

MicroRNA miR-371a-3p – A Novel Serum Biomarker of Testicular Germ Cell Tumors: Evidence for Specificity from Measurements in Testicular Vein Blood and in Neoplastic Hydrocele Fluid

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Key Words

microRNA · Testicular neoplasms · Serum · Biomarker · Hydrocele

Abstract

Background: microRNAs (miRs)-371-3 are suggested to be novel biomarkers of germ cell tumors (GCTs), but their specificity is unresolved. We aimed at clarifying the origin of miR 371a-3p by measuring this miR in peripheral vein blood, and in fluids present in the vicinity of GCTs. **Methods:** miR-371a-3p levels were measured by quantitative PCR in 9 tumor surrounding hydroceles and in cubital vein blood (CVB) and testicular vein blood (TVB) of 64 GCT patients, 51 with clinical stage (CS) 1, 13 with CS2–3. Thirty three CS1 cases had also postoperative CVB measurement. TVB miR levels were compared with those of CVB. Associations with clinical factors were analyzed statistically. **Results:** TVB miR levels were 294-fold, 80-fold and 4.6-fold higher than those in CVB of CS1 patients, CS2–3 patients and controls, respectively. Neoplastic hydrocele fluid comprised of very high miR levels. In CS1, miR levels dropped to normal postoperatively. Statistically,

CVB miR levels are significantly associated with tumor size ($p = 0.0211$) and testis length ($p = 0.0493$). TVB miR levels are associated with testis length ($p = 0.0129$). **Conclusions:** This study provides evidence for the origin of circulating miR 371a-3p molecules from GCT cells. miR-371a-3p represents a specific serum biomarker for germ cell cancer.

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Background

Clinical management of testicular germ cell tumors (GCTs) is largely based on the monitoring of serum tumor markers [1–3]. However, the markers beta human chorionic gonadotropin (bHCG), alpha fetoprotein (AFP) and lactate dehydrogenase (LDH) are expressed only by 60% of GCTs [4–6]. Particularly, seminoma expresses bHCG in less than 20% of cases and AFP in none [7]. Therefore, more sensitive markers are needed. So far,

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none of the suggested new markers could qualify for clinical employment [8, 9]. Recently, microRNAs (miRs) have been suggested to be a novel class of serum biomarkers [10–12]. Regarding testicular GCT, miRs-371-3 as well as miR-302 and miR-367 represent promising candidates [13–20].

miRs are small molecules of ribonucleic acid consisting of about 20 base pairs. They are released from the cellular nucleus and remain stable in body fluids. They can be measured there by quantitative real time polymerase chain reaction (qPCR). miRs-371-3 and miR-302 have been detected in GCT tissue [14, 21–23] and elevated serum levels have been documented in several pilot studies [15, 16, 24]. miR-371a-3p appears to be the most sensitive marker because it showed the greatest decrease of levels in response to treatment [16, 17].

Evidence for the specificity of serum levels of miRs-371-3 for testicular GCT is still equivocal despite the following observations: (1) high serum levels of these miRs in the majority of patients, (2) very low levels in healthy men, (3) in men suffering from non-testicular malignancies and (4) a significant decrease of levels after cure [25]. Another way of confirming the specificity of miRs-371-3 for testicular GCT would be to show particularly high levels of these miRs in body fluids being in close contact to the testicular neoplasm.

Blood in the venous drainage of testicular tumors comprises higher concentrations of bHCG than peripheral blood [26] and the origin of these high levels from the tumor represents settled knowledge [27]. Likewise, bHCG and AFP have been documented in tumor surrounding hydrocele fluid (HY) in higher concentrations than in the peripheral circulation [28]. The difference was explained by the direct leakage of marker molecules into surrounding compartments [29].

This study aimed at ascertaining the specificity of miR-371a-3p for GCT. We measured peripheral serum (cubital vein blood; CVB) levels of this miR preoperatively and compared these levels with postoperative findings and with those found in testicular vein blood (TVB). We also assessed the fluid of tumor surrounding hydroceles of testicular GCTs.

Methods

Patients and Samples

From June 2011 to December 2014, all consecutive patients undergoing surgery for suspected testicular malignancy provided CVB samples preoperatively. TVB samples were obtained during surgery from veins of the spermatic cord by puncture with a gauge

18 needle. Usually, around 2 ml TVB was aspirated; however, some cases had vessels inaccessible for aspiration. CVB and TVB samples were collected in serum separation tubes (Sarstedt, Nümbrecht, Germany) that were maintained at room temperature for approximately 60 min to allow for complete coagulation after blood aspiration. After that the samples were centrifuged (10 min, 2,500 g) to separate serum, and aliquots were frozen at -80°C until further processing. Sixty-four patients were eligible, 51 with clinical stage (CS) 1 disease and 13 with systemic disease (CS2–3). All these patients provided both, preoperative CVB and TVB samples (clinical details in online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000444303); 6 had been reported earlier [24, 25]. In 33 CS1 patients, postoperative CVB samples were available, additionally.

