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Discriminating metastasised from non-metastasised seminoma based on transcriptional changes in primary tumours using NGS

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Background: We aimed to better discriminate (occult) metastasised from non-metastasised seminoma based on transcriptional changes of small RNAs in the primary tumour.

Methods: Total RNAs including small RNAs were isolated from five testicular tumours of each, lymphogenic, occult and non-metastasised patients. Next-generation sequencing (SOLID, Life Technologies) was used to examine transcriptional changes. Small RNAs showing $\geqslant 50$ reads and a significant $\geqslant 2$-fold difference using non-metastasised tumours as the reference group were examined in univariate logistic regression analysis and combinations of two small RNAs were further examined using support vector machines.

Results: On average, 1.3×10^7, 1.4×10^7 and 1.7×10^7 small RNA reads were detectable in non-metastasised, occult and lymphogenic metastasised seminoma, respectively, of which 30–32% remained after trimming. Between 59 and 68% represented annotated reads and between 8.6 and 11% were annotated small RNA tags. Of them, 137 small RNAs showed > 50 reads and a two-fold difference to the reference. In univariate analysis, 32–38 small RNAs significantly discriminated lymphogenic/occult from non-metastasised seminoma, and among these different comparisons, it were the same small RNAs in 51–88%. Many combinations of two of these small RNAs allowed a complete discrimination of metastasised from non-metastasised seminoma irrespective of the metastasis subtype.

Conclusions: Metastasised and non-metastasised seminoma can be completely discriminated with a combination of two small RNAs.

Testicular tumour, as the most common tumour in young men, is associated with a 5-year survival rate close to 100% in early stages. Pure seminoma are the most frequent histological subtype (55%) nowadays and up to 70% were present without visible metastasis at primary staging (Ruf *et al*, 2013a). Gold standard for primary staging is computed tomography (CT) of the chest, abdomen and pelvis to detect metastases. In truly non-metastasised clinical stage I (cSI), patients are cured by orchidectomy alone; however, despite modern staging and classification procedures, up to 30% of cSI seminoma patients bear occult metastasis in primary staging and

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relapse after orchidectomy alone (Krege *et al*, 2008; Albers *et al*, 2012). Until now, no reliable biological parameters exist and the clinical parameter shows a concordance of 65% only in differentiating occult metastasised stages from non-metastasised seminoma (Ruf *et al*, 2013b). Identification of occult metastasised patients is one of the main goals to prevent toxicity

(e.g. cardiovascular and kidney disease, secondary malignancies and decreased fertility) caused by unnecessary adjuvant treatment or diagnostic procedures during follow-up (Kollmannsberger *et al*, 2011).

Recent publications facilitate the idea of certain demographic/ clinical histological risk factors to be associated with both

Table 1. Characteristics of patients, their biopsies and RNA isolates

No.	Metastasis detection at time of primary tumour's diagnosis	Descriptive statistics	Age at diagnosis (years)	Tumour size (mm)	pL	pV	pT	Infiltration rete testis	Initial clinical stage	Total RNA (μg)	RIN
1	Non-metastasised		38	14	0	0	1	N	cSI	16.8	7.9
2			50	22	0	0	1	N	cSI	10.2	8.8
3			31	19	1	0	2	N	cSI	25.7	8.2
4			42	45	0	0	1	Y	cSI	31.2	8.4
5			35	19	0	0	1	Y	cSI	41.8	8.6
		Mean	39.1	23.8						25.1	8.4
		s.d.	7.2	12.2						12.3	0.3
1	Lymphogenic metastasised		32	40	0	0	1	N	cSIIb	59.9	8.8
2			50	12	0	0	3	N	cSIIc	30.1	9.2
3			43	50	0	0	1	N	cSIIb	57.0	8.6
4			42	45	1	0	2	Y	cSIIb	30.3	8.9
5			61	42	0	0	1	Y	cSIIc	46.5	8.7
		Mean	45.2	37.8						44.8	8.8
		s.d.	10.8	14.9						14.2	0.2
1	Occult metastasised		33	55	1	0	2	N	cSI	68.8	9.4
2			37	35	0	0	1	Y	cSI	67.9	9.1
3			37	30	0	0	1	N	cSI	13.8	9
4			31	18	0	0	1	N	cSI	30.9	8.7
5			23	55	0	0	1	N	cSI	41.1	8.4
		Mean	32.1	38.6						44.5	8.9
		s.d.	5.4	16.2						23.9	0.4

