Identification of MicroRNAs From the miR-371–373 and miR-302 Clusters as Potential Serum Biomarkers of Malignant Germ Cell Tumors

Matthew J. Murray, MB, BChir,1,2 David J. Halsall, MSc, PhD,3 C. Elizabeth Hook, MB, BChir, PhD,4 Denise M. Williams, MB, BCh,2 James C. Nicholson, MB, BChir, DM,2 and Nicholas Coleman, MB, ChB, PhD1,5

Key Words: Biomarker; Blood; Diagnosis; Germ cell tumor; Malignant; MicroRNA; miR-302; miR-371–373; Serum

Abstract

Current serum biomarkers for diagnosis and monitoring of malignant germ cell tumors (GCTs) show limited sensitivity and specificity. We previously observed that microRNAs of the miR-371–373 and miR-302 clusters are overexpressed in all malignant GCTs, regardless of patient age, histologic subtype, or anatomic site, but are not reported to be coordinately up-regulated in other tumor types or disease states. Herein we show that levels of all 8 main members of the miR-371–373 and miR-302 clusters were elevated in the serum of a 4-year-old boy at the time of diagnosis of yolk sac tumor. Levels returned to normal during an uneventful clinical follow-up, with kinetics similar to those of the conventional marker α-fetoprotein.

We describe in detail the multiplex polymerase chain reaction protocol used to quantify serum microRNA levels, which is highly robust and reproducible. Our study indicates that miR-371–373 and miR-302 cluster microRNAs are promising candidate biomarkers for improved disease monitoring (and potentially diagnosis) in malignant GCTs.

Germ cell tumors (GCTs) are clinically and pathologically heterogeneous. They arise across a broad age spectrum, encompass a range of histologic subtypes, and occur at numerous anatomic sites, both gonadal and extragonadal.1 Malignant GCTs, which include yolk sac tumors (YSTs), germimomas, and embryonal carcinomas, are generally treated by a combination of surgery and chemotherapy, with additional radiotherapy in some cases. Teratomas (mature or immature) are benign GCTs and should be treated by surgical excision alone, with close clinical follow-up.

There is no universal biomarker of malignant GCTs. The serum markers α-fetoprotein (AFP) and human chorionic gonadotropin (HCG) are used to assist diagnosis but have limitations in sensitivity and specificity. Neither marker is raised in all cases of malignant GCT, and both show elevations in nonmalignant states.1 A biomarker that offered greater sensitivity and specificity for diagnosing or monitoring malignant GCTs would, therefore, be of considerable clinical value. There is increasing interest in the diagnostic potential of microRNAs, which are short, non–protein-coding RNAs that critically regulate gene expression.2 MicroRNAs are dysregulated in cancer,3 and expression profiles can identify the developmental lineage of numerous tumors.4 Consistent with the origin of all malignant GCTs from a common progenitor (the totipotent primordial germ cell1), Palmer et al5 recently demonstrated that miR-371–373 and miR-302 clusters are highly overexpressed in all malignant GCTs (compared with normal control tissues and benign GCTs), irrespective of patient age, histologic subtype, or anatomic site of the tumor.
Indeed, in combined analyses, the 8 main microRNAs from these 2 clusters robustly distinguished 74 malignant GCTs from 27 nonmalignant samples,\(^2\) with only a single adult malignant GCT being misclassified. These microRNA clusters may, therefore, be of clinical value, for example, in diagnosing malignant GCTs (or malignant GCT elements within teratomas), monitoring response to treatment, and detecting early disease recurrence during clinical follow-up.

Recently, microRNAs have been shown to be remarkably stable in the blood, with altered serum levels reflecting dysregulated expression in malignancy.\(^6\)-\(^10\) Indeed, a panel of 4 serum microRNAs was recently demonstrated to be an independent predictor of overall survival in non–small cell lung cancer.\(^11\) We reasoned that members of the miR-371~373 and miR-302 clusters may be universal serum biomarkers for improving clinical management of malignant GCTs and thereby offer advantages over currently available markers. Herein we report that all 8 main microRNAs from the miR-371~373 and miR-302 clusters could be detected at highly elevated levels in the serum at the time of diagnosis of a malignant GCT in a 4-year-old boy. We found that serum levels of miR-372 fell during treatment with chemotherapy and surgery and remained low during an uneventful clinical follow-up. We describe in detail the protocol used to make these observations, which is technically straightforward and scalable. Our observations are the first demonstration of the potential value of these microRNAs as serum biomarkers of malignant GCTs.

