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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Abstract

Background

MicroRNAs (miRs) 371-3 are suggested as novel biomarkers of germ cell tumor (GCTs), but their specificity is unresolved. We aimed to clarify the origin of miR 371a-3p by measuring this miR in peripheral vein blood, and in fluids in vicinity to GCTs.

Methods

miR-371a-3p levels were measured by quantitative PCR in nine 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. 33 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.

key words: microRNA, testicular neoplasms, serum, biomarker, hydrocele

Background

The clinical management of testicular germ cell tumors (GCTs) is largely based on monitoring of serum tumor markers [1], [2],[3]. However, the markers beta human chorionic gonadotropin (bHCG), alpha fetoprotein (AFP) and lactate dehydrogenase (LDH) are expressed by only 60% of GCTs [4],[5],[6]. Particularly, seminoma expresses bHCG in less than 20% of cases and AFP in none [7]. Therefore, more sensitive markers are needed. So far, none of the suggested new markers could qualify for clinical employment [8],[9]. Recently, microRNAs (miRs) have been suggested as a novel class of serum biomarkers [10],[11],[12]. Regarding testicular GCT, miRs-371-3 as well as miR-302 and miR 367 represent promising candidates [13],[14],[15],[16],[17],[18],[19],[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],[22],[23] and elevated serum levels have been documented in several pilot studies [16],[24],[15]. 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 and (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 of 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 in higher concentrations than in the peripheral circulation [28]. The difference was explained by direct leakage of marker molecules into surrounding compartments [29].

This study aimed to ascertain 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). Also, we looked to 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 kept at room temperature for approximately 60 min to allow for complete coagulation after blood aspiration. After that the samples were centrifuged (10 min, 2500 x g) to separate serum and aliquots were frozen at -80°C until further processing. Sixty-four patients were eligible, 51 with clinical stage 1 disease (CS1) and 13 with systemic disease (CS2-3). All of whom provided both, preoperative CVB and TVB samples (clinical details in supplementary table 1), six 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, ten patients undergoing scrotal surgery for non-malignant diseases but who were otherwise healthy provided TVB and CVB samples (supplementary 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 (supplementary table 3). All patients had given informed consent. Ethical approval was given by Ärztekammer Bremen (ref. 301, 2011).

For the present study on specificity we restricted all laboratory analyses to miR-371a-3p of the miR-371-3 cluster, because this miR appears to be the most promising marker, clinically. 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 two clusters are biologically interrelated [22]. The expression of these two clusters represents an embryonic pattern of microRNA 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 hydrocele fluid 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 two 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 quantitative real-time PCR

For preamplification miRNA assays represented an equal mixture of the two 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 sec and 60°C for 4 min using the GeneAmp PCR-System 2700 (Applied Biosystems). The preamplification product was diluted 1:2 in nuclease-free water and used for quantitative real time polymerase chain reaction (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 Master (Roche, Mannheim, Germany). A negative control of amplification was performed for each sample without reverse transcriptase. Non-template negative controls were included in every plate. PCR conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. Cycle threshold (CT) values were normalized to miR-93 as an internal control. Data were analysed using the 7500 software v2.0.6 (Applied Biosystems). Normalized miR-371a-3p expression levels (RQ values) were calculated using the $2^{-\Delta\Delta CT}$ method. ($\Delta\Delta CT = \Delta CT - \Delta CT_{calibrator}$ where $\Delta CT = CT_{miR\text{-}371a\text{-}3p} - CT_{miR\text{-}93}$) [31].

The upper limit of the normal range of peripheral serum levels was considered to be RQ=10 because controls ranged up to this value.

Statistical analysis

Individual relative quantitification (RQ) values measured in CVB and TVB were tabulated along with clinical data using commercially available data base software (MS Excel, Microsoft Corp., Redmond, USA). Correlation of RQ values found in TVB with those in preoperative CVB was analyzed by employing the Pearson product-moment correlation coefficient. Comparison of mean RQ values of the various groups was performed with Wilcoxon and Mann Whitney U-tests using InStat software (GraphPad Software, Inc., San Diego, USA). A *p* value of less than 0.05 was considered significant.