Abbreviations: cSI = clinical stage I; N = no; pL = lymphatic vessel invasion; pT = tumour stage; pV = blood vessel invasion; RIN = RNA integrity number; Y = yes. Mean values of age and tumour size of the metastasised groups are not statistically different from the non-metastasised seminoma reference group ($P > 0.1$, *t*-test).

Table 2. Descriptive statistics of the number of reads before and after trimming and the percentage of annotated small RNAs for non-metastasised, lymphogenic and occult metastasised seminoma

| Seminoma metastasis status | Total no. of reads | Reads after trimming | | Annotated reads | | Small RNA tags | | |
		Abs.	%	Abs.	%	Abs.	Abs. annotated	%
Non-metastasised, $n = 5$								
Mean	1 266 4336.2	3 986 210.6	41 517	2 767 766.2	68.22	444 222.2	36 390.2	—
S.d.	3.9E + 14	2.6E + 14	—	2.0E + 13	—	2.1E + 14	1.3E + 13	—
Min	7.1E + 06	1.2E + 06	41 469	8.3E + 05	60.4	2.1E + 05	2.1E + 04	7.0
Max	1.6E + 07	8.0E + 06	63.2	6.0E + 06	75.4	7.1E + 05	5.0E + 04	—
Lymphogenic metastasised, $n = 5$								
Mean	16 996 933.6	5.4E + 06	30.36	3 379 924.4	62.46	643 430.4	56 876.6	—
S.d.	2.3E + 14	3.1E + 14	—	2.2E + 14	—	3.4E + 14	3.1E + 14	—
Min	1.4E + 07	1.7E + 06	41 317	1.1E + 06	53.3	2.6E + 05	2.6E + 04	—
Max	2.0E + 07	9.2E + 06	47.2	6.4E + 06	69.6	1.1E + 06	9.8E + 04	—
Occult metastasised, $n = 5$								
Mean	13 943 654.4	4 347 341.6	41 638	2 631 565.4	59.16	4.9E + 05	49 741.4	—
S.d.	2.0E + 14	2.6E + 14	—	1.8E + 14	—	2.2E + 14	1.9E + 14	—
Min	1.1E + 07	1.2E + 06	41 463	7.1E + 05	53.2	2.4E + 05	3.2E + 04	—
Max	1.7E + 07	8.3E + 06	49.6	5.5E + 06	66.1	7.9E + 05	8.0E + 04	—

Abbreviations: Abs. = absolute; Min = minimum; max = maximum; s.d. = standard deviation.

detectable and occult seminoma metastasis (Warde *et al*, 1997, 2002; Valdevenito *et al*, 2007; Krege *et al*, 2008; Albers *et al*, 2012; Ruf *et al*, 2013b). We also showed both metastasis subtypes to be indistinguishable on the transcriptional level using a whole genome screening, suggesting that they are not representing different metastasis subtypes (Ruf *et al*, 2014). Recently, other authors started to examine whether a certain set of microRNAs (gnostic approach) might be suitable for discriminating between seminoma-bearing patients and healthy persons (Gillis *et al*, 2007; Palmer *et al*, 2010; Dieckmann *et al*, 2012).

This to our knowledge provides first indications on the diagnostic potential of microRNAs in the field of urology and eventually in the discrimination of metastasised from non-metastasised urological tumours, although not shown so far. Following this approach, we used an agnostic approach where we searched the whole genome for any kind of small RNA species

suitable to discriminate metastasised (either lymphogenic, occult or a combination of both subtypes) from non-metastasised seminoma using next-generation sequencing (NGS).

