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A pipeline to quantify serum and cerebrospinal fluid microRNAs for diagnosis and detection of relapse in paediatric malignant germ-cell tumours

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Background: The current biomarkers alpha-fetoprotein and human chorionic gonadotropin have limited sensitivity and specificity for diagnosing malignant germ-cell tumours (GCTs). MicroRNAs (miRNAs) from the miR-371-373 and miR-302/367 clusters are overexpressed in all malignant GCTs, and some of these miRNAs show elevated serum levels at diagnosis. Here, we developed a robust technical pipeline to quantify these miRNAs in the serum and cerebrospinal fluid (CSF). The pipeline was used in samples from a cohort of exclusively paediatric patients with gonadal and extragonadal malignant GCTs, compared with appropriate tumour and non-tumour control groups.

Methods: We developed a method for miRNA quantification that enabled sample adequacy assessment and reliable data normalisation. We performed qRT-PCR profiling for miR-371-373 and miR-302/367 cluster miRNAs in a total of 45 serum and CSF samples, obtained from 25 paediatric patients.

Results: The exogenous non-human spike-in cel-miR-39-3p and the endogenous housekeeper miR-30b-5p were optimal for obtaining robust serum and CSF qRT-PCR quantification. A four-serum miRNA panel (miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p): (i) showed high sensitivity/specificity for diagnosing paediatric extracranial malignant GCT; (ii) allowed early detection of relapse of a testicular mixed malignant GCT; and (iii) distinguished intracranial malignant GCT from intracranial non-GCT tumours at diagnosis, using CSF and serum samples.

Conclusions: The pipeline we have developed is robust, scalable and transferable. It potentially promises to improve clinical management of paediatric (and adult) malignant GCTs.

Germ-cell tumours (GCTs) are characterised by clinical and pathological heterogeneity. They occur from the neonatal period through to late adulthood and arise at gonadal or extragonadal

sites, including the central nervous system. Malignant GCTs are classified into germinoma and non-germinomatous tumours (yolk sac tumour (YST), embryonal carcinoma (EC) and choriocarcinoma

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(CHC)), while teratomas (which show extensive somatic differentiation) are generally considered benign (Palmer *et al*, 2010). The utility of the conventional body fluid protein biomarkers alpha-fetoprotein (AFP) and human chorionic gonadotropin (HCG) for diagnosis and follow-up is restricted to specific malignant GCT subtypes, as levels are raised predominantly in tumours containing YST and CHC, respectively (Murray and Nicholson, 2011). Moreover, both AFP and HCG can be elevated in non-malignant conditions (Murray *et al*, 2015a). The initial management of primary gonadal GCTs is complete resection where possible, regardless of serum AFP/HCG levels, thus enabling a histopathological diagnosis. For extragonadal primary sites of disease (e.g., the retroperitoneum and mediastinum), typical radiological findings and raised AFP/HCG markers alone may be sufficient for diagnosis, and indeed for intracranial GCTs, this is the recommended practice in Europe and North America (Nicholson *et al*, 2002). For 'marker-negative' extragonadal cases, however, biopsy is required to establish a formal histopathological diagnosis. Such procedures carry risks of morbidity, due to the difficulties in surgical access to these anatomical sites. Furthermore, disease-monitoring after malignant GCT diagnosis to detect early non-symptomatic relapse currently relies heavily on serial radiological imaging, with associated cumulative radiation exposure and second malignant neoplasm risk (Tarin *et al*, 2009). Consequently, body fluid biomarkers that offer greater sensitivity and specificity for diagnosing and/or monitoring malignant GCTs would be of major clinical benefit (Murray *et al*, 2015a).

The ideal biomarker for malignant GCTs would be: stable within body fluids; abundant compared with levels in individuals who were healthy, had benign GCTs or non-GCT pathology; and present regardless of patient age (paediatric or adult), anatomical site of disease (gonadal or extragonadal, including intracranial) or histological subtype (e.g., YST, germinoma and EC) (Murray and Coleman, 2012). Recent years have seen the emergence of microRNAs (miRNAs) as a new generation of biomarkers in malignant GCTs (Murray and Coleman, 2012). MicroRNAs are short, non-protein coding RNAs that are highly stable and well-suited for disease diagnosis and monitoring (Mitchell *et al*, 2008). We previously demonstrated that the eight main miRNAs from the miR-371-373 and miR-302/367 clusters were overexpressed in all malignant GCT tissues (Palmer *et al*, 2010). By using a multiplexed qRT-PCR protocol, we identified that levels of miRNAs from these two clusters were elevated in the serum of a paediatric patient at the time of diagnosis of an extragonadal malignant GCT, with levels falling and remaining low during uneventful clinical follow-up (Murray *et al*, 2011b). In the same year, we confirmed these serum findings in extracranial malignant GCTs across a range of representative ages (paediatric and adult), anatomical sites (gonadal and extragonadal) and histological subtypes (YST, germinoma and EC), the majority of which were AFP/HCG marker-negative (Murray *et al*, 2011a; Murray and Coleman, 2012).

Although our serum miRNA findings in malignant GCTs have since been confirmed and extended, the published studies have exclusively (Belge *et al*, 2012; Dieckmann *et al*, 2012; Rijlaarsdam *et al*, 2015; Spiekermann *et al*, 2015a; Syring *et al*, 2015) or predominantly (Gillis *et al*, 2013) involved adult testicular tumours. In such cases, a consensus panel of four serum miRNAs from the miR-371-373 and miR-302/367 clusters (namely miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p) has been shown to be highly sensitive and specific for detecting malignant GCTs (Gillis *et al*, 2013; Syring *et al*, 2015). The present study represents the first systematic interrogation of expression of these miRNA clusters in serum/cerebrospinal fluid (CSF) samples from patients with paediatric malignant GCTs. Our analysis included serum levels at diagnosis for both ovarian and testicular disease,

early detection of relapse and levels in CSF and serum in intracranial disease. Our investigation of the utility of these miRNAs is underpinned by the development of a robust pipeline for their quantification, including essential quality control (QC) steps. The pipeline is scalable, transferable and suitable for widespread adoption into routine clinical practice.

MATERIALS AND METHODS

The study received approval from the Multicentre Research Ethics Committee (reference 02/4/71) and Cambridge 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; Bustin *et al*, 2009). Full details are available on request.

Patient demographics and tumours analysed. In total, we analysed 45 body fluid samples (serum $n = 41$, CSF $n = 4$), from a cohort of 25 paediatric patients (0–16 years; Table 1). The cohort comprised a malignant GCT group ($n = 8$; extracranial ($n = 5$) and intracranial ($n = 3$)); a combined group comprising benign GCTs and non-GCT tumours ($n = 12$; extracranial ($n = 10$) and intracranial ($n = 2$)), and a group of control subjects without tumours ($n = 5$). The five extracranial malignant GCTs represented gonadal (three testicular, one ovarian) and extragonadal (sacrocoxygeal) disease. The three intracranial malignant GCTs represented two suprasellar site (one germinoma, one non-germinoma) and one pineal site (germinoma). The benign GCTs ($n = 5$) comprised three immature teratomas (ITs) and two mature teratomas (MTs), from both gonadal ($n = 4$) and extragonadal ($n = 1$) sites. The sites of the non-GCT tumours ($n = 6$) were testicular/paratesticular ($n = 2$; one sex cord stromal tumour, one rhabdomyosarcoma), ovarian ($n = 2$; one simple cyst, one small cell carcinoma of the ovary, hypercalcaemic type (Bailey *et al*, 2015)) and intracranial ($n = 2$; one suprasellar WHO grade 1 ganglioglioma and one disseminated *MYCN*-amplified neuroblastoma). The five samples in the control group were from five age- and gender-matched anonymised subjects, as described previously (Murray *et al*, 2015b). There were no statistical differences in age between the three patient groups. Full clinicopathological details are given in Table 1.

