The novel biomarker of germ cell tumours micro-RNA-371a-3p has a very rapid decay in patients with clinical stage 1

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Abstract

Background: Accumulating evidence suggests serum levels of microRNA-371a-3p to be a novel tumour marker of testicular germ cell tumours (GCTs). Presently, there is only limited information regarding the velocity of decline of serum levels in response to treatment.

Patients, methods: Twenty-four patients with testicular GCT (20 seminoma, 4 nonseminoma, median age 40 years) with clinical stage 1 had measurements of serum levels of miR-371a-3p preoperatively and repeatedly on the following 3 days. Three had additional testings within 24 h after surgery. Measurement results were analysed using descriptive statistical methods.

Results: Serum levels dropped to 2.62%, 1.27% and 0.47% of the preoperative level within one, two and three days, respectively. The computed half-life amounts to 3.7 to 7 hours. The velocity of decay is significantly associated with tumour-size.

Conclusions: Serum-levels of miR-371a-3p have a short half-life of less than 12 hours. The rapid decay after treatment represents a valuable feature confirming the usefulness of miR-371a-3p as a valuable serum biomarker of GCT.

Key words

Germ cell tumour, seminoma, nonseminoma, microRNA-371a-3p, serum biomarker

Introduction

The clinical management of testicular germ cell tumours (GCTs) largely relies on the monitoring of the serum tumour markers alpha fetoprotein (AFP), beta human chorionic gonadotropin (bHCG), and lactate dehydrogenase (LDH) [1]. However, clinical decision-making is often hampered by the fact that only 50-60% of GCTs express one of these markers [2], [3], [4]. In the cases with absent marker expression radiological methods e.g. computed tomography (CT) are employed for monitoring of treatment. Recently, serum levels of microRNAs (miRs) of the clusters miR 371-3 and miR 302/367 have been suggested as novel biomarkers of GCT [5], [6], [7], [8], [9]. Among the candidate miRs, miR-371a-3p proved to be most promising marker with a sensitivity of 88.7- 90% and a specificity of 86 - 93.4% [10], [11], [12], [13]. However, before the implementation of this marker in clinical practice, validation of its usefulness in a larger international patient cohort is required [14],[15]. Also, for employing miR-371a-3p serum levels as a tool for clinical decision making information about the velocity of decay of this substance in serum is required. According to the well-defined prerequisites of a useful tumour marker outlined by Lange & Winfield [16], the marker substance should be cleared quite rapidly from serum after removing the source of marker production. If the decay of a candidate substance would involve longer than one week that substance would probably not qualify as a useful tumour marker because changes of the disease status would be mirrored with undue delay [17]. Regarding miR-371a-3p, preliminary data suggest a very rapid half-life [18], [19]. However, that information is based on only very few patients and no further information is available to date. We therefore evaluated the decay of serum levels of miR-371a-3p in an extended patient sample. We also looked to any associations of the decay kinetics with histology of the GCT and with other factors. The goal of our study was to further qualify serum levels of miR-371a-3p as useful tumour marker of GCT.

Methods

Patients and samples

Twenty-four patients with GCT confined to the testis (i.e clinical stage 1) were enrolled for the present study, 20 with pure seminoma, four with nonseminomatous tumours. Median age was 40 years (interquartile range 17.25 years). In all patients, the size of the primary tumour was registered for correlation with serum miR levels. The median tumor size was 31.5 mm (interquartile range 31.5 mm). All of the patients underwent repeated blood aspirations for measurement of serum levels of miR-371a-3p at consecutive time-points after surgery. All had preoperative examinations, as well as blood aspirations on the first and second day after surgery. On the third day after orchiectomy, only 18 patients were available. Of the 24 patients, three underwent multiple testings during the first 24 hours after surgery, one patient (#22, suppl. table 1) had additional serum samples taken at four and eight hours, respectively, after surgery, and two others (#23 and #24, suppl. table 1) had each one additional sample taken at ten and four hours, respectively. Twenty male patients with non-malignant testicular disease were recruited as controls (suppl. table 2). Median age of these patients was 33 years (interquartile range 26.5 years). All participants gave informed consent. The study received Ethical approval (Ärztekammer Bremen, reference 301, 2011).

