Received: 8 October 2019 | Revised: 3 June 2020 | Accepted: 4 June 2020

DOI: 10.1111/andr.12838

ARTICLE

ANDROLOGY WILEY

The metastatic potential of seminomatous germ cell tumours is associated with a specific microRNA pattern

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Funding information
This work was funded by the Southwest German Society of Urology (SWDGU).

Abstract

Background: Seminomatous germ cell tumours (SGCT) are the most frequent malignancy in young men. Reliable prognostic biomarkers for the prediction of metastasis at diagnosis and the risk of relapse in clinical stage I (CSI) are lacking. Adjuvant therapies carry a risk of overtreatment, whereas salvage therapies have a risk of high toxicities. Thus, the identification of reliable prognostic biomarkers is highly desirable to identify patients who will benefit from early adjuvant treatment. MicroRNAs (miRNAs) regulate tumour development and progression, and their potential as biomarkers has already been proven in a variety of malignancies.

Objectives: The aim of our study was to define a specific miRNA expression pattern that discriminates metastatic from non-metastatic primary SGCT.

Materials and methods: Total RNA was isolated from 24 formalin-fixed paraffin-embedded (FFPE) primary SGCT tumours (10 non-metastatic, five metachronously and nine synchronously metastatic) and from 10 normal testicular tissue samples. Microarray analysis was performed for global miRNA expression profiling. The results were validated by quantitative real-time polymerase chain reaction (qRT-PCR). Statistical analysis was performed using SPSS.

Results: Microarray analyses revealed a specific miRNA pattern that distinguishes metastatic from non-metastatic SGCT. Sixty-three miRNAs were differentially expressed in metastatic compared to non-metastatic tumours ($P < .01$). Microarray results were confirmed by qRT-PCR for three out of five selected miRNAs (miR-29c-5p, miR-506-3p and miR-371a-5p; $P < .05$). All five miRNAs (miR-29c-5p, miR-506-3p, miR-1307-5p, miR-371a-5p and miR-371a-3p) showed differential expression between tumour and normal tissues ($P < .05$).

Conclusion: Metastatic primary SGCTs are characterized by a specific miRNA expression pattern. Therefore, specific miRNAs could represent a new tool to predict the metastatic potential in SGCT patients.

KEYWORDS

miRNA, prognostic biomarkers, seminoma, testicular cancer

Kerstin Junker and Julia Heinzelbecker are contributed equally to this work.

1 | INTRODUCTION

Testicular cancer is the most frequent solid malignancy in young men, and pure seminoma (seminomatous germ cell tumour (SGCT)) is the most common histological subtype.[1,2] According to current guidelines, surveillance is the option of choice for clinical stage I (CSI) SGCT patients.[3] However, up to 20% have occult metastatic disease at the time of diagnosis and will relapse on a surveillance regimen.[4] These patients are treated according to clinical stage at relapse, and cure rates close to 100% are reached. Nevertheless, significant acute and long-term toxicity remains an outcome for successful salvage treatment.[5] Carboplatin chemotherapy and radiation are adjuvant treatment options in CSI SGCT. Nevertheless, when applied in every patient, they result in an up to 80% risk of overtreatment. Tumour size and rete testis invasion have often been described as possible predictors of relapse. However, they have never been validated in a prospective setting, and their role as prognostic parameters remains doubtful.[6,7] The detection of reliable prognostic biomarkers that accurately identify patients with CSI seminoma at risk of relapse would therefore be highly desirable.

MicroRNAs (miRNAs) are promising biomarkers in cancer research. These small noncoding RNAs of approximately 20-25 base pairs in length regulate many cellular processes, such as cell development, differentiation, apoptosis and proliferation.[8] They play an important role in tumour development and progression.[9] As miRNAs are stable, they are suitable biomarkers that can be analysed from several sources, such as formalin-fixed paraffin-embedded (FFPE) tissue samples and liquid biopsies.[10] Recently, miRNAs of the miR-371-73 cluster have been described as promising potential diagnostic and prognostic serum biomarkers in germ cell tumours.[11-13] Nevertheless, our knowledge concerning the role of miRNAs in germ cell tumour development and progression is still limited. The aim of our study was to define a specific prognostic miRNA expression pattern in primary tumour tissue of SGCT that allows the differentiation between metastatic and non-metastatic SGCT.

2 | MATERIALS AND METHODS

2.1 | Patient samples

FFPE tumour samples were obtained from 24 primary SGCTs. Samples were from 10 patients who did not show metastasis for at least 24 months after diagnosis under surveillance (non-metastatic, median follow-up of 26 months [24-156]), five patients who presented with CSI at diagnosis and developed metachronous metastases under surveillance, and nine patients who presented with synchronous metastases. Staging was performed within four weeks after diagnosis. Surveillance protocols were used according to European Association of Urology guidelines.[3] In 10 patients, normal FFPE testicular tissue from tumour-distant areas was obtained. All tissue samples were reviewed by a pathologist. Tumour classification was performed according to current TNM classification systems.[14]

2.2 | Total RNA isolation

Total RNA was isolated from the FFPE samples using the miRNeasy FFPE Isolation Kit (Qiagen) according to the manufacturer's instructions. For determination of RNA quantity, a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher) was used.

2.3 | MiRNA expression analyses

MiRNA expression analyses were performed using the human miRNA microarray technique (Agilent Technologies; Version 16). Total RNA (100 ng) was labelled and hybridized on a miRNA microarray (miRNA complete labelling and hybridization kit, Agilent human microarrays; Agilent Technologies) according to the manufacturer's instructions. After extraction of raw data using Feature Extraction (Agilent Technologies), the data were analysed using Qlucore (Qlucore; Version 3). The total gene signal was normalized to the 75th percentile of signal intensity.