Overview of pipeline development. The pipeline developed for robust serum miRNA quantification is shown in Figure 1. Components of this pipeline are described in the following sections, with reference to the various colour-coded boxes in Figure 1.

Sample processing and RNA extraction. As part of each patient's standard clinical care, blood was sampled in serum separator tubes and CSF was collected in plain tubes (Table 1). All specimens were sent to the routine clinical biochemistry diagnostic laboratories at Addenbrooke's Hospital, Cambridge, UK, where they were processed and centrifuged within 4 h of receipt. After being used for routine clinical measurements, samples were kept refrigerated at 4 °C for up to 3 days before residual serum/CSF was retrieved and then frozen and stored at –80 °C. At each stage, all samples were processed according to good clinical and laboratory practice, including the use of disposable pipette tips. Only samples without macroscopic evidence of haemolysis were used (Murray *et al*, 2015b), to avoid substantial technical variation due to the release of intracellular miRNAs from red blood cells (Kirschner *et al*, 2011; Figure 1, box A).

RNA isolation. RNA from 200 μ l of thawed serum or CSF samples was isolated using the miRNeasy serum kit (Qiagen, Crawley, UK), according to the manufacturer's instructions.

Table 1. Clinicopathological details of samples and patients analysed

Sample	Cancer type	Code	Gender	Age (years) at diagnosis	Site	Days since diagnosis	Serum sample	CSF sample
1	N/A	CONT_1	Male	13	N/A	0	Yes	No
2	N/A	CONT_2	Female	2	N/A	0	Yes	No
3	N/A	CONT_3	Female	11	N/A	0	Yes	No
4	N/A	CONT_4	Male	1	N/A	0	Yes	No
5	N/A	CONT_5	Female	7	N/A	0	Yes	No
6	MT	BGCT_ExC#1	Male	4	Testis	0	Yes	No
7	IT	BGCT_ExC#2	Female	14	Ovary	0	Yes	No
8	SCST	BGCT_ExC#3	Male	3	Testis	0	Yes	No
9	IT	BGCT_ExC#4	Female	15	Ovary	0	Yes	No
10	Simple cyst	BGCT_ExC#5	Female	13	Ovary	0	Yes	No
11	MT	BGCT_ExC#6	Female	0	Sacrocoxygeal	0	Yes	No
12	IT	BGCT_ExC#7	Female	11	Ovary	0	Yes	No
13	MT	BGCT_ExC#8	Female	11	Ovary	0	Yes	No
14	Ganglioglioma	B-non-GCT_IC#1	Female	15	CNS	0	Yes	Yes
15	Paratesticular RMS	M-non-GCT_ExC#1	Male	13	Testis	0	Yes	No
16	SCCOHT	M-non-GCT_ExC#1	Female	15	Ovary	0	Yes	No
17	YST	MGCT_ExC#1	Male	2	Testis	0	Yes	No
18	MMGCT	MGCT_ExC#2	Male	15	Testis	0	Yes	No
19	MMGCT	MGCT_ExC#3_01	Male	14	Testis	0	Yes	No
20	MMGCT	MGCT_ExC#3_02	Male	14	Testis	14	Yes	No
21	MMGCT	MGCT_ExC#3_03	Male	14	Testis	28	Yes	No
22	MMGCT	MGCT_ExC#3_04	Male	14	Testis	33	Yes	No
23	MMGCT	MGCT_ExC#3_05	Male	14	Testis	42	Yes	No
24	MMGCT	MGCT_ExC#3_06	Male	14	Testis	49	Yes	No
25	MMGCT	MGCT_ExC#3_07	Male	14	Testis	63	Yes	No
26	MMGCT	MGCT_ExC#3_08	Male	14	Testis	70	Yes	No
27	MMGCT	MGCT_ExC#3_09	Male	14	Testis	76	Yes	No
28	MMGCT	MGCT_ExC#3_10	Male	14	Testis	83	Yes	No
29	MMGCT	MGCT_ExC#3_11	Male	14	Testis	101	Yes	No
30	MMGCT	MGCT_ExC#3_12	Male	14	Testis	105	Yes	No
31	MMGCT	MGCT_ExC#3_13	Male	14	Testis	126	Yes	No
32	MMGCT	MGCT_ExC#3_14	Male	14	Testis	160	Yes	No
33	MMGCT	MGCT_ExC#3_15	Male	14	Testis	189	Yes	No
34	MMGCT	MGCT_ExC#3_16	Male	14	Testis	278	Yes	No
35	MMGCT	MGCT_ExC#3_17	Male	14	Testis	342	Yes	No
36	MMGCT	MGCT_ExC#3_18	Male	14	Testis	782	Yes	No
37	YST	MGCT_ExC#4	Female	11	Ovary	4	Yes	No
38	YST	MGCT_ExC#5	Male	1	Sacrocoxygeal	0	Yes	No
39	Germinoma	MGCT_IC#1	Male	10	CNS	0	Yes	No
40	Non-germinoma	MGCT_IC#2	Female	13	CNS	0	Yes	Yes
41	Germinoma	MGCT_IC#3	Female	12	CNS	0	Yes	Yes
42	MYCN-amplified neuroblastoma	M-non-GCT_IC#1	Male	2	CNS	At relapse	No	Yes

Abbreviations: B-non-GCT_IC = benign non-germ-cell tumour pathology (intracranial); BGCT_ExC = benign germ-cell tumour (extracranial); CNS = central nervous system; CONT = non-tumour control samples; CSF = cerebrospinal fluid; M-non-GCT_ExC = malignant non-germ-cell tumour pathology (extracranial); IT = immature teratoma; MMGCT = mixed malignant germ-cell tumour; MGCT_ExC = malignant germ-cell tumour (extracranial); MGCT_IC = malignant germ-cell tumour (intracranial); MT = mature teratoma; N/A = not applicable; RMS = rhabdomyosarcoma; SCST = sex cord stromal tumour; SCCOHT = small cell carcinoma of the ovary, hypercalcaemic type; YST = yolk sac tumour.

In order to increase RNA yield, we first added MS2 carrier RNA (Roche, Welwyn Garden City, UK) to QIAzol to give a final concentration of $1.25 \mu\text{g ml}^{-1}$ (Murray *et al*, 2015b). To measure the RNA extraction efficiency, we also made a master mix of the exogenous non-human spike-in cel-miR-39-3p from the kit

and added a fixed quantity (5.6×10^8 copies) to each sample (Murray *et al*, 2014), as per the manufacturer's instructions. This fixed quantity resulted in subsequent cel-miR-39-3p qRT-PCR levels within the expected range for endogenous human serum miRNA levels (Murray *et al*, 2014). For samples where 200 μl was

