Is Relative Quantification Dispensable for the Measurement of MicroRNAs as Serum Biomarkers in Germ Cell Tumors?

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Abstract. *Background: Classical biomarkers α-fetoprotein, β-human chorionic gonadotropin and lactate dehydrogenase (AFP, bHCG and LDH) are elevated in only 60% of all testicular germ cell tumor (TGCT) patients. microRNAs (miRNAs) are a novel class of useful biomarkers in cancer and miRNAs of the miR-371-3 cluster were proven to be valuable markers for TGCT patients. Materials and Methods: We compared the Ct and ΔCt values of miR-371-3 by real time PCR (qPCR) with and without 18S rRNA for normalization. Expression of miR-371a-3p, miR-372 and miR-373-3p was measured in 25 TGCTs, 4 non-TGCTs and 17 age-matched male controls. Results: A highly positive correlation between Ct and ΔCt values was found in all samples. The highest correlation was found for miR-371a-3p (R2: 0.956). Conclusion: Results show that qPCR can be used without endogenous control for analyzing miR-371-3 in the serum of patients with testicular cancer and male controls if the technical procedure is performed under controlled conditions.*

microRNAs (miRNAs) are short nucleic acid molecules synthesized in the cellular nucleus. Their main function is to modify post-transcriptional gene activity by targeting mRNA molecules. Once released from the cellular nucleus, miRNAs can enter almost all biological fluids *e.g.* serum, saliva, urine and milk where they are thought to mediate intercellular communication (1-5). After departure from the cell of origin, miRNAs show a remarkable stability because of their inclusion into membrane vesicles called exosomes (6) or their attachment to particular proteins as *e.g.* Argonaute (AGO) proteins.

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The presence in various body fluids in conjunction with the high chemical stability in these fluids renders miRNAs potential biomarkers in a variety of diseases notably in the clinical management of cancer. Clinical management of the disease is widely based on monitoring of serum biomarkers *e.g.* α-fetoprotein (AFP), β-human chorionic gonadotropin (bHCG) and lactate dehydrogenase (LDH) (7). Unfortunately, these classical markers are elevated in only 60% of all testicular germ cell tumor (TGCT) patients (8). miRNAs encoded by the small miR-371-3 cluster mapping to the terminal region of the long arm of chromosome 19 are among the most promising candidates in that field. miRNAs of that cluster are predominantly expressed in embryonic stem cells and, to a lesser extent, in the placenta (9-13). Notably, a strong expression has been detected in TGCTs probably because the genetic and biochemical features of these tumors are very much related to embryonic stem cells and other embryonal structures. In the tissue of TGCTs, the expression of miR-372 and miR-373-3p, but not miR-371a-3p, represents a sensitive marker (14-16). In contrast, miR-371a-3p seems to hold the greatest promise as a biomarker in the serum of TGCT patients with high sensitivity and specificity (17-19). However, before adoption into wide-spread clinical practice, details of the miRNA miR-371a-3p serum test have to be elaborated. One unresolved question, with respect to the performance of the test, is whether or not quantification of serum levels can be accomplished with the marker alone by measuring cycle threshold values (Ct values) or if the analysis is also feasible by quantification relative to the amount of another cell-free RNA used as a reference (ΔCt method) (20). Ideally, the endogenous control RNA should be unrelated to type and stage of disease and should show very little variation among individuals. Performing real time-polymerase chain reaction (PCR) without an endogenous control would imply less laboratory technical effort and shorter analytical time, likewise. Clearly, for large scale use of measuring miR-371-3 serum levels, the least-expensive and least-time-consuming method would be

preferable. Thus, we compared the C_t and ΔC_t values, respectively, of miR-371-3 serum levels in TGCT patients measured by real time PCR with and without using 18S ribosomal RNA (rRNA) as endogenous control.

Materials and Methods

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

Serum samples. Serum levels of miR-371-3 were determined in samples obtained from 25 TGCT patients (13 seminoma, 12 non-seminoma) of clinical stage I, II and III (Lugano classification) and median age 37 (18-60) years. Cubital vein blood samples were obtained during routine blood examinations usually three days before orchiectomy. To study miRNAs serum levels in non-germ cell tumors (GCTs), four patients with Leydig cell tumors were examined. The median age was 38 (23-50) years. Seventeen male patients, aged 23-51 years (median=37), with non-malignant scrotal diseases (hydrocele, spermatocele, epididymitis) served as controls.