2.4 | Quantitative real-time polymerase chain reaction

Quantitative qRT-PCR was performed on 100ng/µl total RNA using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies), and specific TaqMan primers for miR-29c-5p, miR-371a-3p, miR-1307-5p and RNU48 and TaqMan Gene expression Master Mix (Life Technologies) were used according to the manufacturer's protocol. Since no specific TaqMan primers were available for miR-371a-5p and miR-506-3p, qRT-PCR for these miRNAs was performed using the miRcury LNA miRNA PCR kit (Qiagen) according to the manufacturer's instructions (total RNA used: 50 ng/µL). In both methods, qRT-PCR was performed in triplicate, and the average value was calculated. For used primers and probes, see Table S1. The expression values of tissue samples were normalized using RNU48 (TaqMan) or SNORD48 (miRcury LNA).

2.5 | Statistical analyses

For the miRNA expression analysis, we compared non-metastatic, metachronously and synchronously metastatic patients. For our further analysis, we compared non-metastatic to metastatic SGCT. Clinicopathological data were analysed using the chi-square test for categorical data and the Mann-Whitney U test for non-parametric data. Microarray data were analysed using Student's t test. Unpaired (two-group comparison) unsupervised hierarchical clustering was performed using Qlucore software. The parameters were

TABLE 1 Clinicopathological data

	Non-metastatic	Metachronously metastatic	Synchronously metastatic
Patient number, n (%)	10 (42)	5 (21)	9 (37)
Clinical stage at primary diagnosis, n (%)			
CS I	10 (100)	5 (100)	
IA	9 (90)	3 (60)	
IB	1 (10)	2 (40)	
CS II	0 (0)	0 (0)	8 (90)
IIA			0 (0)
IIB			4 (44)
IIC			4 (44)
CS III			1 (10)
IIIA			1 (11)
IIIB			0 (0)
Age at diagnosis (y)			
Median [IQR]	38 [34.0-41.8]	39 [36.5-43.0]	46 [38.0-49.5]
β-HCG preoperative (Norm: <2.0 mIU/mL)			
Median [IQR]	0.8 [0.1-10.9]	0.6 [0.2-1.2]	10.4 [1.2-25.3]
AFP preoperative (Norm: <5.8 IU/mL)			
Median [IQR]	2.4 [1.6-2.9]	3.2 [1.7-7.3]	1.9 [1.2-2.2]
LDH preoperative (Norm: 0.0-262.0 U/L)			
Median [IQR]	188.0 [165.8-242.0]	290.5 [228.0-362.0]	360.0 [328.5-742.0]
S Stage			
S0	10 (100)	5 (100)	8 (89)
S1	0 (0)	0 (0)	1 (11)
S2	0 (0)	0 (0)	0 (0)
S3	0 (0)	0 (0)	0 (0)
pT-Stadium, n (%)			
pT1	9 (90)	3 (60)	5 (56)
pT2	1 (10)	2 (40)	4 (44)
pT3	0 (0)	0 (0)	0 (0)
pT4	0 (0)	0 (0)	0 (0)
Invasion of rete testis, n (%)			
No	7 (70)	4 (80)	3 (33)
Yes	3 (30)	1 (20)	6 (67)
Tumour size (cm)			
Median [IQR]	2.0 [1.7-3.1]	3.0 [3.0-5.8]	5.6 [4.3-7.8]
Death, n (%)	0 (0)	0 (0)	2 (22)
Relapses, n (%)	-	5 (21)	-
Time to relapse (mo)			
Median [IQR]	-	12 [9-17]	-

Abbreviations: AFP, alpha-fetoprotein; IQR, interquartile range; LDH, lactate dehydrogenase; β-HCG, beta-human chorionic gonadotropin.

set to fold change (FC)> 1.5, $P < .05$ or < 0.01, respectively, and variance $\sigma = 0.2$. The false discovery rate (FDR) was defined using the Benjamini-Hochberg method for multiple testing, and q was adjusted at < 0.25 based on FDR. qRT-PCR data were analysed using REST 2009 (Technical University Munich, Germany & Qiagen, Version 2009) software and the Mann-Whitney U test. For the validation with qRT-PCR, we chose the respective miRNAs on the basis of low p-values, large fold changes, few outliers, a well-balanced

distribution between up- and downregulated miRNAs and biological relevance. Fold changes were calculated using Qlucore (miRNA expression analyses) and REST 2009 (qRT-PCR) software. To analyse qRT-PCR data, Mann-Whitney U test and boxplot diagrams were generated with SPSS (SPSS 23; IBM).

3 | RESULTS

3.1 | Clinicopathological parameters

Clinicopathological data are summarized in Tables 1 and 2. In the five patients who relapsed in CSI (= metachronous metastases), the median tumour size was 3.0 cm [interquartile range (IQR): 3.0-5.8] (see Table 1). Only one patient showed rete testis invasion. In terms of potential prognostic clinicopathological parameters, only the median level of preoperative lactate dehydrogenase (LDH) and the median tumour size differed significantly between non-metastatic and metastatic tumours (LDH: 188.0 vs 350.0, $P < .001$; tumour size: 2.0 vs 5.0, $P = .001$; see Table 2). Two patients with synchronous metastases (clinical stage at diagnosis IIB and IIC) died. One patient died of bleomycin-induced pulmonary fibrosis, and the other died of generalized sepsis during chemotherapy.

3.2 | Microarray analysis

We identified 176 ($P < .05$) and 63 ($P < .01$) miRNAs that were differentially expressed in metastatic compared to non-metastatic SGCT (Figure 1, Table 3). In the next step, we investigated synchronously and metachronously metastatic tumours separately. 398 miRNAs were expressed significantly different between non-metastatic (n = 10) and synchronously (n = 9) metastatic SGCT ($P < .05$). Sixty-six miRNAs ($P < .05$) showed significantly different expression between non-metastatic (n = 10) and metachronously metastatic (n = 5) tumours (Figure 1). Of those, 36 miRNAs distinguished both synchronously and metachronously metastatic SGCT from non-metastatic SGCT (Figure 2, $P < .05$). Thereof, 25 miRNAs showed increased expression, and 11 miRNAs showed decreased expression in metastatic compared to non-metastatic tumours. We identified 93 ($P < .05$) and 3 ($P < .01$) miRNAs (miR-22-5p, miR-625-5p, miR-4252) that were differentially expressed between metachronously (n = 5) and synchronously (n = 9) metastatic tumours ($P < .05$).

