British Journal of Cancer

ARTICLE

Clinical Studies

Detection of recurrences using serum miR-371a-3p during active surveillance in men with stage I testicular germ cell tumours

Christian D. Fankhauser[1,12]✉, Ailsa J. Christiansen[1,2,12], Christian Rothermundt[3], Richard Cathomas[4], Marian S. Wettstein[1], Nico C. Grossmann[1], Josias B. Grogg[1], Arnoud J. Templeton[5], Anita Hirschi-Blickenstorfer[6], Anja Lorch[7], Silke Gillessen[3,8,9,10], Holger Moch[2], Joerg Beyer[11] and Thomas Hermanns[1]

BACKGROUND: MiR-371a-3p predicts the presence of a macroscopic non-teratomatous germ cell tumour (GCT). We hypothesised that miR-371a-3p can also detect recurrence during active surveillance (AS) of stage I GCT.
METHODS: We prospectively collected serum samples of 33 men. Relative expression of serum miR-371a-3p levels was determined at each follow-up visit using real-time quantitative reverse transcription-polymerase chain reaction.
RESULTS: Recurrence was detected using standard follow-up investigations in 10/33 patients (30%) after a median of 7 months. Directly after orchiectomy, miR-371a-3p levels were not elevated in any of the 15 patients with available post-orchiectomy samples. However, all ten recurring patients exhibited increasing miR-371a-3p levels during follow-up, while miR-371a-3p levels remained non-elevated in all but one patient without recurrence. MiR-371a-3p detected recurrences at a median of 2 months (range 0–5) earlier than standard follow-up investigations.
CONCLUSIONS: MiR-371a-3p levels immediately post orchiectomy are not predictive for recurrences and unfortunately cannot support decision-making for AS vs. adjuvant treatment. However, miR-371a-3p detects recurrences reliably and earlier than standard follow-up investigations. If this can be confirmed in larger cohorts, monitoring miR-371a-3p could replace surveillance imaging in seminomatous GCT and reduce the amount of imaging in non-seminomatous GCT. Earlier detection of disease recurrence may also reduce the overall treatment burden.

British Journal of Cancer (2022) 126:1140–1144; https://doi.org/10.1038/s41416-021-01643-z

INTRODUCTION

Around 60% of patients with testicular germ cell tumours (GCTs) present with localised, non-metastatic disease. After orchiectomy, most men are included in active surveillance (AS) programmes with regular measurements of the serum tumour markers alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG) and lactate dehydrogenase (LDH). As these serum markers only detect around 60% of non-seminoma and <5% of seminoma recurrences [1], additional cross-sectional imaging (computerised tomography (CT) or magnetic resonance imaging (MRI)) is required during AS. However, the use of CT can result in considerable cumulative radiation exposure in this young patient population [2] and both CT and MRI add relevant health-care costs [3]. Serum miR-371a-3p has recently emerged as a blood-based biomarker predicting macroscopic GCT, whereas pure teratomatous GCT can still not be detected using miR-371a-3p [4]. Based on previous reports, miR-371a-3p could be an ideal biomarker for early and reliable detection of recurrences in men undergoing AS of stage I GCT.

MATERIALS AND METHODS

Blood collection and serum processing

Between April 2019 and January 2021, we prospectively collected 143 serum samples from 33 men with stage I testicular GCT undergoing AS after orchiectomy without adjuvant chemotherapy who were registered in the Swiss Austrian German Testicular Cancer Cohort Study (SAG TCCS; NCT02229916) [5]. All participants in the study provided written informed consent. One site also collected pre- and post-orchiectomy serum samples from 15 men with GCT. In addition, ten men who underwent orchiectomy but had non-GCT histology were used as controls.