Laboratory methods

Serum levels of miR-371a-3p were measured by quantitative PCR as reported earlier [10], [20]. Briefly, total RNA was isolated from 200 µl of cubital vein serum using the miRNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was conducted with the TaqMan MicroRNA Revese Transcription Kit (Thermo Fisher Scientific, Schwerte, Germany) and stem-loop primers for miR-371a-3p (assay ID 002124) and the endogenous control miR-30b-5p (assay ID 000602). The cDNA was preamplified by a standard PCR and quantified in a qPCR using the aforementioned TaqMan assays and a 7500 Fast Real Time PCR System (Thermo Fisher Scientific, Schwerte,

Germany). The relative quantity (RQ) of miR-371a-3p was calculated using the $\Delta\Delta C_T$ method [21].

Statistical analysis

The miR-371a-3p expression of the various subgroups is presented as median with interquartile ranges (IQR). The difference between independent samples (tumour patients and controls) was analysed with the Mann Whitney U-test. Differences between dependent samples (expression at different time points in the same patient cohort) were assessed with the Wilcoxon ranked sign test. The miR-371a-3p half-life was estimated by calculation of the linear equation of a straight line between the two data points surrounding the y-value of 50% miR-371a-3p expression by employing the measurement results of the three patients with additional blood aspirations within the first 24 h after orchiectomy. The Pearson correlation was used for correlation analysis of interval scaled data. Significant differences were assumed at $p < 0.05$.

Results

Within 24 hours after orchiectomy the serum miR-371a-3p levels dropped to 2.62% of the preoperative level to decline further to 1.27% after 48 h, and to 0.47% after 72 h, respectively (fig.1a). The differences between the median RQ values measured preoperatively and the median miRNA expressions at 24 h, 48 h or 72 h after orchiectomy, respectively, were statistically significant ($p < 0.001$ for all comparisons), as were the differences between the median levels measured after 24 h and after 48 h, as well as the difference between the RQ values after 24 h and 72 h ($p = 0.001$ and $p < 0.001$, respectively). The difference between the levels at 48 h and 72 was no longer significant. but the median levels of preoperative tumour samples, as well as those at 24 h, 48 h and 72 h, were significantly different from the median level of controls ($p < 0.001$, $p < 0.001$, $p = 0.009$ and $p = 0.043$, respectively). There

was no statistical significant difference between the miR-371a-3p expression in seminoma and non-seminoma at the different time points (suppl. fig. 1).

In the first patient with multiple testings during the first day after surgery (#22) (fig. 1b), the miR-371a-3p expression declined to 87.05% after 4 h and to 37.82% after 8 h, arriving at 1.62% after 24 h and 2.1% after 48 h. The second patient (#23) dropped to 1.8% of the pre-operative value after 10 h, to reach at undetectable levels of miR-371a-3p after 24 h and 48 h. MiR-371a-3p values of the third patient (#24) dropped to 45.52% after 4 h, to further decrease to 3.38% after 24 h and to 1.38% after 48 h. The computed half-lifes of miR-371a-3p serum levels amount to 7.01 h (#22), 5.09 h (#23), and 3.67 h (#24) in the three patients with multiple blood aspirations within the first 24 h after surgery.

We observed a significant correlation of preoperative RQ-values with tumour-size ($p = 0.001$, $r^2=0.38$) (fig. 2), with larger tumours featuring higher expressions than smaller ones.

To further assess the velocity of miRNA decay, a cut-off of RQ = 1.933 representing the upper level of norm (ULN) of miR serum levels was calculated by employing the preoperative miR expressions of the 24 GCT patients and 20 controls in an receiver operating characteristics (ROC) analysis (suppl. fig. 2). The time needed to fall below that cut off value was used as an estimate for the velocity of miRNA decay. Sixteen of the 24 patients dropped below the cut off within 72 h (fig. 3). There was a significant correlation between tumour size and the velocity of decay, with larger tumours needing more time to drop below the cut off value ($p = 0.016$, $r^2 = 0.348$). No significant difference was observed between the velocities of decay of seminomas and nonseminomas, respectively. Likewise, patients´ age was not correlated with the velocity of decay.

Discussion

The crucial result of the present study is the very short half-life of miR-371a-3p serum levels of 3.67 to 7.01 hours. This result is in line with preliminary findings [18], [19]. As outlined in

classical reviews regarding the role of tumour markers, serum levels should mirror the activity of the disease allowing clinical decisions to be taken in relation to the serum levels actually measured [16]. Thus, a rapid decay after treatment is a quite favourable feature of a tumour marker because any changes of the extent of disease will be immediately highlighted by the marker [22]. Among the markers currently in use, bHCG also has a short half-life with about 36 hours [23]. AFP involves a considerably longer half-life of 5 – 7 days [24], [25], [26]. Particularly in CS1 patients with elevated AFP levels, it is sometimes inconvenient to wait for appropriate marker decline to be sure about the stage 1 condition [27]. A half-life of less than 24 hours as found for miR-371a-3p thus represents a highly useful feature of a serum tumour marker [15].