3.3 | Results of quantitative real-time PCR

Microarray results were validated in the same cohort using qRT-PCR. As described in the material and methods part, we chose four miRNAs, that were either significantly down- (miR-29c-5p, miR-506-3p and miR-1307-5p) (Figure 3A,C,E; Table 3) or upregulated (miR-371a-5p) in metastatic tumours (Figure 3G, Table 3). Because of its recently described promising role as a diagnostic serum biomarker for testicular cancer, miR-371a-3p was also included.[13]

We found significantly different expression of miR-29c-5p, miR-506-3p and miR-371a-5p in metastatic compared to non-metastatic

TABLE 2 Differences in clinicopathological data between non-metastatic and metastatic tumours

	Non-metastatic (n = 10)	Metastatic (n = 14)	P-value
Age at diagnosis (years)			
Median [IQR]	38 [34.0-41.8]	42 [37.8-49.0]	0.235
β-HCG preoperative			
(Norm: <2.0 mIU/mL)			
Median [IQR]	0.8 [0.1-10.9]	1.6 [0.5-18.4]	0.312
AFP preoperative			
(Norm: <5.8 IU/mL)			
Median [IQR]	2.4 [1.6-2.9]	2.0 [1.6-2.8]	0.666
LDH preoperative			
(Norm: 0.0-262.0 U/L)			
Median [IQR]	188.0 [165.8-241.5]	350.0 [278.5-558.5]	<0.001
Invasion of rete testis, n (%)			
No	7 (70)	7 (50)	
Yes	3 (30)	7 (50)	0.421
Tumour size (cm)			
Median [IQR]	2.0 [1.7-3.1]	5.0 [3.0-6.5]	0.001

Abbreviations: AFP, alpha-fetoprotein; IQR, interquartile range; LDH, lactate dehydrogenase; β-HCG, beta-human chorionic gonadotropin.

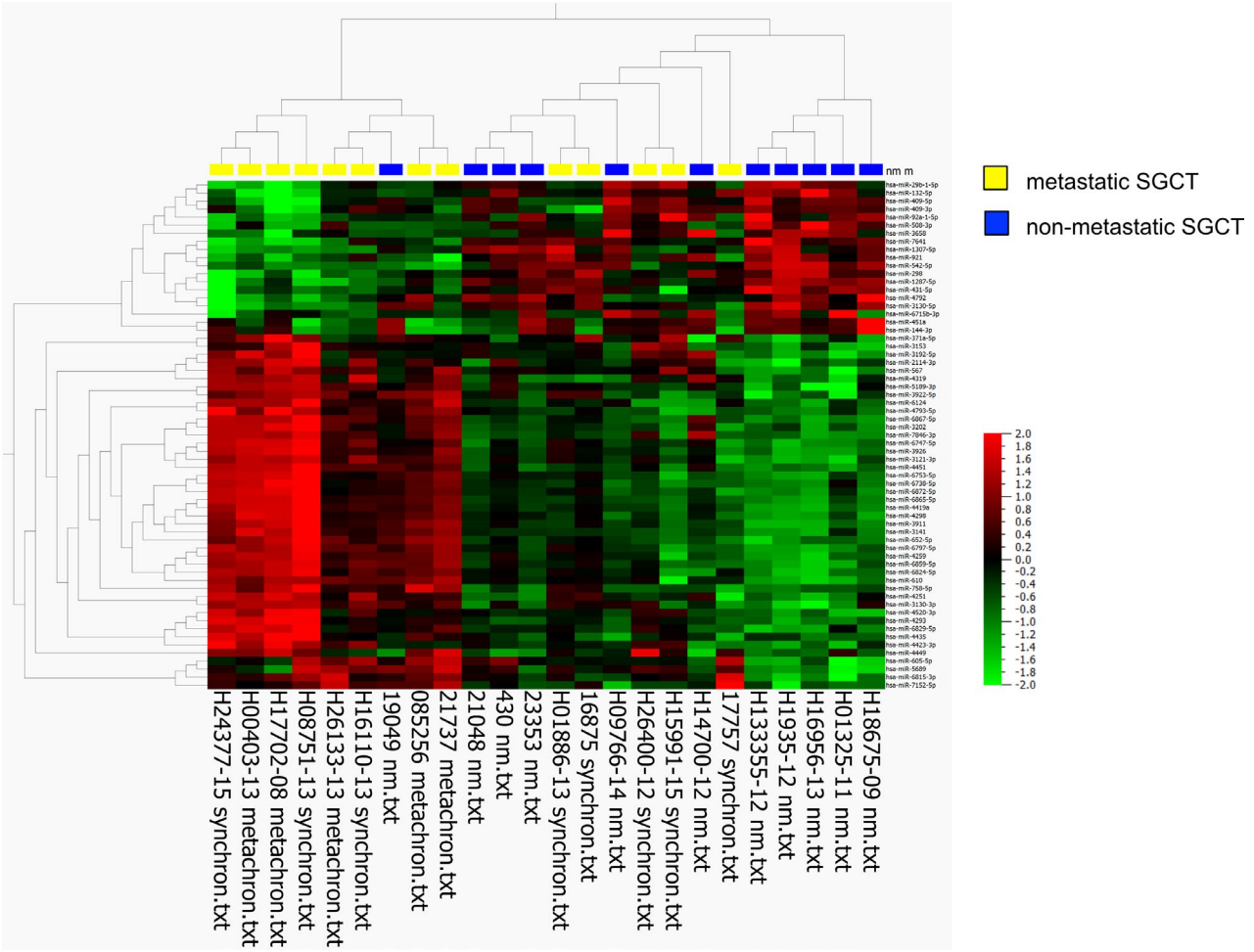

FIGURE 1 The heat map shows the 63 miRNAs ($P < .01$) that are significantly different expressed in metastatic compared to non-metastatic SGCT

tumours by qRT-PCR, with miR-29c-5p and miR-506-3p being significantly downregulated and miR-371a-5p being significantly upregulated in metastatic tumours (Figure 3B,D,H). There was no significant difference between synchronously and metachronously metastatic tumours (miR-29c-5p: $P = .560$, FC=−1.03; miR-506-3p: $P = .800$; FC=−1.03; miR-371a-5p: $P = .620$; FC = 1.07).