Blood samples were collected in serum-separation tubes that were kept at room temperature for approximately 60 min to allow for complete coagulation after blood aspiration. The samples were then centrifuged at 4000 U/min for 10 min to separate serum. The serum aliquots were transferred into cryotubes and immediately stored at 34˚C for 5 to 30 days. Subsequently, the serum samples were frozen at –80˚C for long term storage.

RNA extraction. For RNA isolation, 200 μl frozen serum was thawed on ice and total RNA extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the protocol supplied by the manufactures. As the only modifications relative to the manufacturer's instructions, 400 μl of the upper aqueous phase were employed instead of 600 μl and 600 μl 100% ethanol were used instead of 900 μl. RNA was quantified by spectrophotometry prior to further use.

Reverse transcription. For cDNA synthesis, the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) was employed for reverse transcription (RT) of miRNA. Fifty-five ng total RNA from each sample were used. RT primers represented an equal mixture of three miRNA specific stem-loop primers (miR-371a-3p, miR-372 and miR-373-3p) from the relevant TaqMan miRNA assays (Applied Biosystems, Darmstadt, Germany). The reactions with the final volume of 15 μl were incubated in the GeneAmp PCR System 2700 (Applied Biosystems, Darmstadt, Germany) for 30 min at 16˚C, 30 min at 42˚C, and 5 min at 85˚C, respectively.

For the 18S rRNA, 55 ng total RNA of each sample was reverse transcribed in the presence of 200 U/μl of M-MLV reverse transcriptase (Invitrogen, Karlsruhe, Germany), RNase Out, 150 ng random hexamers, 0.1 M DTT, 5x first strand buffer and 100 mM dNTPs according to the manufacturer's instructions. RNA was initially denatured at 65˚C for 5 min and subsequently kept on ice for 1 min. Reverse transcription was performed at 37˚C for 50 min followed by inactivation of the reverse transcriptase at 70˚C for 15 min in the GeneAmp PCR System 2700 (Applied Biosystems, Darmstadt, Germany).

Pre-amplification. A pre-amplification step was used prior to real time PCR. For pre-amplification, miRNA assays represented an equal mixture of three miRNAs (miR-371a-3p, miR-372 and miR-373-3p) from the relevant TaqMan miRNA assays (Applied Biosystems, Darmstadt, Germany). 3 μl miRNA assays was diluted in 12 μl nuclease-free water. Twelve and one-half μl of this solution was combined with 12.5 μl of RT product, plus 25 μl TaqMan Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany), to make a final volume of 50 μl.

For 18S rRNA preamplification, 1.2 μl forward primer, 1.2 μl reverse primer and 0.2 μl probe were diluted in 10.4 μl nuclease-free water. Twelve and one-half μl of this solution was combined with 12.5 μl of RT product, plus 25 μl TaqMan Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany), to make a final volume of 50 μl.

The PCR was performed for miRNAs and 18S rRNA at 95˚C for 10 min, followed by 14 cycles of 95˚C for 15 sec and 60˚C for 4 min using the GeneAmp PCR System 2700 (Applied Biosystems, Darmstadt, Germany). The preamplification product was diluted 1:5 in nuclease-free water. This product was used directly for real time PCR.

Quantitative real time PCR (qRT-PCR). For miRNA quantification using real time PCR, 9 μl of the preamplification product was added to 10 μl TaqMan Universal PCR Master Mix and 1 μl of 20x TaqMan miRNA assay. For 18S rRNA quantification using real time PCR, 7.4 μl of the preamplification product was added to 10 μl TaqMan Universal PCR Master Mix and 2.4 μl primer and 0.2 μl probe. Serum levels of miRNAs and 18S rRNA were measured by the 7300 real time PCR System (Applied Biosystems, Darmstadt, Germany).

For each sample the reactions were performed in triplicate. A negative control of amplification was performed for each sample without reverse transcriptase. Non-template negative controls of cDNA synthesis and real time PCR for each miRNA were included in every plate. PCR conditions were 10 min at 95˚C, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Data were analyzed using the 7300 System software (Applied Biosystems, Darmstadt, Germany). Cycle threshold values were normalized to the internal control, 18S rRNA (21-23).