For comparison of TVB miR levels of patients with those of healthy males, 10 patients undergoing scrotal surgery for non-malignant diseases but who were otherwise healthy provided TVB and CVB samples (online suppl. table 2).

Nine GCT cases had a tumor surrounding hydrocele large enough for harvesting 2 ml of the fluid for analysis. Three patients with idiopathic hydrocele served as controls (online suppl. table 3). All patients had given informed consent. Ethical approval was given by Ärztekammer Bremen (ref. 301, 2011).

For this study on specificity, we restricted all laboratory analyses to miR-371a-3p of the miR-371-3 cluster because clinically, this miR appears to be the most promising marker. All of the candidate miRs are located in the clusters miR-371-3 and miR 302/367 on closely related chromosomal regions [30] and accordingly, the miRs of these 2 clusters are biologically interrelated [22]. The expression of these 2 clusters represents an embryonic pattern of miRs expression. It is therefore rational to assume that if one of these miRs is verified to specifically derive from GCT, then very probably the others do so too.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from 200 μl serum or HY using the miRNeasy Mini kit (Qiagen, Hilden, Germany) and RNA was quantified by spectrophotometry (Eppendorf, Hamburg, Germany). Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Darmstadt, Germany). RT primers represented an equal mixture of 2 miRNAs (miR-371a-3p, assay ID 002124 and miR-93, assay ID 000432)-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.

Preamplification and qPCR

For preamplification, miRNA assays represented an equal mixture of the 2 miRNAs, and RealTime ready cDNA Pre-Amp Master (Roche, Mannheim, Germany) was used. The PCR was performed at 95°C for 1 min, followed by 14 cycles of 95°C for 15 s and 60°C for 4 min using the GeneAmp PCR-System 2700 (Applied Biosystems). The preamplification product was diluted in the ratio 1:2 in nuclease-free water and used for qPCR.

Serum levels of miR-371a-3p were measured by qPCR with the TaqMan miRNA assay using the Applied Biosystems 7500 real-time PCR System (Applied Biosystems). All PCR experiments were carried out in triplicate using the FastStart Universal Probe

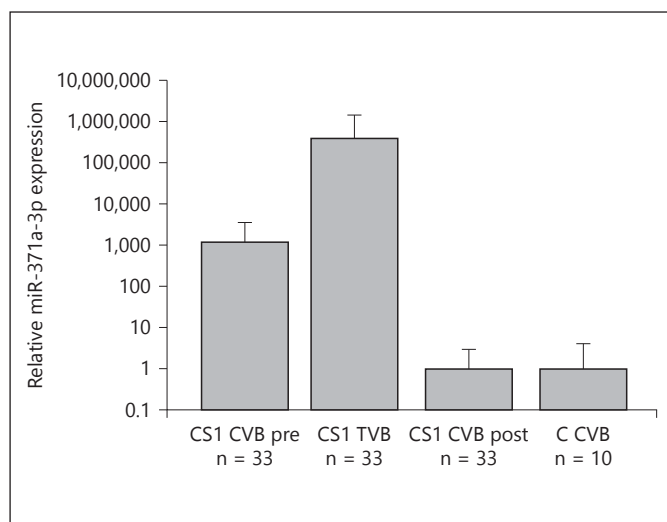


Fig. 2. Postoperative decrease of miR-371a-3p levels in CS1 patients. Columns show mean miR 371a-3p levels prior to surgery and corresponding postoperative levels in 33 CS1 patients. For comparison, mean values of controls and testicular vein measurements are given additionally. C = Controls; pre = preoperative; post = postoperative. The y axis is plotted on a log₁₀ scale.

comparisons of the various groups are summarized in table 2. In brief, mean peripheral serum miR levels of GCT patients are significantly higher than those of controls. The same is true for TVB miR levels. Postoperatively, the mean serum miR level of 33 CS1 patients dropped to RQ <9 (fig. 2 and online suppl. table 1).

There was a weak correlation of individual CVB miR levels with those of TVB. The Pearson product-moment correlation coefficient was $R^2 = 0.62$ in the CS1 group and $R^2 = 0.63$ in the metastasized group (fig. 3a, b). For each group, one outlier was omitted.