The relation of RQ values of TVB and preoperative CVB (TVB/CVB) was calculated in patients and controls. The mean results of TVB/CVB relations found in the clinical stages CS1 and CS2-3 were compared to each other and to controls. The following factors were analyzed for potential associations with miR levels: histology (seminoma vs. non-seminoma), age, tumor size, testis length, pathological tumor (pT) stage and localization of the tumor (left vs. right). Associations of these factors were analyzed for TVB/CVB relations and for miR expression in TVB and CVB, respectively. Statistical evaluations were performed with univariate and multivariate analyses using the R software version 3.01 [32].

Results

Mean miR-371a-3p expression in various groups

miR-371a-3p levels were significantly higher in TVB than in corresponding CVB, both in controls and in patients (figure 1, supplementary tables 1-2). The difference was 294-fold in CS1 patients, 80-fold in CS2-3 patients and 4.6-fold in controls (table 1).

Median RQ values of the various groups with quartile ranges are presented in figure 1. Results of statistical cross 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 (figure 2 and supplementary 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 (figures 3 a,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 (figure 1 and supplementary table 3).

Association with clinico-pathological factors

The multivariate (table 3) and univariate (table 4) analysis of possible associations of RQ values in CVB and TVB with clinical factors revealed a complex pattern of results. Because of 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 three patient groups analyzed (CS1, CS2-3, and entire GCT group). However, peripheral serum miR 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 with testis length, only.

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 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 (more details table 4).

Discussion

The key results of the present study are the findings of significantly higher levels of miR-371a-3p in testicular vein blood 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 hydrocele fluid. 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 hydrocele fluid of two patients [33]. We found high levels of miR-371a-3p in the tumor surrounding hydrocele fluid 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 Lactate dehydrogenase in tumor surrounding hydroceles in 20 GCT patients [29]. The rational explanation for the great difference between the levels in hydrocele fluid 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. We found a much higher expression of miR-371a-3p in TVB than in CVB in all cases. In healthy males, there is 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],[35], [36].

The relation of miR-371a-3p levels in TVB versus 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, (figures 3 a,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 testicular vein blood 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 accords 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, two 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 worth to be highlighted 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 only achieved on the univariate level.

Conclusions

The present 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 hydrocele fluid than in peripheral serum. Further evidence comes from the distinct drop of miR evels 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 (ns. 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.

List of abbreviations

AFP	alpha fetoprotein
bHCG	beta human chorionic gonadotropin
CS	clinical stage
CVB	cubital vein blood
GCT	germ cell tumor
miR	microRNA
pT stage	pathological tumor stage
R^2	Pearson product-moment correlation coefficient
RQ	relative quantity
TVB	testicular vein blood

Competing interests

The authors declare that they have no competing interests

Authors' contributions

KPD, MS, JB and GB designed the study, interpreted the data, and wrote the manuscript.
MS and GB performed the miR measurements.
KPD, TB, RI, and PA collected the human samples and ascertained the clinical data.
WW carried out the statistical analyses.
TL performed the histological examinations of orchiectomy specimens.
All authors read and approved the final version of this manuscript.

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Legends to figures

Figure 1:

miR-371a-3p expression in CVB, TVB and hydrocele fluid in CS1 and CS2-3 patients and controls.

Boxes show the median miR expression (bold bar) and quartile ranges (extension of box) of the patient group, whiskers denote variation within 1.5 fold of interquartile range, outliers are documented as dots. C: controls. CS1: clinical stage one. CS2-3: clinical stage two and three. CVB: cubital vein blood. TVB: testicular vein blood. HY: hydrocele fluid. The y axis is plotted on a $\log_{10}$ scale

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. CS1: clinical stage one. C: controls. CVB: cubital vein blood. TVB: testicular vein blood. pre: preoperative. post: postoperative. The y axis is plotted on a $\log_{10}$ scale

Figure 3a: Statistical correlation of testicular vein miR levels with corresponding peripheral vein miR levels: CS1 patients

R^2: Pearson product-moment correlation coefficient, CVB: cubital vein blood, TVB: testicular vein blood

Figure 3b: Statistical correlation of testicular vein miR levels with corresponding peripheral vein miR levels: patients with metastases (CS 2-3)

R^2: Pearson product-moment correlation coefficient, CVB: cubital vein blood, TVB: testicular vein blood

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. CS1: clinical stage one. CS2-3: clinical stage two and three. RQ: relative quantity. CVB: cubital vein blood. TVB: testicular vein blood.

