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Serum Levels of MicroRNA miR-371a-3p: A Sensitive and Specific New Biomarker for Germ Cell Tumours

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Article info

Abstract

Background: Clinical management of germ cell tumours (GCTs) relies on monitoring of serum tumour markers. However, the markers α-fetoprotein (AFP), the β-subunit of human chorionic gonadotropin (bHCG), and lactate dehydrogenase (LDH) are expressed in <60% of GCT cases.

Objective: To test the utility of the microRNAs (miRNAs) miR-371a-3p, miR-372-3p, miR-373-3p, and miR-367-3p as sensitive and specific GCT serum biomarkers.

Design, setting, and participants: Serum levels of miRNAs were measured in 166 consecutive patients with GCT before and after treatment and in 106 male controls. In the first 50 consecutive patients, all four miRNAs were measured. In the main study, only the most sensitive miRNA was further analysed.

Outcome measurements and statistical analysis: The specificity and sensitivity of the four miRNAs were studied using receiver operating characteristic curves. miRNA sensitivities were compared to those of classical markers. Statistical cross-comparisons of miRNA levels for GCT subgroups and controls were performed at various time points during treatment.

Results and limitations: Overall, miR-371a-3p performed best, with 88.7% sensitivity (95% confidence interval [CI] 82.5–93.3%) and 93.4% specificity (95% CI 86.9–97.3%) and an area under the curve of 0.94, outperforming AFP, bHCG, and LDH (combined sensitivity 50%). According to Kernel density estimation, the sensitivity and specificity were 86.3% and 92.5%, respectively. miR-371a-3p levels dropped to normal after completion of treatment. The miRNA levels correlated with treatment failure and relapse. Teratoma did not express miR-371a-3p.

Conclusions: The miRNA miR-371a-3p is a specific and sensitive novel serum GCT biomarker that accurately correlates with disease activity. Validation of this test in a large-scale prospective study is needed.

Patient summary: miR-371a-3p is a novel serum marker for germ cell tumours that is expressed by 88.7% of patients and thus is far more sensitive and specific than classical serum markers. It correlates with tumour burden and treatment results. Validation in a large patient cohort is needed.

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1. Introduction

Monitoring of the serum biomarkers α-fetoprotein (AFP), the β-subunit of human chorionic gonadotropin (bHCG), and lactate dehydrogenase (LDH) is a cornerstone of clinical management of testicular germ cell tumours (GCTs) [1]. However, these markers are expressed in <60% of cases, so novel sensitive markers are needed [2]. Although many substances have been suggested as biomarkers for GCT, none have qualified for clinical use [3].

In 2011, microRNAs (miRNAs) of the clusters miR-371-3 and miR-302/367 were suggested as new serum biomarkers [4]. The miRNA molecules represent a particular class of small RNAs consisting of approximately 20 base pairs [5]. After release from the cell, these molecules remain stable in extracellular fluids [6] and can be measured by quantitative polymerase chain reaction (qPCR).

The miRNAs of the miR-371-3 and miR-302/367 clusters were originally detected in GCT tissue [7–10] and four independent pilot studies confirmed elevated serum levels [11–15]. Furthermore, circulating miRNAs of the two clusters are clearly specific for GCT because it was demonstrated that they are absent in other malignancies [16], and much higher levels of these miRNAs were found in testicular vein blood than in the peripheral circulation [17]. The goal of the present study was to further evaluate the usefulness of miR-371a-3p, miR-372-3p, miR-373-3p, and miR-367-3p as serum biomarkers of GCT in an unselected large patient sample. To determine whether the four miRNAs would be equally appropriate as serum biomarkers, all were tested in a preliminary study consisting of 50 GCT patients. The miRNA with the highest discriminatory power was then further evaluated in a cohort of 166 patients. We explored the utility of that miRNA as a serum biomarker by comparing its sensitivity to that of classical markers and by monitoring the response of miRNA levels to treatment.

2. Patients and methods

2.1. Patients

From June 2011 to September 2015, a total of 166 patients with GCT and 12 patients with Leydig cell tumour (LCT) who were aged 18–60 yr were prospectively enrolled from four institutions (Albertinen-Krankenhaus Hamburg, Bundeswehrkrankenhaus Hamburg, Universitätsklinikum Hamburg-Eppendorf, Klinikum Bremen-Mitte). Sixty-four participants were briefly mentioned previously in relation to a specific analysis of miRNA levels in testicular vein blood (Table 1) [12,17].

