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MicroRNA miR-371a-3p in serum of patients with germ cell tumours: evaluations for establishing a serum biomarker

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SUMMARY

As only 60% of the patients with germ cell tumour (GCT) express the classical markers, new markers as for example microRNAs (miRNAs) are required. One promising candidate is miR-371a-3p, but data are sparse to date. We measured serum levels of miR-371a-3p in GCT patients, in controls, and in cases with other malignancies. We also assessed the expression in other body fluids and we looked to the decline of serum miR-371a-3p levels after treatment. miR-371a-3p levels were measured by quantitative polymerase chain reaction in serum samples of 25 GCT patients, 6 testicular intraepithelial neoplasia (TIN) patients, 20 healthy males and 24 non-testicular malignancies (NTMs). Testicular vein blood (TVB) was examined in five GCT patients and five controls. Five GCT patients had serial daily measurements after orchiectomy. Five seminal plasma samples, three urine specimens and one pleural effusion fluid were processed likewise. GCT patients had significantly higher miR-371a-3p serum levels than controls and NTMs. Serum levels of controls, TINs and NTMs were not significantly different. TVB samples of GCT patients had 65.4-fold higher serum levels than peripheral blood. Malignant pleural effusion fluid had extremely high levels of miR-371a-3p, seminal plasma had strongly elevated levels by comparison with serum levels of controls. In urine of GCT patients, no miR-371a-3p expression was detected. Daily measurements after orchiectomy in stage 1 patients revealed a decline by 95% within 24 h. Serum levels of miR-371a-3p appear to be a promising specific biomarker of GCTs as is suggested by high serum levels in GCT patients, the rapid return of elevated levels to normal range after treatment, the association of serum levels with tumour bulk, the non-expression in NTMs and the much higher levels of miR-371a-3p in TVB. This potential marker deserves further exploration in a large-scale clinical study.

INTRODUCTION

microRNAs (miRNAs) are small, non-coding RNA molecules, which are involved in post-transcriptional gene regulation thus playing an essential role in many biological processes as, for example cell differentiation, apoptosis and tumour development (Bartel, 2004; Esquela-Kerscher & Slack, 2006; Farazi *et al.*, 2011). Some miRNAs are abundantly expressed in cancer tissues (Catto *et al.*, 2011). In general, they are characterized by a high stability in body fluids once being released from tumour cells (Reis *et al.*, 2010; Weber *et al.*, 2010). Three members of the cluster miR-371-3 (miR-371a-3p, miR-372 and miR-373-3p) have shown a strong association with testicular germ cell tumours (GCTs) (Gillis *et al.*, 2007; Palmer *et al.*, 2010; Murray *et al.*, 2011; Ruf *et al.*, 2014; Syring *et al.*, 2014). These characteristics fuelled the hope that these miRNAs could serve as serum biomarkers of GCTs, particularly in light of the clinical need for more sensitive markers in this disease (Bezan *et al.*, 2014). Accordingly, in a pilot study, we found the serum levels of all three miRNAs to be significantly higher in GCT patients than in controls and to drop to the level of controls immediately after orchiectomy (Belge *et al.*, 2012; Dieckmann *et al.*, 2012). Of the three miRNAs evaluated, miR-371a-3p performed most favourably, because it revealed a considerably high expression in seminoma and non-seminoma and it also showed the highest post-operative decrease (Belge *et al.*, 2012; Dieckmann *et al.*, 2012). Thus, miR-371a-3p serum levels seem to be a promising biomarker of GCTs. Notwithstanding, before making its way into clinical practice more biological and biochemical characteristics of this putative serum biomarker need to be elaborated. If, in fact, miR-371a-3p originates from the testicular GCT, one must assume higher serum levels in testicular vein blood (TVB) than in peripheral blood. Also, if this marker is specific for GCT, then a slight elevation should also be expected in patients who are only afflicted with testicular intraepithelial neoplasia (TIN), the

uniform progenitor of GCTs (Dieckmann & Skakkebaek, 1999). From a clinical point of view it is important to know, how fast the elevated serum levels of the presumed marker will decrease subsequent to treatment. Another important question with respect to the specificity of this miRNA is its expression in other malignancies. Accordingly, we performed measurements of serum levels of miR-371a-3p in TVB and compared these levels with those found in peripheral blood. We also looked to miR-371a-3p serum levels in patients with TIN only and in a variety of non-testicular malignancies (NTMs). Finally, we looked to the velocity of serum level decrease after treatment by performing repeat measurements in GCT patients clinical stage 1 (CS1).

