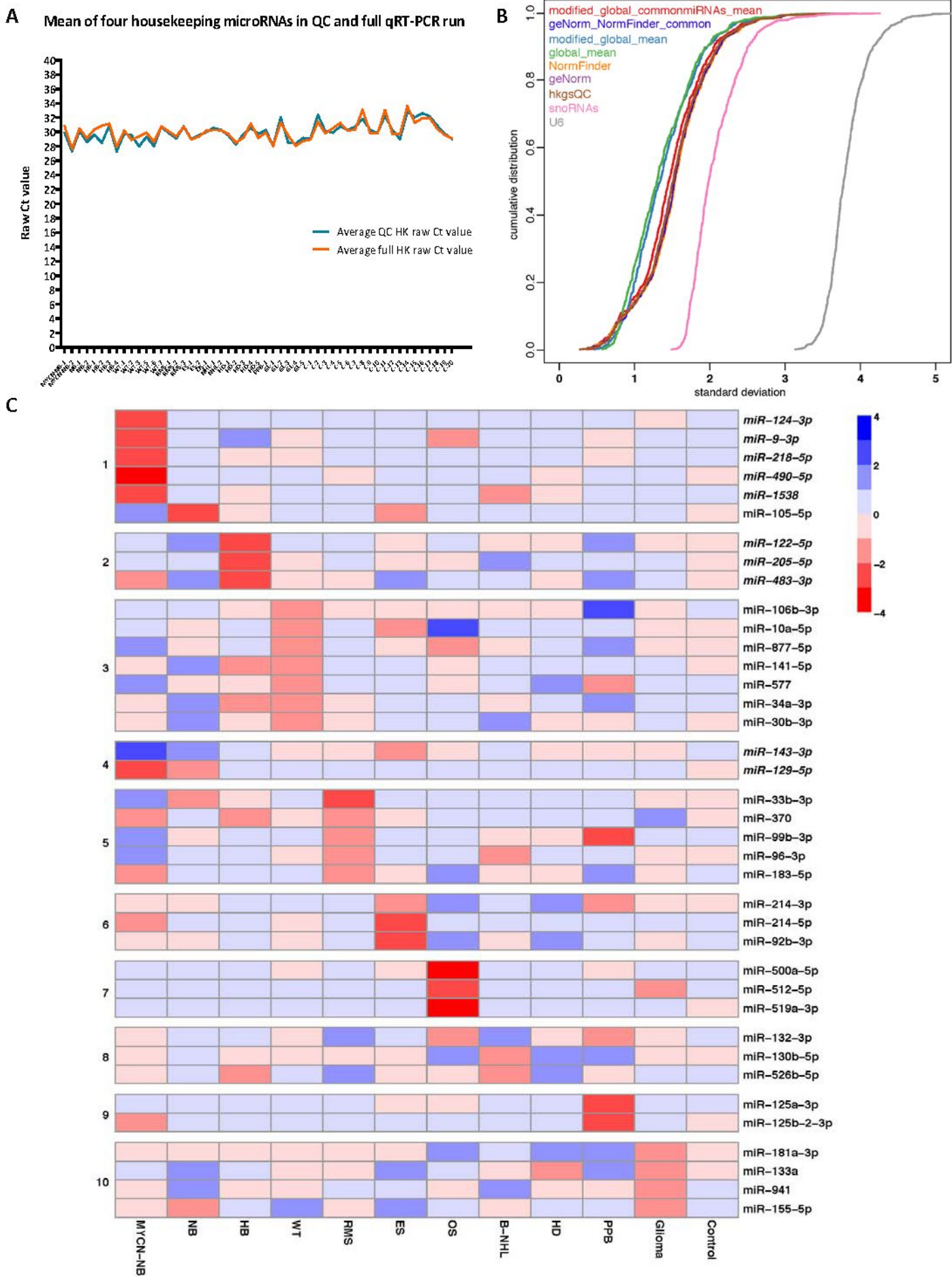
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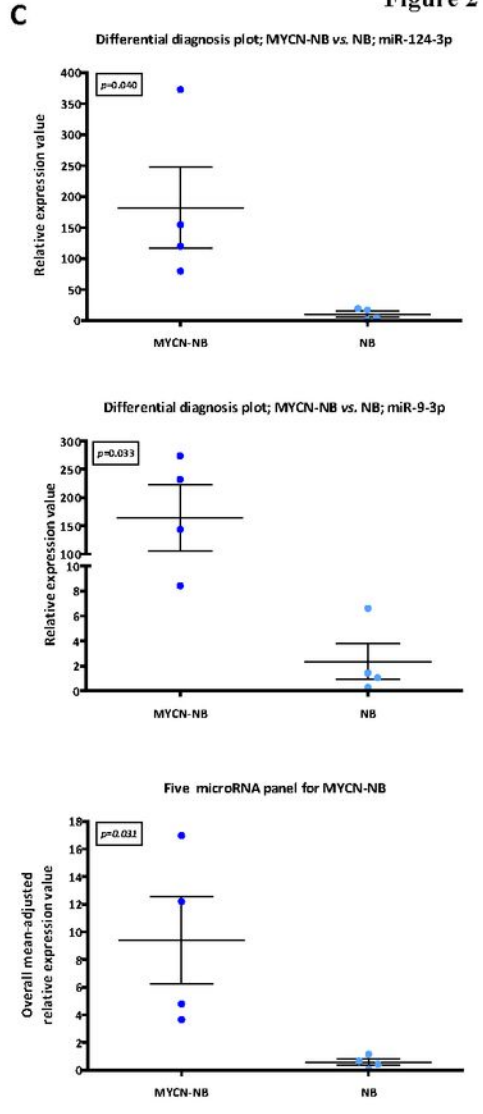
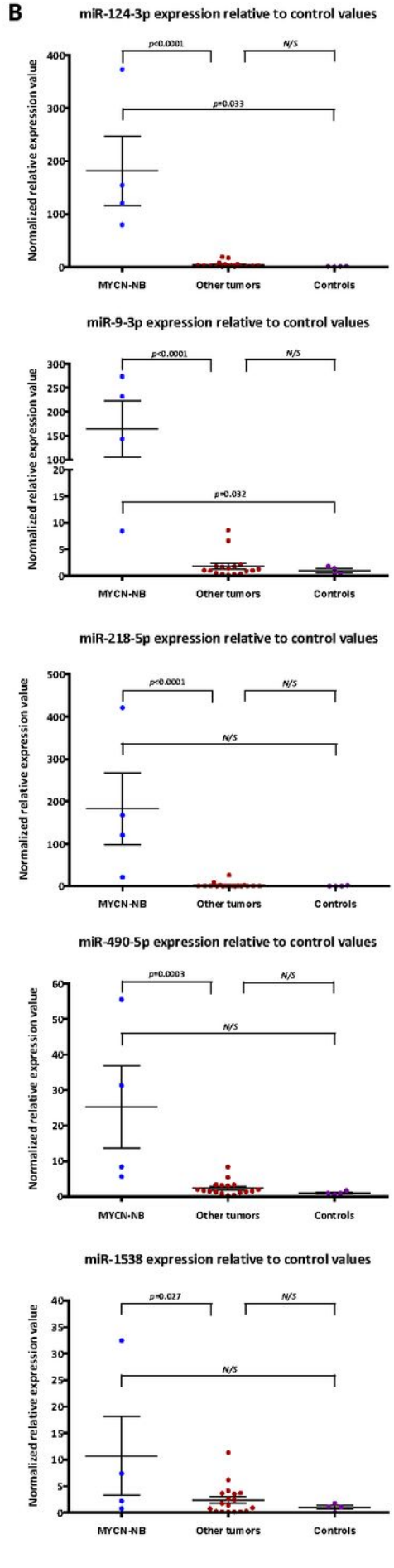
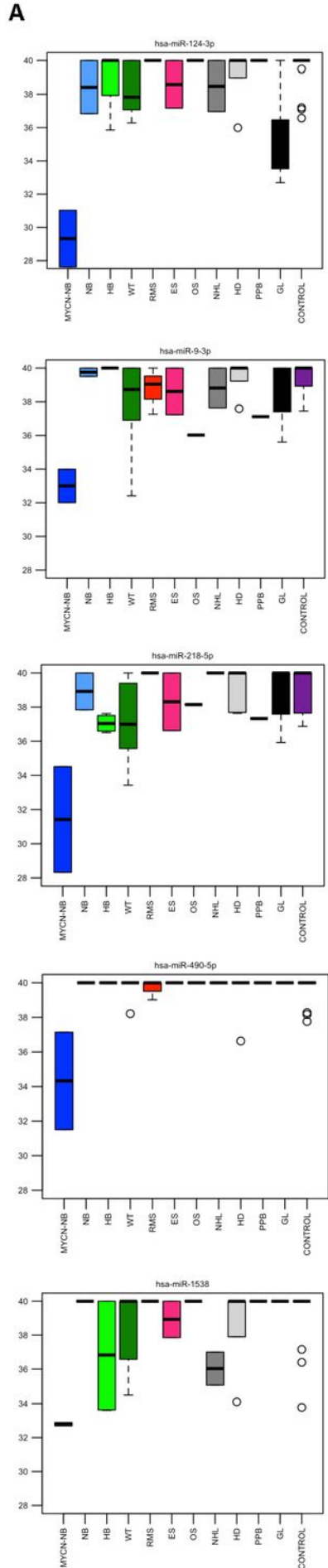
represent outlier samples. B) The graphs show the mean normalized relative expression values (horizontal bars) and SEM (vertical bars) for the microRNAs listed in A) above, in HB samples versus the ‘other tumors’ and control samples, in the confirmatory qRT-PCR study. C) The ‘differential diagnosis’ graphs show the mean normalized relative expression values for the microRNAs listed in A) above, in MYCN-NB samples versus NB samples, in the technical confirmation qRT-PCR study. In addition, an overall mean-adjusted relative expression value for the specific three microRNA panel was calculated, where each of the three microRNAs listed in A) above was given an equal weighting. Confirmatory qRT-PCR data were normalized and plotted as described in Figure 2. Error bars=SEM. For tumor abbreviations see Table 1.

Figure 4. Serum microRNA expression in Wilms tumor (WT) vs. all neuroblastomas (MYCN-NB/NB). A) The boxplots show the median (black bar) and inter-quartile range (colored bar) raw Ct values (y-axis) of two microRNAs (miR-129-5p and miR-143-3p) relative to other tumors and controls, across the 53 samples in the full qRT-PCR study (discovery-phase test set). Whiskers represent 1.5x inter-quartile range. Dots represent outlier samples. B) and C) The graphs show the mean normalized relative expression values (horizontal bars) and SEM (vertical bars) 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 ‘differential diagnosis’ plots, in the technical confirmation qRT-PCR study. Confirmatory qRT-PCR data were normalized and plotted as described in Figure 2. Error bars=SEM. For tumor abbreviations see Table 1.