As controls, 106 male participants from the same age group were recruited (12 healthy men and 94 patients with benign scrotal conditions such as hydrocele, spermatocele, epididymitis, and varicocele) (Table 1). The first consecutive 50 GCT patients and 20 controls were participants in a preliminary study that was conducted separately. In the main study, serum samples before orchiectomy were available for 150 of the 166 GCT patients. To monitor changes in miRNA levels secondary to chemotherapy, serum samples were repeatedly collected (once per cycle) from 18 patients with clinical stage 2 (CS2) disease, nine patients with CS3 disease, and ten patients experiencing relapse. Serum aliquots were frozen and stored at −80 °C before further processing (Supplementary methods). All patients gave informed consent. Ethics approval was given by Ärztekammer Bremen (reference 301, 2011). Further clinical details are shown in Supplementary Tables 1–7.

2.2. Laboratory methods

For RNA isolation, an miRNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract total RNA from 200 μl of serum. Reverse transcription (RT) was performed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). The RT product was preamplified, and levels of miR-371a-3p (assay 002124), miR-372-3p (assay 000560), miR-373-3p (assay 000561), and miR-367-3p (assay 000555) were measured by qPCR using a TaqMan miRNA assay (Applied Biosystems). Cycle threshold (C_t) values were normalised to miR-93-5p (assay 000432) as an internal control, and the relative quantity (RQ) was calculated using the $2^{-\Delta\Delta Ct}$ method [18]. Details of the measurement methods are described in the Supplementary methods [19].

The classical serum tumour markers AFP, bHCG, and LDH were measured according to laboratory guidelines [20]. Preoperative values were available for 139 patients.

Table 1 – Clinical data for patients with germ cell tumours, patients with Leydig cell tumours, and control subjects for the preliminary and main studies

Group	Preliminary study			Main study		
	n	Age (yr)	Tumour diameter (mm)	n	Age (yr)	Tumour diameter (mm)
Total GCT patients	50	37.0 (28.0–46.0)	27.0 (15.3–40.0)	166	38.5 (30.3–46.0)	29.0 (18.0–45.0)
CS1, total	40	39.0 (32.5–46.0)	25.5 (15.0–40.0)	107	40.0 (32.0–46.5)	25.0 (15.0–38.0)
CS1, seminoma	24	44.5 (38.8–48.3)	26.5 (15.0–40.0)	78 [a]	43.0 (35.0–47.8)	25.0 (15.0–38.0)
CS1, nonseminoma	16	31.0 (23.0–35.3)	25.5 (17.5–35.8)	29	30.0 (23.0–35.0)	26.5 (19.5–39.3)
CS2, total	6	27.0 (26.3–28.5)	19.5 (16.0–26.0)	38	39.5 (31.0–47.0)	33.5 (20.0–61.3)
CS2, seminoma	–	–	–	17	36.0 (31.0–46.0)	40.0 (24.0–65.8)
CS2, nonseminoma	6	27.0 (26.3–28.5)	19.5 (16.0–26.0)	21	41.0 (31.0–48.0)	30.0 (17.3–52.5)
CS3, total	4	38.5 (28.8–46.5)	52.5 (41.3–61.3)	11	36.0 (25.0–44.5)	60.0 (45.0–78.0)
CS3, seminoma	1	48.0	–	1	48.0	–
CS3, nonseminoma	3	31.0 (26.5–38.5)	60.0 (52.5–62.5)	10	33.5 (25.0–41.5)	62.5 (52.5–82.3)
Patients with relapse	–	–	–	10 [a]	29.0 (26.8–39.3)	–
Leydig cell tumours	–	–	–	12	46.0 (33.3–50.3)	–
Control subjects	20	36.0 (28.3–48.3)	–	106	38.0 (26.0–48.0)	–

GCT = germ cell tumour; CS = clinical stage. Data are presented as median (interquartile range).
[a] One patient is included in the CS1 seminoma group and in the group of relapsing patients because of relapse 2 yr after carboplatin therapy.