**Case Report**

A 4-year-old boy was examined because of a 2-month history of abnormal gait and severe constipation, with 2 weeks of a swollen right buttock and fecal soiling. There were no urinary symptoms. Examination revealed a 10 × 10-cm firm swelling of the right buttock. The rest of the examination was unremarkable, save for palpable fecal loading per abdomen.

Magnetic resonance imaging demonstrated a large pelvic mass, which engulfed the coccyx and distal sacrum to the level of the S2 vertebra, displaced the rectum, and infiltrated the subcutaneous paraspinal tissues and right gluteal musculature.\(\text{Image 1A}\) and \(\text{Image 1B}\). The colon and bladder were grossly distended. A staging chest computed tomography scan revealed a number of 6- to 7-mm peripheral lung nodules\(\text{Image 1C}\) consistent with metastases. Conventional serum tumor markers revealed a markedly raised AFP level (82,430 IU/mL; reference range, 0-10 kU/L) with a normal HCG level (<2 IU/L). Renal function and estimation of glomerular filtration rate were normal.

Open biopsy of the mass showed a mixed composition, with benign glandular formations and pleomorphic large malignant cells forming lace-like arrangements. By immunohistochemical analysis, the malignant cells expressed AFP and pancytokeratins, as well as integrase interaction 1 (excluding malignant rhabdoid tumor)\(\text{Image 1D}\) and \(\text{Image 1E}\). Together, these findings were consistent with the diagnosis of an extracranial, stage 4YST arising within a sacrococcygeal teratoma. According to the UK Children’s Cancer and Leukaemia Group extracranial GCT protocol (GC-III), the patient was stratified into the high-risk group owing to the degree of AFP elevation.\(^12\)

He received 6 courses of “JEB” chemotherapy per protocol (carboplatin, mg intravenously, equating to area under the curve [AUC] of 7.9 mg/mL/min, on day 2; etoposide, 120 mg/m\(^2\) intravenously on days 1, 2, and 3; and bleomycin, 15 IU/m\(^2\) intravenously on day 3), at 3-week intervals, except for course 5, which was delayed due to infection. Serum AFP estimation had normalized by the start of the sixth course. Reassessment imaging at the end of chemotherapy confirmed resolution of the lung metastases and significant reduction in size of the pelvic primary tumor to 3.4 × 3.3 × 2.6 cm. At surgery, on day 139, the coccyx was removed en bloc with the tumor. Ninety percent macroscopic excision was possible, with residual tumor adherent to the rectum and sciatic nerve. The patient made an uncomplicated recovery and entered clinical follow-up with regular AFP estimation (which remains <2 kU/L, now 10 months after the end of treatment) and unremarkable magnetic resonance imaging findings.

**Materials and Methods**

**Study Rationale**

In view of our previous findings, we investigated whether the 8 main members of the miR-371~373 and miR-302 clusters could be detected in the serum at the time of malignant GCT diagnosis and also monitored changes in levels of a representative microRNA during treatment and follow-up. 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.

**Determination of Cross-reactivity Between Individual MicroRNA Assays**

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) measurement of microRNA levels using TaqMan reagents (Applied Biosystems, Warrington, England) is routinely performed in 2 steps using a “singleplex” approach. First, RT is undertaken to produce complementary DNA (cDNA) from an individual microRNA, using a specific stem-loop primer. Second, PCR is performed with a specific
Radiologic and histopathologic features of the germ cell tumor in the index patient. A, Coronal magnetic resonance imaging (MRI) demonstrating a large central pelvic mass with contrast enhancement. B, Axial MRI of pelvis showing infiltration of the gluteal muscles by the tumor. C, Axial computed tomographic chest scan revealing a metastatic nodule in the left peripheral lung field (arrow). D, Yolk sac tumor component in which pleomorphic malignant cells form lace-like arrangements (H&E, ×200). E, Immunohistochemical staining for α-fetoprotein showing strong positivity in the malignant cells (×200). The images in D and E were taken using an Olympus BX51 microscope with Infinity II camera and capture software (Olympus, Southend, England).
probe to quantify expression of each individual microRNA. In initial work, we sought to determine whether TaqMan qRT-PCR steps could be multiplexed to enable high-throughput quantification (with technical replicates) of all main members of the miR-371~373 and miR-302 clusters using small serum volumes. In particular, we investigated whether multiplexing would be limited by cross-reactivity, given the very similar nucleotide sequences of the individual microRNAs; for example, miR-302b and miR-302d differ from miR-302c at only 2 nucleotide positions (Figure 1A).