Furthermore, the expression levels of miR-29c-5p and miR-506-3p were significantly lower in tumour tissue than normal testicular tissue, whereas miR-371a-5p expression was significantly higher (Figure 3 B,D,H).

The expression of miR-1307-5p did not significantly differ between metastatic and non-metastatic tumours (Figure 3 F). However, we found a significantly different expression level between normal testicular tissue and tumour tissue, with the expression level being significantly higher only in non-metastatic tumours (Figure 3E-F).

We found no significantly different expression levels for miR-371a-3p in metastatic compared to non-metastatic tumours (Figure 3I). There was a significantly higher expression level of miR-371a-3p in both non-metastatic and metastatic tumour tissue compared to normal testicular tissue (Figure 3I).

4 | DISCUSSION

The development of valid prognostic markers in early stage SGCT would allow a more precise identification of patients who relapse and thus offer possibilities for early adjuvant therapies. Consequently, the toxicity of relapse treatment could be avoided, and long-term toxicities in these young cancer patients could be reduced.[5] Thus, far, clinicopathological parameters failed to predict tumour progression in early stage SGCT, and reliable prognostic parameters are still lacking.[6,7] The promising role of miRNAs as potential biomarkers has already been shown for numerous tumour entities, including urological carcinomas.[10,15]

To the best of our knowledge, our study was the first to develop a miRNA expression tool for the identification of patients at high risk of relapse in SGCT. Hereby, we confirm a potential role of miRNAs as prognostic biomarkers in SGCT. We found a panel of miRNAs that clearly discriminate between metastatic and non-metastatic SGCTs. We confirmed three of them, miR-29c-5p and miR-506-3p and miR-371a-5p, by qRT-PCR. Within these, we found no difference between synchronously and metachronously metastatic tumours, suggesting a common role of these miRNAs in metastasis

TABLE 3 Overview of the results of the microarray assay of miRNAs that discriminated between metastatic and non-metastatic SGCT. On the basis of low *P*-values, large fold changes, few outliers, a well-balanced distribution between up- and downregulated miRNAs and biological relevance four miRNAs (in bold letters) were chosen for qRT-PCR

miRNA	*P*-value	*q*-value	Fold change
hsa-miR-4449	<.001	0.109	1.828
hsa-miR-1307-5p	**<.001**	**0.146**	**0.481**
hsa-miR-6815-3p	<.001	0.181	2.040
hsa-miR-371a-5p	**<.001**	**0.181**	**1.573**
hsa-miR-6829-5p	<.001	0.181	1.660
hsa-miR-4293	<.001	0.181	2.324
hsa-miR-4520-3p	<.001	0.181	1.931
hsa-miR-6824-5p	<.001	0.181	1.968
hsa-miR-4423-3p	<.001	0.181	2.177
hsa-miR-6747-5p	.001	0.181	1.744
hsa-miR-567	.001	0.181	1.550
hsa-miR-6797-5p	.001	0.181	1.832
hsa-miR-758-5p	.001	0.181	1.769
hsa-miR-542-5p	.001	0.181	0.454
hsa-miR-6859-5p	.001	0.181	1.940
hsa-miR-3130-5p	.001	0.181	0.588
hsa-miR-3926	.002	0.181	1.526
hsa-miR-3911	.002	0.181	1.899
hsa-miR-3922-5p	.002	0.181	1.851
hsa-miR-451a	.002	0.181	0.219
hsa-miR-5189-3p	.002	0.181	1.962
hsa-miR-3121-3p	.002	0.185	1.803
hsa-miR-431-5p	.002	0.185	0.553
hsa-miR-4419a	.002	0.185	1.745
hsa-miR-3153	.002	0.185	1.661
hsa-miR-409-5p	.002	0.185	0.461
hsa-miR-7641	.002	0.185	0.521
hsa-miR-6867-5p	.002	0.185	1.540
hsa-miR-921	.003	0.197	0.551
hsa-miR-7152-5p	.003	0.223	1.684
hsa-miR-610	.004	0.277	1.540
hsa-miR-5689	.004	0.277	1.660
hsa-miR-652-5p	.004	0.277	2.018
hsa-miR-7846-3p	.004	0.277	1.615
hsa-miR-6865-5p	.005	0.281	1.649
hsa-miR-1287-5p	.005	0.281	0.504
hsa-miR-4251	.005	0.281	2.101
hsa-miR-4298	.005	0.281	1.714
hsa-miR-3658	.005	0.281	0.582
hsa-miR-3130-3p	.006	0.281	2.152
hsa-miR-92a-1-5p	.006	0.281	0.448
hsa-miR-605-5p	.006	0.281	1.770
hsa-miR-4259	.006	0.281	1.830
hsa-miR-6872-5p	.006	0.281	1.563
hsa-miR-6124	.006	0.281	1.676
hsa-miR-4451	.006	0.281	1.547
hsa-miR-2114-3p	.006	0.281	1.622
hsa-miR-4792	.006	0.281	0.553
hsa-miR-3141	.006	0.281	1.626
hsa-miR-4319	.007	0.288	1.689
hsa-miR-29b-1-5p	.007	0.288	0.478
hsa-miR-144-3p	.007	0.288	0.172
hsa-miR-4793-5p	.007	0.288	1.795
hsa-miR-298	.007	0.289	0.649
hsa-miR-6753-5p	.008	0.289	1.680
hsa-miR-508-3p	.008	0.308	0.266
hsa-miR-6738-5p	.009	0.308	1.614
hsa-miR-132-5p	.009	0.308	0.542
hsa-miR-3202	.009	0.308	1.531
hsa-miR-6715b-3p	.009	0.320	0.604
hsa-miR-3192-5p	<.010	0.322	1.525
hsa-miR-4435	<.010	0.322	1.639
hsa-miR-409-3p	<.010	0.322	0.386
hsa-miR-509-3-5p	.011	0.329	0.382
hsa-miR-664b-5p	.011	0.329	0.640
hsa-miR-4732-5p	.011	0.329	1.632
hsa-miR-711	.011	0.329	1.544
hsa-miR-506-3p	**.012**	**0.329**	**0.324**
hsa-miR-2276-3p	.012	0.329	1.783
hsa-miR-128-2-5p	.012	0.329	0.467
hsa-miR-6777-5p	.012	0.329	1.617
hsa-miR-4646-5p	.012	0.329	1.528
hsa-miR-1301-5p	.013	0.329	1.595
hsa-miR-4743-5p	.013	0.329	1.548
hsa-miR-1183	.013	0.329	1.536
hsa-miR-4801	.013	0.329	1.800
hsa-miR-138-1-3p	.013	0.329	1.702
hsa-miR-302c-5p	.013	0.329	1.676
hsa-miR-4296	.013	0.329	1.931
hsa-miR-4738-3p	.014	0.329	1.508
hsa-miR-1295b-3p	.014	0.329	1.877
hsa-miR-1288-3p	.014	0.329	0.625
hsa-miR-6862-5p	.014	0.329	1.574
hsa-miR-2467-3p	.015	0.329	1.547
hsa-miR-3197	.015	0.329	0.647
hsa-miR-508-5p	.015	0.329	0.600