[1]Department of Urology, University Hospital Zurich, University of Zurich, Zurich, Switzerland. [2]Department of Pathology and Molecular Pathology, University Hospital Zurich, University of Zurich, Zurich, Switzerland. [3]Department of Oncology, Kantonsspital, St. Gallen, Switzerland. [4]Department of Oncology, Kantonsspital Chur, Chur, Switzerland. [5]Department of Medical Oncology, St. Claraspital Basel and Faculty of Medicine, University of Basel, Basel, Switzerland. [6]Onkozentrum Hirslanden, Klinik Hirslanden, Zürich, Switzerland. [7]Department of Oncology, University Hospital Zurich, University of Zurich, Zurich, Switzerland. [8]EOC Oncology Institute of Southern Switzerland, Bellinzona, Switzerland. [9]Universita della Svizzera Italiana, Lugano, Switzerland. [10]University of Bern, Bern, Switzerland. [11]Department of Medical Oncology, Inselspital, University Hospital, University of Bern, Bern, Switzerland. [12]These authors contributed equally: Christian D. Fankhauser, Ailsa J. Christiansen. ✉email: cdfankhauser@gmail.com

Received: 29 August 2021 Revised: 25 October 2021 Accepted: 17 November 2021
Published online: 15 December 2021

Table 1. Summary of active surveillance programmes for patients with Sem, non-Sem LR and non-Sem HR.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Sem																								
Blood analysis			x			x			x			x			x			x			x			x
X-ray chest						x						x						x						x
CT/MRI abdomen						x						x						x						x
Non-Sem LR																								
Blood analysis		x		x		x		x		x		x		x		x		x		x		x		x
X-ray chest		x		x		x		x		x		x		x		x		x		x		x		x
CT/MRI abdomen				x								x												x
Non-Sem HR																								
Blood analysis	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
X-ray chest		x		x		x		x		x		x		x		x		x		x		x		x
CT/MRI abdomen			x			x			x			x			x			x			x			x

Blood analysis corresponds to standard HCG, AFP and LDH analysis in addition to serum miR-371a-3p analysis. Schedules that are more detailed are shown in Supplementary data.
CT computerised tomography, *MRI* magnetic resonance imaging, *Sem* seminoma stage I, *non-Sem LR* non-seminoma stage I low risk, *non-Sem HR* non-seminoma stage I high risk.

Serum samples were collected at each patient visit during standard follow-up (Table 1 and Appendices 1–3 in Supplementary File). Whole blood was collected by venepuncture into BD Vacutainer SST II tube using a needle with a minimum of 21 gauge. Collection tubes containing whole blood were gently inverted five times and then stored in the upright position at room temperature for a minimum of 30 min and up to a maximum of 60 min. Collection tubes were centrifuged at $2000 \times g$ in a swinging bucket rotor for 10 min at room temperature. The resulting serum was aliquoted in an RNase-free workspace into RNAse-free cryovials. Aliquots were subsequently stored at −80 °C.

RNA extraction

RNA was extracted from 200 µL of serum using the miRNeasy Serum Kit (Qiagen) into a final volume of 100 µL nuclease-free water as described previously [6]. All isolated RNA was stored at −80 °C. Briefly, to increase RNA yield, MS2 carrier RNA (Roche, final concentration 1.25 µg mL^{-1}) was added to Qiazol prior to isolation and the exogenous spike-in microRNA (miRNA) cel-miR-39-3p (5.6×10^8 copies) was used as an initial quality control for extraction efficiency. Resulting real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) Ct values for cel-miR-39-3p fell within the expected range for endogenous human serum miRNA levels as previously described [6, 7]. All samples underwent quality control analysis prior to subsequent target miRNA qRT-PCR analyses.

Sample quality control

Quality control analysis was performed for cel-miR-39-3p, the endogenous housekeeper miR-30b-5p and the haemolysis control miRNAs miR-451a and miR-23a-3p as previously described [6]. Consistency of extraction was acceptable for all samples analysed (cel-MiR-39-3p Ct value range 18.1–23.9, miR-30b-5p value range 24.8–27.9). Haemolysis assessment was performed by calculating the ΔCt values for miR-23a-3p minus miR-451a. Sixty-four samples had a ΔCt of <8 and therefore no substantial evidence of haemolysis;[8] 57 samples had a borderline ΔCt value of 8–9 and 33 samples demonstrated ΔCt values indicative of haemolysis (ΔCt >9). Target miRNA analyses were performed on all samples; however, caution was exhibited in interpreting the results of haemolysis suspicious samples (Supplementary Fig. 1).