Hydrocele miR levels were much higher than corresponding levels in peripheral blood. In hydrocele controls, no miR-371a-3p expression was detected (fig. 1 and online suppl. table 3).

Association with Clinico-Pathological Factors

The multivariate (table 3) and univariate (table 4) analyses of possible associations of RQ values in CVB and TVB with clinical factors revealed a complex pattern of results. Because of the wide variation of miR levels in the TVB samples, logarithmical RQ values were employed to look for associations.

Upon multivariate analysis, the relation of RQ values TVB/CVB was not associated with any of the factors tested in any of the 3 patient groups analyzed (CS1, CS2–3, and entire GCT group). However, peripheral serum miR

Table 1. Mean miR-371a-3p expression in CVB and in corresponding TVB in patients and controls

| Group | Source of serum | n | Mean RQ values | Relation TVB/CVB |
|--------------|-----------------|----|----------------|------------------|
| C | CVB | 10 | 0.9 | 4.58 |
| C | TVB | 10 | 4.3 | |
| CS1 | CVB | 51 | 1,843.6 | 294.02 |
| CS1 | TVB | 51 | 542,064.6 | |
| CS2–3 | CVB | 13 | 6,186.9 | 80.29 |
| CS2–3 | TVB | 13 | 496,766.6 | |
| All patients | CVB | 64 | 2,725.8 | 195.49 |
| All patients | TVB | 64 | 532,863.4 | |

C = Controls.

Table 2. Statistical cross comparisons (p values) of miR-371a-3p levels of the groups documented in figure 2. Groups in the first column are compared to those in the second column

| Patient sample/fluid examined | p value |
|-------------------------------|---------|
| C/CVB | |
| CS1/CVB | <0.0001 |
| CS2–3/CVB | <0.0001 |
| C/TVB | |
| CS1/TVB | <0.0001 |
| CS2–3/TVB | <0.0001 |
| CS1/CVB | |
| CS1/TVB | <0.0001 |
| CS2–3/CVB | 0.0277 |
| CS1/HY | |
| CS1/CVB | 0.0313 |
| C/CVB | 0.0004 |
| C/TVB | 0.0007 |
| CS2–3/CVB | 0.0456 |
| CS2–3/CVB | |
| CS2–3/TVB | 0.0002 |

C = Controls.

levels were found to be significantly associated with tumor size and testis length in the entire group of patients. Testicular vein miR levels were significantly associated only with the testis length.

In CS1 patients, peripheral serum levels were not associated with any factor while TVB levels proved associated with testis length. In metastasized patients, peripheral miR levels were associated with tumor size, whereas TVB levels had no association with any factor.

Upon univariate analysis of the entire group of patients, there were significant associations of peripheral

Table 3. Significant associations of miR levels with clinical factors in TVB and CVB stratified for CS1 and CS2–3: multivariate analyses

| Patient group | Source of serum | Clinical parameter with significant association | p value |
|---------------|------------------|---|------------------|
| All patients | Relation CVB/TVB | None | |
| All patients | CVB | Tumor size Testis length | 0.0211 0.0493 |
| All patients | TVB | Testis length | 0.0129 |
| CS1 | Relation CVB/TVB | None | |
| CS1 | CVB | None | |
| CS1 | TVB | Testis length | 0.0068 |
| CS2–3 | Relation CVB/TVB | None | |
| CS2–3 | CVB | Tumor size | 0.012 |
| CS2–3 | TVB | None (log values) | |