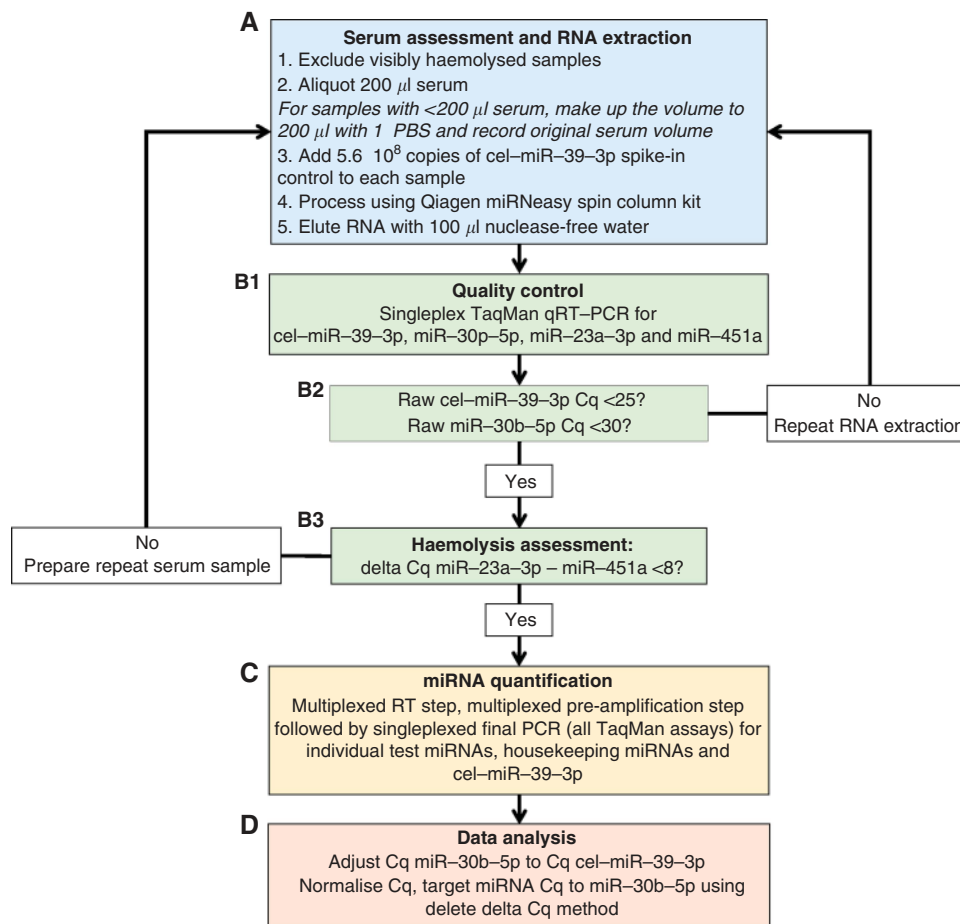


Figure 1. Pipeline for quantifying miRNA levels in serum using qRT-PCR. For each box (**A**, **B1**, **B3**, **C** and **D**), the text in bold describes the relevant stage of the pipeline. Box **B2** describes assessment of RNA extraction efficiency.

not available, volumes were made up to 200 µl with an appropriate volume of 1 × PBS. Total RNA was eluted from columns with 100 µl of nuclease-free water and stored at −80 °C.

Initial QC assessment. Samples were screened for suitability for use in subsequent qRT-PCR analyses. Initial QC miRNA assessment was performed for: cel-miR-39-3p (non-human spike-in); miR-30b-5p (housekeeping miRNA); miR-23a-3p and miR-451a (used together for haemolysis assessment (Blondal *et al*, 2013)). For this initial QC step, miRNA RT and PCRs were performed in singleplex. Five µl of RNA was reverse transcribed using the TaqMan miRNA reverse transcription kit (Life Technologies, Paisley, UK) using the miRNA-specific stem-loop primer from the relevant TaqMan miRNA assay kit (Life Technologies; Supplementary Table S1), as per the manufacturer's instructions. The final volume of 15 µl for each reaction underwent RT using a GeneAmp PCR System 9700 (Applied Biosystems, Warrington, UK) at 16 °C for 30 min, 42 °C for 30 min, followed by a final step of 85 °C for 5 min, as described previously (Murray *et al*, 2011b). A singleplex final PCR was then performed as per the manufacturer's instructions. This PCR was performed on a Mastercycler ep_gradient/S realplex (Eppendorf, Stevenage, UK) at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for one minute, as described previously (Murray *et al*, 2014). To exclude non-specific amplification, a no-template control (NTC) was run for each assay in the QC step. In all cases, no product was detectable (Figure 1, boxes B1–B3).

Consistency of RNA extraction efficiency between samples was satisfactory, as measured by cel-miR-39-3p levels (Cq range 19.4–23.7; s.d. 1.28) (Figure 1, box B2 and Supplementary

Figure S1A). Serum levels of the housekeeping miRNA miR-30b-5p (Murray *et al*, 2014) were also within the expected range (Cq range 23.7–28.8; s.d. 1.40; Figure 1, box B2 and Supplementary Figure S1B). Haemolysis assessment was performed using both miR-451a Cq (Supplementary Figure S1C) and delta Cq (miR-23a-3p minus miR-451a) values (Figure 1, box B3 and Supplementary Figure S1D). This assessment showed that 37 of 41 samples (90.2%) had a delta Cq of <8 and therefore no substantial evidence of haemolysis (Blondal *et al*, 2013); three samples had a borderline delta Cq value of 8–9 and only a single sample demonstrated haemolysis (delta Cq 10.2; Supplementary Figure S1D). In the absence of further replicate non-haemolysed serum samples, we proceeded to full analysis of these four cases in our evaluation study, but with appropriate caution regarding the results. This approach allowed us to study the potential effects of sample haemolysis on candidate biomarker levels.

MicroRNA quantification. Following initial QC assessment, the next phase of the pipeline was to quantify expression of a larger panel of target and housekeeping miRNAs (Supplementary Table S1). We first performed a multiplexed RT step. The TaqMan miRNA-specific stem-loop RT primers included those for the eight main miRNAs from the miR-371–373 and miR-302/367 clusters (Murray *et al*, 2011b), plus three miRNA housekeeping genes previously shown to be most stable in paediatric serum (namely miR-30b-5p, miR-30c-5p and miR-191-5p (Murray *et al*, 2014, 2015b), and the non-human spike-in technical control cel-miR-39-3p. To ensure sufficient product for the final PCRs, we performed two identical multiplexed RT reactions for each sample, using 5 µl of RNA eluate in each,

and an equal mixture of the relevant RT primers, adapted from (Murray *et al*, 2011b; Supplementary Table S1). The final volume of 15 μ l for each reaction underwent RT, as described above for the initial QC phase (Figure 1, box C).

Multiplexed pre-amplification of RT product. For the pre-amplification step, reactions were multiplexed using the relevant TaqMan assay probes, as described (Murray *et al*, 2011b). As pre-amplification of specific RT products increases target abundance without introducing bias (Murray *et al*, 2011b), this allowed greater assay sensitivity and increased the number of target miRNAs measured from the small starting volume. Briefly, an equal mix of all 20 \times TaqMan assay probes was prepared for each reaction and diluted to 0.2 \times with 1 \times Tris-EDTA buffer (pH 8.0), as described previously (Murray *et al*, 2011b). Two identical pre-amplification reactions (each 50 μ l) for each sample contained 25 μ l of 2 \times pre-amplification master mix (Life Technologies), 25 μ l of the diluted TaqMan assay probe mix and 12.5 μ l of multiplexed cDNA product (Murray *et al*, 2011b). After heating to 95 $^{\circ}$ C for 10 min, 14 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 4 min were run on the GeneAmp PCR System 9700 (Applied Biosystems; Murray *et al*, 2011b). The resulting reaction products were diluted 1:5 with nuclease-free water, and then the two pre-amplification reaction products for each individual sample were pooled, to make a final volume of 500 μ l, sufficient to perform all subsequent singleplex final miRNA PCRs in triplicate (Figure 1, box C).

Final singleplexed PCR of pre-amplification product. Nine μ l of the diluted pre-amplified cDNA product was added to 10 μ l of TaqMan 2 \times Universal PCR master mix, No AmpErase UNG (Applied Biosystems) and 1 μ l of each individual 20 \times TaqMan assay probe, thereby maintaining ratios identical to those previously described (Murray *et al*, 2011b). All reactions were performed in triplicate. PCR was performed using the conditions described above for the initial QC phase. For both the QC (Figure 1, boxes B1–B3) and the miRNA quantification steps (Figure 1, box C), the Cq threshold was set manually to 2000 fluorescence units across all PCR plates. NTC samples were also run in the miRNA quantification step; raw mean NTC Cq values are listed in Supplementary Table S1. Test miRNAs with results within two Cq values of the NTC sample or greater were classed as non-expressing (Figure 1, box C).