MATERIAL AND METHODS

Ethics statement

The study was approved by the ethical committee of the Ärztekammer Bremen, (reference number 301). The guidelines of the declaration of Helsinki are followed.

Sample collection

All patients and control persons enrolled in this study were adult. Cubital vein blood samples (CVB) were obtained from patients during routine blood examinations. Blood samples were collected in serum separation tubes (Sarstedt, Nümbrecht, Germany) that were kept at room temperature for approximately 60 min to allow for complete coagulation after blood aspiration. The samples were then centrifuged to separate serum and aliquots were frozen at −80°C until further processing. In one patient with widespread metastases, the expression of miR-371a-3p was analysed in CVB and additionally in aspirated fluid from pleura effusion of this patient. To prove the expression level of miR-371a-3p in other body fluids, five seminal plasma samples from healthy individuals and three urine specimens from GCT patients were investigated additionally.

The patient sample consisted of six patients with TIN only, 24 patients with the following NTMs: 7 prostatic carcinoma, 4 renal cell carcinomas, 8 bladder carcinomas, 1 thyroid carcinoma, 1 oesophageal carcinoma, 1 hepatocellular carcinoma, 1 rectal carcinoma and 1 colonic carcinoma. For comparison, 25 patients with GCT all with CS1 (Lugano classification) were included. Twenty male patients with non-malignant scrotal diseases (hydrocele, spermatocele and epididymitis) served as controls. Twenty of the GCT patients and 17 of the controls had been reported previously (Dieckmann *et al.*, 2012). All of the GCT patients had measurements prior to surgery and again 2–6 days post-operatively. Five of the patients had serial daily measurements post-operatively for 3–6 days to study the decay of serum levels after treatment. In five GCT patients and in five patients without malignant disease, TVB samples were analysed. Pleural effusion aspirate, seminal plasma and urine specimens were obtained during routine clinical examinations.

RNA Isolation

For RNA isolation from serum, pleura, urine and seminal plasma samples, 200-μL frozen body fluid was thawed on ice, total RNA extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and RNA was quantified by spectrophotometry (Eppendorf, Hamburg, Germany).

cDNA synthesis

For all samples, reverse transcription (RT) was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). 55 ng total RNA from each sample was used.

RT primers represented an equal mixture of two miRNAs (miR-371a-3p, Assay ID 002124 and miR-20a, Assay ID 000580)-specific stem-loop primers from the relevant miRNA assays (Applied Biosystems). The reactions with a final volume of 15 μL were incubated in the GeneAmp PCR-System 2700 (Applied Biosystems) at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min respectively.

Pre-amplification of RT products

For pre-amplification, 0.75 μL of each miRNA assay was diluted in 13.5 μL nuclease-free water. The PCR with a final volume of 50 μL (12.5 μL of this solution, 12.5 μL of RT product, 25 μL TaqMan Universal PCR Master Mix (Applied Biosystems) was performed at 95°C for 10 min, followed by 14 cycles of 95°C for 15 sec and 60°C for 4 min using the GeneAmp PCR-System 2700 (Applied Biosystems). The pre-amplification product was diluted 1:5 in nuclease-free water.