Results

For the study we compared C_t values and ΔC_t values (C_t gene of interest minus C_t internal control) of the three miRNAs of the miR-371-3 cluster in 25 GCTs (13 seminoma and 12 non-seminoma) with clinical stage I, II and III, in four non-GCTs (Leydig cell tumors) and in 17 controls. Expression of all three miRNAs was measured in all 25 GCTs serum samples. In one serum sample of Leydig cell tumors and in six control serum samples miR-371a-3p expression was not detectable, while miR-372 and miR-373-3p expression could be measured in all non-GCTs and controls. The miR-371-3 C_t values of the Leydig cell tumors were in the range of the C_t values of controls (Figure 1A, B, C).

When comparing the C_t (without endogenous control) values with the ΔC_t (C_t sample minus C_t endogenous control) values of miR-371a-3p from all serum samples,

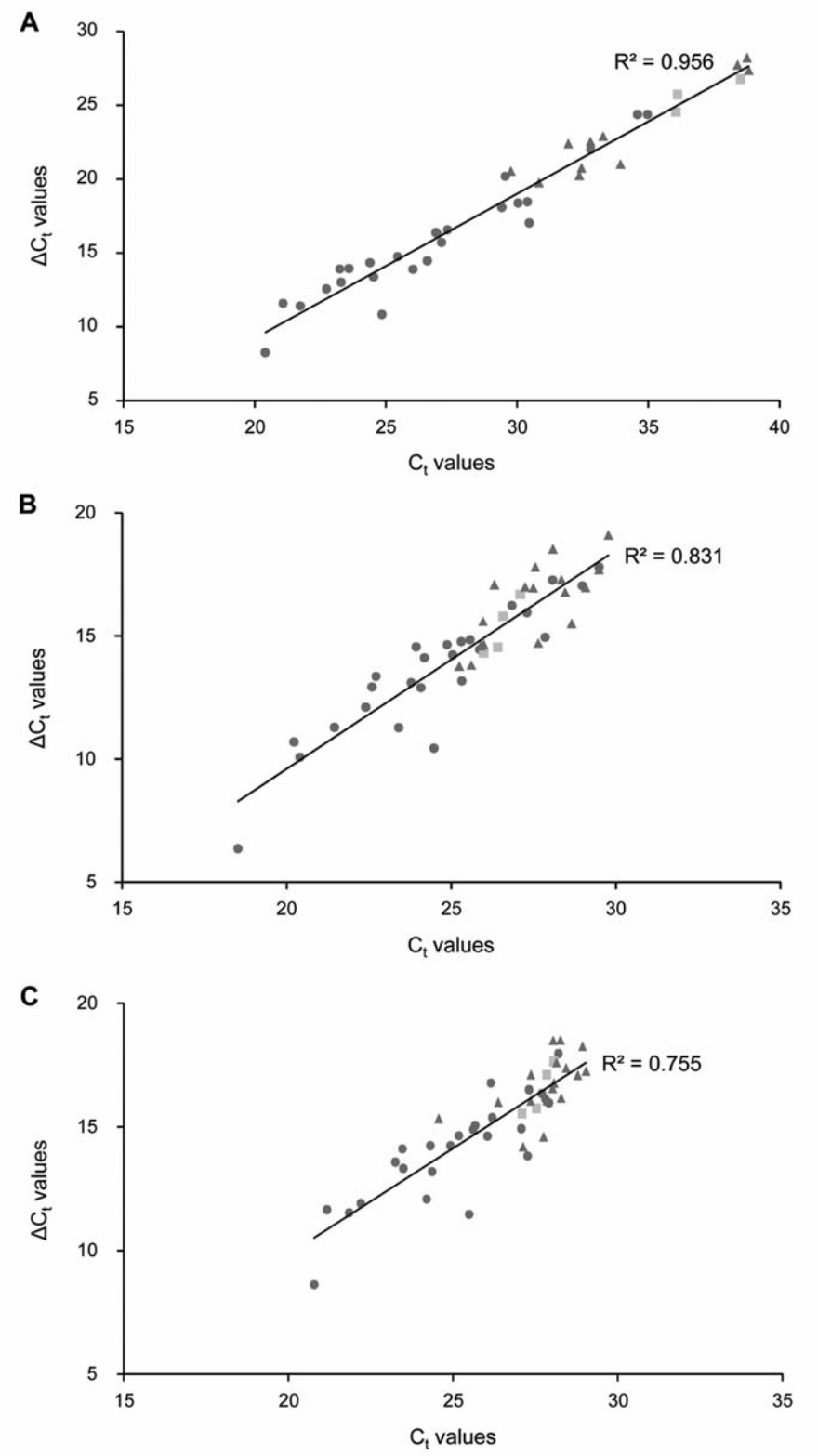

Figure 1. *Ct values vs. ΔC_t values of miR-371-3 in serum. C_t values vs. ΔC_t values of miR-371a-3p (A) in 25 GCTs (●) with clinical stage I, II and III, three Leydig cell tumors (■) and 11 controls (▲). C_t values vs. ΔC_t values of miR-372 (B) and miR-373-3p (C) in 25 GCTs with clinical stage I, II and III, four Leydig cell tumors and 17 controls. R^2 (Pearson product-moment correlation coefficient) was calculated for all miRNAs: 0.956 (miR-371a-3p), 0.831 (miR-372) and 0.755 (miR-373-3p).*

there is a high correlation (Pearson product-moment correlation coefficient (R2): 0.956). The R^2 of miR-372 (R^2=0.831) and miR-373-3p (R^2=0.755) were lower than miR-371a-3p (Figure 1A, B, C). Overall, a highly positive correlation between C_t and ΔC_t values was found in all samples. In matching the miRNA C_t values with the corresponding ΔCt values of the three miRNAs, the highest correlation was found for miR-371a-3p (Figure 1A).

Discussion

The central result of the present investigation is the highly positive correlation between C_t and ΔC_t values measured for all three miRNAs. The highest R^2 was found for miR-371a-3p. The technical procedure of serum preparation, as well as RNA extraction, reverse transcription, pre-amplification and real time-PCR was always performed under controlled conditions and consistent methods. Because of this identical processing of all samples the results are reliable.