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	**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. CS1: clinical stage one. CS2-3: clinical stage two and three. CVB: cubital vein blood. TVB: testicular vein blood. HY hydrocele fluid

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

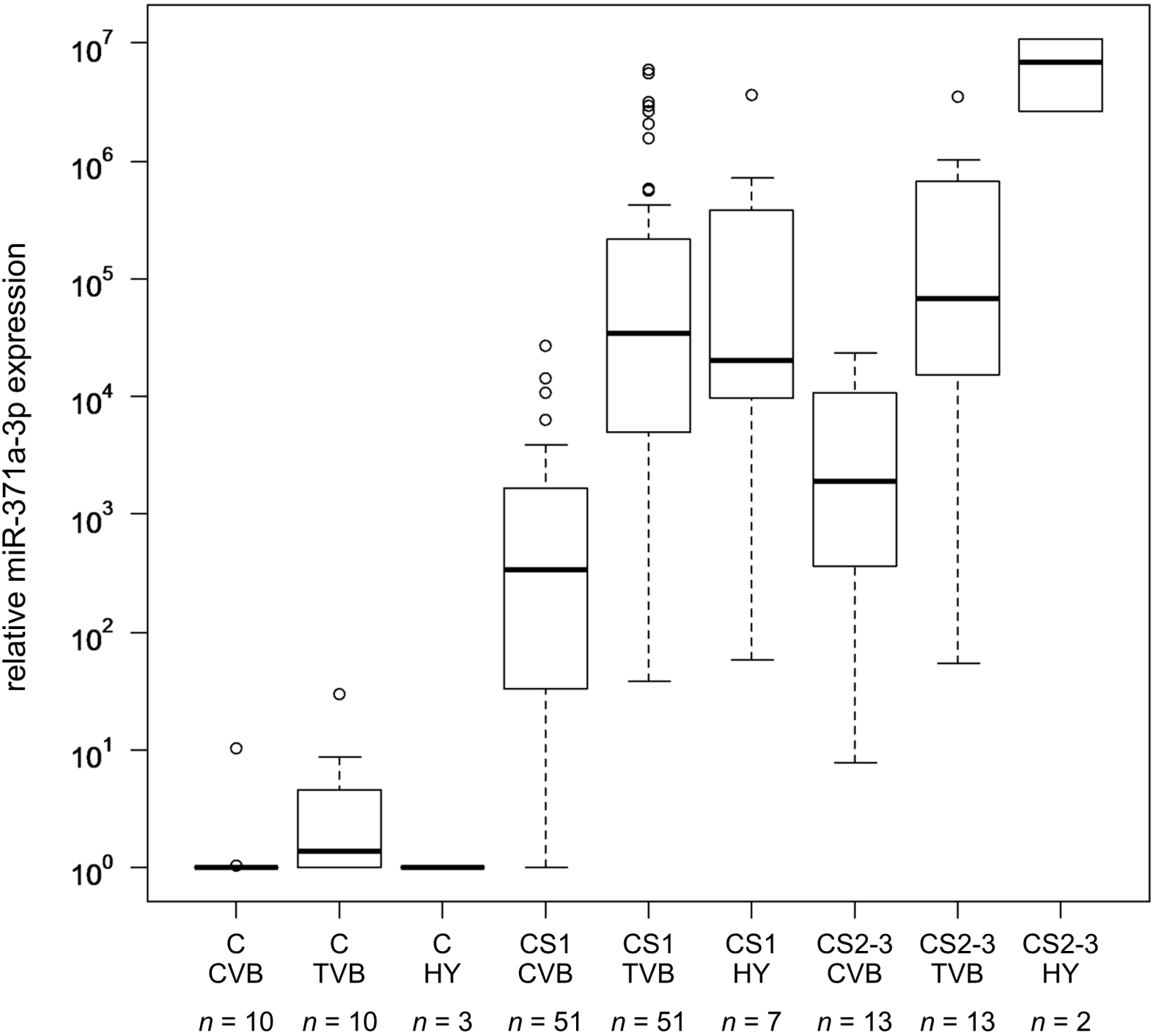
Patient group	source of serum	clinical parameter with significant association	*p* value
all patients	Relation CVB/TVB	none	
all patients	CVB	tumor size	0.0211
		testis length	0.0493
all patients	TVB	testis length	0.0129
CS1	Relation CVB/TVB	none	
CS1	CVB	none	
CS1	TVB	testis length	0.0068
CS2-3	Relation CVB/TVB	none	
CS2-3	CVB	tumor size	0.012
CS2-3	TVB	none (log values)	