Quantitative real-time PCR

For the quantitative real-time PCR, 9 μL of the pre-amplification product was added to 10 μL TaqMan Universal PCR Master Mix and 1 μL of 20× TaqMan microRNA assay using the Applied Biosystems 7300 real-time PCR System (Applied Biosystems). The relative quantification was performed with miR-20a as endogenous control. For each sample, the reaction was performed in triplicate. A negative control of amplification was performed for each sample without reverse transcriptase. Non-template negative controls for each miRNA were included in every plate. PCR conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Data were analysed using the 7300 system software (Applied Biosystems). Cycle threshold (Ct) values were normalized to the internal control, miR-20a (Livak & Schmittgen, 2001). Ct values later than 39 were noted as not detectable.

Statistical analysis

Descriptive statistical analysis was performed using Excel software (Microsoft Corp., Redmond, USA). The two-sided Mann–Whitney *U*-test was employed for statistical comparisons of mean serum levels of various groups. A p-value of less than 0.05 was considered being significant.

RESULTS

The mean RQ-value of NTMs was in the range of the mean RQ-value of controls (Fig. 1). The difference was statistically not significant. Moreover, the individual RQ-values of patients with NTM varied similarly to the controls from 0 (not detectable) to 14 (Fig. 2). GCT patients CS1 had significantly higher mean miR-371a-3p serum levels than controls ($p < 0.0001$) and NTMs ($p < 0.0001$), respectively. TIN patients had slightly higher serum levels than controls and NTMs; however, this difference was not statistically significant (Fig. 1). The mean RQ-value of GCTs was significantly higher than that of TIN patients ($p < 0.05$). Individual data are provided in Table S1 (A, B, E and H) and standard deviation in Figure S1.

Figure 1 Synopsis of mean levels of miR371a-3p in various groups. Comparison of mean RQ-values in GCTs, TIN patients, other malignancies and controls. In addition, results of miR-371a-3p expression in serum and pleural effusion of one CS3 GCT patient is shown. C, controls; CS1, clinical stage 1; CS3, clinical stage 3; NTM, non-testicular malignancy; pt, patient; TIN, testicular intraepithelial neoplasia; [1], RQ-value; *, significant; ***, extremely significant. The *y* axis is plotted on a $\log_{10}$ scale.

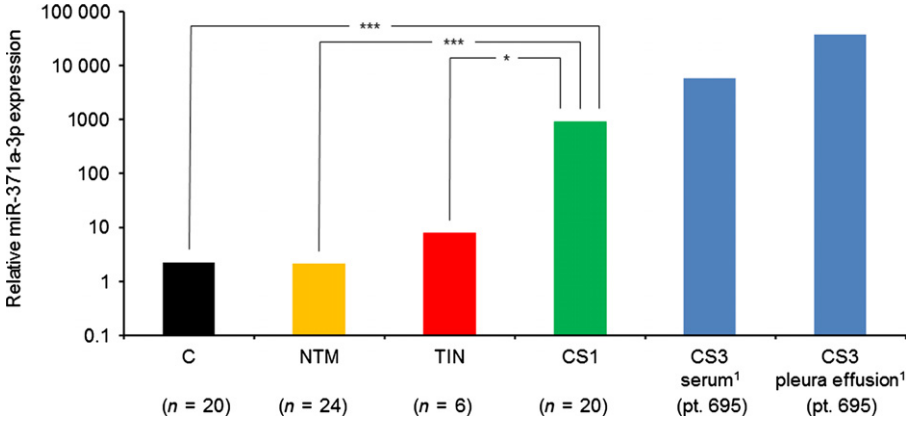
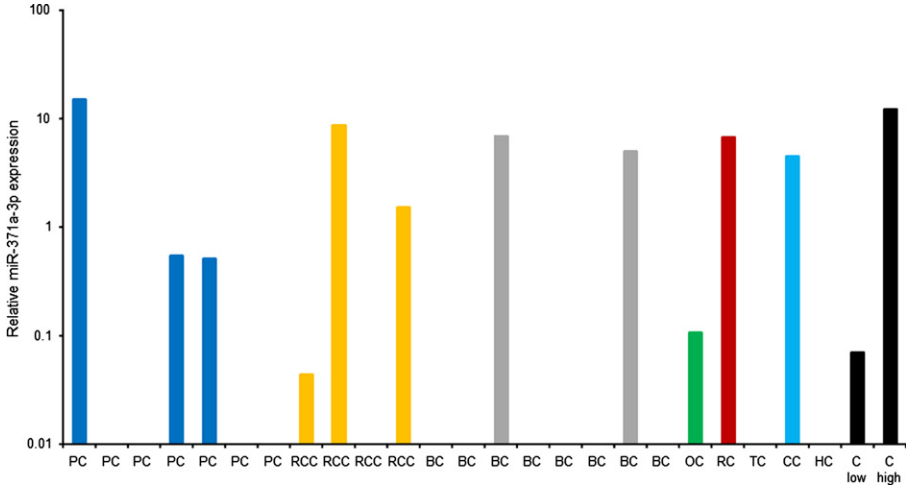