**Figure 1** Serum levels of microRNAs from the miR-371~373 and miR-302 clusters. **A**, Top, The graph shows mean miR-302c expression (y-axis) in samples prepared using individual reverse transcription (RT) primers for each of the 5 main members of the miR-302 cluster (x-axis). This was performed in 3 yolk sac tumors in triplicate. In all cases, products were quantified using the miR-302c probe. Mean expression values in the samples prepared using the miR-302c RT primer were assigned an arbitrary value of 100%. No signal was detected in samples prepared using RT primers specific for the other 4 main members of the miR-302 cluster. Bottom, Listed are the nucleotide sequences of the 5 main members of the miR-302 cluster. Nucleotides identical to those in miR-302c are shown in black, and differences are in red. **B**, Fold change overexpression of the 8 main microRNAs from the miR-371~373 and miR-302 clusters in the serum at diagnosis of the index malignant germ cell tumor, relative to the overall mean expression value for normal serum from 3 subjects. miR-371~373 cluster members are depicted in red and miR-302 cluster members in blue. Error bars represent the standard deviation. **C**, Serial measurements of serum α-fetoprotein (AFP; circles) and miR-372 (triangles) expression from diagnosis (day 0) to day 410 after diagnosis. Note that the y-axis is plotted on a log10 scale. Black arrows indicate the timing of the 6 chemotherapy courses, and the red arrow indicates the timing of surgical excision of the primary tumor.
We used total RNA from 3 randomly selected YSTs from a previous study.\(^5\) We initially performed standard singleplex RT steps for each of the 5 main members of the miR-302 cluster (miR-302a~302d and miR-367) using specific TaqMan RT primers (Applied Biosystems), according to the manufacturer’s instructions. Each RT product then underwent the standard PCR quantification step, but using only the TaqMan probe for miR-302c in all cases. The specificity of miR-302c quantification was determined from the amounts of each microRNA detected using this approach. All reactions were performed in triplicate. The overall mean miR-302c expression for the 3 YST samples (based on 9 data points) was assigned an arbitrary value of 100%. Expression levels of miR-302c determined in each of the other specimens (prepared using RT primers specific for miR-302a, miR-302b, miR-302d, and miR-367, respectively) were given relative percentage values.

**Processing of Blood Samples**

As part of the patient’s standard clinical care, blood was sampled in serum separator tubes. All specimens were processed and centrifuged within 4 hours of receipt. We compared microRNA levels with those in normal human serum, using anonymized specimens from 3 healthy subjects. After being used for routine clinical measurements, residual serum was frozen, stored at –80°C, and thawed only for RNA isolation. For certain time points, the volume of residual serum was insufficient for RNA extraction (ie, <400 \(\mu\)L\(^13\)).

**RNA Isolation From Serum**

For this step, 400 \(\mu\)L of serum was thawed on ice and small RNAs (<200 nucleotides in length) isolated using the mirVana PARIS isolation kit (Ambion, Warrington, England), according to the manufacturer’s instructions for liquid samples, but with 2 acid-phenol chloroform extractions owing to the high protein content of serum.\(^6\) The resultant 80-\(\mu\)L eluate from each sample underwent small RNA quantification using the NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE). The range of concentrations observed (2.1-17.8 ng/\(\mu\)L) was similar to that reported previously.\(^9\)

**Multiplexed RT of Serum RNA**

For a multiplexed RT step, 5 \(\mu\)L of eluate from each sample was used, using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems). The RT primers represented an equal mixture of eight 5× microRNA-specific stem-loop primers from the relevant TaqMan microRNA assay kits (all Applied Biosystems), namely miR-371-3p (Assay ID 002124), miR-372 (000560), miR-373 (000561), miR-302a (000529), miR-302b (000531), miR-302c (000533), miR-302d (000535), and miR-367 (000555). Otherwise, the reaction was performed as per the manufacturer’s instructions. The final volume of 15 \(\mu\)L underwent RT using a GeneAmp PCR System 9700 (Applied Biosystems) at 16°C for 30 minutes, 42°C for 30 minutes, and followed by a final step of 85°C for 5 minutes.