Real-time PCR analysis

Five microliters of RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription Kit (Life Technologies, Paisley, UK) using the miRNA-specific stem-loop primer from the relevant TaqMan miRNA Assay Kit (Life Technologies; Supplementary Table 1), as per the manufacturer's instructions. The final volume of 15 µL for each reaction underwent reverse transcription using an Eppendorf Mastercycler pro S thermocycler at 16 °C for 30 min, 42 °C for 30 min, followed by a final step of 85 °C for 5 min, as described previously [9]. qRT-PCR was performed on un-amplified singleplex complementary DNA using TaqMan Fast Advanced Master Mix as per the manufacturer's instructions and run on an Applied Biosystems ViiA 7 System. RT-PCR conditions were to hold at 95 °C for 2 min, followed by 45 cycles of 95 °C for 1 s and 60 °C for 20 s. To exclude non-specific amplification, a non-template control was run for each assay. In all cases, no product was detectable. A standard positive sample (miRNA extracted from the TCam-2 seminoma cell line, a kind gift from Professor Matthew Murray, University of Cambridge, UK) was also routinely assayed as an internal experimental control. The miR-371a-3p results were not communicated to the treating clinicians and did not influence follow-up management.

Statistical analysis

Relative expression of the target miRNA (miR-371a-3p) was calculated via standard ΔCt calculations compared to the housekeeper miR-30b-5p ($rE = 2^{-(\Delta Ct)}$, where ΔCt = Ct (miR-371a-3p) − Ct (miR-30b-5p) as previously described [10]. For visualisation, the rE was transformed to log 10. Statistical analyses were performed using R Studio version 1.4.1103 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Quality control

When assessing the follow-up serum samples, a ΔCt of <8 and therefore no substantial evidence of haemolysis was detected in 43 samples [3]. Borderline ΔCt values of 8–9 were found in 39 samples and high ΔCt values, indicating that haemolysis (ΔCt >9) were found in 35 samples.

In our cohort, we found no evidence that haemolysis impacted the relative expression of miR-371a-3p (Supplementary Fig. 1). In the 74 samples that had delta haemolysis values >8, 66 (89 %) had no miR-371a-3p detected, and of the remaining eight samples that did have detectable miR-371a-3p expression, we would have predicted this given the samples were from patients where

Table 2. Clinical cohort characteristics of patients with known recurrence.

#	Primary tumour histology and risk factors for recurrence	Days from first miR-371a-3p elevation to confirmed recurrence	Days from orchiectomy to recurrence	Site of recurrence and size of largest lesions in short axis
1	Seminoma, 35 mm Rete testis invasion	0	549	Iliac lymph nodes 20 mm
2	Seminoma, 28 mm Rete testis invasion	97	406	Retroperitoneum 22 mm
3	Seminoma 50 mm Rete testis invasion	98	465	Retroperitoneum 13 mm
4	80% embryonal, 10% seminoma, 10% yolk sac Lymphovascular invasion	49	210	Multiple nodes in the chest, mediastinal and retrocrural 18 mm
5	Seminoma, 77 mm No rete testis invasion	154	210	Retroperitoneum 29 mm
6	Seminoma, 27 mm Rete testis invasion	109	421	Retroperitoneum 26 mm
7	60% embryonal carcinoma, 10% seminoma, 30% yolk sac No lymphovascular invasion	33	189	HCG elevation, no visible disease
8	Seminoma, 55 mm No rete testis invasion	89	134	Iliac lymph nodes 20 mm
9	Seminoma, 50 mm Rete testis invasion	0	217	Retroperitoneum 17 mm
10	Seminoma, 24 mm No rete testis invasion	7	110	Iliac lymph nodes 30 mm

Histology of primary tumour, time to recurrence detection post orchiectomy via imaging (#1–4 and #6–10) or elevated HCG (#5), and the number of days prior to conventional method detection that miR-371a-3p elevation was observed are shown.
HCG human chorionic gonadotrophin.

recurrence was known to occur. Importantly, the only 'false-positive' serum miR-371a-3p value obtained had a delta haemolysis value within the normal range (7.15) and there were no samples with unpredicted miR-371a-3p values when the delta haemolysis value was >8. This is in agreement with previous studies showing that haemolysis does not increase relative expression values for miR-371a-3p and reporting that lysed red blood cells do not contain detectable amounts of miR-371a-3p [11].