Figure 2 Individual results in non-testicular malignancies. Each column represents the measurement of the relative miR-371a-3p value in an individual patient/person. BC, bladder cancer; CC, colon cancer; C, controls (showing the lowest and highest detectable expression among 20 controls); HC, hepatocellular carcinoma; OC, oesophageal carcinoma; PC, prostatic cancer; RC, rectal cancer; RCC, renal cell carcinoma; TC, thyroid carcinoma;. The *y* axis is plotted on a $\log_{10}$ scale.



The pleural effusion fluid had a 6.5-fold higher miRNA expression level than the simultaneously examined corresponding CVB of the patient. Both, the miR-371a-3p serum level and the pleural fluid level of this patient with large metastatic load are much higher than the mean serum level of GCT CS1 patients (Fig. 1). Individual data are provided in Table S1 (D).

In the urine specimens of three GCT patients, no miR-371a-3p was identified, while miR-20a was detected within Ct value 22 and 24.

The mean seminal plasma expression of miR-371a-3p in healthy individuals was 177.2-fold higher than the mean level found in serum of controls ($p < 0.01$) but it was 2.3-fold lower than the mean serum expression of GCT CS1 patients (Fig. 3).

In TVB, miR-371a-3p levels were higher than in corresponding CVB, both in controls and in patients with GCT. The difference was 27.3-fold in GCT patients ($p < 0.0001$) and 3.4-fold in the controls (Fig. 3). Comparing mean CVB levels of CS1 patients with TVB levels of controls, the difference was 118.3-fold. Individual data are provided in Table S1 (A, C, E, F and G) and standard deviation in Figure S2.

The miR-371a-3p expression in each group relative to the control group for Figs 1 & 3 is shown in Table 1.

The serial daily measurements in five patients after orchiectomy revealed a rapid decay of serum miR-371a-3p levels. All the patients showed a decrease to less than 5% of pre-operative levels within 24 h. Further measurements revealed a complete clearance within 1–6 days in all of the patients. The quantitative decay curves of miR-371a-3p expression for all of the five CS1 GCT patients are given in Fig. 4(A–E) showing the RQ-values from before surgery to 72 h (A–D) and to 144 h (E) thereafter. The mean RQ-values of the five patients at the different time points after surgery are shown in Fig. 4(F).

DISCUSSION

The central result of this study is the accumulation of further evidence for the value of circulating serum miR-371a-3p as a biomarker of GCTs.

Figure 3 Comparison of miR-371a-3p measurements in testicular vein blood, cubital vein blood and seminal plasma. Comparison of mean RQ-values of CVB and TVB in controls and CS1 GCT patients. In addition, mean miR-371a-3p expression in five seminal plasmas is given. For better understanding, the mean serum levels of CS1 patients are shown (same as in Fig. 1). C, controls; CS1, clinical stage 1; CVB, cubital vein blood; TVB, testicular vein blood; *, significant; **, highly significant; ***, extremely significant. The *y* axis is plotted on a $\log_{10}$ scale.