(Continues)

(Continues)

TABLE 3 (Continued)

miRNA	*P*-value	*q*-value	Fold change
hsa-miR-6895-5p	.015	0.329	1.551
hsa-let-7b-5p	.015	0.333	0.479
hsa-miR-3173-3p	.016	0.333	1.689
hsa-miR-204-5p	.016	0.333	0.348
hsa-miR-6802-5p	.016	0.333	1.705
hsa-miR-675-3p	.016	0.333	1.795
hsa-miR-4518	.016	0.333	0.554
hsa-miR-99a-5p	.016	0.333	0.357
hsa-miR-664a-5p	.016	0.333	0.497
hsa-miR-411-5p	.017	0.334	0.318
hsa-miR-4485-3p	.017	0.336	0.537
hsa-miR-377-5p	.017	0.336	0.560
hsa-let-7c-5p	.018	0.337	0.429
hsa-miR-29c-5p	**.018**	**0.337**	**0.486**
hsa-miR-1973	.018	0.341	0.565
hsa-miR-98-5p	.019	0.346	0.396
hsa-miR-323a-3p	.019	0.346	0.314
hsa-miR-378j	.019	0.346	1.802
hsa-miR-4726-3p	.019	0.347	1.720
hsa-miR-3925-5p	.021	0.358	0.589
hsa-miR-6780b-5p	.021	0.358	1.738
hsa-miR-542-3p	.021	0.361	0.427
hsa-miR-892b	.021	0.361	0.626
hsa-miR-8087	.021	0.361	1.551
hsa-let-7a-5p	.022	0.362	0.479
hsa-miR-127-3p	.022	0.362	0.396
hsa-miR-373-5p	.022	0.362	1.607
hsa-miR-140-5p	.023	0.378	0.575
hsa-miR-2392	.025	0.393	1.917
hsa-miR-379-5p	.025	0.393	0.338
hsa-miR-513c-3p	.025	0.393	0.577
hsa-miR-410-3p	.025	0.393	0.359
hsa-miR-509-3p	.026	0.393	0.376
hsa-miR-382-5p	.026	0.393	0.375
hsa-miR-4638-5p	.026	0.393	0.559
hsa-miR-507	.026	0.393	0.333
hsa-miR-6821-5p	.027	0.393	1.664
hsa-miR-6749-3p	.027	0.393	1.674
hsa-miR-424-5p	.027	0.393	0.377
hsa-miR-487a-3p	.027	0.393	0.384
hsa-miR-514a-3p	.027	0.393	0.215
hsa-miR-6789-5p	.028	0.401	1.782
hsa-miR-511-3p	.030	0.410	0.475
hsa-miR-487b-3p	.030	0.410	0.537
hsa-miR-7157-3p	.030	0.410	0.659
hsa-miR-150-3p	.030	0.410	1.526
hsa-miR-583	.030	0.410	1.661
hsa-miR-6804-3p	.031	0.410	1.610
hsa-miR-10a-3p	.031	0.410	0.612
hsa-miR-338-3p	.031	0.410	0.458
hsa-miR-4286	.031	0.410	0.571
hsa-miR-10b-3p	.032	0.410	0.617
hsa-miR-4327	.032	0.410	1.519
hsa-miR-432-5p	.032	0.410	0.630
hsa-miR-135a-5p	.032	0.410	0.423
hsa-let-7f-5p	.032	0.410	0.497
hsa-let-7d-5p	.033	0.410	0.507
hsa-miR-6781-5p	.033	0.410	1.719
hsa-miR-6754-3p	.033	0.410	1.764
hsa-miR-376b-3p	.033	0.410	0.301
hsa-miR-299-5p	.034	0.411	0.364
hsa-miR-493-5p	.034	0.411	0.349
hsa-miR-154-5p	.034	0.411	0.445
hsa-miR-136-3p	.034	0.411	0.357
hsa-miR-503-5p	.035	0.415	0.474
hsa-miR-10b-5p	.036	0.415	0.460
hsa-miR-143-5p	.036	0.415	0.505
hsa-miR-376a-5p	.036	0.415	0.459
hsa-miR-4519	.036	0.415	2.027
hsa-miR-489-3p	.036	0.415	0.636
hsa-miR-299-3p	.037	0.418	0.350
hsa-miR-485-5p	.037	0.419	0.637
hsa-miR-200a-5p	.038	0.419	1.501
hsa-miR-758-3p	.039	0.419	0.426
hsa-miR-455-5p	.039	0.419	0.584
hsa-miR-497-5p	.039	0.419	0.570
hsa-miR-4520-2-3p	.040	0.428	0.665
hsa-miR-1180-3p	.041	0.438	0.618
hsa-miR-195-5p	.042	0.439	0.563
hsa-miR-21-3p	.043	0.443	0.558
hsa-miR-369-5p	.044	0.443	0.469
hsa-miR-6775-5p	.044	0.443	1.552
hsa-miR-1248	.045	0.443	0.622
hsa-miR-144-5p	.045	0.443	0.546
hsa-miR-1185-5p	.045	0.443	0.565
hsa-miR-125b-5p	.045	0.443	0.467
hsa-miR-376a-3p	.047	0.451	0.395
hsa-miR-140-3p	.047	0.451	0.664
hsa-miR-4315	.048	0.461	1.527
hsa-miR-6090	.049	0.464	1.637