SAG TCCS cohort characteristics

The median age of the 33 men in the SAG TCCS cohort was 36 years (interquartile range [IQR] 30–45). Eighteen men had pure seminoma and 15 mixed or non-seminomatous testicular GCTs. In men with seminoma, rete testis infiltration was present in 9/18 (50%) and median tumour size was 3 cm (IQR 2–4). In men with non-seminoma, the lymphovascular invasion was present in 2/15 (13%). During a median follow-up time of 15 months (IQR 7–20), 10 of the 33 patients (30%) recurred after a median of 7 months (IQR 6–14; Table 2).

MiR-371a-3p levels

Early after orchiectomy (range 1–38 days, median 4 days), miR-371a-3p levels were not elevated in any patient of the institution collecting early post-orchiectomy serum samples ($n = 15$; Supplementary Fig. 2). However, 3 of those 15 patients recurred during follow-up and were then found to be positive for miR-371a-3p.

In the SAG TCCS cohort of 33 patients, all ten patients with recurrences (nine of which were detected via imaging, while one was detected solely through elevated HCG levels; Table 2) exhibited increasing miR-371a-3p levels (Fig. 1). Patients without recurrence did not display this elevation except one seminoma patient, who had a single elevated level (miR-371a-3p relative expression value of 0.0006 at 12 months, other blood markers AFP, LDH and HCG remained unchanged), which was no longer detectable at the next follow-up measurement.

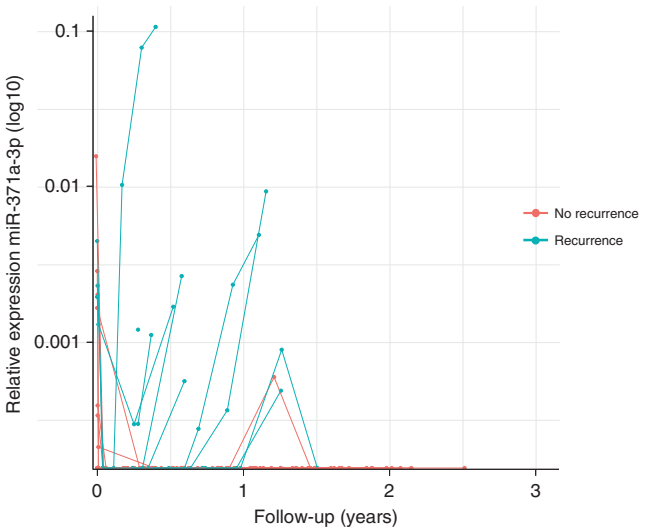

Fig. 1 Relative miRNA expression (log 10) of miR-371a-3p compared to the housekeeper miRNA (miR-30b-5p) during follow-up, stratified by recurrence status. Relative expression was calculated by ($rE = 2^{-(\Delta Ct)}$, where $\Delta Ct = Ct$ (MiR-371a-3p) − Ct (miR-30b-5p). No recurrence (red) and recurrence (blue) patients are shown. The single blue dot corresponds to a patient where only one serum sample was obtained. The patient with a solely increased red dot and subsequently non-measurable value is regarded as a false-positive finding.

Recurrences were detected at a median of 2 months (IQR 0.5–3, range 0–5) earlier using miR-371a-3p than using the standard

follow-up investigations (Table 2). All miR-371a-3p levels pre- and post orchiectomy of the ten men with non-GCT histology were negative.

DISCUSSION

Increasing serum miR-371a-3p levels during follow-up reliably predicted disease recurrence in all relapsing patients. Furthermore, recurrences were detected up to five months earlier compared to the standard follow-up investigations, and miR-371a-3p levels remained undetectable in all but one patient without recurrence.

Our results are in line with previous studies regarding the failure to detect microscopic non-teratomatous GCT early after orchiectomy using serum miR-371a-3p [12–14]. van Agthoven and Looijenga [14] showed that germ cell neoplasia in situ (GCNIS) cannot be detected using measurements of serum miR-371a-3p. However, Radtke et al. suggested that the presence of microscopic GCNIS may result in detectable serum miR-371a-3p levels in a subset (approximately 50%) of cases [15]. The same group reported that small GCT can be detected, but that the diagnostic accuracy depends on the size of the tumour [12]. Lobo et al. collected serum samples after orchiectomy and reported that early after orchiectomy serum miR-371a-3p levels were not predictive for a recurrence later during follow-up [13]. However, at relapse, Lobo et al. [13] as well as Terbuch et al. [16] confirmed that men with macroscopic, mainly retroperitoneal recurrences, show elevated serum miR-371a-3p levels.