Data normalisation and analysis. In the miRNA quantification step (Figure 1, box C), cel-miR-39-3p and miR-30b-5p were again measured, to allow assessment of the technical reproducibility of the pipeline. A highly significant correlation was observed between the Cq values obtained in the initial QC (Figure 1, box B) and the miRNA quantification steps (Figure 1, box C), for both cel-miR-39-3p and miR-30b-5p ($R^2 = 0.937$, $P < 0.0001$ and $R^2 = 0.867$, $P < 0.0001$, respectively; Supplementary Figure S1E; Figure 1, box D).

When analysing the miRNA quantification data (Figure 1, box C), we first normalised expression of the three endogenous housekeeping miRNAs miR-30b-5p, miR-30c-5p and miR-191-5p (Murray *et al*, 2014, 2015b) to cel-miR-39-3p, in order to account for any differences in efficiency of the RNA extraction and RT steps. All three housekeeping miRNAs were found to be stably expressed across all 41 serum samples (Figure 2A). Importantly, all the miRNAs used for housekeeping purposes are transcribed from independent chromosomal loci (miR-30b-5p (chromosomal locus 8q24); miR-30c-5p (6q13); miR-191-5p (3p21.31) and miR-23a-3p (19p13.3) (Murray *et al*, 2015b)). Furthermore, all have previously been shown to be abundant and stably expressed in serum from both paediatric and adult (Blondal *et al*, 2013) patients. For the four serum samples that showed evidence of haemolysis (grey arrows in Figure 2A), expression levels of these

three miRNAs still fell within the normal range, demonstrating their suitability for housekeeping purposes.

By linear regression analysis, levels of cel-miR-39-3p correlated with each of the three endogenous housekeeping miRNAs, miR-30b-5p, miR-30c-5p and miR-191-5p (Figure 2B), although R^2 values were only modest, at 0.679, 0.418 and 0.421, respectively. To confirm that cel-miR-39-3p provided additional stringency when using the housekeeping miRNAs for data normalisation, we compared levels of all three with each other, both before (Figure 2C) and after (Figure 2D) cel-miR-39-3p correction. Although the three housekeeping miRNAs showed positive correlations with each other in all comparisons ($P < 0.0001$), addition of cel-miR-39-3p normalisation increased the R^2 values in all cases (miR-30b-5p vs miR-30c-5p, $R^2 = 0.857-0.925$; miR-30b-5p vs miR-191-5p, $R^2 = 0.824-0.877$; miR-30c-5p vs miR-191-5p, $R^2 = 0.888-0.904$; Figure 2C and D).

The s.d. of the serum Cq values across the whole cohort of 41 samples was lowest for miR-30b-5p (1.16), when compared with miR-30c-5p (1.52) and miR-191-5p (1.51). Consequently, miR-30b-5p was selected for normalisation of target miRNA quantification data, following normalisation of the miR-30b-5p Cq values to the cel-miR-39-3p levels determined in the miRNA quantification step (Figure 1, box D). Target miRNA levels were quantified relative to miR-30b-5p using the delta Cq method, as described previously (Murray *et al*, 2014, 2015b). For further details, see Supplementary Materials and Methods. In this evaluation study we applied very stringent criteria for defining miRNA overexpression, whereby only serum miRNA levels that showed a ≥ 2.0 -fold change in expression (Murray *et al*, 2014; Murray *et al*, 2015b) compared with the highest level from the non-tumour control group were considered positive. Differences in serum miRNA levels between experimental groups were assessed using the Mann-Whitney *U*-test ($P < 0.05$, significant).

RESULTS

A four-serum miRNA panel (miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p) enables sensitive diagnosis of paediatric extracranial malignant GCTs. In group comparisons, normalised levels of four miRNAs were significantly higher in the serum of paediatric patients with extracranial malignant GCT, compared with both the combined benign GCT/non-GCT tumour group and the non-tumour control group (Figure 3A). This panel comprised the three main miRNAs from the miR-371-373 cluster (miR-371a-3p, miR-372-3p and miR-373-3p), plus miR-367-3p from the miR-302/367 cluster. Normalised serum levels of miR-302a-c were not consistently detected above the control threshold in malignant GCT patients (data not shown). As miR-302d-3p levels in all test samples were within two Cq values of the NTC sample, this miRNA was excluded from subsequent analyses.

When assessing cases individually, using our highly stringent criteria for defining miRNA overexpression, positive results were seen in four out of five extracranial malignant GCTs for miR-371a-3p; five of five for miR-372-3p; two of five for miR-373-3p and three of five for miR-367-3p (Figure 3B and Supplementary Figure S2A–D). There was no obvious association between test miRNA levels, and the presence of haemolysis in this small sample set. The greatest dynamic range in levels was seen for miR-371a-3p, which also showed the clearest separation between positive and negative samples – for example, the lowest level of miR-371a-3p observed in a positive sample (MGCT_ExC#3) was 10-fold higher than the highest non-tumour control (CONT_5; Supplementary Figure S2A). Interestingly, we did not detect miR-371a-3p overexpression in a serum sample reportedly taken