(Continues)

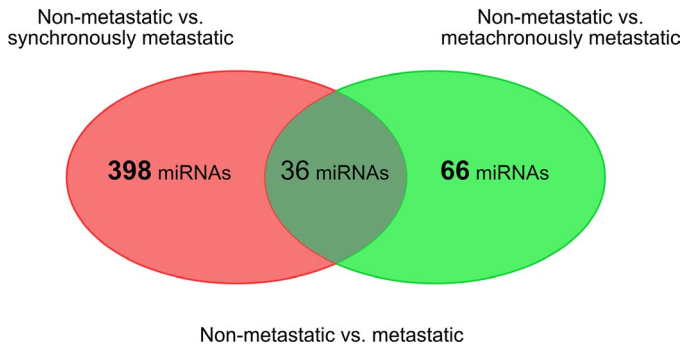

FIGURE 2 The Venn diagram shows the total number and the overlapping number (bold) of significantly differentially expressed miRNAs to discriminate non-metastatic from synchronously metastatic SGCT (red), non-metastatic from metachronously metastatic SGCT (bright green) and non-metastatic from metastatic SGCT (dark green)

irrespective of the time of diagnosis. These results are consistent with the findings of others that report almost indistinguishable results for the transcriptional levels between synchronously and metachronously metastatic SGCT.[16,17] In summary, our results indicate that non-metastatic and metastatic SGCT can be distinguished by specific miRNA expression patterns.

Members of the miR-29 family have been identified as tumour suppressors in a variety of malignancies.[18] In gall bladder cancer, miR-29c-5p was significantly downregulated. Its overexpression in vitro led to repressed proliferation and to the induction of apoptosis by inhibition of the mitogen-activated protein kinases pathway.[19] When evaluating the prognostic value of miRNAs in seminomas by using next generation sequencing, Ruf et al identified 38 miRNAs that significantly distinguished non-metastatic from metastatic SGCT. A two-miRNA combination (eg miR-29c + Enst00000387347; miR-29a + Enst00000387347) was defined that discriminated metastatic from non-metastatic tumours.[17] Consistent to our work miR-29c was expressed at significantly different levels in metastatic compared to non-metastatic tumours. Unfortunately, Ruf et al did not further describe if −3p or −5p was examined. Furthermore, they did not validate their results by qRT-PCR.[17] Additionally, to miR-29c and in contradiction to our work Ruf et al showed that miR-29a was significantly differentially expressed in metastatic compared to non-metastatic tumours. However, none of the other in our study identified miRNAs has been described by Ruf et al Our study results, together with the results of Ruf et al, suggest that miR-29c might be involved in the metastatic process of SGCTs.

We found that the expression level of miR-506-3p was lower in metastatic than non-metastatic tumours as well as in tumour tissue compared to normal testicular tissue. The role of the miR-506 family and its pivotal role in regulating cell proliferation, differentiation, migration and invasion is well described.[20] In the majority of tumour entities, this miRNA acts as a tumour suppressor, for example, in ovarian cancer or clear cell renal cell carcinoma.[21,22] In respect of our study results, thus miR-506-3p might have a possible role as a tumour suppressor in SGCT.

We could not confirm a significant difference in miR-1307-5p expression in metastatic compared to non-metastatic tumours. However, we found a significantly higher expression of this miRNA in tumour tissue compared to normal testicular tissue. The function of miR-1307 is still unknown. Upregulation of miR-1307 plays a role in chemotherapy resistance of ovarian cancer cell lines. Death-associated protein kinase 3 was identified as a target of miR-1307, which increases the antiapoptotic and survival abilities of tumour cells.[23]

Furthermore, we found that miR-371a-5p was significantly overexpressed in metastatic compared to non-metastatic tumours. The results are similar to those in hepatocellular carcinoma, where miR-371a-5p is upregulated and its overexpression is associated with poor survival.[24]

Another promising member of this cluster is miR-371a-3p, which was found to be overexpressed in the serum of testicular cancer patients compared to controls.[11] Dieckmann et al recently demonstrated that serum levels correlate with disease stage and response to treatment. Again, they postulate its role as an important prognostic parameter and tumour marker that can be easily analysed from liquid biopsies.[12,13] In terms of SGCT, only 15%-20% of patients express serum beta-HCG, displaying an important lack of efficacy of the classic tumour markers. In contrast, miR-371a-3p is generally detectable in the serum of SGCT patients.[12,13] Interestingly, when examining miR-371a-3p in the primary tumour tissues of our study cohort, we did not find a difference between non-metastatic and metastatic tumours. This finding is in line with the results reported by Dieckmann et al that also failed to prove a difference concerning primary tumour tissue.[11] Nonetheless, we found a significant difference between tumour tissue and normal testicular tissue for both miR-371a-3p and miR-371a-5p. MiR-371a-5p belongs to the miR-371-73 cluster that was first described by Palmer et al to be overexpressed in germ cell tumours. Palmer et al hypothesized that several members of this cluster might serve as diagnostic biomarkers for testicular cancer.[25]

In addition, miR-302 and miR-367 clusters have been identified as putative serum markers, too.[26] Nevertheless, miR-371a-3p proved to be the most sensitive and specific miRNA serum marker.[27] We did not find different miRNA expression of miR-302 and miR-367 clusters between non-metastatic and metastatic SGCT in primary SGCT tissues.