2.3. Statistical analysis

Median RQ values for independent subgroups were compared using the Mann-Whitney U test, whereas related groups were compared using the Wilcoxon signed rank test. Bonferroni correction was applied in the preliminary study to adjust for multiple testing. In addition to empirical calculations, the distribution of RQ values was modelled using Kernel density estimation to obtain a more realistic assessment of the distribution in a larger sample size. Receiver operating characteristic (ROC) analysis was performed to evaluate the discriminatory power of the markers analysed. We chose RQ = 5 as the cutoff value to evaluate sensitivity and specificity in the main study. The frequency of categorical data was compared using the Pearson χ^2 test. Multiple regression analysis was performed to analyse the association between marker expression and tumour diameter or pT stage. Exact 95% confidence intervals (CIs) were calculated. For values based on Kernel density estimation, 95% CIs were calculated by bootstrapping with $n = 2500$ simulations. All tests were two-sided, and significance was assumed at $p < 0.05$. Statistical analysis was performed using SPSS version 22 (IBM, Armonk, NY, USA) and R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria) [21].

3. Results

3.1. Preliminary study

The preliminary study revealed a significantly higher level of expression for each of the four miRNAs in GCT patients compared to controls (Fig. 1A–D), with significance retained after correction for multiple testing ($p < 0.001$ for all miRNAs). Patients with metastases had higher expression levels than those with CS1 disease, but after Bonferroni correction this difference was only significant for miR-371a-3p ($p = 0.001$) and miR-367-3p ($p = 0.006$). Of the four miRNAs, miR-371a-3p had the highest ability to discriminate between patients and controls. The difference in median RQ values for controls and patients was greatest for miR-371a-3p, whereas the interquartile ranges for miR-372-3p and miR-373-3p in patients overlapped considerably with those for controls. Thus, of the four miRNAs tested, miR-371a-3p had the highest sensitivity and specificity (area under the curve [AUC] 0.943; 95% CI 0.874–0.982) according to ROC analyses with density estimation (Fig. 1E–H).

When the four miRNAs were tested together as one marker panel with the assumption that one increased miRNA constituted an overall positive score, maximum efficiency (highest Youden index) was obtained, with sensitivity of 92% and specificity of 80%. miR-371a-3p reached specificity of 84.7% when set to the same sensitivity of 92%. Because the discriminatory power of miR-371a-3p alone was at least similar to that of the miRNA panel, only miR-371a-3p was selected for further analysis in the extended patient sample.

3.2. Main study

Figure 2A shows the median expression of miR-371a-3p in controls, in patients with LCTs, and in the three clinical GCT stages in conjunction with postoperative measurements in patients with CS1 and CS2. We observed highly significant differences in preoperative median values between GCT subgroups and controls or patients with LCTs. Significant differences were also noted between preoperative and postoperative values in CS1 and CS2 patients.

Stratifying GCT patients into various histologic subgroups (Fig. 2B) revealed that teratoma had very low median expression, close to the normal range, in patients with CS1 and CS2 disease. Among CS1 patients, miRNA levels in seminoma and nonseminoma were significantly different from each other and from teratoma. The median miRNA levels for the subgroups featured in Figure 2A,B are listed in Supplementary Table 8 with p values related to the various cross-comparisons shown in Supplementary Table 9.

The diagnostic sensitivity of miR-371a-3p was calculated to as 88.7% (95% CI 82.5–93.3%) using empirical data for all 150 preoperative samples and 106 controls, and the specificity was 93.4% (95% CI 86.9–97.3%), with an AUC of 0.945 (asymptotic 95% CI 0.916–0.974). Using the density estimation model, sensitivity was 86.3% (95% CI 79.7–90.4%) and specificity was 92.5% (95% CI 89.0–95.9%), with an AUC of 0.939 (95% CI 0.907–0.965) (Fig. 2C, Supplementary Fig. 1). We also found dissimilar miR expression between localized and disseminated disease, with sensitivity of 81.4% (95% CI 72.1–87.0%) and specificity of 92.5% (95% CI 89.0–96.1%) in CS1 ($n = 107$), and sensitivity of 98.6% (95% CI 94.8–99.9%) and specificity of 92.5% (95% CI 88.9–96.3%) in CS2–3 ($n = 43$).

In CS1 seminoma, multiple regression analysis revealed a highly significant association between miR-371a-3p expression and tumour diameter ($p < 0.001$), but no association with pT stage (Fig. 3).