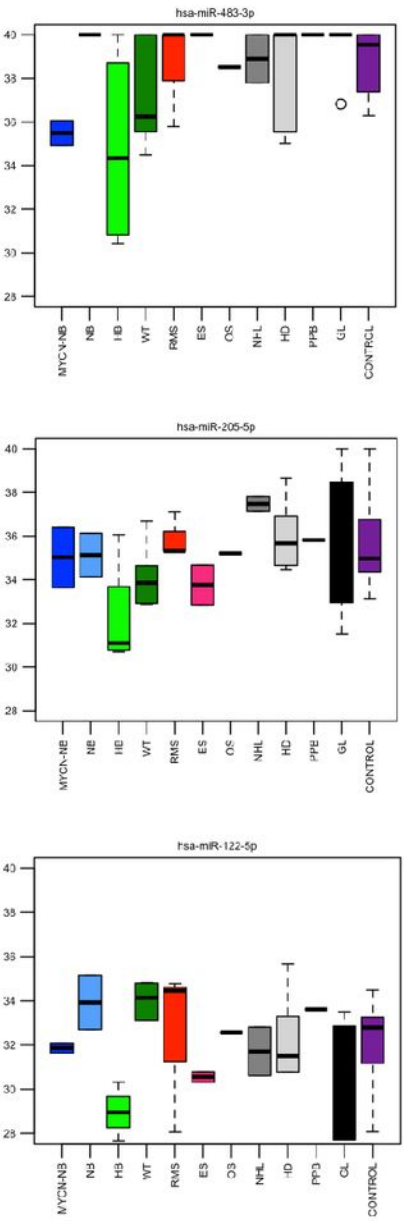
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Figure 5. Serum microRNA expression in sarcoma subtypes. A) The boxplots show the median (black bar) and inter-quartile range (pink bar) raw Ct values (y -axis) of three selected microRNAs (miR-214-3p, miR-214-5p and miR-92b-3p) in ES (Ewings sarcoma) samples