Our findings support and extend those results as we prospectively and repeatedly collected numerous serum samples during follow-up, generating a unique cohort for serum miR-371a-3p analyses. Our results indicate that recurrences can indeed be detected earlier by serum miR-371a-3p compared to standard follow-up schedules. However, our data and that of most other groups suggest that with current miRNA methodologies, only macroscopic but not microscopic non-teratomatous GCT can be detected. Thus, measurements of miR-371a-3p do not support decision-making regarding AS vs. adjuvant treatment in stage I disease immediately after orchiectomy. Moreover, given the extensive experience and relatively low costs associated with the conventional blood markers AFP and HCG, it is unlikely that miR-371a-3p would be added as a replacement diagnostic tool. Rather, initially, it would be added to the currently accepted methodologies. Given there is currently no available blood-based marker for pure teratoma, cross-sectional imaging will also remain an important element in the follow-up of mixed or non-seminomatous GCTs.

However, the reliable detection of disease recurrence and the absence of increasing miR-371a-3p elevation in men without disease recurrence have the potential to significantly change the management of patients undergoing AS. If our results are confirmed in larger prospective cohorts (NCT04435756, NCT04914026), measurements of miR-371a-3p could decrease the number of regular cross-sectional imaging investigations during AS that, in turn, would decrease health-care costs and radiation exposure. Particularly, men with pure seminomatous GCT might only require cross-sectional imaging when miR-371a-3p levels increase. However, in men with non-seminomatous GCT regular imaging cannot be omitted completely as recurrences with pure teratomatous GCT are not detected by serum miR-371a-3p. Given our relatively small cohort, it is not yet possible to assess the efficacy of serum miR-371a-3p for relapse detection when comparing seminoma and non-seminoma.

Furthermore, serum miR-371a-3p analysis also has the potential to detect recurrences earlier compared to standard follow-up investigations. Earlier detection of disease recurrence can result in a lower stage of disease at the time of recurrence detection. This can positively influence the overall treatment burden, as some men with non-seminomatous GCT may not require post-chemotherapy residual retroperitoneal lymph node dissection, while others with low volume metastatic GCT may qualify for evolving treatment options including radiochemotherapy or robotic retroperitoneal lymph node dissection [17–19].

In our cohort, serum miR-371a-3p detected recurrences reliably and earlier than the standard follow-up investigations. Although limited by a small sample size and short follow-up period, our results are promising and support the hypothesis that serum miR-371a-3p analyses may prove beneficial in the surveillance of localised testis cancer patients. Further research is needed to standardise miRNA measurements across laboratories and to define miRNA levels in the normal populations before miRNA monitoring can be incorporated into routine clinical practice.

DATA AVAILABILITY

Data are available upon request.

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ACKNOWLEDGEMENTS

We thank Davide Ardizzone and Lisa Roth for managing clinical data, our study nurses Yvonne Döring and Uta Bonitz for their support in this project and Jordi Nicola Fronzaroli for sample collection.

AUTHOR CONTRIBUTIONS

Conception and design: CDF, AJC, JB, TH and CR. Acquisition of data: AJC, CDF, CR, RC, NCG, JBG, AJT and AH-B. Analysis and interpretation of data: AJC and CDF. Drafting of the manuscript: CDF, AJC, JB, TH. Critical revision of the manuscript for important intellectual content: all authors. Statistical analysis: CDF, AJC and MSW. Obtaining funding: CDF, JB, TH and CR. Administrative, technical or material support—supervision: JB, TH and CR.

FUNDING

This study was funded by the Swiss Cancer League (KLS-4297-08-2017), Stiftung zur Krebsbekämpfung, Hanne Liebermann Stiftung, Alfred und Anneliese Sutter-Stöttner Stiftung, Dr. Hans Altschüler Stiftung, Padella Stiftung.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the local ethical committee of St. Gallen (EKSG 13/08/L). All patients provided written consent. The study was performed in accordance with the Declaration of Helsinki.

CONSENT FOR PUBLICATION

All patients provided written consent.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41416-021-01643-z.

Correspondence and requests for materials should be addressed to Christian D. Fankhauser.

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