Figure 4 shows the superior sensitivity of miR-371a-3p compared to the classical GCT markers in both histologic subgroups (all comparisons $p < 0.001$). For the entire group of 139 GCT patients for whom all values were available, the overall sensitivity of miR-371a-3p was considerably higher than that of the combined AFP, bHCG, and LDH markers (87.8% vs 50.4%). Further details are given in Supplementary Table 10.

The individual repeated measurements of miR-371a-3p among 18 CS2 patients during chemotherapy are shown in Figure 5A. Expression of miR-371a-3p decreased in 12 patients after orchiectomy and dropped to the normal range in the majority of patients after the first cycle of chemotherapy, remaining there during the later course. In one patient (case #118) miR-371a-3p expression was approximately 20% of the starting value until cycle 3, but then dropped to zero. On completion of therapy, most patients had miR-371a-3p expression of almost zero; two patients had expression of 1.5% and 2.2%, respectively, of the starting value.

In the nine CS3 patients, miR-371a-3p expression decreased markedly for all except one after the first cycle of chemotherapy (Fig. 5B). No further information is available for this patient. miR-371a-3p expression levels in the other patients remained low until completion of treatment.
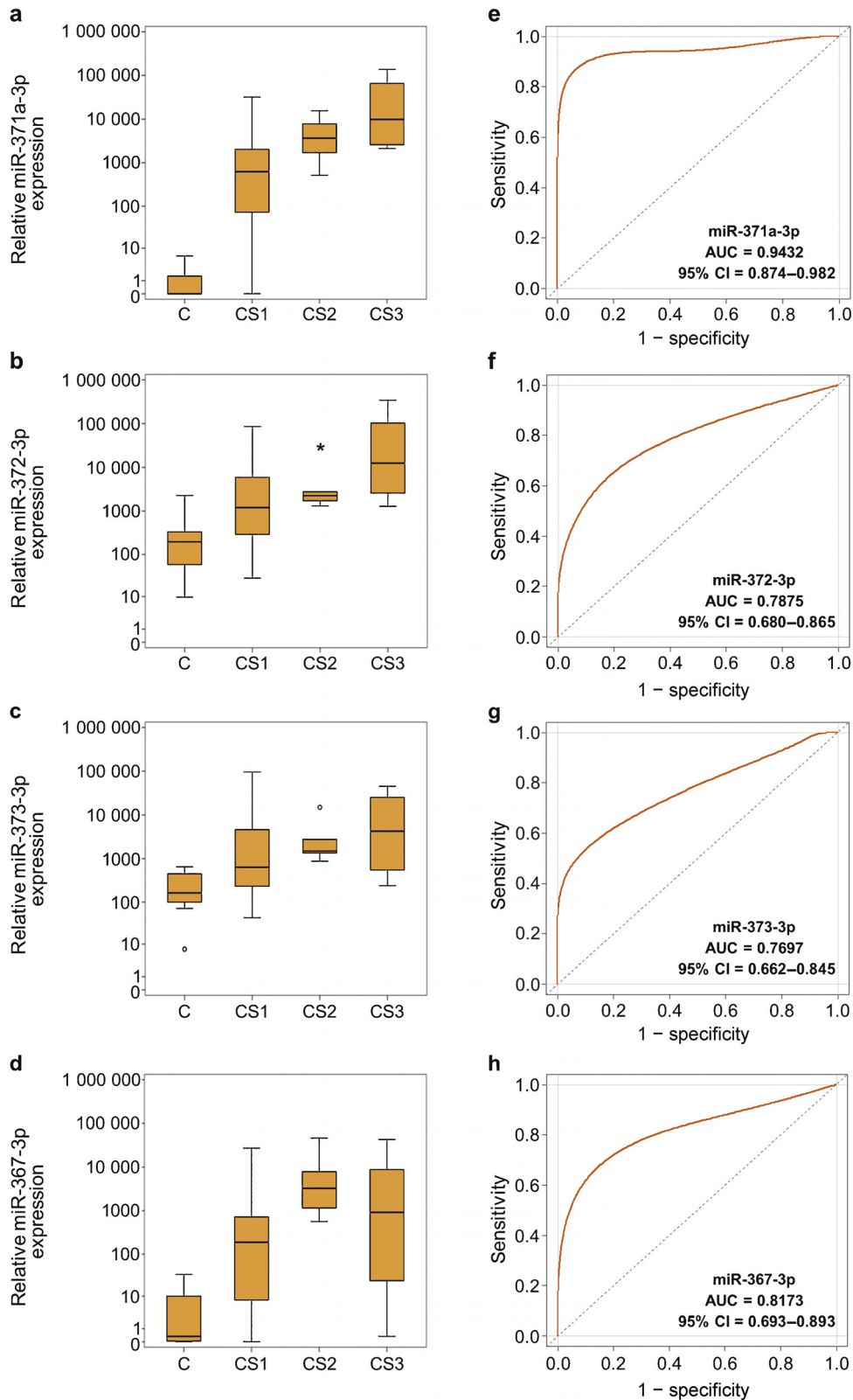
Fig. 1 – Box plots of relative microRNA (miRNA) expression in 50 patients with germ cell tumours and 20 controls (preliminary study) for (A) miR-371a-3p, (B) miR-372-3p, (C) miR-373-3p, and (D) miR-367-3p. The bold lines within the boxes show the median miRNA expression in patients with CS1, CS2, and CS3 disease and controls, with box size indicating the interquartile range. The y-axis is plotted on a log$_{10}$ scale. (E–H) Receiver operating characteristic curve with value for area under the curve (AUC) and 95% confidence interval (CI) for the corresponding box plot. C = controls; CS = clinical stage.