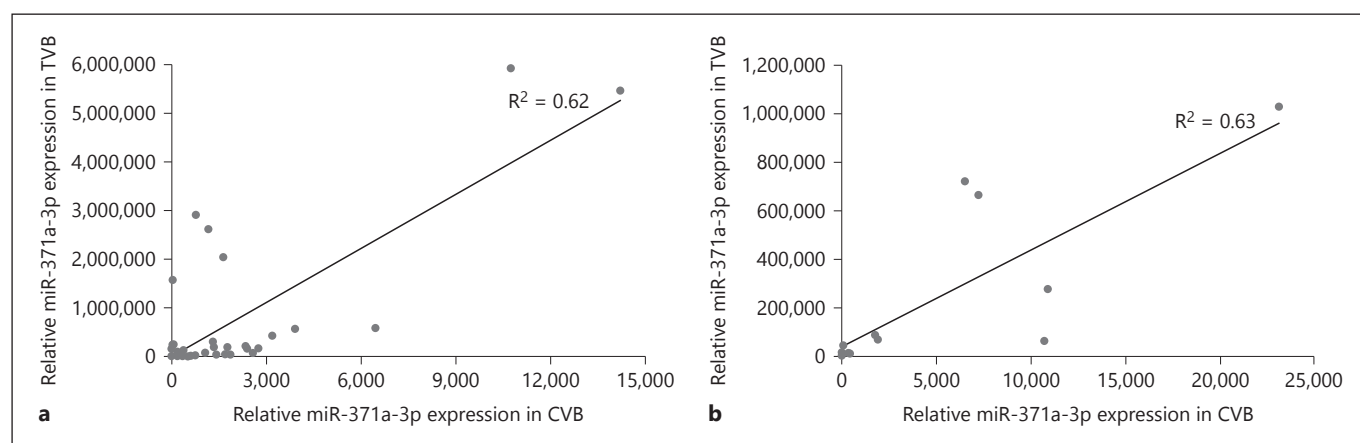


Fig. 3. **a** Statistical correlation of testicular vein miR levels with corresponding peripheral vein miR levels: CS1 patients. **b** Statistical correlation of testicular vein miR levels with corresponding peripheral vein miR levels: patients with metastases (CS2–3). R^2 = Pearson product-moment correlation coefficient.

miR levels with all parameters tested except for age. In the entire group, TVB miR levels were associated with tumor size, testis length and pT stage (table 4).

Discussion

The key results of this study are the findings of significantly higher levels of miR-371a-3p in TVB than in the peripheral circulation, the drop of miR levels into the normal range after treatment, and finally, the high expression of miR-371a-3p in neoplastic HY. These results strongly suggest that circulating miR-371a-3p

molecules in serum do specifically originate from GCT cells.

Hydrocele formation is a common benign anomaly caused by excess production of the peritesticular fluid from the layers of the tunica vaginalis. Some testicular neoplasms are surrounded by a neoplastic hydrocele. As early as in 1932, Zondek detected endocrine products of testicular tumors in the neighboring HY of 2 patients [33]. We found high levels of miR-371a-3p in the tumor surrounding HY in all of our cases, and hydrocele miR levels were much higher than those in the peripheral circulation. These results mirror the findings of elevated bHCG and LDH in tumor surrounding hydroceles in 20 GCT patients [29]. The rational explanation for the great

Table 4. Significant associations of miR levels in TVB and CVB with clinical parameters in CS1 and CS2–3 patients: univariate analyses

| Patient group | Source of serum | Significant associations | p value |
|---------------|-----------------|--------------------------|---------|
| All patients | CVB | Testis length | <0.0001 |
| | | Tumor size | <0.0001 |
| | | pT stage | 0.0005 |
| | | Localization | 0.0327 |
| | | Histology | 0.0415 |
| All patients | TVB | Testis length | 0.0005 |
| | | Tumor size | 0.0406 |
| | | pT stage | 0.0312 |
| CS1 | CVB | Testis length | 0.0010 |
| | | Tumor size | 0.0028 |
| | | pT stage | 0.0046 |
| | | Localization | 0.0262 |
| CS1 | TVB | Testis length | 0.0006 |
| | | pT stage | 0.028 |
| CS2–3 | CVB | Tumor size | 0.0001 |
| | | Testis length | 0.0080 |
| CS2–3 | TVB | Tumor size (log values) | 0.0300 |
| | | pT stage (log values) | 0.0165 |

difference between the levels in HY and peripheral circulation, respectively, is direct penetration of miR molecules from the tumor into the neighboring compartments.

The spermatic vein plexus represents the only venous effluent from the testis. This drainage system contains all metabolic products of the testicle in much higher concentrations than the peripheral circulation where the testicular output is diluted with the total blood volume of the body. In all cases, we found a much higher expression of miR-371a-3p in TVB than in CVB. In healthy males, there was also a small difference between TVB and CVB levels, suggesting a limited release of miR-371a-3p also in healthy testicles. However, in GCT patients, the difference between TVB and CVB is manifold higher. In view of the vascular anatomy of the scrotum, the rational explanation for the high miR-371a-3p levels in TVB of patients is that these molecules are released from testicular GCT cells. Analogous experience has been reported with the classical markers where higher concentrations of bHCG and AFP were documented in TVB than in peripheral blood [27, 34–36].