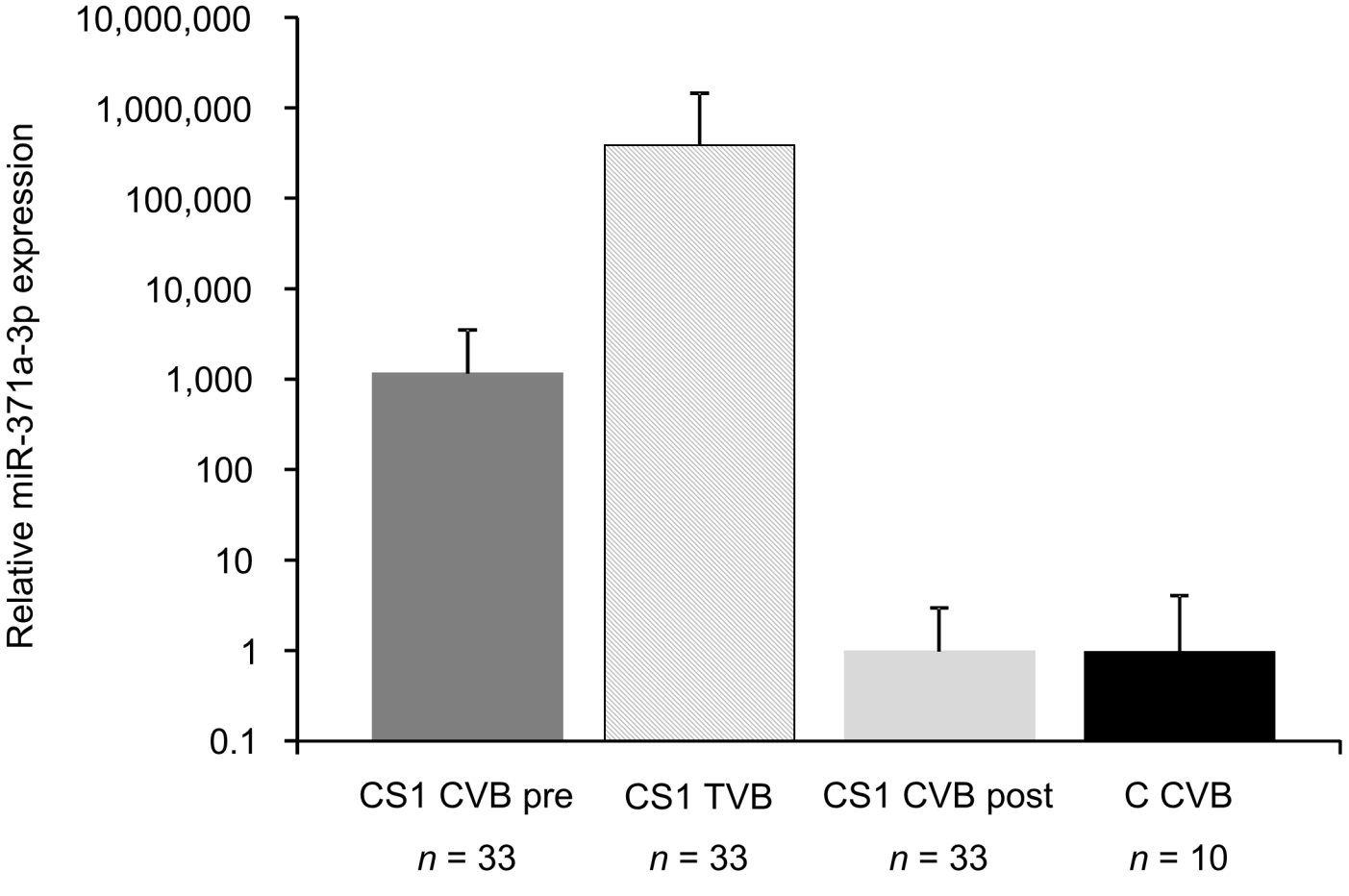
CS1: clinical stage one. CS2-3: clinical stage two and three. CVB: cubital vein blood. TVB: testicular vein blood.