a

b

c
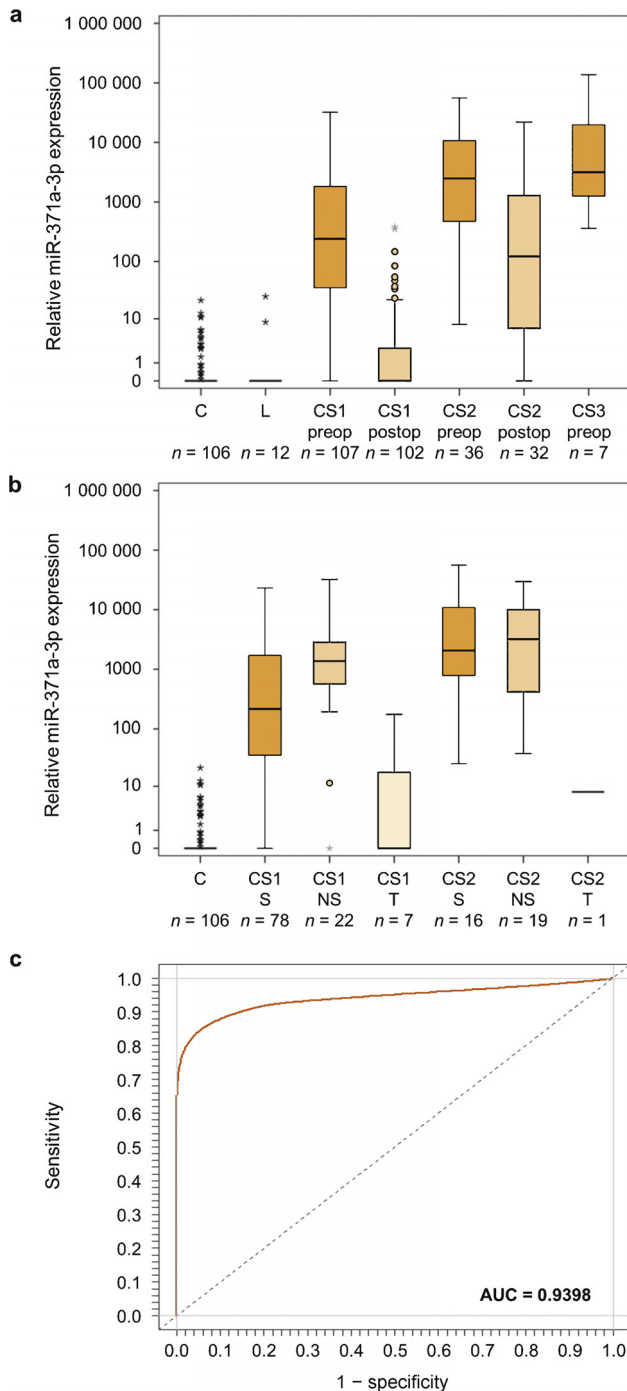
Fig. 2 – Box plots and receiver operating characteristic (ROC) curves of miR-371a-3p expression in patients with germ cell tumours and controls (main study). (A) Box plot of miR-371a-3p expression in controls, in patients with Leydig cell tumours, and in patients with clinical stage 1 (CS1), CS2, or CS3 disease. Data for preoperative patient cohorts are shown in orange, and for postoperative cohorts in light orange. (B) Box plot of miR-371a-3p expression in patients with tumours of different histologic subtypes and in controls. Data for seminomas are shown in orange, for nonseminomas in light orange, and for teratomas in light light orange. (C) ROC curve with area under the curve (AUC) for miR-371a-3p expression in 150 preoperative GCT serum samples and 106 controls. The y-axis in A and B is plotted on a $\log_{10}$ scale. C = controls; L = Leydig cell tumours; NS = nonseminoma; postop = postoperative; preop = preoperative; S = seminoma; T = teratoma.
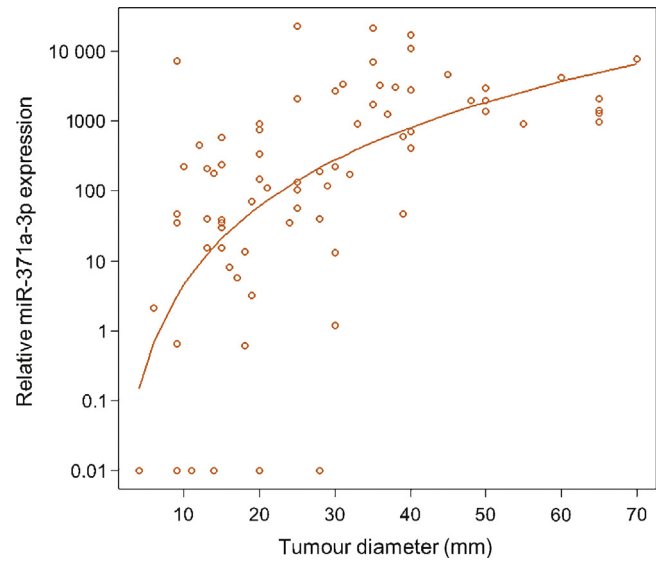