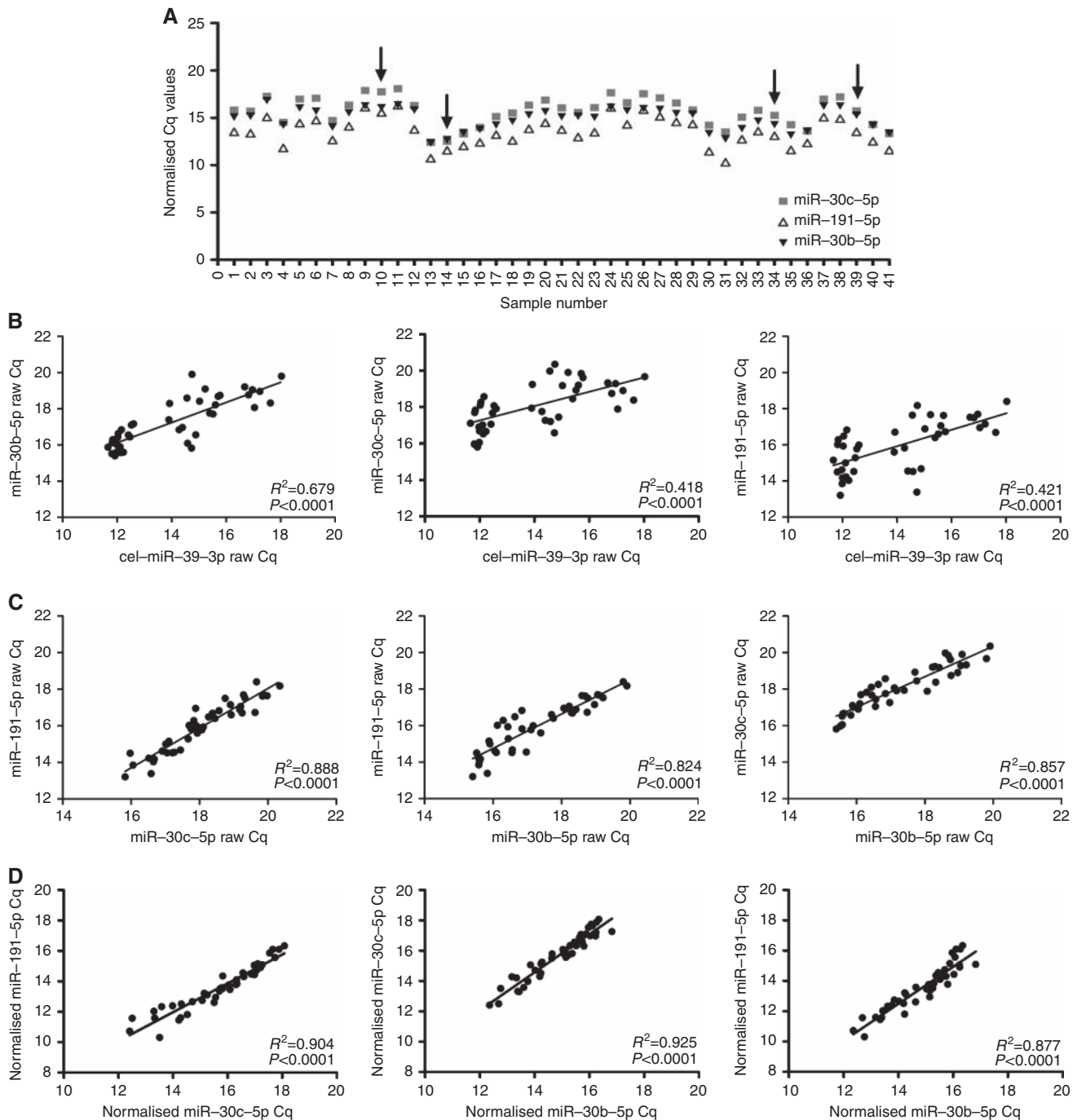


Figure 2. Levels of housekeeping miRNAs in the quantification phase of the pipeline (Figure 1, box C). **(A)** Cq values for the housekeeping miRNAs miR-30b-5p, miR-30c-5p and miR-191-5p, obtained after normalisation to cel-miR-39-3p, in all 41 serum samples tested in this study. Sample numbers correspond to those in Table 1. The four samples that had a delta Cq (miR-23a-3p minus miR-451a) value > 8 in the QC step (Figure 1, box B3) are indicated by arrows. **(B)** Correlations between the raw Cq values for the housekeeping miRNAs in **(A)** above and the raw cel-miR-39-3p Cq values. **(C and D)** Correlations between the raw **(C)** and cel-miR-39-3p-normalised **(D)** Cq values of the three housekeeping miRNAs in all serum samples tested: miR-30b-5p vs miR-191-5p (left), miR-30c-5p vs miR-191-5p (centre) and miR-30b-5p vs miR-30c-5p (right).

at the time of diagnosis of an ovarian YST (MGCT_Exp#4; Supplementary Figure S2A). Further enquiry revealed that the sample had in fact been taken 4 days following complete resection of the ovarian primary tumour, rather than prior to surgery. This is consistent with the described very short half-life for miR-371a-3p in the serum, where the major source has been removed (Dieckmann *et al*, 2015; Spiekermann *et al*, 2015a). However, other members of the four-miRNA panel identified remained

elevated in this serum sample (Supplementary Figure S2). Together, these observations demonstrated the value of using the whole panel in detecting extracranial malignant GCTs, including where the timing of the 'diagnostic' serum sample was suboptimal. In our dataset, miR-372-3p was the only serum miRNA that was elevated in all cases of extracranial malignant GCT.

Receiver operator characteristic (ROC) analysis, based on varying cutoffs for defining miRNA overexpression, showed that

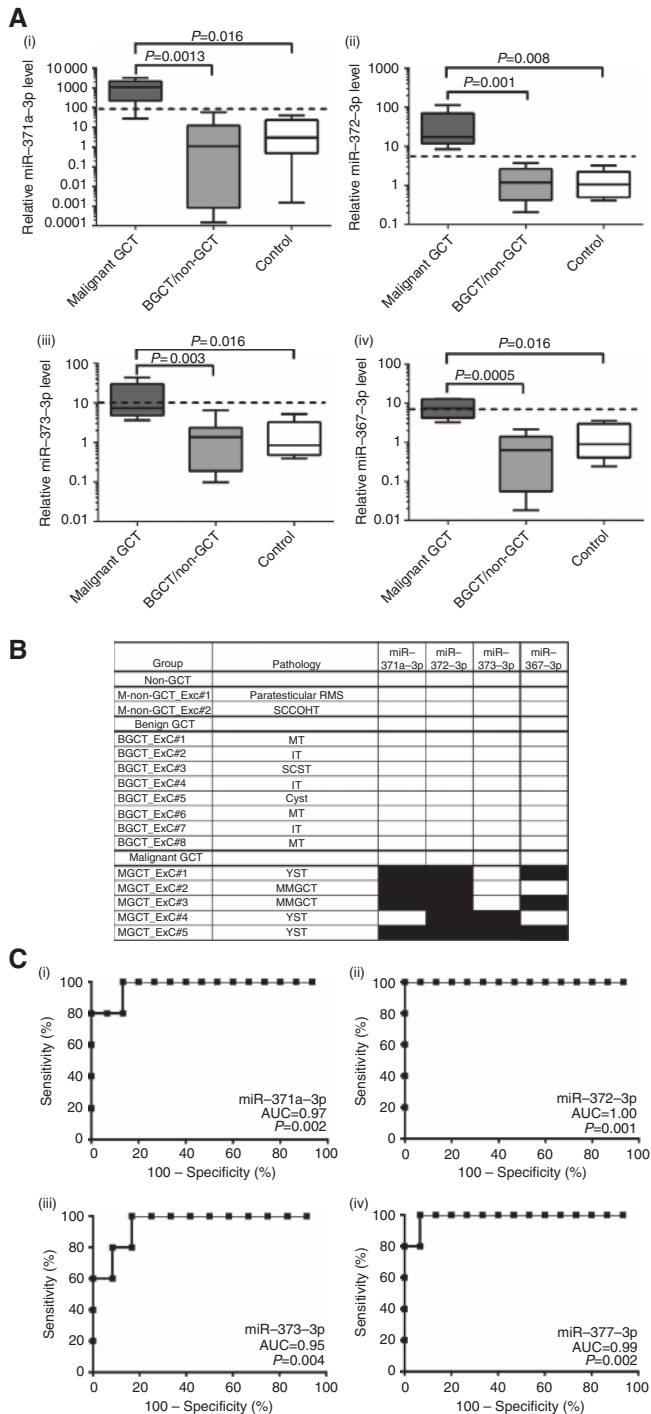


Figure 3. Relative levels of serum miRNAs in the quantification phase of the pipeline (Figure 1, box C) based on samples from patients with extracranial disease. **(A)** Levels in the three patient groups of: (i) miR-371a-3p, (ii) miR-372-3p, (iii) miR-373-3p and (iv) miR-367-3p by box-and-whisker plot. The broken horizontal line in each graph represents the cutoffs derived from the serum samples, that is, 2.0 times the highest level seen in the non-tumour control group. Box = interquartile range; whiskers = full range of data. **(B)** Summary heatmap of levels of each member of the four-miRNA panel in serum samples from patients at the time of diagnosis of extracranial disease. Positive results (i.e., miRNA levels ≥ 2.0 -fold above the highest level observed in the non-tumour control group) are displayed as black boxes. For abbreviations, see Table 1. **(C)** Individual ROC curves for each member of the four-miRNA panel, namely: (i) miR-371a-3p, (ii) miR-372-3p, (iii) miR-373-3p and (iv) miR-367-3p. Abbreviation: AUC = area under the curve; ROC = receiver operator characteristic.

each of the four members of the miRNA panel, miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p, were independently highly sensitive and specific for differentiating paediatric extracranial malignant GCT cases ($n = 5$) from a combined group ($n = 15$) consisting of both the extracranial benign GCT/non-GCT tumour cases and the non-tumour control samples (Figure 3C). We also compared raw Cq (non-normalised) and delta Cq (normalised) values for the four-serum miRNA panel (Supplementary Figure S2E), following recent reports suggesting that relative quantification using normalisation to a housekeeping miRNA may not be required when quantifying serum miRNAs for detection of malignant GCTs (Spiekermann *et al*, 2015b). We observed a close correlation between raw Cq and delta Cq values for miR-371a-3p ($R^2 = 0.969$), but for miR-372-3p, miR-373-3p and miR-367-3p, the correlation values were lower ($R^2 = 0.792$, 0.824 and 0.773, respectively; Supplementary Figure S2E).

Serum miRNA levels allow early detection of relapse during longitudinal follow-up for extracranial (testicular) malignant GCT. We assessed the value of the miR-371-373 and miR-302/367 cluster miRNAs for early detection of relapse in malignant GCT. We examined 18 samples obtained during diagnosis, treatment and follow-up (to day (d) 782) from a patient with a stage 1 testicular mixed malignant GCT (Figure 4). For full clinical details of the case, see Supplementary Results (clinical case 1).