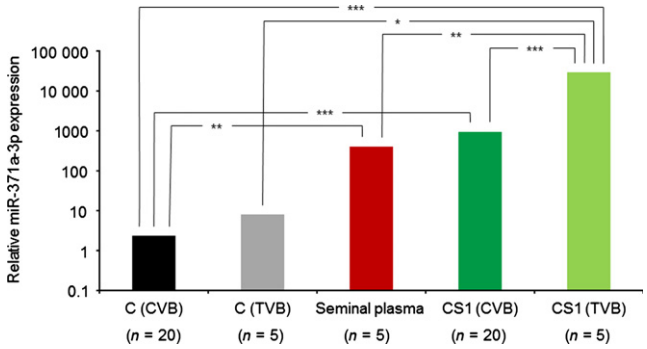


Table 1 Mean expression of miR 371a-3p in the groups examined

Samples	*n*	Relative miR-371a-3p expression	
C (CVB)	20	Mean RQ value	1.00
NTM	24	Mean RQ value	1.75
TIN	6	Mean RQ value	1.95
C (TVB)	5	Mean RQ value	3.42
Seminal plasma	5	Mean RQ value	177.23
CS1 (CVB)	20	Mean RQ value	404.14
pt. 695 CS3 serum	1	RQ value	2590.11
pt. 695 CS3 pleural effusion	1	RQ value	16 806.81
CS1 (TVB)	5	Mean RQ value	26 415.28

miR-371a-3p expression in each group relative to the control group for Figs 1 & 3. C, controls; CS1, clinical stage 1; CS3, clinical stage 3; CVB, cubital vein blood; n, number of samples; NTM, non-testicular malignancy; pt, patient; TIN, testicular intraepithelial neoplasia; TVB, testicular vein blood.

Recently, a method for analysing this miRNA in serum of GCT patients using real-time PCR was developed (Murray *et al.*, 2011; Belge *et al.*, 2012). miR-371a-3p has the potential of becoming a serum biomarker of malignant GCTs (Murray & Coleman, 2012) because, as a rule, substantially increased levels of miR-371a-3p have been detected in serum of patients with GCTs compared to controls. Also, after orchiectomy there was a significant decline into the range of controls (Belge *et al.*, 2012; Dieckmann *et al.*, 2012; Murray & Coleman, 2012; Gillis *et al.*, 2013).

If serum levels of miR-371a-3p expression are to be used as specific biomarkers of GCT, the potential of false-positive results needs to be explored. As a great number of circulating serum miRNAs are released by various types of cancer, for example prostate cancer, bladder cancer and renal cell carcinoma (Mahn *et al.*, 2011; Wulfken *et al.*, 2011; Sanders *et al.*, 2012; Scheffer *et al.*, 2012; Bezan *et al.*, 2014), it was mandatory to exclude that miR-371a-3p is released from malignancies other than GCT. Accordingly, in all 24 malignancies investigated herein, the miR-371a-3p expression in serum was in the range of the 20 controls. The NTM mean RQ-value does not significantly differ from the mean RQ-value of controls and both of the mean RQ-values (NTMs and controls) are extremely different from the mean RQ-value of GCTs CS1. In all, this result clearly accords with the view of miR-371a-3p being specific for GCTs.

The finding of significantly higher levels of miR-371a-3p in TVB than in peripheral blood indicates that the primary source causing the elevation of this miRNA in serum of GCT patients must be the testicular tumour itself. A slight difference between miR-371a-3p levels in TVB and CVB was also found in controls; however, this difference was only 3.4-fold, whereas the difference was 27.3-fold in GCT patients. Thus in all, the present results further underscore the specificity of miR-371a-3p for GCTs.