Fig. 3 – Association between miR-371a-3p expression and tumour diameter among 77 patients with clinical stage 1 seminoma. The association is highly significant ($p < 0.001$). The adjusted R^2 is 0.386. The y-axis is plotted on a $\log_{10}$ scale.
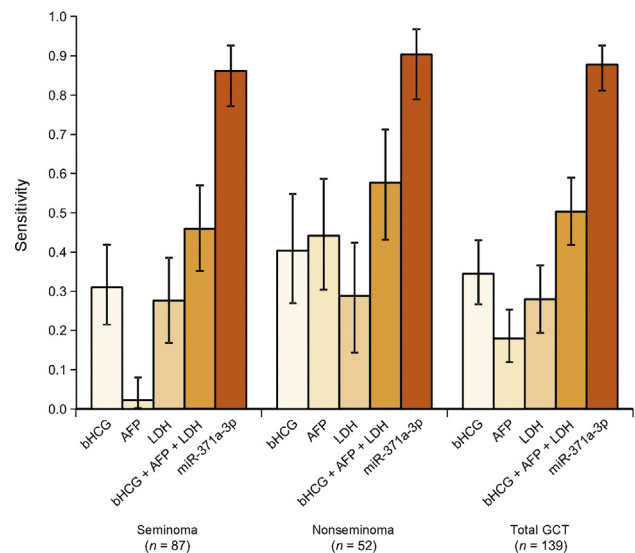

Fig. 4 – Comparison of the classical markers α-fetoprotein (AFP), β-subunit of human chorionic gonadotropin (bHCG), and lactate dehydrogenase (LDH) with miR-371a-3p according to preoperative measurements. The bars represent the sensitivity of bHCG (Pale orange bars), AFP (light light orange bars), LDH (light orange bars), combined bHCG + AFP + LDH (orange bars), and miR-371a-3p (dark orange bars) among patients with seminoma and nonseminoma and in the entire germ cell tumour (GCT) cohort. Error bars represent 95% confidence intervals.