The primary tumour contained YST, EC, CHC and immature teratoma (IT) components. At the time of diagnosis, the serum levels of miR-371a-3p, miR-372-3p and miR-367-3p were elevated (10.1, 2.6- and 2.1-fold, respectively, compared with the highest non-tumour control (Supplementary Figure S2A-D)). Seven of the eight main miRNAs (miR-302d-3p excluded) from the miR-371-373 and miR-302/367 clusters were profiled in 17 further longitudinal serum samples. After resection of the primary tumour, levels of miR-371a-3p and miR-372-3p decreased to baseline by d14 (Figure 4C and D, respectively). At the time of radiologically detected relapse of malignant disease on d28 (Figure 4E), AFP did not increase, while HCG levels showed only a modest increase of 35.4% from their previous values at d14 (Figure 4A and B, respectively). In striking contrast, miR-371a-3p increased from a relative level of 25.2 on d14, to 100.3 at d28 (398% increase) and 220.7 at d33 (876% increase; Figure 4C). Levels of miR-372-3p also increased at this time, albeit more modestly (Figure 4D).

Once treatment of the malignant relapse commenced (d33), levels of miR-371a-3p and miR-372-3p decreased rapidly to baseline (Figure 4C and D), whereas both AFP and HCG showed an initial 'flare' (observed at d42; Figure 4A and B), before falling to baseline. Further lymph node enlargement detected radiologically at the end-of-treatment at d101 was not associated with elevations of these miRNAs (nor of AFP/HCG; Figure 4). Following retroperitoneal lymph node dissection (at d140), histopathology showed a diagnosis of growing teratoma syndrome (a non-malignant condition; (Logothetis *et al*, 1982)), rather than further malignant relapse. In uneventful subsequent clinical follow-up from d140 to d782, AFP/HCG, miR-371a-3p and miR-372-3p levels all remained negative (Supplementary Figure S3A). By linear regression analysis of all 18 longitudinal samples, levels of AFP, HCG, miR-371a-3p and miR-372-3p each correlated significantly with the other three markers (Supplementary Figure S3B), with the strongest correlation being between miR-371a-3p and miR-372-3p levels ($R^2 = 0.909$; $P < 0.0001$). Outlier points in the serum miRNA and AFP/HCG correlations reflected the more pronounced changes in serum miRNA levels at the time of malignant relapse (at d28; red arrows in Supplementary Figure S3B) and the lack of a serum miRNA flare immediately following administration of chemotherapy (d42; green arrows in Supplementary Figure S3B).

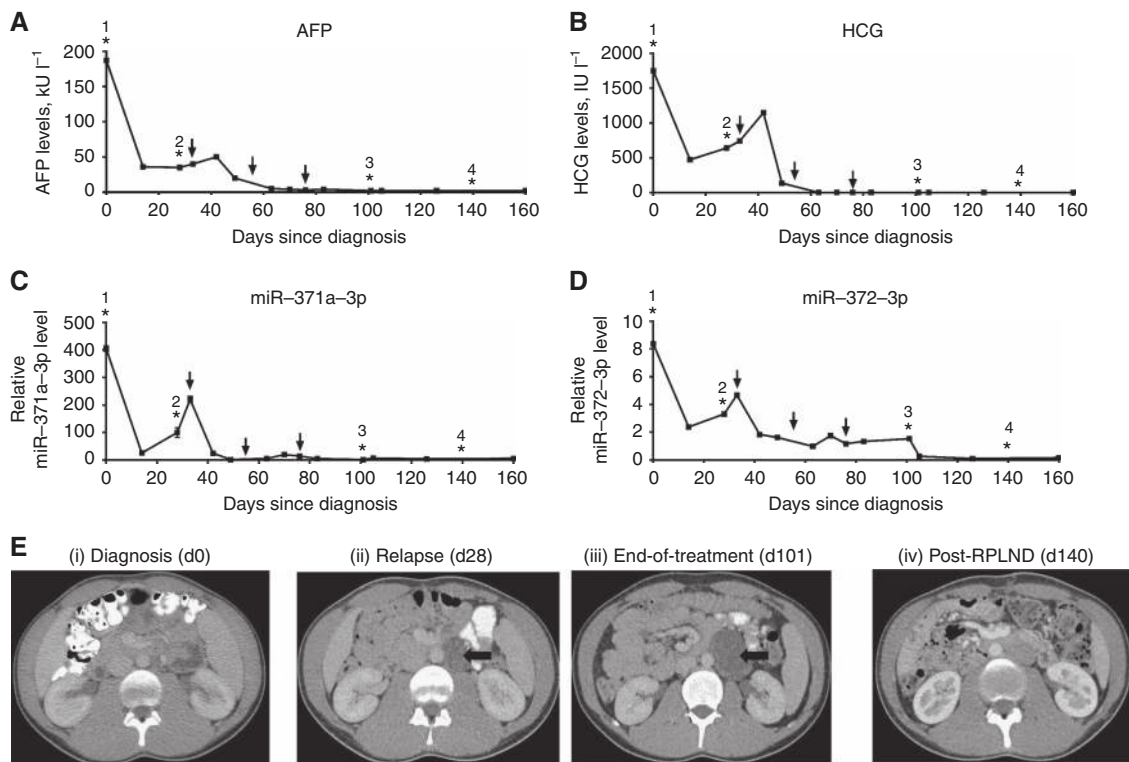


Figure 4. Follow-up of a patient with a testicular mixed malignant GCT (MGCT_Exc#3). **(A and B)** Show levels of the serum protein biomarkers AFP and HCG, respectively, while **(C and D)** show levels of miR-371a-3p and miR-372-3p, respectively, from d0 (diagnosis) to d160 of follow-up. Asterisks denote the clinical stages of: diagnosis (i), malignant relapse (ii), end-of-treatment (iii) and retroperitoneal lymph node dissection (RPLND, iv). The arrows show the timing of each chemotherapy cycle. **(E)** shows representative CT scan images at the four time points described above. The arrows show the retroperitoneal disease.

Serum and CSF miRNA levels distinguish intracranial malignant GCTs from intracranial non-GCT pathology in paediatric patients. To determine if the four-miRNA panel comprising miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p could be used to detect intracranial malignant GCT disease, we measured the levels of these miRNAs in four serum and four CSF samples, obtained from a total of five patients (Table 1 and Figure 5). For full clinical details of these cases, see Supplementary Results (clinical cases 2).

Serum levels of miR-371a-3p and miR-373-3p were positive at diagnosis in the intracranial malignant GCT patients MGCT_IC#3 and MGCT_IC#1, respectively (Figure 5B), both of whom had negative serum and CSF AFP/HCG levels. None of the other miRNAs tested was positive in the other serum samples, using the previously specified serum cutoffs. We extracted RNA from CSF of four patients (MGCT_IC#2, MGCT_IC#3, B-non-GCT_IC#1 and M-non-GCT_IC#1). The M-non-GCT_IC#1 case was a 2-year-old male with *MYCN*-amplified neuroblastoma (Murray *et al*, 2015b), who developed intracranial metastatic disease during treatment (Table 1). In each case, a fixed quantity of non-human spike-in cel-miR-39-3p was added, as for the serum samples. We quantified the levels of cel-miR-39-3p, the three housekeeping miRNAs that we had shown to be stable in the serum (miR-30b-5p, miR-30c-5p and miR-191-5p), and miR-124-3p, which had previously been suggested as a suitable CSF housekeeping miRNA (Exiqon, 2013).