compared with other sarcoma samples [OS (osteosarcoma) and RMS (rhabdomyosarcoma)], across the 53 samples in the full qRT-PCR study (discovery-phase test set). Whiskers represent 1.5x inter-quartile range. Dots represent outlier samples. B) ‘Differential diagnosis’ plots for ES vs. RMS/OS in the full qRT-PCR study (discovery-phase test set). Data were normalized using the global mean method and are relative to the mean expression levels in the control group. In addition, an overall mean-adjusted relative expression value for the specific three microRNA panel was calculated, where each of the three microRNAs listed in A) above was given an equal weighting. Error bars=SEM. For tumor abbreviations see Table 1.

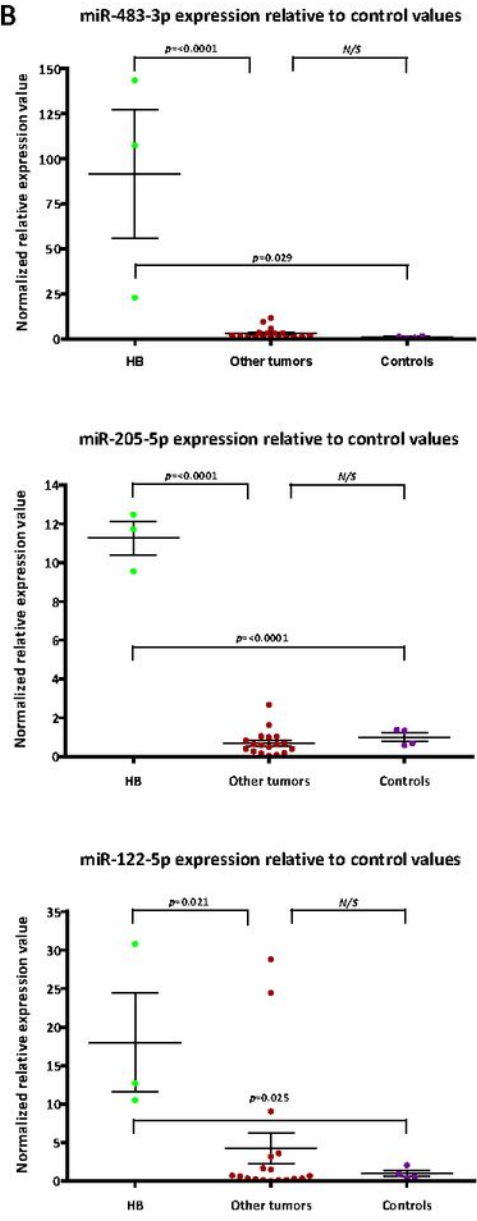




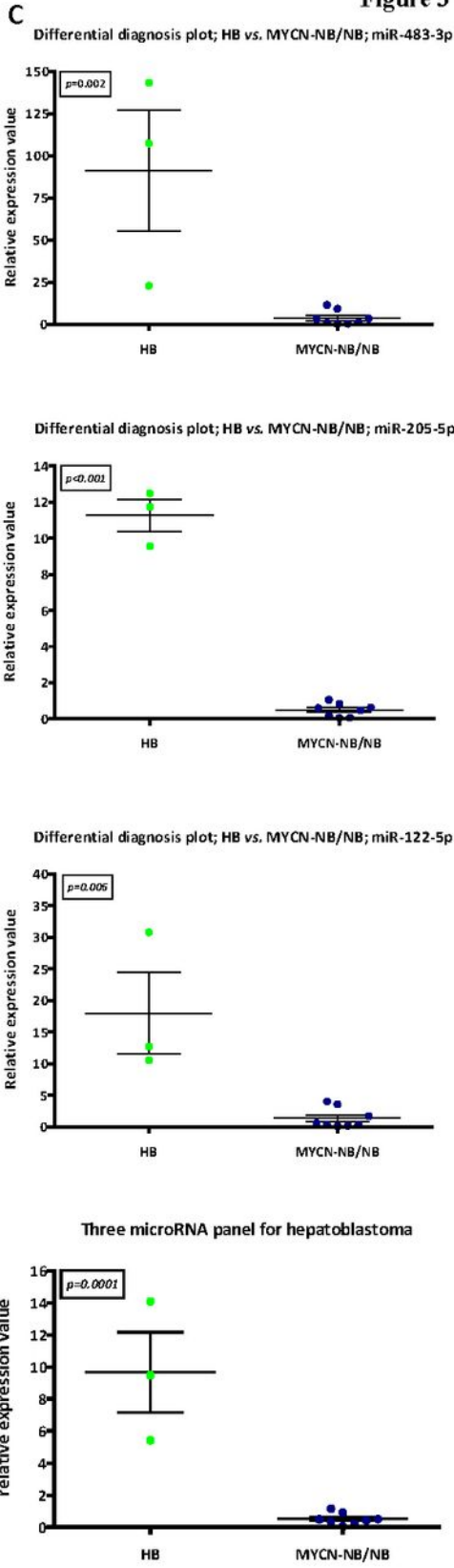
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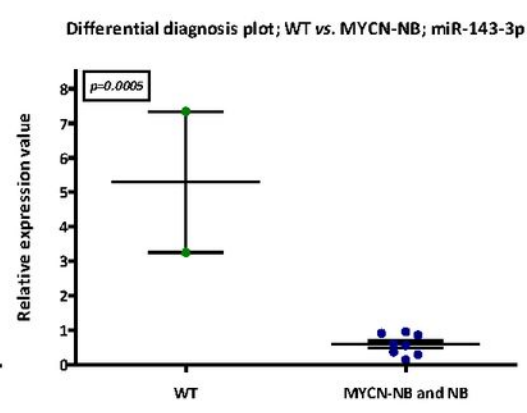
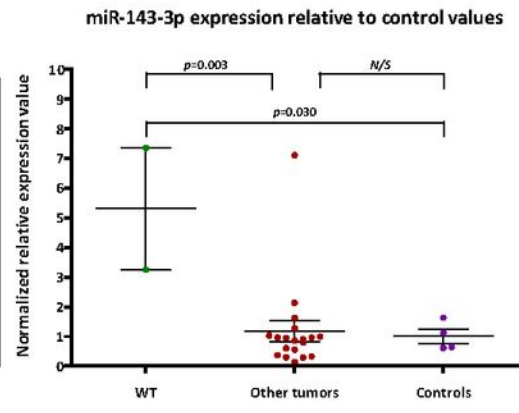
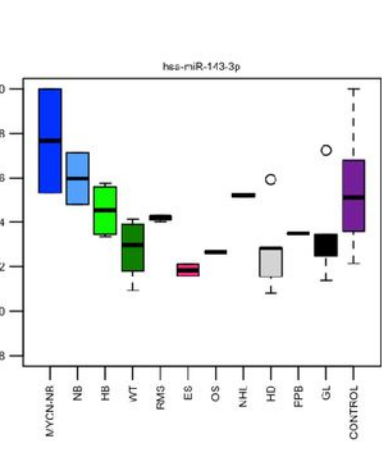
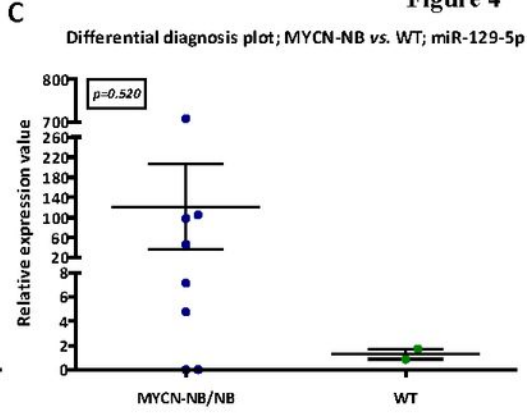
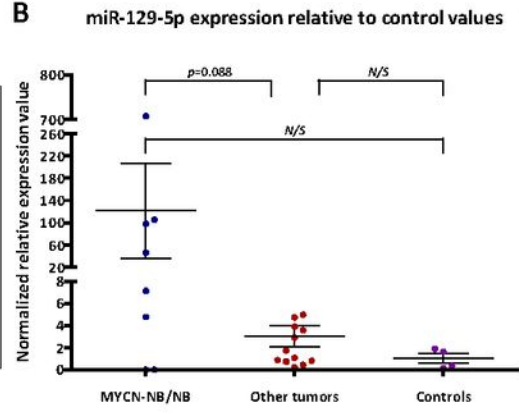