**Multiplexed Preamplification of RT Product**

This step was performed using the TaqMan PreAmp 2× Master Mix Kit (Applied Biosystems), according to the manufacturer’s instructions, but using an equal mixture of 8 individual 20× PCR probes obtained from the TaqMan microRNA assay kits described in the preceding paragraph. This pooled stock was diluted to 0.2× with 1× Tris-EDTA buffer (pH 8.0) as described,\(^8\) and 12.5 \(\mu\)L was combined with an equal volume of RT product, plus 25 \(\mu\)L of TaqMan PreAmp Master Mix, to make a final volume of 50 \(\mu\)L. After heating to 95°C for 10 minutes, 14 cycles of 95°C for 15 seconds and 60°C for 4 minutes were run on the GeneAmp PCR System 9700. The resultant 50 \(\mu\)L preamplification product was then diluted 1:5 in nuclease-free water to make a final 250-\(\mu\)L volume, which allowed singleplexed final PCRs to be undertaken, in triplicate, for all 8 microRNAs.

**Final Singleplexed PCR of Preamplification Product**

For this step, 9 \(\mu\)L of the diluted preamplification product was added to 10 \(\mu\)L of TaqMan 2× Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), and 1 \(\mu\)L of an individual 20× TaqMan microRNA probe, thereby maintaining ratios identical to those previously described.\(^8\) All reactions were done in triplicate. PCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The automatic program setting was used for calculating cycle threshold (Ct) values. Means were calculated from the triplicate Ct values; for the 3 normal serum samples, an overall mean was taken from the 9 relevant values. Relative quantification for each of the 8 microRNAs being studied was performed using the \(2^{\Delta\Delta Ct}\) method, with fold change = \(2^{(\text{pooled normal Ct} - \text{mean patient Ct})}\). All Ct values were normalized for small RNA input into the initial RT reaction, to allow for the variation in RNA concentration in the eluates used.

**Results**

**Determination of Cross-reactivity Between Individual MicroRNA Assays**

We first investigated the specificity of detection of individual microRNAs by TaqMan qRT-PCR. The TaqMan probe for miR-302c gave no signal with samples prepared following RT steps specific for miR-302a, miR-302b, miR-302d, or miR-367, despite miR-302a, miR-302b, and miR-302d having
only 2 or 3 differences in nucleotide sequence compared with miR-302c (Figure 1A). As miR-302c is 23 nucleotides in length, we conclude that TaqMan qRT-PCR produced no cross-reactivity between microRNAs sharing up to 91% sequence homology. The members of the miR-371~373 cluster display much greater sequence variation than that seen for those of the miR-302 cluster and are, therefore, even less likely to produce cross-reactivity by TaqMan qRT-PCR. In view of the observed assay specificity, we concluded that multiplexing the RT and preamplification steps of TaqMan qRT-PCR would be suitable for high-throughput quantification of all 8 main members of the miR-371~373 and miR-302 clusters, using small-volume serum samples.

Detection of Serum miR-371~373 and miR-302 Clusters by qRT-PCR

In initial work, we tested the value of 6 standard small nuclear and nucleolar RNAs as housekeeping genes for normalizing microRNA qRT-PCR analyses. The RNAs were RNU6b, RNU24, RNU38b, RNU43, RNU44, and RNU48 (all assays from Applied Biosystems). Levels were quantified according to the manufacturer’s instructions in normal serum samples. We observed marked variation in expression, which in some cases was undetectable (data not shown). These small nuclear and nucleolar RNAs were therefore deemed unsuitable for normalization of serum microRNA expression levels, consistent with previous reports.6 Accordingly, in keeping with the findings of others,6 microRNAs were quantified using a fixed volume of RNA isolation eluate (5 μL), obtained from a fixed volume of initial serum (400 μL), with normalization to initial input small RNA concentration. The resultant adjusted Ct values were used for relative microRNA quantification using the δCt method.7 We observed a linear relationship between Ct values in samples that had undergone a multiplexed preamplification step and those that had not (data not shown), indicating that no amplification bias was introduced by preamplifying the RT product.