Levels of cel-miR-39-3p recovery from the four CSF samples obtained in the miRNA quantification step were equivalent to those observed with the serum sample cohort (mean Cq cel-miR-39-3p CSF $13.27 \pm \text{s.d. } 0.12$ vs serum $13.98 \pm \text{s.d. } 0.26$; Supplementary Figure S4A). However, miR-124-3p levels showed a > 6 Cq difference across the CSF samples. The greatest miR-124-3p abundance in the CSF was in the *MYCN*-amplified neuroblastoma

sample (M-non-GCT_IC#1; Supplementary Figure S4A), consistent with our previous findings that miR-124-3p is a disease-associated serum biomarker (Murray *et al*, 2015b). Together, these data indicate that miR-124-3p is not a good candidate for CSF normalisation. In contrast, all three of the other housekeeping miRNAs tested were very stable across the small number of CSF samples examined, albeit with higher mean Cq values than in the serum samples (miR-30b-5p, 17.0 vs 14.9; miR-30c-5p, 19.0 vs 15.7 and miR-191-5p, 16.5 vs 13.6, respectively; Supplementary Figure S4A). Similar to our observations in the serum samples, we identified that the optimal CSF housekeeping miRNA was miR-30b-5p, which had a lower s.d. (0.18) than miR-30c-5p (0.53), miR-191-5p (0.21) and miR-124-3p (3.0; Supplementary Figure S4A).

As with the serum samples, we also quantified CSF levels of miR-23a-3p and miR-451a to assess haemolysis (Supplementary Figure S4B). Despite all four CSF samples being clear and colourless on visual inspection (i.e., showing no evidence of haemolysis), two were above the serum haemolysis threshold ($\Delta \text{Cq} > 8$) using standard methodology (Blondal *et al*, 2013; Supplementary Figure S4B). For both miR-451a and miR-23a-3p, levels in CSF were lower than in haemolysis-negative serum samples ($\Delta \text{Cq} < 8$, $n = 37$), as indicated by higher mean Cq values (Supplementary Figure S4B). For several samples, the elevations in miR-23a-3p Cq values were substantially greater than those of miR-451a. This indicated that ΔCq (miR-23a-3p – miR-451a) levels were of limited use in indicating haemolysis in CSF.

The levels of miR-371-373 and miR-302/367 were then measured in the three available diagnostic CSF samples from patients with primary intracranial tumours (MGCT_IC#2, MGCT_IC#3 and B-non-GCT_IC#1; Figure 5C). The normalisation and stringent cutoff procedures used for serum samples

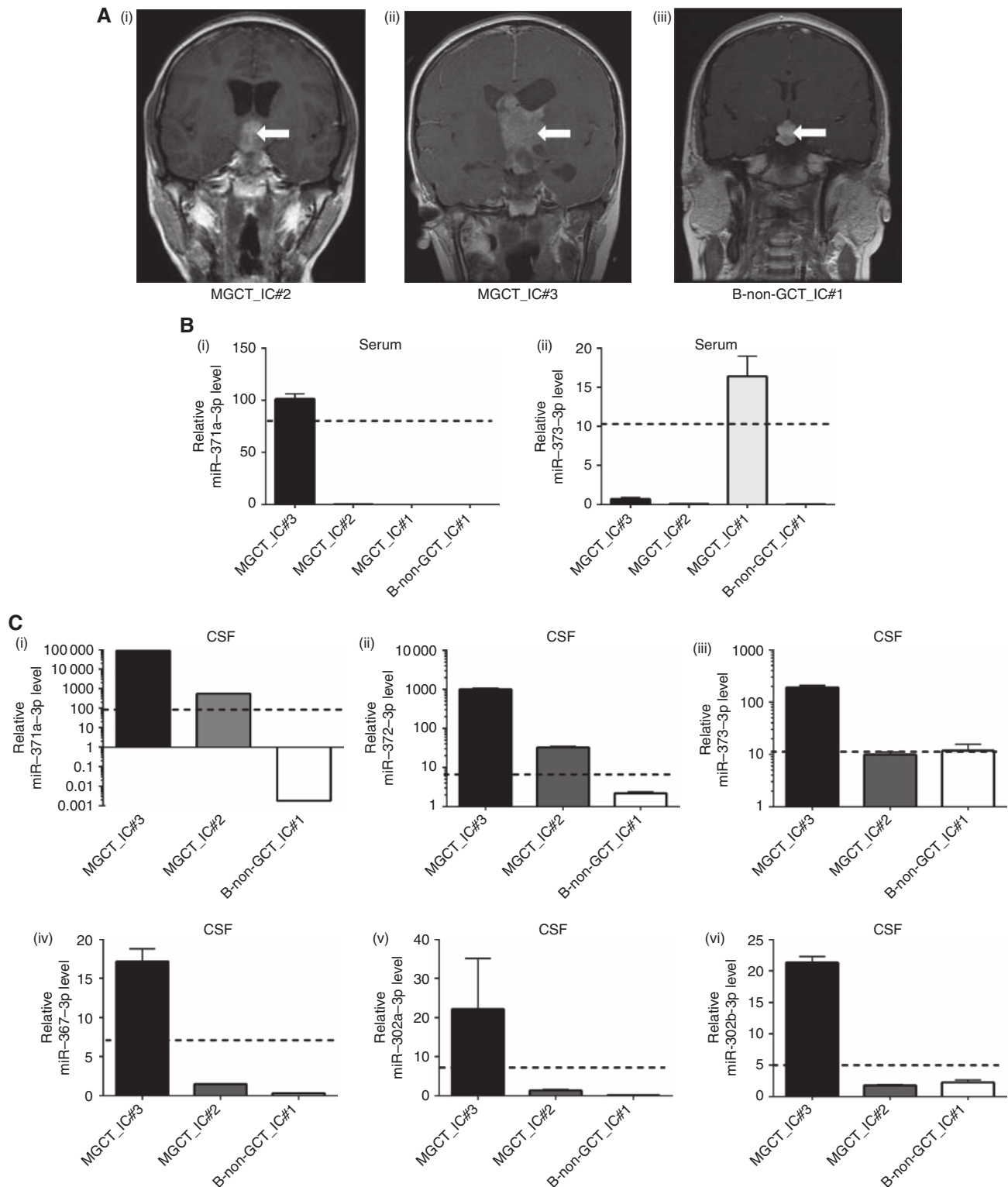


Figure 5. Relative levels of serum and CSF miRNAs for diagnosis of primary intracranial GCTs. (A) MR images for three patients with primary intracranial tumours, namely: (i) a suprasellar non-germinomatous GCT (MGCT_IC#2), (ii) a suprasellar germinoma (MGCT_IC#3) and (iii) a ganglioglioma (B-non-GCT_IC#1). (B) Serum levels of: (i) miR-371a-3p and (ii) miR-373-3p, measured in the three patients with primary intracranial disease shown in (A), plus a fourth with a primary intracranial pineal germinoma (MGCT_IC#1). (C) CSF levels of: (i) miR-371a-3p, (ii) miR-372-3p, (iii) miR-373-3p, (iv) miR-367-3p, (v) miR-302a-3p and (vi) miR-302b-3p in the three patients with primary intracranial disease shown in (A). Thresholds (dotted lines) are as in Figure 3. Error bars = s.e.m.

were applied, with miRNAs that had a ≥ 2.0 -fold change in expression compared with the highest serum level from the non-tumour control group being considered positive. Using these criteria, CSF miR-371a-3p and miR-372-3p levels clearly

distinguished the intracranial malignant GCTs from the non-malignant (ganglioglioma) case (Figure 5C). In addition, CSF levels of miR-373-3p, miR-367-3p, miR-302a-3p and miR-302b-3p were also positive in the MGCT_IC#3 germinoma case

(Figure 5C). The raw (non-normalised) Cq values for the four miRNAs in the panel identified from serum analysis were similar in the positive and negative CSF samples to those in the positive and negative serum samples, respectively (Supplementary Figure S4C), suggesting there was approximate equivalence in the overall abundance of these miRNAs in serum and CSF.