MATERIALS AND METHODS

Patient selection. Non-metastasised seminoma ($n = 5$) received no adjuvant treatment and were free of relapse/progress for at least 2 years of follow-up. Occult metastasised patients ($n = 5$) presented without visible metastasis at primary staging, received no adjuvant treatment and developed retroperitoneal tumour progress during follow-up. For patients with detectable metastasis at primary staging ($n = 5$), we focused on clinical stage IIb and IIc to include lymphogenic metastatic spread only and to provide a high level of

Table 3. Summary of significantly associated small RNAs with metastasis status per group

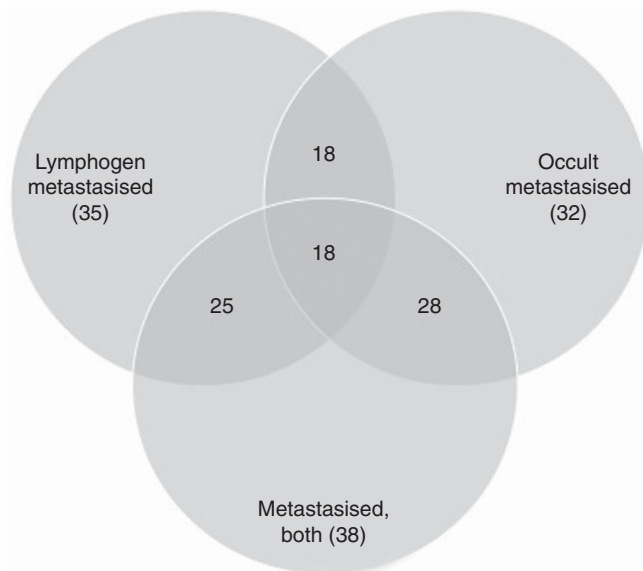| Non- *vs* metastasised (lymphogenic and occult, $n = 38$)					Non- *vs* lymphogenic metastasised ($n = 35$)					Non- *vs* occult metastasised ($n = 32$)				
Small RNA	*P*-value	Fold change	95% Confidence interval		Small RNA	*P*-value	Fold change	95% Confidence interval		Small RNA	*P*-value	Fold change	95% Confidence interval	
Let-7b	0.043	0.48	0.25	0.91	Mir-18a	0.003	5.00	2.31	10.82	Mir-18a	0.034	3.19	1.31	7.79
Mir-17	0.044	2.34	1.11	4.96	Mir-25	0.001	3.57	2.17	5.89	Mir-25	0.004	3.81	1.97	7.38
Mir-18a	0.008	4.10	1.70	9.85	Mir-29a	0.005	0.42	0.27	0.65	Mir-29a	0.019	0.36	0.18	0.71
Mir-25	0.0004	3.69	2.15	6.34	Mir-92a-1	0.017	2.65	1.41	5.00	Mir-92a-1	0.043	2.10	1.15	3.84
Mir-29a	0.001	0.39	0.25	0.60	Mir-92a-2	0.015	2.73	1.44	5.18	Mir-92a-2	0.042	2.14	1.16	3.95
Mir-92a-1	0.007	2.38	1.41	4.02	Mir-93	0.050	6.24	1.32	29.48	Mir-99a	0.029	0.18	0.05	0.64
Mir-92a-2	0.006	2.43	1.42	4.16	Mir-99a	0.011	0.18	0.06	0.50	Mir-29b-1	0.046	0.37	0.16	0.85
Mir-99a	0.001	0.18	0.08	0.40	Mir-197	0.011	2.38	1.42	4.00	Mir-29b-2	0.044	0.36	0.16	0.84
Mir-29b-1	0.012	0.45	0.27	0.77	Mir-148a	0.036	2.51	1.23	5.13	Mir-182	0.018	3.43	1.52	7.76
Mir-29b-2	0.012	0.46	0.27	0.77	Mir-30c-2	0.033	2.81	1.28	6.17	Mir-125b-1	0.040	0.22	0.07	0.74
Mir-30c-2	0.038	2.41	1.14	5.08	Mir-30d	0.024	2.27	1.27	4.05	Mir-128-1	0.029	3.19	1.36	7.49
Mir-182	0.008	3.35	1.57	7.14	Mir-7-1	0.028	3.64	1.41	9.40	Mir-125b-2	0.040	0.22	0.07	0.74
Mir-183	0.037	5.22	1.29	21.04	Mir-182	0.015	3.26	1.54	6.90	Mir-320a	0.035	2.59	1.24	5.41