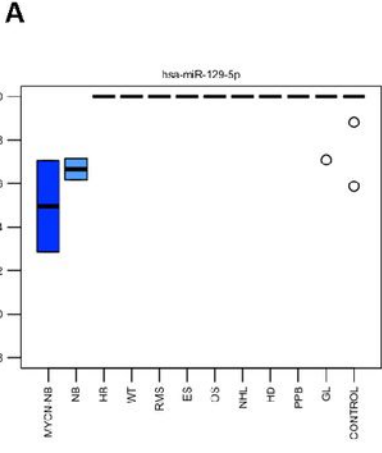


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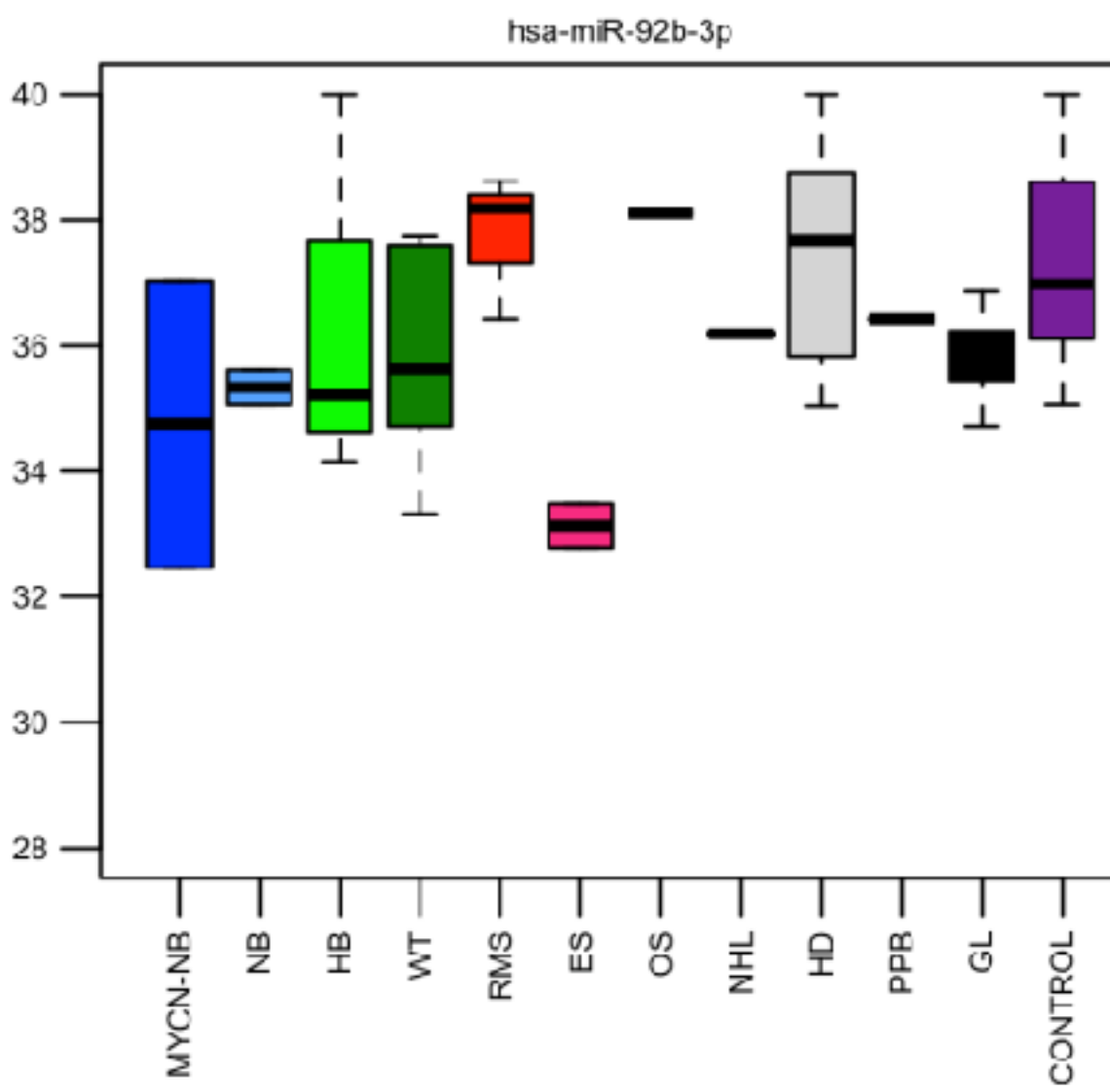
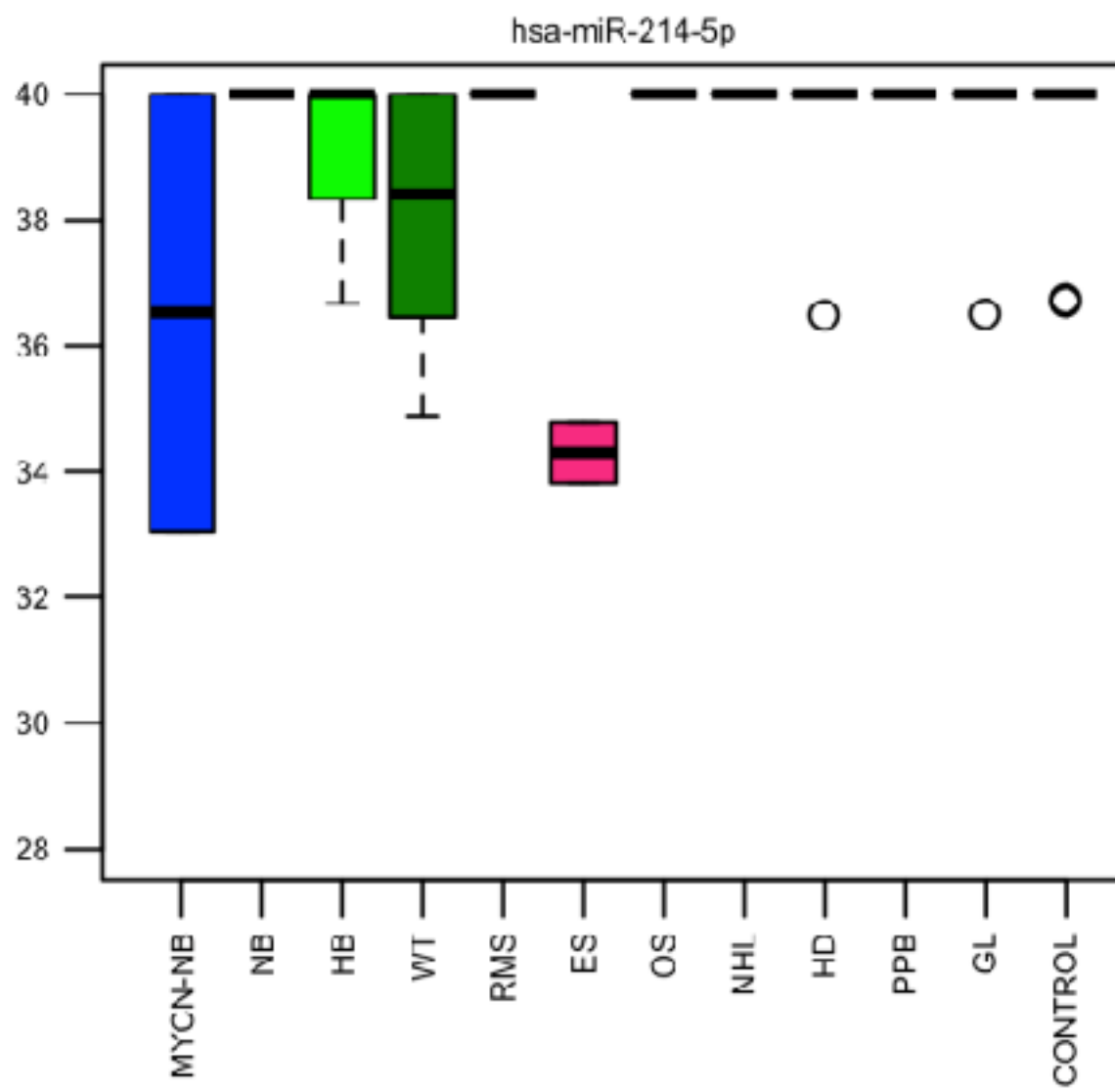
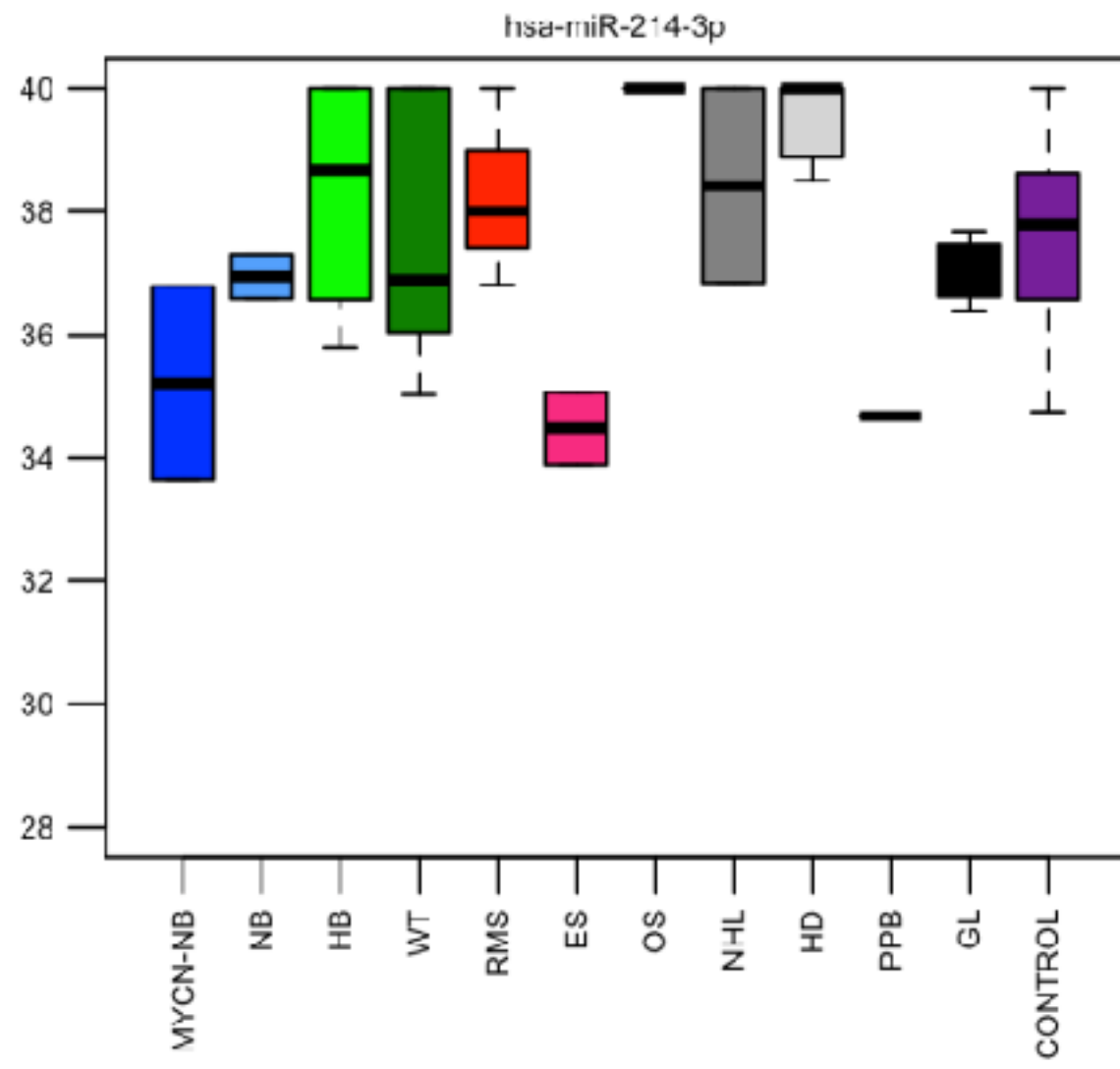


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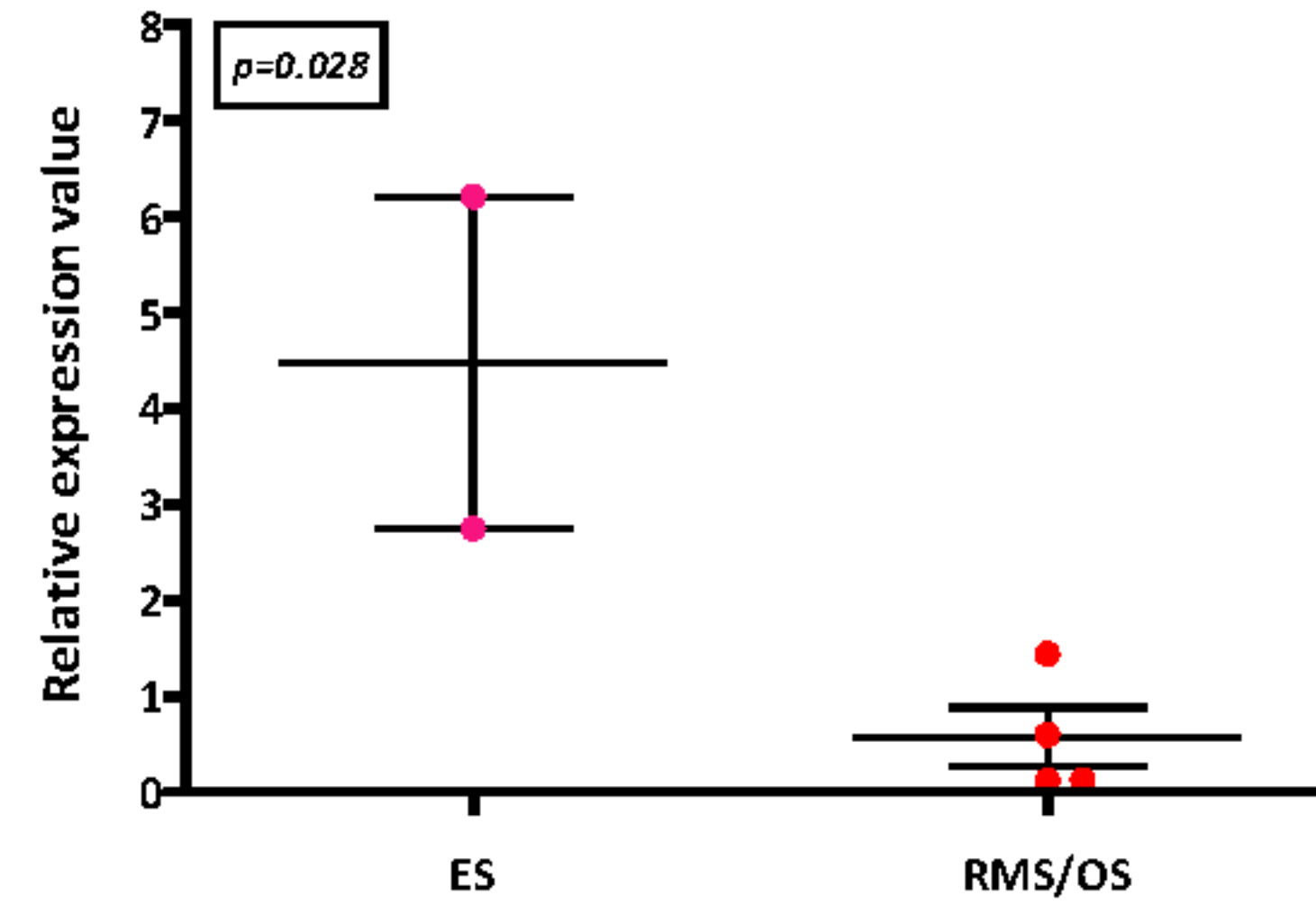


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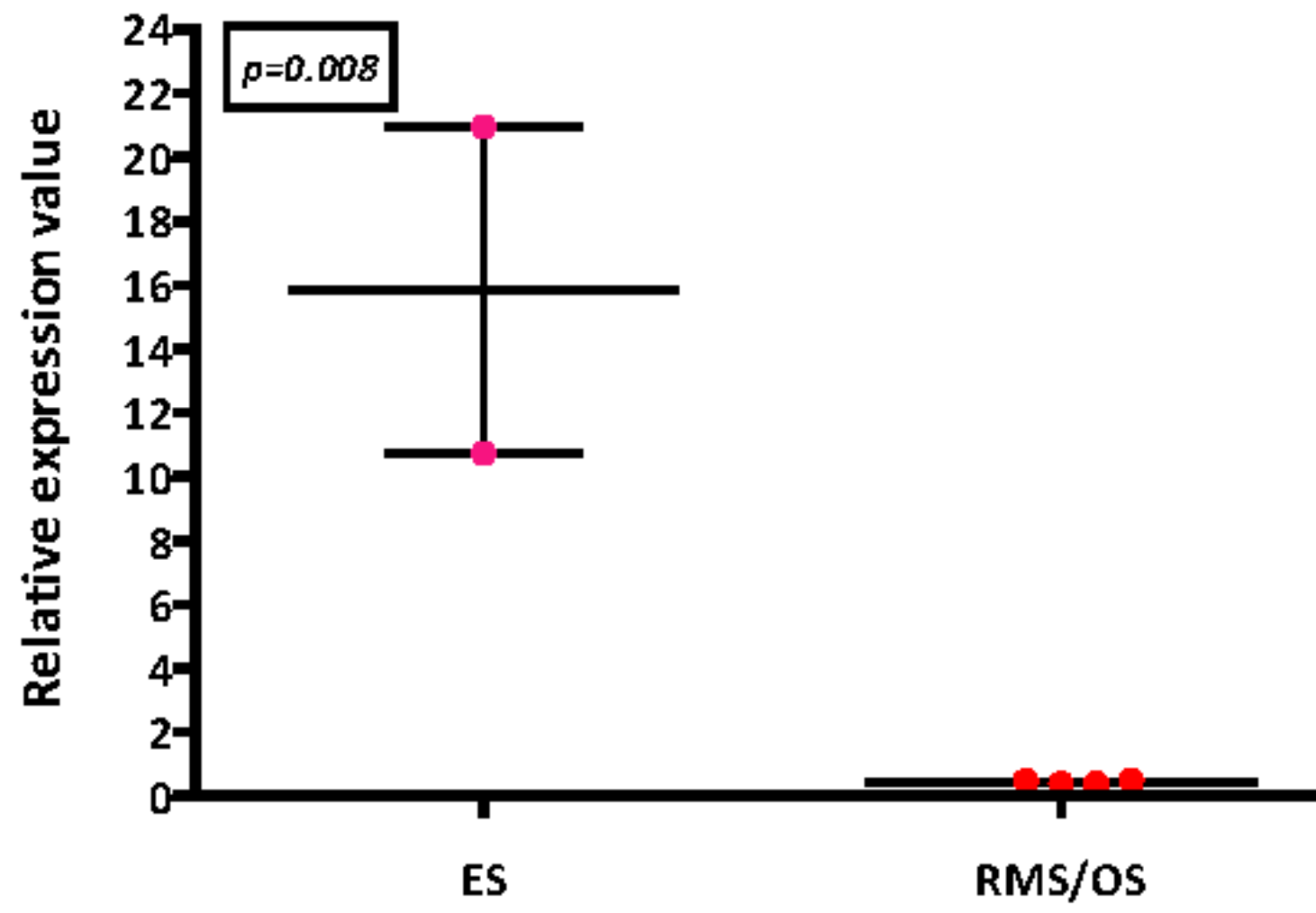