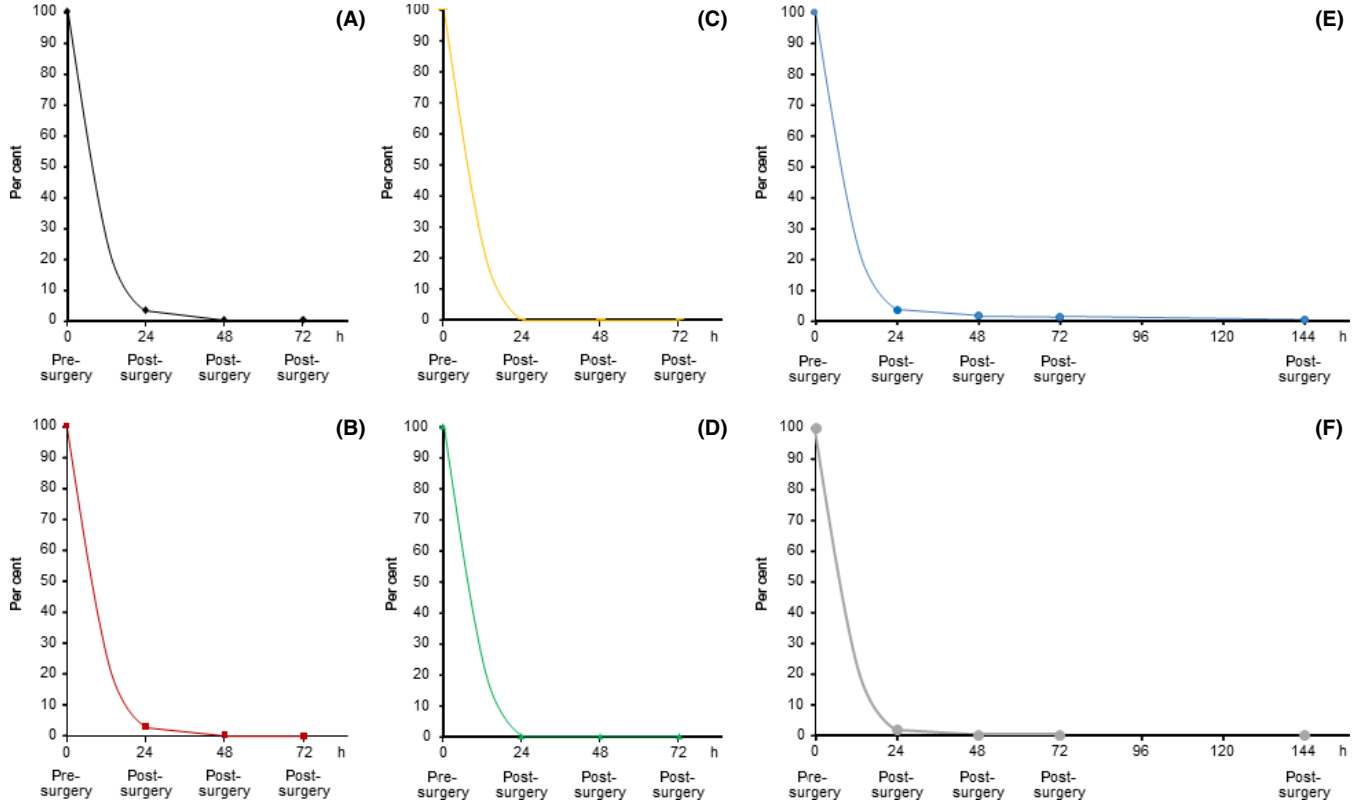
Reportedly, miR-367 is another promising biomarker for testicular GCTs (Murray *et al.*, 2011; Murray & Coleman, 2012; Gillis *et al.*, 2013; Syring *et al.*, 2014). Simultaneous measurement of both miR-367 and miR 371a-3p could further increase the specificity of the test.

A very high miR-371a-3p expression was measured in the serum of one patient with systemic dissemination of non-seminoma. This result closely accords with miR-371a-3p measurements of four patients with GCT clinical stages 2 and 3, as reported previously (Dieckmann *et al.*, 2012). The miR-371a-3p expression of these five patients with advanced disease is obviously higher than the mean RQ-value of CS1 patients (Dieckmann *et al.*, 2012). These findings suggest that the miRNA level is associated with tumour load and with clinical stage, accordingly. This assumption is supported by the extremely high level of miR-371a-3p found in the pleural effusion fluid of the same GCT patient. As large-volume metastatic deposits were located at the pleural walls of this patient, the aspirated fluid from the effusion had been in direct contact with the miRNA releasing tumour cell population. So, a higher expression of miR-371a-3p in the aspirated fluid is an analogous finding to the higher levels found in TVB than in CVB, and is again another piece of evidence for germ cell cancer being the origin of miR-371a-3p in body fluids.