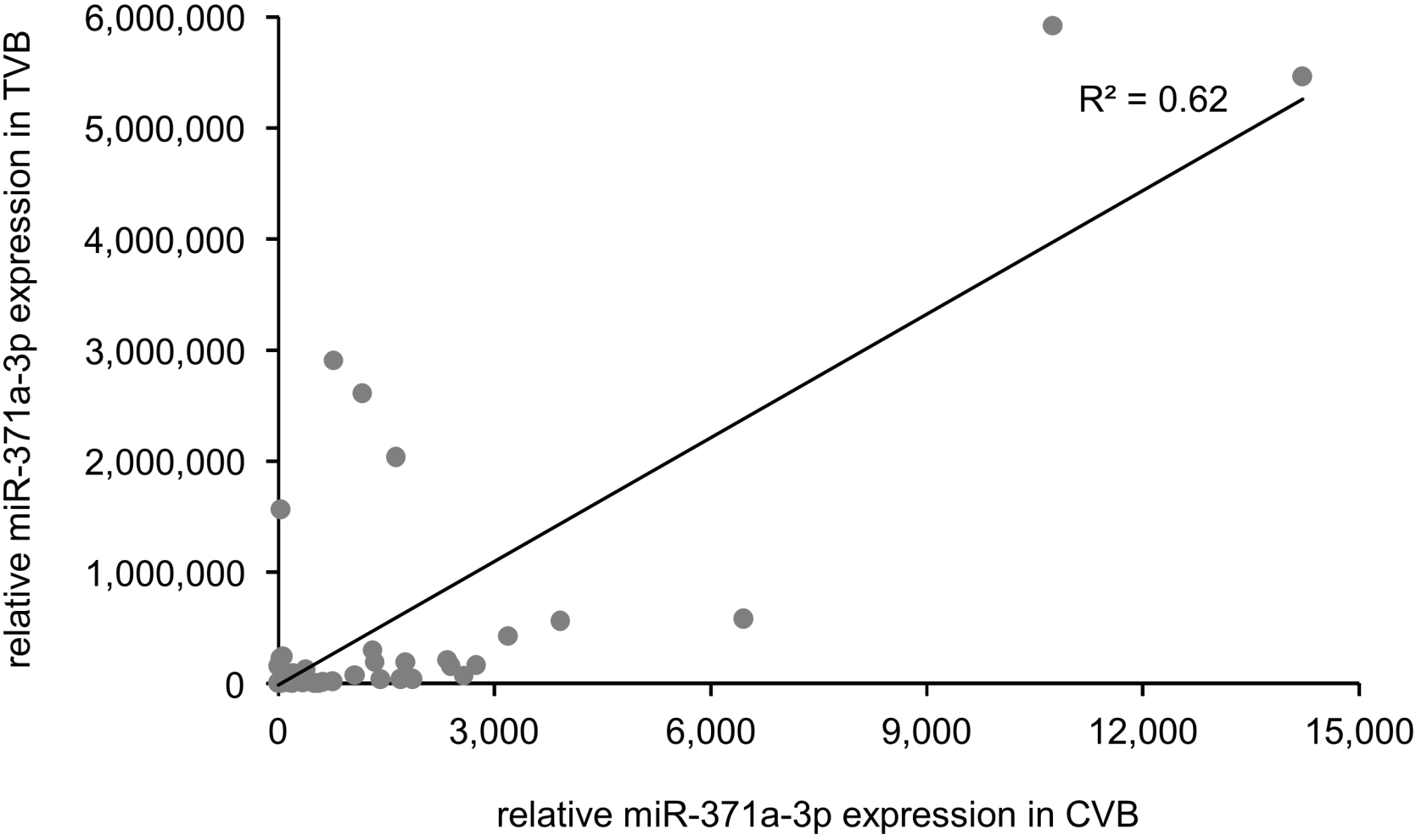
Table 4: Significant associations of miR levels in TVB and CVB with clinical parameters in CS1 and CS2-3 patients: univariate analyses.