With respect to the four Leydig cell tumors investigated, the miR-371a-3p C_t and ΔC_t values of three patients were in the range of the mean C_t and ΔC_t value of the 17 controls, while the miR-371a-3p expression was too low for detection in the fourth patient. In general, expression of miRNAs of the miR-371-3 cluster appear to be a specific feature of GCTs rather than a common one of testicular neoplasms (24).

miRNAs of the miR-371-3 cluster, and in particular miR-371a-3p, have been suggested as novel biomarkers for the clinical management of TGCT patients (17-19, 24). Analyzing miRNAs in serum by using real time-PCR is a non-invasive, standardized and reproducible method (1, 5, 25) that is technically robust enough to be implemented in a routine clinical laboratory.

Real time PCR is the most common method for quantification of miRNAs in body fluids. As a rule, for relative quantification of miRNAs an endogenous control is used for normalization. The expression of the endogenous control should show only a low variation in serum samples. The major advantages of real time PCR without endogenous control are time saving and cost saving alike, because of less technical efforts.

Our results provide convincing evidence that real time- PCR can be used without endogenous control for analyzing miRNAs miR-371-3 in serum of TGCT patients and male controls in the same age, if the technical procedure is performed under controlled conditions. A quantification of miR-371-3 without an endogenous control leads to adequate results.

Therefore, technically, the method of measuring of miRNA miR-371a-3p serum levels by real time PCR could soon be implemented in the clinical laboratory routine. Testicular cancer patients will probably benefit from this novel method. As shown previously, the test appears to be so specific and sensitive that reducing diagnostic radiation exposure to the young patients might be envisioned by abandoning computed tomographic examinations in a significant number of patients.

Based on detection of miRNAs of the miR-371-3 cluster by quantitative real time-PCR, a method is described to follow the clinical course of patients with testicular germ cell tumors (TGCTS). Under controlled conditions the simultaneous quantification of an endogenous control is dispensable. Overall, the test offers a suitable tool for "liquid biopsies" for the follow-up of TGCT patients after surgery.

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