There are several studies that examined the expression of miRNAs in primary SGCT tissue compared to normal testicular tissue using microarrays. Novotny et al found miR-9, miR-105, miR-182 and miR-183 to be highly expressed in SGCT[28] whereas Bing et al found miR-21, miR-221, miR-222, miR-372 and miR-373 to be upregulated in primary SGCT tissue.[29] Nevertheless, both studies did not examine differences between non-metastatic and metastatic SGCT. In our study, none of the before mentioned miRNAs were differentially expressed between metastatic and non-metastatic SGCT.

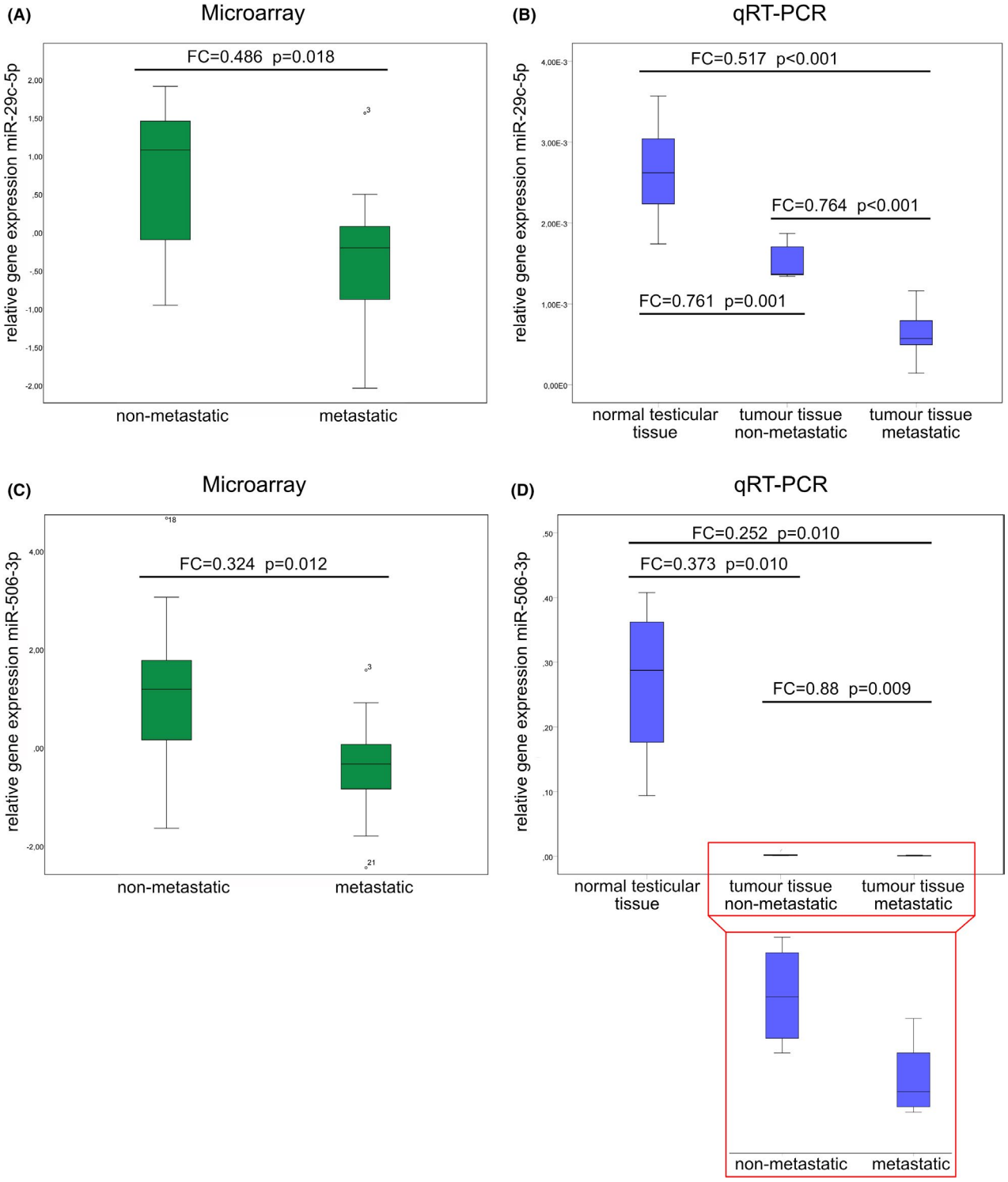

FIGURE 3 The box plots illustrate the expression changes of miR-29c-5p (A, B), miR-506-3p (C, D), miR-1307-5p (E, F) and miR-371a-5p (G, H) in normal testicular tissue, non-metastatic and metastatic tumours based on the array data (green boxes) and the PCR analyses (blue boxes); for miRNA-371-3p (I) based only on the PCR data (blue boxes)

Examining the primary tumour tissue or the serum of testicular cancer patients offers different possibilities. FFPE tissue from orchiectomy specimens is easy to access because it is already part of routine pathological examination. The analysis of original tumour specimens offers important insights into the functional characteristics of the primary tumour, and its prognostic value has already been proven in a variety of genitourinary cancers.[10,30] Liquid biopsies, such as patient serum samples, allow the monitoring of biomarkers in a dynamic time-dependent manner, thus offering important information