TIN is the uniform precursor of GCTs and it may be present in a testicle many years before the GCT becomes invasive (Dieckmann & Skakkebaek, 1999). There is evidence for the expression of miR-371a-3p (and others) in TIN cells (Novotny *et al.*, 2012; McIver *et al.*, 2013), however so far, no serum studies of patients with TIN have been documented. This study is the first to report measurements of serum levels of miR-371a-3p in six such patients. There appears to be a slight trend towards a higher mean RQ-value in TIN patients than in controls; however, this difference is not significantly different, statistically. The mean RQ-value of TIN patients is significantly lower than the mean RQ-value of GCTs CS1. As the expression of miR-371a-3p in body fluids is apparently associated with tumour bulk (vide supra), one must assume that the number of cells in TIN-bearing testicles secreting miR-371a-3p is not sufficient to achieve high (measurable) levels of circulating miRNAs in the peripheral blood. Also, the present negative result could be explained by the assumption that TIN cells release lesser amounts of miRNAs into body fluids than full-blown germ cell tumour cells do. Thus, it would be interesting to look to testicular vein blood of a patient with TIN only. However, this would be a very rare clinical situation and can hardly be expected to be available for investigation.

The velocity of decay of miR-371a-3p after elimination of the source of circulating molecules has been shown in five GCT patients CS1. In fact, there is a very rapid decline to lower than 5% of the initial value within 24 h after orchiectomy. After

Figure 4 Decay of serum levels of miR 371a-3p after treatment. Daily measurements of miR-371a-3p expression in five individual CS1 GCT patients. The starting value was always measured before orchiectomy. A–D: RQ-values up to 72 h after surgery. E: RQ-values up to 144 h after surgery. F: mean RQ-values of all five patients are shown up to 72 h after surgery (summary).



another 1–5 days, clearance of miR-371a-3p has been completed. The rapid decline of miR-371a-3p levels as a response to curative treatment is an indispensable feature of any clinically valuable biomarker. So, this feature may substantially aid qualifying miR-371a-3p as a new biomarker of GCTs.

miR-371a-3p is predominantly expressed in embryonic stem cells, GCTs, and in the placenta (Bar *et al.*, 2008; Laurent *et al.*, 2008; Ren *et al.*, 2009; Bullerdiek & Flor, 2012; Gillis *et al.*, 2013). We analysed the miRNA expression in five seminal plasma of healthy young men and found an increased expression akin to the extent found in serum of GCT patients CS1. The increased expression of miR-371a-3p in seminal plasma may be explained by the fact that the germ cells directly release the miRNAs into the seminal plasma.

In three GCT patients, we analysed miR-371a-3p expression concomitantly in serum and urine. While serum levels were found expectedly high, no expression was detectable in urine. This finding is somehow surprising at first glance, because other body fluids, for example seminal plasma and pleural fluid evidently harbour these miRNAs (vide supra). Putatively, miRNAs at least miR-371a-3p are cleared from the urine during the filtration processes of the kidney.

There are certainly limitations of our study relating to methodological-technical and to statistical problems. With respect to our laboratory technology, we used only miR 20a for normalization although the employment of more than a single microRNA has been widely adopted for serum studies. With regard to statistical analysis, we acknowledge that larger sample sizes are clearly needed for more meaningful conclusions. However, the results obtained so far are very much promising and thus worth to be reported.

CONCLUSION

miR-371a-3p appears to be an auspicious biomarker of GCTs as is suggested by high serum levels in GCT patients, the association of serum levels with tumour bulk, the rapid return of elevated levels to normal range after treatment, the non-expression in non-testicular malignancies and the much higher levels of miR-371a-3p in testicular vein blood. This presumed marker clearly deserves further exploration in a large-scale clinical study.