Clinically important is the association of the velocity of decay with the size of the primary tumour and with the initial extent of marker elevation [28]. Thus, patients with larger tumours and with higher miR levels have a somewhat delayed decline although the decay is still faster than that of the classical markers. Probably, the association of marker decay with tumour bulk will not considerably impact the clinical utility of the test.

No associations of the marker decay have been observed with patient age and this finding is the same with the classical markers [29]. Also, no association of miR decay was found with histology of the primary. However, this finding might be premature because it rests on only four patients with nonseminoma, and particularly nonseminoma may involve a number of various histologies including teratoma. To obtain safe information about the velocity of decay of miR levels in nonseminoma a larger patient sample needs to be evaluated.

Although there is now clear evidence regarding the very rapid decline of miR levels in CS1 patients, there is still limited information about the velocity of decay in metastasized cases in response to therapy. Principally, the regression of tumour marker levels in metastasized cases may be different from CS1 cases depending on tumour bulk and on the response to therapy [24], [30]. In a previous report we found the majority of CS2-3 patients to reach normalization of miR levels already after the first cycle of chemotherapy upon repeat

measurements during treatment [10]. This would signify a very rapid decline in metastasized cases, too. As a matter of fact, a similarly rapid decline should be expected from a biological point of view, likewise, because the biological clearance mechanisms of microRNAs are most probably identical in all patients with GCT. Clearly, this understanding requires confirmation in a larger prospective series.

Limitations of the present study involve the still low number of patients analysed. Further, the conclusions are limited by the very low number of patients with nonseminoma, particularly in light of the histologic variability of nonseminomatous GCTs. An over-all appraisal of the decay of miR-371a-3p is limited by the lack of metastasized cases in the present series. Also, the half-life was computed from only three patients with varying time points of examination, therefore these values should be considered with care. In light of the obviously very rapid decline of miR levels more measurements within the first 24 hours after orchiectomy would have been desirable to compute a more accurate decay curve. However, such data are hard to accomplish because multiple blood aspirations within one day are rather inconvenient for patients and represent ethical problems on the one side and create logistic-practical problems on the other side.

Conclusion

The very rapid decay of miR-371a-3p serum levels after removal of the source of this substance with a half-life of less than 12 hours is a highly favourable feature and adds further support to the value of this miR as a serum biomarker of GCTs.

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Disclosure Statement

Gazanfer Belge, and Klaus-Peter Dieckmann each possess 10% ownership shares of miRdetect, GmbH, Bremen, a company aiming to develop a commercially available test for measuring microRNAs in serum. MiRdetect holds a patent for the measurement of miRNA in body fluids at the limit of detection. A further patent for the use of miR-371a-3p as a marker for GCNis was filed by miRdetect/University of Bremen on November, 2nd 2016.

None of the other authors declare any conflict of interests with publishing this report.

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Legends to figures

Fig. 1. a Box plot showing the different relative miR-371a-3p expressions in serum samples of patients with testicular germ cell tumours at consecutive time points following orchiectomy. See methods section for clinical details. Horizontal line within box denotes median serum RQ value, upper and lower limits of boxes denote inter quartile ranges (IQRs). Whiskers show lowest and highest values within a range of 1.5 IQR. The y-axis represents a logarithmic scale.

b Line graph showing the rapid decay of serum miR-371a-3p levels in three patients with multiple testings during the first 24 hours after surgery. See methods section for clinical details. The y-axis represents percentages of the preoperative serum values. The horizontal dashed line depicts the 50% reduction level.

Fig. 2. Scatter plot showing the correlation between the preoperative relative expression of miR-371a-3p and the tumour size in 24 patients with testicular GCT. The y-axis is displayed in a logarithmic scale.

Fig. 3. Scatter plot showing the velocity of miR-371a-3p decay in relation to the tumour size. X-axis denotes tumour-size (mm), y-axis denotes the time needed to reach the cut-off level. Only those patients whose miRNA levels dropped below the cut-off value within 72 h (n = 16) are included. The figure clearly illustrates that smaller tumours arrive more rapidly at the cut-off level.

Supplemental Fig. 1. Box plot showing the miR-371a-3p expression in seminoma (n = 20) and nonseminoma (n = 4) at different time points of examination. Left-sided boxes (grey) represent seminoma, right-sided boxes (dark) on the left represent nonseminoma. Horizontal line within box denotes median serum RQ value, upper and lower limits of boxes denote inter quartile ranges (IQRs). Whiskers show lowest and highest values within a range of 1.5 IQR. The y-axis is depicted in a logarithmic scale.