The relation of miR-371a-3p levels in TVB vs. CVB was higher in CS1 patients (TVB/CVB = 294) than in metastasized cases (TVB/CVB = 80). This difference in the

relations cannot be explained by different statistical correlations in the stage groups because the correlation coefficients are identical in both groups ($R^2 = 0.62$ in CS1 and 0.63 in CS2–3 (fig. 3a, b)). Also, the TVB/CVB relation is not influenced by any clinical factor. However, there is a simple biological explanation for the different relations: in CS1, the primary GCT is the only source releasing miR-371a-3p molecules, whereas in systemic disease, the metastatic deposits represent additional sources increasing the peripheral miR expression. So, the denominator of the TVB/CVB relation is greater in metastasized than in localized disease reducing the relation in these cases. These findings underscore the perception of GCT being the origin of circulating miR-371a-3p.

A possible confounding factor of measurements of TVB is compression or mechanical manipulation of the tumor-bearing testicle upon surgery. However, this factor would only increase miR levels in TVB to a certain degree in some cases and most probably, it cannot account for the large differences between TVB and CVB that were found in the entire patient cohort.

In CS1 patients, we noted a distinct drop of serum miR levels after surgery. Again, this observation is in accordance with the understanding that these miRs are specifically released from the tumor. Once the source of production is eliminated, serum levels are supposed to clear. Similar results have been reported previously [15, 16, 24], and the present report is a confirmation based on a larger patient number.

The search for associations of serum miR levels with clinical parameters revealed a complex array of results upon univariate analysis (table 4), and the biological mechanisms resulting in this intricate pattern of statistical findings remain elusive. The results might be confounded by multiple testing because many factors were tested in many different groups. Also, due to small patient numbers in several subgroups, some results might have come by chance. However, 2 significant associations were noted upon multivariate analysis of the entire group of patients and in various subgroups alike, and these factors appear to be important clinically: testis length and tumor size.

Both factors are proxies for the number of tumor cells. So, the association of serum miR expression with testis length and tumor size does probably reflect the specific production of these miR molecules in GCT cells. The well-recognized association of tumor size with metastatic risk in seminoma [37] might contribute to the association observed because seminoma cases clearly outnumber non-seminoma in this series. Surprisingly, miR levels of

TVB in localized disease are not associated with tumor size. This negative finding could relate to the heterogeneous composition of many GCTs [38] and apparently, not all of the compartments of GCTs do equally express miR-371a-3p. Necrotic zones, cystic areas or teratoma may represent areas with lacking miR expression. Also, as reported earlier, the extent of miR expression in tumor tissue does not seem to correlate with corresponding serum levels [24]. Obviously, miR production is confined to specific areas or cell populations of the neoplasm and thus, tumor size does only indirectly affect serum levels. Moreover, the biological pathways regarding the release of miR molecules from the tumor into serum appear to be different from the mechanisms governing the release of the classical markers AFP and bHCG where rather close correlations of tissue expression and serum levels are recognized [39].

The only association revealed by univariate analysis that is worth highlighting is the possible effect of higher pT stage on miR levels. This association was observed for TVB in all stages and for CVB in all patients together and in CS1 cases alone (table 4). As pT stage 2 denotes vascular invasion of the tumor [40], direct drainage of released miR molecules into serum is probably facilitated in this setting. In light of this rational biological explanation, the association of miR levels with local tumor stage deserves credit, although statistical evidence is achieved only on the univariate level.

Conclusions

This study provides evidence for the understanding that circulating miR-371a-3p molecules specifically derive from GCT cells. The strongest support comes from the findings of much higher levels of this miR in TVB and in tumor surrounding HY than in peripheral serum. Further evidence comes from the distinct drop of miR levels

after surgery in CS1 patients and from the associations of miR levels with testicular length and with higher pT stage.

Although our data exclusively relate to miR-371a-3p, it is rational to assume that the other candidate miRs (No. 372, 373, 367, 302) are likewise specific for GCT because they are closely related genetically and their clinical features are similar to the miR evaluated here. With regard to sensitivity, it is probably useful to employ a panel of these miRs in a clinical test rather than miR-371a-3p alone.

In all, circulating miR-371a-3p molecules in serum represent a highly specific biomarker of GCT. Further clinical studies are warranted to evaluate the usefulness of this novel marker in daily practice.

Disclosure Statement

The authors declare that they have no competing interests.

Authors' Contributions

K.-P.D., M.S., J.B. and G.B. designed the study, interpreted the data, and wrote the manuscript. M.S. and G.B. performed the miR measurements. K.-P.D., T.B., R.I. and P.A. collected the human samples and ascertained the clinical data. W.W. carried out the statistical analyses. T.L. performed the histological examinations of orchiectomy specimens. All authors read and approved the final version of this manuscript.

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