Figure 5C shows individual miR-371a-3p expression in nine patients with relapse, all of whom had elevated expression levels. As with the other patients with metastases, miR-371a-3p levels decreased after the first cycle of chemotherapy, with one exception (case #159). Levels remained low for all patients except one (case #163), in whom miR-371a-3p expression increased to >300% of the starting value. In this case, the course of miR-371a-3p
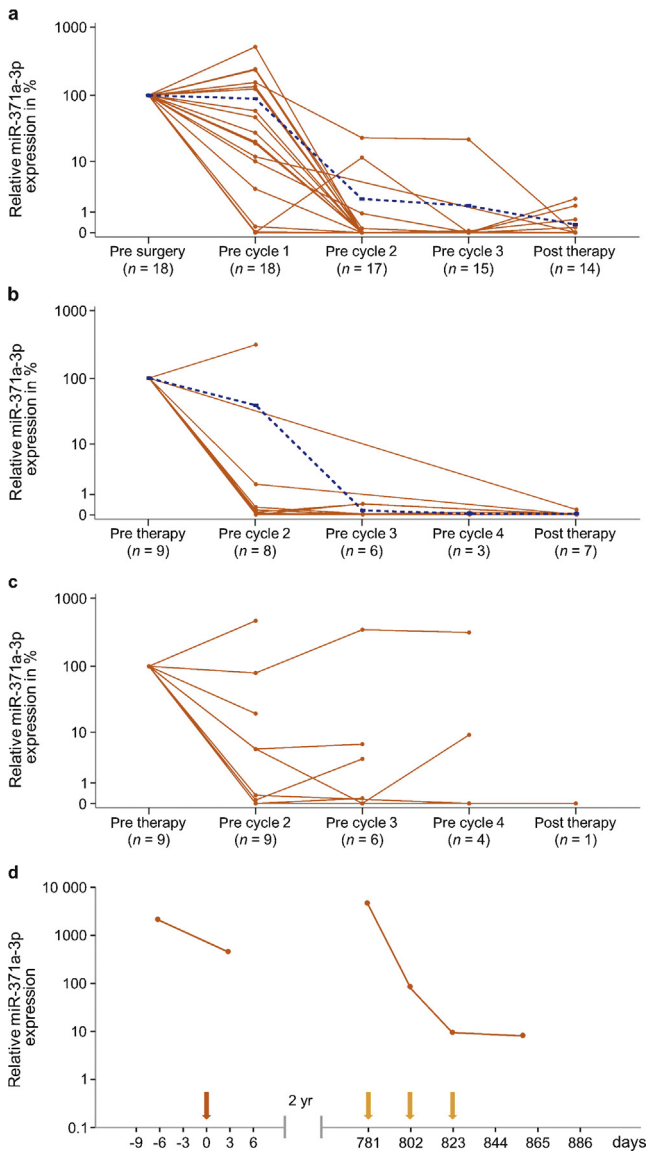
Fig. 5 – Changes in miR-371a-3p levels in individual patients with systemic disease during the course of chemotherapy. Each line denotes an individual patient. Dotted lines represent mean values for the cohort. The values represent percentages of the starting value. The *y*-axis shows values for relative quantity and is plotted on a $\log_{10}$ scale in all diagrams. (A) Patients with clinical stage 2 (CS2) disease, (B) CS3 disease, (C) relapsing disease, and (D) an individual patient with CS1 seminoma who relapsed 2 yrs after adjuvant carboplatin therapy. The orange arrow indicates the time point of orchiectomy, and the light orange arrows the chemotherapy courses for relapse.

expression mirrored that of AFP levels, which likewise rose to >300%, indicating tumour progression.

One patient with CS1 seminoma relapsed 2 yr after adjuvant carboplatin treatment (Fig. 5D). Notably, this patient had persistently high miRNA expression after orchiectomy. The level had further increased at relapse, but dropped to normal on remission after chemotherapy.

4. Discussion

The study results reveal that serum levels of miR-371a-3p apparently fulfil all prerequisites for a valuable biomarker.