Mir-15b	0.024	2.65	1.26	5.58	Mir-183	0.003	4.39	2.18	8.86	Mir-128-2	0.034	3.20	1.31	7.81
Mir-125b-1	0.006	0.31	0.16	0.63	Mir-15b	0.002	2.77	1.77	4.32	Mir-106b	0.001	5.22	2.69	10.13
Mir-128-1	0.012	3.86	1.56	9.55	Mir-128-1	0.009	4.53	1.91	10.74	Mir-29c	0.019	0.30	0.13	0.67
Mir-145	0.023	0.50	0.29	0.85	Mir-128-2	0.009	4.77	1.97	11.54	Mir-296	0.017	4.01	1.62	9.90
Mir-125b-2	0.006	0.31	0.16	0.63	Mir-106b	0.003	6.63	2.71	16.23	Mir-326	0.004	2.61	1.63	4.17
Mir-150	0.028	0.36	0.16	0.81	Mir-30c-1	0.033	2.80	1.28	6.12	Mir-151a	0.015	2.35	1.37	4.04
Mir-128-2	0.014	3.98	1.53	10.36	Mir-296	0.019	3.78	1.55	9.20	Mir-331	0.033	2.58	1.25	5.30
Mir-106b	0.001	5.92	2.49	14.11	Mir-130b	0.045	2.95	1.21	7.18	Mir-484	0.016	2.06	1.29	3.27
Mir-29c	0.005	0.40	0.24	0.68	Mir-378a	0.015	2.66	1.43	4.95	Mir-505	0.006	2.65	1.59	4.42
Mir-30c-1	0.037	2.41	1.15	5.06	Mir-340	0.0002	3.42	2.34	4.98	Mir-92b	0.014	3.10	1.53	6.31
Mir-296	0.007	3.89	1.71	8.87	Mir-326	0.002	3.63	2.12	6.21	Mir-652	0.022	3.23	1.44	7.24
Mir-378a	0.028	2.32	1.19	4.53	Mir-331	0.001	3.05	1.96	4.74	ENST00000516461	0.045	2.53	1.18	5.44
Mir-340	0.006	3.12	1.58	6.18	Mir-345	0.035	3.26	1.31	8.12	ENST00000516775	0.045	2.54	1.18	5.47
Mir-326	0.001	3.12	1.82	5.33	Mir-423	0.001	2.24	1.65	3.02	ENST00000517209	0.046	7.07	1.39	35.98
Mir-151a	0.023	2.32	1.22	4.39	Mir-484	0.015	2.28	1.35	3.85	ENST00000387347	0.050	2.77	1.17	6.57
Mir-331	0.003	2.81	1.61	4.92	Mir-505	0.002	3.62	2.03	6.47	ENST00000474885	0.001	3.38	2.05	5.56
Mir-484	0.003	2.17	1.43	3.29	Mir-92b	0.015	4.76	1.76	12.89	ENST00000459522	0.001	3.35	2.04	5.52
Mir-505	0.002	3.13	1.72	5.71	Mir-625	0.041	3.47	1.28	9.42	ENST00000488123	0.039	2.89	1.24	6.72
Mir-92b	0.016	3.93	1.48	10.40	Mir-421	0.025	2.77	1.34	5.70	ENST00000463796	0.013	4.19	1.73	10.17
Mir-652	0.035	2.77	1.19	6.45	Mir-744	0.023	2.14	1.26	3.63					
ENST00000387347	0.013	2.84	1.39	5.79	ENST00000387347	0.005	2.91	1.68	5.06					
ENST00000474885	0.014	2.99	1.40	6.41	ENST00000466665	0.027	2.50	1.28	4.87					
ENST00000459522	0.015	2.97	1.39	6.37										
ENST00000463796	0.034	3.79	1.26	11.44										
ENST00000466665	0.046	2.27	1.09	4.71										