Supplemental Fig. 2. Receiver operating characteristic (ROC) curve for the discrimination of 24 patients with testicular germ cell tumours and 20 controls. The area under the curve is 0.969.

Supplemental table 1. Clinical data and RQ values of testicular germ cell tumour patients

Patient ID	Age [years]	S/NS	Tumour size [mm]	RQ miR-371a-3p pre-operative	RQ miR-371a-3p 4 h postop.	RQ miR-371a-3p 8 h postop.	RQ miR-371a-3p 10 h postop.	RQ miR-371a-3p 24 h postop.	RQ miR-371a-3p 48 h postop.	RQ miR-371a-3p 72 h postop.
1	49	S	18	20.64	n.a	n.a.	n.a.	0.43	0.0	0.0
2	34	S	34	2035.26	n.a	n.a.	n.a.	48.47	5.79	2.54
3	31	S	68	1728.14	n.a	n.a.	n.a.	108.91	47.28	62.21
4	28	NS	9	12.3	n.a	n.a.	n.a.	0.0	0.0	0.0
5	53	S	48	182.91	n.a	n.a.	n.a.	7.25	0.0	0.0
6	47	S	8	0.0	n.a	n.a.	n.a.	0.0	0.0	0.0
7	66	S	7	58.53	n.a	n.a.	n.a.	1.61	0.0	0.0
8	41	S	56	950.14	n.a	n.a.	n.a.	14.58	4.86	3.12
9	34	S	47	1620.25	n.a	n.a.	n.a.	32.72	9.7	1.94
10	n.a.	S	54	4347.59	n.a	n.a.	n.a.	78.79	1.47	5.61
11	47	NS	14	45.86	n.a	n.a.	n.a.	3.92	2.71	n.a.
12	29	S	16	54.38	n.a	n.a.	n.a.	0.0	0.0	0.0
13	47	NS	24	354.83	n.a	n.a.	n.a.	39.56	24.61	11.69
14	52	S	15	2.57	n.a	n.a.	n.a.	0.0	0.0	0.0
15	34	S	15	2.29	n.a	n.a.	n.a.	0.0	0.0	n.a.
16	41	S	45	607.61	n.a	n.a.	n.a.	14.18	2.1	0.63
17	33	S	44	712.63	n.a	n.a.	n.a.	22.53	18.8	4.37
18	39	S	62	919.69	n.a	n.a.	n.a.	2.4	0.89	0.41
19	60	S	31	71.11	n.a	n.a.	n.a.	0.0	0.0	0.0
20	57	S	40	101.83	n.a	n.a.	n.a.	4.59	5.71	n.a.
21	34	S	32	282.09	n.a	n.a.	n.a.	14.44	3.84	0.46
22	62	S	35	1081.69	941.63	409.07	n.a.	17.47	22.76	n.a.
23	35	S	14	45.8	n.a.	n.a.	0.82	0.0	0.0	n.a.
24	27	NS	16	154.07	70.13	n.a.	n.a.	5.2	2.12	n.a.

NS: Nonseminoma; S: Seminoma; RQ: Relative quantity; n.a.: not available

Supplemental table 2. Clinical data and RQ values of controls

Control ID	Age [years]	RQ miR-371a-3p
1	52	0.0
2	26	0.0
3	28	0.0
4	23	0.0
5	43	0.0
6	18	1.58
7	49	0.0
8	36	0.0
9	18	0.0
10	33	0.0
11	18	0.37
12	22	0.0
13	50	2.78
14	24	0.0
15	22	0.0
16	33	1.47
17	47	0.36
18	54	0.0
19	54	0.0
20	48	1.16