First, miR-371a-3p is biologically specific for GCT, although, theoretically, an immune response reaction cannot be completely excluded. Healthy control subjects and patients with nonmalignant scrotal disease, as well as those with non-GCT of the testis (LCT), did not express the marker [13,14]. Second, the marker expression correlated with clinical stage. Patients with metastases had significantly higher miRNA levels than CS1 cases. In CS1 seminoma, miR-371a-3p levels correlated with tumour size. Third, miRNA levels correlated with treatment effects. In CS1 disease, elevated miR-371a-3p levels dropped to normal postoperatively, mirroring the tumour-free situation. In patients with metastases, miR-371a-3p expression decreased with chemotherapy to reach normal levels on completion of therapy. Notably, patients with relapsing disease also expressed the marker, as observed in nine cases. In addition, failure of therapy can be highlighted by the marker, as observed in one relapsing patient who had increasing miRNA levels on disease progression despite chemotherapy. It is also noteworthy that one patient with CS1 disease that relapsed after 2 yrs had supranormal postoperative miR-371a-3p expression. Persistent miRNA elevation might have heralded pending clinical relapse. Overall, serum miR-371a-3p levels adequately correlated with the actual state of disease and treatment response.

The association between miRNA expression levels and histologic subtype is unexplained. However, it could be hypothesised that correlation between the degree of morphologic tumour differentiation and miRNA serum levels exists because the miR-371–3 cluster is expressed primarily in undifferentiated stem cells [22]. This would explain the low expression in teratoma, which represents the GCT subtype with the highest degree of morphologic differentiation [23]. Conversely, the higher expression of miR-371a-3p in nonseminoma could be related to the high proportion of embryonal carcinoma in our nonseminoma group. This histologic subtype is biologically close to stem cells [24]. By contrast, seminoma cells have a somewhat higher degree of differentiation, with morphologic similarity to spermatogonia [25], and thus feature lower miR-371a-3p expression than embryonal carcinoma.

Since 2012, pilot studies have documented the applicability of miRNAs of the 302/367 and 371–3 clusters as serum markers of GCT. Accordingly, it was proposed that all miRNAs of the two clusters be used as one diagnostic panel in clinical practice. The present study confirms that miR-371a-3p is the miRNA with the highest ability to discriminate GCT patients from control subjects [14]. MiR-367-3p performed second best, whereas miR-372-3p and miR-373-3p had considerably lower discriminatory power.

The optimised quantification methods used in this study revealed that miR-371a-3p has specificity at least identical to the panel of four miRNAs when the sensitivity was set to an equal value. In practice, it is probably not necessary to use the panel of all miRNAs, as previously suggested [13,15,26,27]. In light of the economic constraints in clinical practice and the results of the present study, it seems rational to use miR-371a-3p exclusively, adding only miR-367-3p for unresolved cases.

The diagnostic specificity of miR-371a-3p is extraordinarily high compared to the classical AFP, bHCG, and LDH markers. More than 86% of GCT patients express this novel marker. The expression is most valuable in seminoma, in which <20% of patients express bHCG [28]. Because miRNA expression is associated with tumour size, small CS1 seminomas may have only slightly increased levels. A minor limitation of the utility of miR-371a-3p is its lack of expression in teratoma. However, this finding is in accordance with the lack of expression of classical markers in this histologic subtype [29].

Limitations of our study include the comparatively low number of patients with metastases. Owing to the somewhat dissimilar expression of the new marker in localized and systemic disease, confounding of the results by selection is conceivable, so the clinical applicability of the test remains uncertain so far. Long-term observational data are missing, and it has still not been formally proven that low miRNA levels on completion of treatment remain low. Nevertheless, preliminary data indicate that levels remain low during follow-up unless the disease recurs.

5. Conclusions

The promising results from pilot studies were confirmed. To the best of our knowledge, this study is the first to use density estimation for a large sample size to gauge the real distribution of RQ values for GCTs and controls. The study also provides evidence of changes in miR-371a-3p expression during chemotherapy in a fairly sizeable patient sample and documents miR expression in relapsing patients. miR371a-3p appears to be a highly sensitive and specific serum GCT biomarker that can aid during monitoring of GCT and could possibly help in sparing radiologic examinations [30]. A prospective large-scale validation study is under way with a view to implementing the test in clinical practice.

Author contributions: Klaus-Peter Dieckmann and Gazanfer Belge had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Supervision: Dieckmann, Belge.
Other: None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2016.07.029.

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