In the serum sample taken at the time of malignant GCT diagnosis in the index patient, we observed clear overexpression of all 8 main members of the miR-371~373 and miR-302 clusters compared with normal serum pooled from 3 healthy subjects (Figure 1B). The fold changes were most marked for miR-372 and miR-373 (703 and 192, respectively; Figure 1B). The standard deviations were low, with a median value of 3.4% of the respective means, demonstrating the technical reproducibility of the method used.

Tracking of Serum miR-372 Expression Levels From Diagnosis

The microRNA that was most overexpressed at the time of malignant GCT diagnosis (miR-372) was selected for tracking of expression levels during the patient’s subsequent treatment and uneventful follow-up (Figure 1C). Unfortunately, insufficient residual serum was available to quantify miR-372 levels on days 22, 31, 39, and 44 after diagnosis. However, at day 73 after diagnosis, miR-372 levels had fallen from 703- to 5.8-fold higher than in normal serum, with AFP reducing from 82,430 to 69 kU/L (range, 0-10 kU/L). By day 91, miR-372 levels were only 2.2-fold higher and AFP was 6 kU/L. At all subsequent time points during the patient’s follow-up, miR-372 levels were 1 or less (ie, less than or equal to levels in normal serum) and AFP levels were 2 kU/L or less (Figure 1C).

Discussion

The value of the conventional tumor markers AFP and HCG in malignant GCTs is restricted by limited sensitivity and specificity. Both markers can be elevated in the absence of malignancy, with physiologic elevations of HCG in pregnancy and of AFP (produced during fetal development) in the neonatal period. In the latter case, levels are very high and variable,14 being influenced, for example, by gestational age, birth weight, and the presence or absence of hyperbilirubinemia.14,15 This can make interpretation of AFP levels difficult, particularly in monitoring patients with neonatal sacrococcygeal teratomas for detection of malignant (YST) recurrence. Further elevations of AFP are seen in gastrointestinal and hepatic disease and hereditary and metabolic disorders.16 There are also issues of sensitivity, as AFP and/or HCG are not raised in all cases of malignant GCT.

Because it is possible to diagnose some malignant GCTs by elevated tumor markers alone, without recourse to surgical biopsy,1 a reliable universal marker may prevent some patients having to undergo such surgical procedures. Moreover, in patients with AFP–/HCG– malignant GCTs, subsequent marker estimation is of no benefit in tumor monitoring or follow-up for early disease recurrence. In view of these issues, identification of novel universal serum biomarkers that are highly sensitive and specific for malignant GCTs would be of considerable clinical value.

The coordinate and universal overexpression of all members of the miR-371~373 and miR-302 clusters has been shown only in malignant GCTs to date,5 demonstrating the potential specificity of these markers for this disease. Furthermore, these microRNAs are overexpressed in malignant GCTs independent of patient age, histologic subtype, and anatomic site of disease and should, therefore, offer high sensitivity as biomarkers.

To be useful as blood-based markers, microRNAs need to be released from solid tumors, protected from endogenous RNase activity, and be robustly detectable in the patient serum or plasma. MicroRNAs released from tumor cells
appear to be protected from RNase degradation by packaging within membrane-bound exosome particles.\textsuperscript{17,18} Furthermore, microRNA levels are stable, even in serum samples subjected to multiple freeze-thaw cycles\textsuperscript{6,8} and those left 24 hours at room temperature before processing.\textsuperscript{8} These properties would be useful in routine clinical practice, where such variations in sample handling are likely to occur. We observed no detectable cross-reactivity between microRNAs from the miR-302 cluster despite very high sequence homology, confirming the suitability of our multiplexed qRT-PCR approach for detecting these microRNAs in the serum. The addition of a pre-amplification step, which does not introduce technical bias, provides enough product to perform final singleplexed PCRs, with 3 technical replicates, for multiple individual microRNAs. Similar multiplex qRT-PCR approaches have been used by others in the detection of short, noncoding RNAs.\textsuperscript{19} For each of the 8 microRNAs studied, we demonstrated minimal variability between expression values in technical replicates, further indicating the reproducibility of our approach.

We conclude that multiplex qRT-PCR is a robust approach for detecting members of the miR-371~373 and miR-302 clusters in serum. These microRNAs may be of clinical value in disease monitoring, as demonstrated for the index case reported herein, and potentially in the diagnostic setting. Further carefully controlled work is now justified to examine systematically the value of these microRNAs in malignant GCT diagnosis, assessment of therapeutic response, and detection of subclinical recurrence.

**References**