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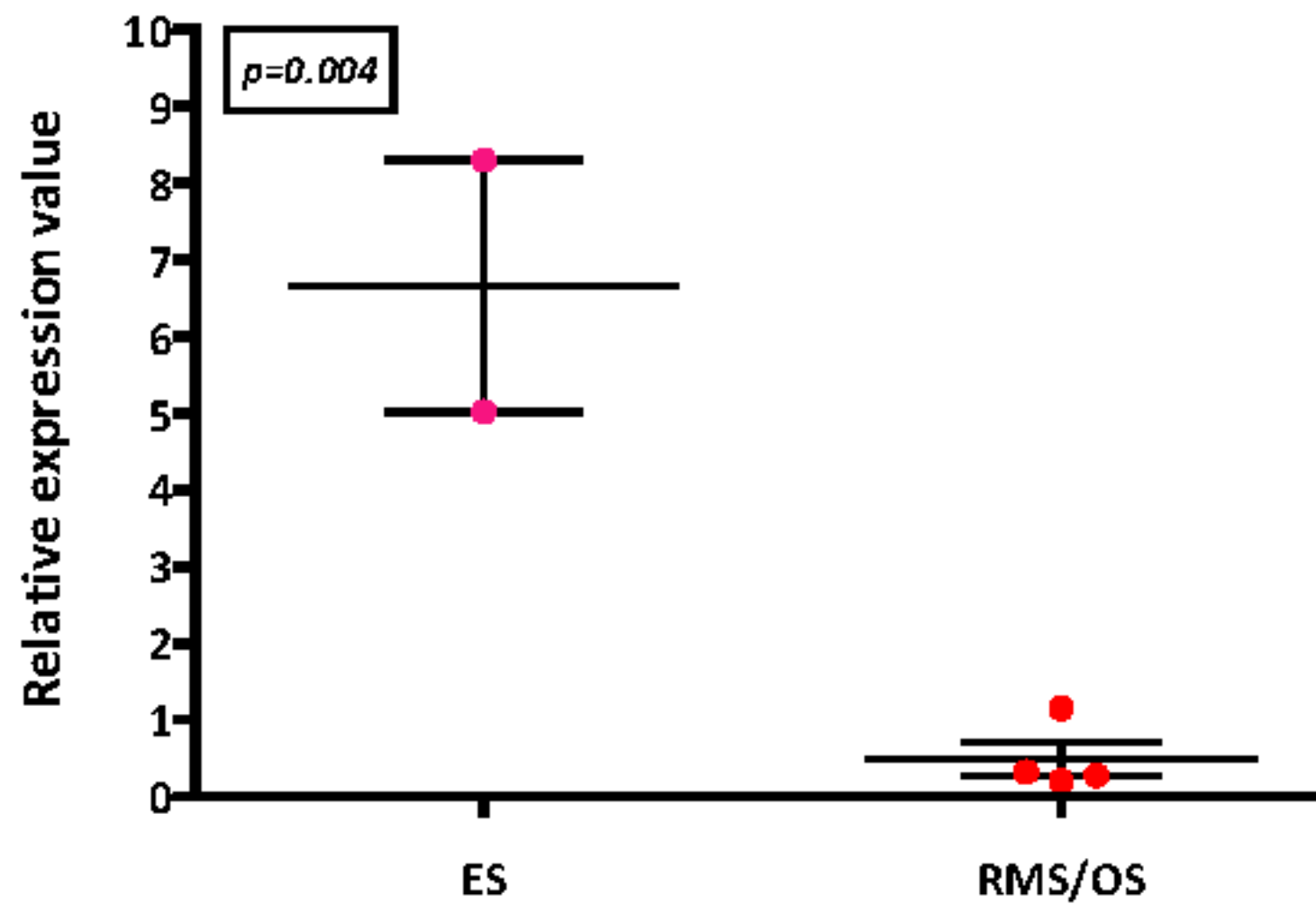
Differential diagnosis plot; ES vs. RMS/OS; miR-214-3p



Differential diagnosis plot; ES vs. RMS/OS; miR-214-5p



Differential diagnosis plot; ES vs. RMS/OS; miR-92b-3p



Three microRNA panel for Ewings sarcoma