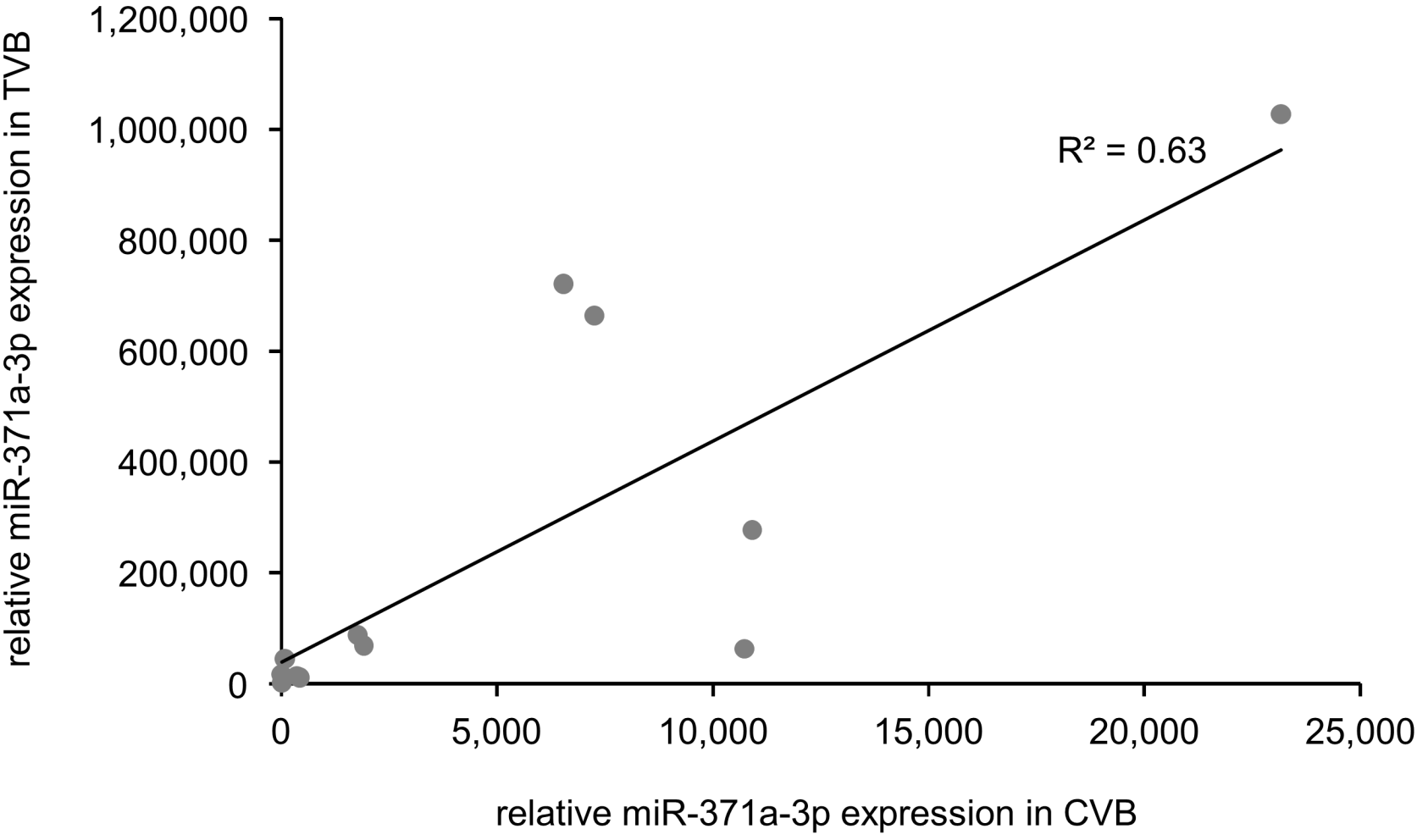
Patient group	source of serum	significant associations	*p* value
all patients	CVB	testis length	< 0.0001
		tumor size	< 0.0001
		pT stage	0.0005
		localization	0.0327
		histology	0.0415
all patients	TVB	testis length	0.0005
		tumor size	0.0406
		pT stage	0.0312
CS1	CVB	testis length	0.0010
		tumor size	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

CS1: clinical stage one. CS2-3: clinical stage two and three. CVB: cubital vein blood. TVB: testicular vein blood.


relative miR-371a-3p expression
10^0
10^1
10^2
10^3
10^4
10^5
10^6
10^7
C CVB
C TVB
C HY
CS1 CVB
CS1 TVB
CS1 HY
CS2-3 CVB
CS2-3 TVB
CS2-3 HY
n = 10
n = 10
n = 3
n = 51
n = 51
n = 7
n = 13
n = 13
n = 2


Relative miR-371a-3p expression
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
0.1
CS1 CVB pre
n = 33
CS1 TVB
n = 33
CS1 CVB post
n = 33
C CVB
n = 10