RQ: Relative quantity

Figures

Fig. 1

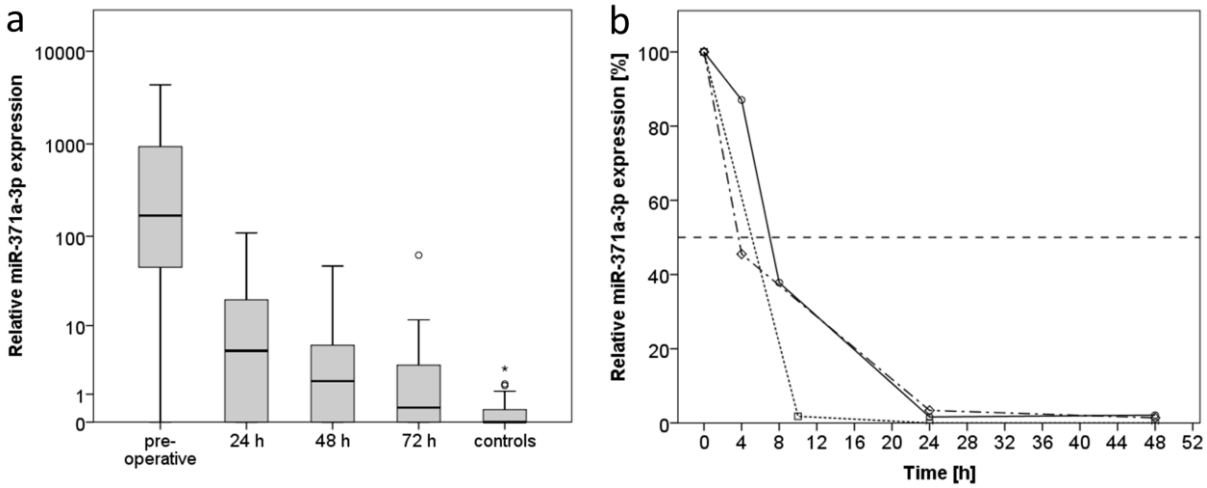


Fig. 2

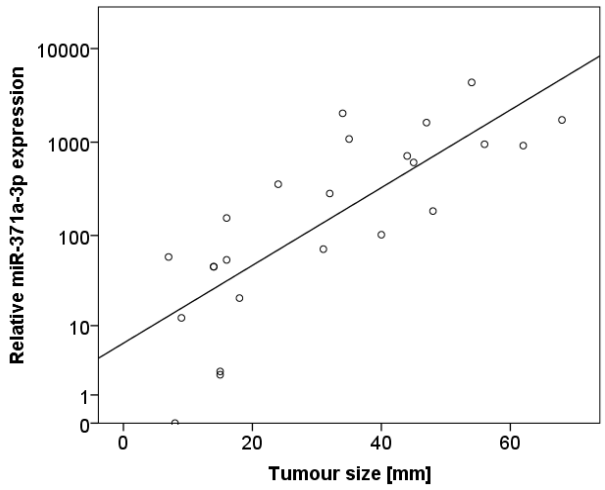
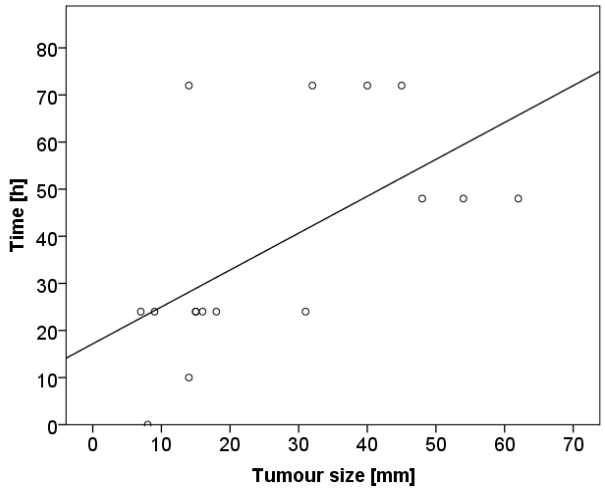
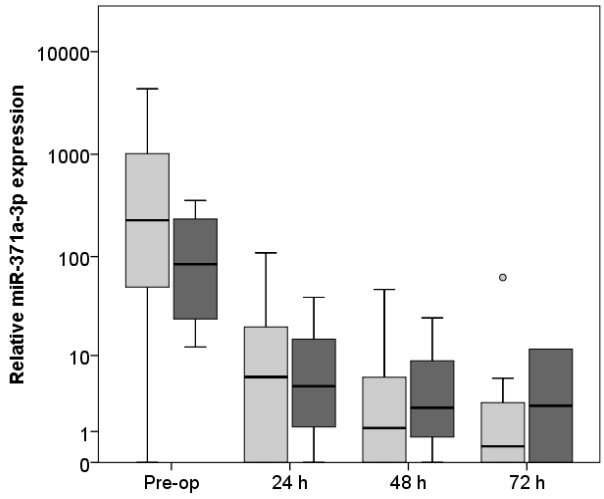

Relative miR-371a-3p expression
10000
1000
100
10
1
0
0
20
40
60
Tumour size [mm]

Fig. 3.



Suppl. Fig.1



Suppl. Fig. 2