DISCUSSION

We report a robust, quality controlled pipeline for measuring levels of specific serum and CSF miRNAs in paediatric patients with extracranial and intracranial malignant GCTs. The pipeline benefits from an exogenous non-human spike-in control (cel-miR-39-3p), normalisation using an endogenous house-keeping miRNA (miR-30b-5p) and haemolysis quantification (Blondal *et al*, 2013). Variation in exogenous cel-miR-39-3p levels between samples may be reported as differences in Cq values (Yamada *et al*, 2014) or recovery rates (Sanders *et al*, 2012). The variation we observed in cel-miR-39-3p levels in our cohort, of ~4 Cq cycles, reflects the genuine difference in RNA recovery between serum samples, which must be normalised to avoid creating false negative/positive results. This variation is consistent with other reports (e.g., Yamada *et al*, 2014) and less than that described in other studies, which for example, report a recovery rate of 1–56% (i.e., variation of ~6 Cq values; Sanders *et al*, 2012).

The correlation data in our relatively small study also indicate an additional benefit of normalising to a stable, endogenously expressed miRNA, that is, miR-30b-5p. This second normalisation step accounts for differences between overall circulating endogenous miRNA levels in individuals, which is not addressed using cel-miR-39-3p levels alone. Our data indicate that this highly stringent pipeline, including two normalisation steps, minimises technical alterations and maximises detection of true biological variation between serum samples. It will be important for prospective studies to evaluate the relative contributions of the two normalisation steps to the avoidance of potential false negative/positive results. In future, once assessed fully, it may be possible to relax the proposed normalisation stringency in circulating miRNA studies.

For CSF analysis, we have shown that miR-30b-5p is stable in a small cohort of samples, demonstrating minimal variation, and may therefore also be suitable for normalisation in future CSF studies. In contrast to previous suggestions (Exiqon, 2013), miR-124-3p does not appear appropriate as a housekeeping gene for CSF normalisation and indeed has been identified as a candidate tumour biomarker in its own right (Murray *et al*, 2015b).

Haemolysis assessment may also be important during initial QC, as variations in red blood cell lysis between samples may result in altered serum expression levels of miRNAs of interest for technical, rather than biological, reasons (Pritchard *et al*, 2012). There are a number of methods for such assessment, including the measurement of free haemoglobin (Kirschner *et al*, 2011), or the established use of delta Cq (miR-23a-3p minus miR-451a) levels (Blondal *et al*, 2013; Exiqon, 2013; Ludwig *et al*, 2015; Murray *et al*, 2015b). The haemolysis results described here, using the latter approach, appear comparable in terms of cutoff thresholds to data obtained utilising different qRT-PCR platforms (Blondal *et al*, 2013; Ludwig *et al*, 2015). Haemolysis did not appear to have any major effect on the levels of our candidate biomarkers from the miR-371-373 and miR-302/367 clusters, albeit based on a low sample size. However, as it will be critical to avoid false positive results when such tests are established in clinical practice, we recommend that haemolysis assessment should be performed prospectively in circulating miRNA studies, to define precisely what effect haemolysis has on candidate biomarkers in borderline

cases. Of note, the miRNA quantification method used to indicate haemolysis in serum samples was not appropriate for CSF, where alternative approaches are required.

From our profiling of all eight main members of the miR-371-373 and miR-302/367 clusters, we derived a panel of four miRNAs that is capable of accurately detecting both extracranial and intracranial malignant GCT disease in paediatric patients (see Supplementary Discussion). Interestingly, the same panel was shown to be of diagnostic value in adults with testicular malignant GCTs (Gillis *et al*, 2013; Syring *et al*, 2015). In contrast to recent reports (Dieckmann *et al*, 2015; Spiekermann *et al*, 2015a,b), miR-371a-3p alone was not sufficient to identify some malignant GCTs in our patient cohort, including in serum from a patient with an intracranial (pineal) germinoma, where only miR-373-3p was positive. Moreover, the particularly rapid reduction in miR-371a-3p levels following tumour removal in stage 1 disease (Dieckmann *et al*, 2015; Spiekermann *et al*, 2015a) will lead to negative results in samples taken more than 1 day post surgery, unlike the other miRNAs in the panel. Our data suggest that expression in body fluids of the four miRNAs in the panel varies across the spectrum of malignant GCT patients and that the full panel provides maximal sensitivity, without affecting specificity. Accordingly, elevated levels of one (or more) member(s) of the panel would represent a positive test result. As miR-367-3p is transcribed from a separate chromosome locus (4q25) to the other three miRNAs from the miR-371-373 cluster (19q13), elevated serum miR-367-3p levels would add confidence to a diagnosis of malignant GCT, as distinct from other malignancies that overexpress miR-371-373 miRNAs but not miR-367-3p. Such tumours include carcinomas of the stomach (Liu *et al*, 2012) and pancreas (He *et al*, 2014), as well as primary intracranial tumours with genomic amplification of 19q13 (Pfister *et al*, 2009; Korshunov *et al*, 2010).

We have shown, for the first time, that miRNAs from the four-serum miRNA panel can sensitively detect malignant GCT relapse during longitudinal follow-up. In the case studied, miR-371a-3p and miR-372-3p detected relapse of an AFP/HCG-secreting (marker-positive) testicular mixed malignant GCT. Indeed, our data suggest that the miRNAs are more sensitive at detecting malignant relapse than conventional protein biomarkers, as they provided a clear positive result at the initial time of radiological relapse (d28), when HCG levels showed only a modest increase and AFP levels had not changed. In addition, miR-371a-3p and miR-372-3p appeared to give a more accurate measure of early response to therapy, as, unlike AFP and HCG, levels did not flare following initiation of chemotherapy. Longitudinal monitoring of further extracranial malignant GCT cases using this panel of serum miRNAs is likely to demonstrate their clinical utility in the follow-up of 'marker-negative' cases. In due course, serum miRNA monitoring in malignant GCT patients may reduce the need for serial CT scans, the associated radiation exposure and second cancer risk (Tarin *et al*, 2009).

It has recently been suggested that, it may not be necessary to perform relative quantification when assessing serum miRNAs as a potential test for testicular malignant GCTs and that raw Cq values provide adequate diagnostic discrimination (Spiekermann *et al*, 2015b). Our data suggest that overall this is a suboptimal approach when using current methodology. Serum miR-371a-3p has a large dynamic range, allowing a greater degree of separation between positive and negative samples in our study. For this miRNA, the raw Ct (non-normalised) and delta Ct (normalised) values demonstrated a very strong correlation ($R^2=0.969$). However, for the other three miRNAs in the four-serum panel (namely miR-372-3p, miR-373-3p and miR-367-3p), the dynamic range was lower. The difference in mean Cq value between positive and negative samples was ~4 and the R^2 correlation for Cq and delta Cq values were also lower at 0.792, 0.824 and 0.773, respectively.

For such miRNAs, differences in RNA extraction efficiency will have a relatively high impact on PCR measurements and will require careful control via relative quantification approaches. Methods that use data normalisation will also enable analysis of low volume samples (<200 µl), for which dilution is required to produce a suitable starting volume. Such a scenario is particularly likely to occur in specimens from paediatric patients and/or certain sample types, such as CSF.

In due course, robust absolute quantification systems may supersede relative quantification when measuring miRNA levels in body fluids (Hindson *et al*, 2013), although the use of a non-human spike-in or other appropriate 'standard' will still need to be employed, to ensure the accuracy of RNA extraction steps and subsequent quantification within and between samples (Sanders *et al*, 2012). Determining absolute miRNA counts per set volume of serum would allow the determination of universal cutoffs, for example, for miR-451a in assessing haemolysis and for the four-miRNA panel in diagnosing malignant GCT. This should improve sensitivity and specificity overall and provide greater confidence in 'borderline' cases, ensuring that diagnostic decisions are not undermined by subtle technical differences. This is important for translation into the clinic, where minimisation of potential false negative/positive results is critical.

In conclusion, we have described a robust pipeline for diagnosis and monitoring of extracranial and intracranial paediatric malignant GCTs. The technical principles and clinical observations described here will underpin the future clinical validation work that is now warranted.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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