6,000,000
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
relative miR-371a-3p expression in TVB
R² = 0.62
0
3,000
6,000
9,000
12,000
15,000
relative miR-371a-3p expression in CVB


1,200,000
1,000,000
800,000
600,000
400,000
200,000
0
relative miR-371a-3p expression in TVB
R² = 0.63
0
5,000
10,000
15,000
20,000
25,000
relative miR-371a-3p expression in CVB

Supplementary Table 1: Clinical details of patients and relative miR-371a-3p expression in CVB and TVB.

case Id	age (years)	histology S/NS	percentage of teratoma	tumor size (mm)	testis length (mm)	pT stage	localization right/left	CS	CVB (RQ) pre-op	TVB (RQ)	CVB (RQ) post-op
14	35	NS	95%	35	40	pT1	ri	1	0.000	669.549	0.000
15	26	S	0	33	45	pT1	ri	1	771.729	2,901,834.938	0.022
17	28	NS	0	9	43	pT1	ri	1	554.700	2,506.816	n.m.
18	48	S	0	15	45	pT1	ri	1	492.184	2,094.250	3.593
19	41	S	0	25	55	pT1	ri	1	1,765.523	189,358.699	0.030
20	41	S	0	55	55	pT2	ri	1	1,162.388	2,607,999.372	n.m.
21	48	S	0	10	50	pT1	ri	1	186.708	1,540.696	0.000
22	22	NS	100%	9	50	pT1	le	1	0.113	695.662	0.000
23	52	S	0	15	32	pT2	ri	1	32.767	5,268.124	0.000
25	45	S	0	37	55	pT1	ri	1	1,062.353	73,131.747	0.293
26	35	NS	0	54	55	pT2	ri	1	2,391.268	151,149.076	n.m.
27	37	S	0	9	43	pT1	ri	1	0.542	5,677.671	0.000
28	55	NS	0	9	70	pT2	le	1	10,754.019	5,913,475.246	3.577
29	32	S	0	38	50	pT3	le	1	2,572.260	67,437.259	n.m.
30	37	S	0	9	38	pT1	ri	1	0.170	579.579	1.130
31	50	S	0	30	54	pT2	ri	1	0.672	36.965	0.000
32	31	S	0	28	50	pT1	le	1	33.209	1,561,473.360	0.013
33	54	S	0	13	35	pT1	le	1	13.014	8,031.733	0.000
34	18	NS	80%	33	55	pT1	le	1	160.518	5,145.532	6.255
36	49	S	0	13	40	pT2	le	1	174.149	8,164.584	0.062
37	36	S	0	15	50	pT1	ri	1	200.198	89,097.046	n.m.
38	33	S	0	4	35	pT1	ri	1	0.379	5,797.373	0.000
39	45	S	0	40	65	pT2	le	1	14,219.408	5,457,765.911	n.m.
41	41	NS	60%	50	50	pT1	le	1	1,337.139	189,940.808	0.000
42	32	NS	80%	45	55	pT1	le	1	9.545	4,816.550	0.000
43	52	S	0	9	35	pT1	le	1	39.062	1,880.759	0.000
44	31	S	0	45	50	pT2	ri	1	3,916.182	558,048.824	0.000
45	34	NS	100%	11	35	pT1	ri	1	31.333	225,293.666	n.m.
47	46	S	0	13	33	pT1	ri	1	33.220	2,823.738	0.000
50	33	S	0	40	65	pT1	ri	1	2,346.870	208,113.811	n.m.
51	30	S	0	28	42	pT2	ri	1	0.000	151,462.180	0.000
53	31	NS	60%	30	40	pT2	le	1	3,187.045	420,303.413	1.780
54	31	S	0	35	55	pT2	le	1	1,421.810	29,555.272	0.000
55	25	S	0	18	38	pT1	le	1	11.377	9,126.662	4.421