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AUTHOR CONTRIBUTIONS

MS carried out the molecular genetic study, analysis and interpretation of the data, drafting of manuscript; GB involved in study concept, analysis and interpretation of data, drafting of manuscript.;TB contributed to patient enrolment, collection of samples for analysis, compilation of clinical data; RI contributed to patient enrolment, collection of samples for analysis, compilation of clinical data; NW carried out the kinetic profiles and

analysis of data; JB provided study concept, analysis and interpretation of data, drafting of manuscript; KPD involved in study concept, patient enrolment, interpretation of data, drafting of manuscript; All authors critically revised and finally approved the manuscript.

MEETING COMMENTS*

Hector Chemes (Buenos Aires, Argentina)

Have you measured serum levels of microRNA 371-3 in young boys with yolk sac tumours?

Klaus-Peter Dieckmann (Hamburg, Germany)

That is an interesting question but we have no young children in our study.

Leendert Looijenga (Rotterdam, Netherlands)

The preliminary data from our studies in Rotterdam, and Matthew Murray's data from Cambridge, UK, include paediatric patients with germ cell tumours. Serum levels of microRNAs are as informative in children as in adults. Childhood yolk sac tumour is associated with raised serum levels, but mature teratomas are not.

William Boellaard (Rotterdam, Netherlands)

If patients have metastatic disease at the time of orchidectomy is there a partial decline in serum microRNA levels following orchidectomy?

Klaus-Peter Dieckmann

Patients with metastatic disease have a small decline in microRNA serum levels after orchidectomy followed by a greater sharp fall in serum levels after the first course of chemotherapy.

Niels E Skakkebæk (Copenhagen, Denmark)

You measured microRNA in the seminal plasma of five patients. What were their diagnoses?

Klaus-Peter Dieckmann

These were five healthy males without testicular cancer.

Hubert Schorle (Bonn, Germany)

MicroRNA 371-3 serum levels are the same in patients with CIS/TIN as in healthy controls but you did not show error bars in your cohort. Serum levels rise with tumour stage for seminomas and non-seminomas. Have you performed statistical analyses and are your data robust?

Klaus-Peter Dieckmann

Our data are still preliminary and have to be analysed further. We shall have statistical data with error bars when more samples have been tested. I am convinced that the test is very robust.

Mark Greene (Rockville, USA)

Biomarker projects often go astray because we focus on patients who already have the disease. It is important to discover if the marker is detectable before the disease is clinically evident, and this might be possible by testing serum collected from large cohort studies. There are hundreds of cohort studies in which individuals are enrolled when they are healthy, blood and serum samples are banked, and they embark on long term follow up. Testicular cancer is a rare tumour therefore only a small number of cases will be detected in a large number of enrolled individuals. We should be collecting samples now in order to discover prediagnostic serum elevations, and to assess the potential for a screening test. Testicular germ cell tumours grow relatively rapidly which might make it difficult to use biomarkers as a screening tool.

Klaus-Peter Dieckmann:

We had 4 patients with CIS/TIN but no invasive tumour at the time of serum sampling. They had only a minimal increase in serum levels of microRNA. Such a marginal increase would be of little value as a screening test.

Carsten Rusner (Halle, Germany)

From a clinical point of view, how expensive is a serum microRNA test compared to the standard serum markers of AFP and hCG? MicroRNA is very sensitive and specific, and would be a useful routine test for testicular tumour patients.

Klaus-Peter Dieckmann

The microRNA test is at the experimental stage carried out in the research laboratory with several steps being performed by hand. Each examination costs about €60 plus lab technician's time. We are improving the test and will produce a "kit" for rapid measurement which should be available at the end of 2015. An AFP test costs about €10. At present, serum tests for AFP and βhCG have a sensitivity of about 50-60% for detecting active germ cell tumour, whereas the microRNA test has a sensitivity of 90%.

*[Correction added on 22 December 2014, after first online publication: Meeting Comments added.]

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. CT values of miR-371a-3p and miR-20a of the samples with miR-371a-3p detection.
Figure S1. Boxplot for Figure 1.
Figure S2. Boxplot for Figure 3.