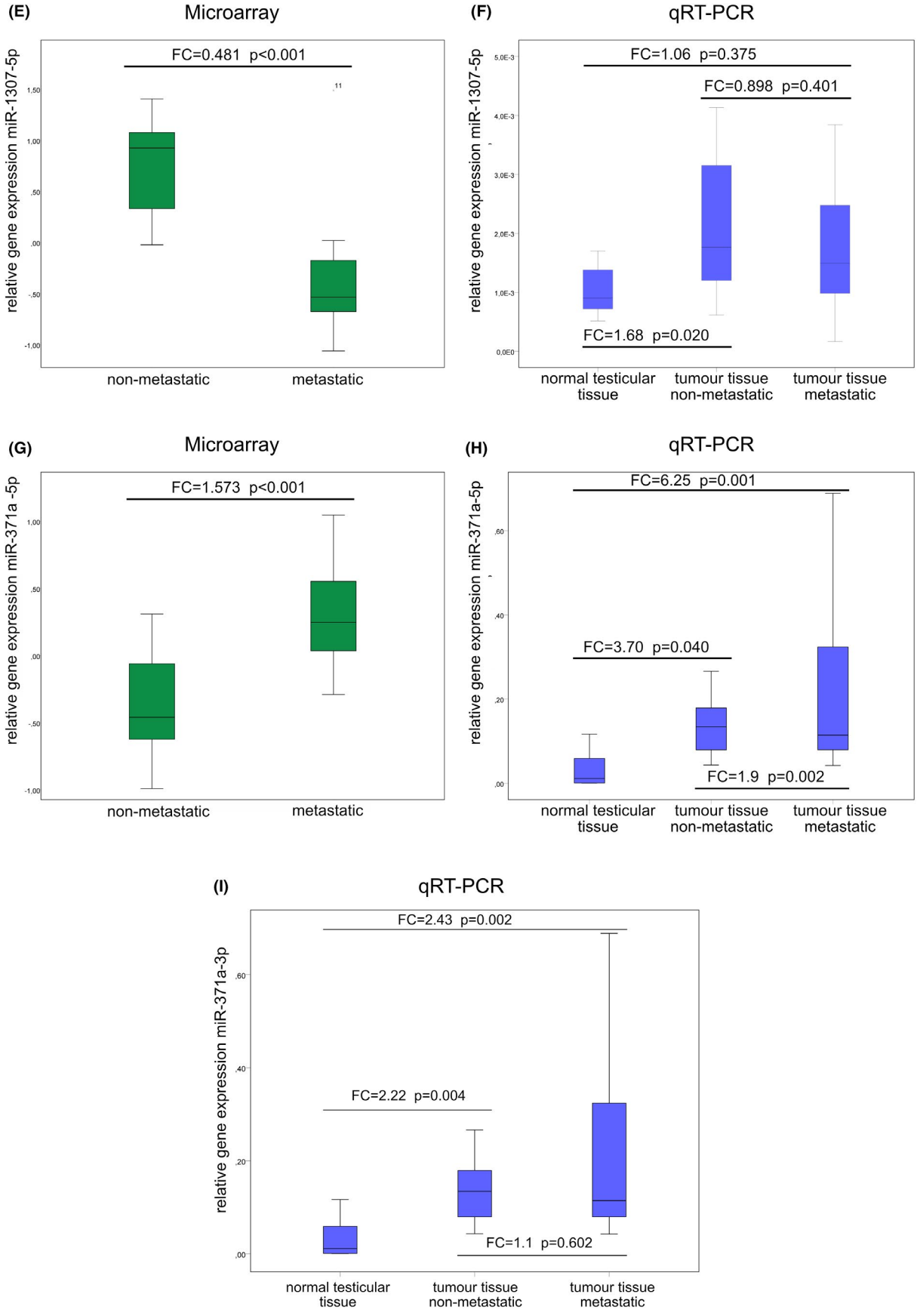

FIGURE 3 (Continued)

about therapy success. The combination of the information from primary tumour tissue together with that of patient serum samples offers a unique opportunity to gain precise insight into the tumour biology and functionality of testicular germ cell cancer.

Regarding the prognostic value of clinicopathological parameters in our study, non-metastatic and metastatic SGCT only differed with respect to tumour size and the preoperative level of LDH. In addition to rete testis invasion, tumour size is considered a possible prognostic factor in SGCT. However, its significance as a prognostic marker remains controversial. In a recently published systematic review, tumour size positively correlated with relapse in ten of the 14 included studies, whereas rete testis invasion was associated with tumour relapse in only four of the 12 included studies.[6] In a review by Boormans et al, there was no sufficient evidence for both factors.[7] These results emphasize the need for the identification of reliable prognostic parameters in SGCT.

This study has several limitations, most notably the small number of cases examined (n = 24). Additionally, molecular biological analysis for global screening, such as microarrays, does not allow for a high number of cases because these analyses are very expensive. Future multicentre studies should validate our promising results in a larger, independent cohort of patients together with the determination of sensitivity and specificity for a single miRNA or a combination of multiple miRNAs (miRNA profiles).

5 | CONCLUSIONS

We showed for the first time that miRNA expression analysis is a promising tool for the identification of patients at high risk of relapse. The specific miRNA expression patterns identified seem to define the individual metastatic risk of SGCTs in a more precise manner than presently possible. A more personalized risk stratification based on miRNA expression could be helpful to reduce unnecessary adjuvant treatment strategies on one hand and the burden of acute and long-term toxicities of salvage treatments on the other hand.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was performed in accordance with the Declaration of Helsinki and was approved by an ethics committee (Ärztekammer des Saarlandes, Saarbrücken, 162/13).

ACKNOWLEDGEMENTS

We thank Alexander Vogt for his skilful technical assistance and are grateful to Sebastian Hölters for his help with the microarray analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Simone Ernst involved in conception and design, data acquisition, data analysis and interpretation, drafting the manuscript, statistical analysis and design of experiments. Joana Heinzelmann involved in data analysis and interpretation, statistical analysis, design of experiments and critical revision of the manuscript. Rainer M. Bohle involved in patho-histological re-evaluation and critical revision of the manuscript. Georg Weber involved in design of experiments and critical revision of the manuscript Michael Stöckle involved in critical revision of the manuscript, data analysis and interpretation Kerstin Junker involved in conception and design, data analysis and interpretation, drafting the manuscript, critical revision of the manuscript, supervision and design of experiments. Julia Heinzelbecker involved in conception and design, data analysis and interpretation, drafting the manuscript, critical revision of the manuscript, statistical analysis, supervision and design of experiments.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Ernst S, Heinzelmann J, Bohle RM, et al. The metastatic potential of seminomatous germ cell tumours is associated with a specific microRNA pattern. *Andrology*. 2020;8:1687–1698. https://doi.org/10.1111/andr.12838