56	47	S	0	70	45	pT2	le	1	6,455.190	578,028.342	6.840
57	35	S	0	14	55	pT1	ri	1	148.968	50,024.153	n.m.
58	41	S	0	12	40	pT2	le	1	375.604	121,497.565	n.m.
59	43	S	0	20	55	pT2	ri	1	751.362	15,619.596	0.000
60	45	S	0	65	70	pT2	le	1	1,696.171	36,117.169	2.216
61	41	S	0	19	50	pT1	ri	1	58.824	236,977.188	n.m.
62	18	NS	40%	60	60	pT2	le	1	26,865.548	3,192,757.666	n.m.
63	38	NS	0	20	45	pT1	le	1	161.033	2,634.268	0.000
64	26	NS	0	17	55	pT2	le	1	1,859.997	34,846.722	0.000
84	48	S	0	15	40	pT1	ri	1	29.599	1,978.289	0.845
85	33	S	0	36	52	pT2	ri	1	2,744.063	159,439.236	n.m.
86	23	NS	0	24	55	pT2	ri	1	1,310.144	295,863.813	n.m.
87	46	S	0	20	45	pT1	ri	1	618.618	7,776.402	0.000
88	44	S	0	40	55	pT2	ri	1	338.361	5,757.536	n.m.
89	51	S	0	19	50	pT1	ri	1	2.682	1,021.958	n.m.
90	33	S	0	21	38	pT1	ri	1	93.625	9,529.994	n.m.
91	49	S	0	50	52	pT2	le	1	1,632.434	2,035,125.560	n.m.
3	20	NS	0	28	50	pT2	ri	2b	6,541.110	720,353.011	n.m.
5	26	NS	0	13	52	pT2	ri	2a	431.569	9,396.291	n.m.
7	31	NS	0	35	40	pT2	le	2c	10,735.459	61,398.650	n.m.
8	31	S	0	65	65	pT2	le	2b	10,923.638	275,641.593	n.m.
9	47	NS	30%	80	80	pT2	ri	2a	23,165.973	1,026,819.036	n.m.
10	32	S	0	25	45	pT1	ri	2b	20.331	52.810	n.m.
11	41	S	0	21	32	pT2	ri	2a	89.679	43,094.204	n.m.
12	50	S	0	20	55	pT2	le	2a	359.082	12,488.574	n.m.
15	38	NS	100%	15	40	pT1	ri	2a	6.894	15,602.878	n.m.
16	46	S	0	60	65	pT2	le	2a	17,209.141	3,476,888.376	n.m.
18	53	S	0	75	75	pT2	le	2a	7,255.988	663,159.012	n.m.
31	46	S	0	45	60	pT2	le	2a	1,914.112	67,292.799	n.m.
4	48	S	0	30	50	pT2	le	3	1,776.780	85,778.392	n.m.

S: seminoma. NS: non-seminoma. CS: clinical stage. pT: pathological tumor. pre-op: preoperative. post-op: postoperative. CVB: cubital vein blood. TVB: testicular vein blood. RQ: relative quantity. n.m.: not measured.

Supplementary Table 2: Individual results of miR-371a-3p measurements in CVB and TVB of controls.

case Id	age (years)	diagnosis	CVB (RQ)	TVB (RQ)
12	42	spermatocele	0.000	0.000
32	56	spermatocele	0.037	0.000
33	51	Posttraumatic fibrosis	0.000	28.737
34	50	Epididymo-orchitis	0.000	7.769
57	18	varicocele	0.000	0.000
59	52	spermatocele	0.000	0.000
63	48	Benign epididymal tumor	0.000	0.000
79	36	varicocele	9.259	0.758
90	41	spermatocele	0.000	1.693
96	40	epididymitis	0.000	3.652

CVB: cubital vein blood. TVB: testicular vein blood. RQ: relative quantity.

Supplementary Table 3: Individual results of miR-371a-3p levels in hydrocele fluid

case Id	age (years)	CS	CVB (RQ)	TVB (RQ)	HY (RQ)
25	45	1	1,062.4	73,131.8	707,459.9
26	35	1	2,391.3	151,149.1	20,033.1
28	55	1	10,754.2	5,913,475.3	3,604,889.4
43	52	1	39.1	1,880.8	39,827.8
50	33	1	2,346.9	208,113.8	17,529.8
64	26	1	1,860.0	34,846.7	1,893.8
89	51	1	2.7	1,022.0	56.2
6	27	2b	13,043.9	n.a.	10,999,951.7
18	53	2a	7,256.0	663,159.0	2,632,363.8
125	86	C	n.d.	n.a.	n.d.
128	46	C	n.a.	n.a.	n.d.
131	75	C	n.d.	n.a.	n.d.

C: control. CS: clinical stage. n.a. not available. n.d.: not detectable. CVB: cubital vein blood. TVB: testicular vein blood. RQ: relative quantity. HY: hydrocele fluid.