Figure 1. Venn diagram showing the number of significantly associated small RNAs (Table 1) to discriminate different metastasis subtypes (lymphogenic and occult) and the combination of both subtypes from non-metastasised seminoma.

diagnostic accuracy (avoiding doubtful lymph nodes). Lymphogenic metastasis and non-metastasised patients were matched with occult metastasised patients considering demographic and histological parameters if possible (Table 1).

Tissue samples and histological examination. Testicular tumour biopsies ($n = 15$) were incubated into RNAlater solution (Qiagen, Hilden, Germany) during the operation and later stored at –20 °C. All tissue samples were examined by an experienced pathologist for histological and TNM classification (Table 1). The local ethical commission approved the study and all human samples were obtained with informed consent.

RNA isolation. Tissues frozen in RNAlater solution (Qiagen) were carefully thawed, homogenised (Homogenizer; Omni, Warrenton, VA, USA) and digested using proteinase K. Total RNA including small RNAs was isolated (mirVana Kit; Life Technologies, Darmstadt, Germany) and remaining DNA digested. RNA was stored at –80 °C until its use. Quality and quantity of isolated total RNA was measured spectrophotometrically (NanoDrop; PeqLab Biotechnology, Erlangen, Germany), whereas RNA integrity was assessed by the 2100 Agilent Bioanalyser (Life Science Group, Penzberg, Germany). For analysis, we used only RNA specimens with a ratio of $A_{260}/A_{280} \geqslant 2.0$ (NanoDrop) and RNA integrity number (RIN) $\geqslant 7.9$ for small RNA NGS (IMGM Laboratories, Martinsried, Germany/CeGat, Tübingen, Germany).

Small RNA NGS and data analysis. We performed a genome-wide small RNA sequencing using the SOLiD5500xl NGS Technology (Life Technologies). In brief, total RNA was purified (PureLink miRNA Isolation Kit, Life Technologies), enriched small RNAs were ligated to SOLiD adaptors, reverse transcribed (SOLiD RT primer and ArrayScript RT, Life Technologies), cDNA was purified (MinElute PCR Purification Kit; Qiagen), a cutoff size of 60–80 nt was selected (Novex precast gel products; Invitrogen, Life Technologies), cDNA was in-gel amplified and samples were barcoded using SOLiD 3′Barcode Primer (Life Technologies) at the same time. Amplified cDNA was purified (PureLink PCR Micro Kit; Life Technologies) and used in emulsion PCR (SOLiD EZ Bead, Life Technologies). Thereafter, the emulsion was broken to recover enriched beads and the so-called di-base probes were used by the SOLiD system in the sequencing-by-ligation procedure.

Besides the SOLiD5500xl inherent software (LifeScope, Life Technologies) used for image and signal processing, software CLC Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark) was used for clustering, counting and annotation of all generated 75 bp reads. After discarding reads without adaptor sequence and being shorter than 15 bp (trimming), reads were assigned to known miRNAs (Sanger miRBase release 18; http://www.mirbase.org/) and known non-coding RNAs (ensembl database Homo_sapiens.GRCh37.67.ncrna.fa; http://www.ensembl.org). Small RNAs showing a significant and $\geqslant 2$-fold differences in gene expression among groups and at least 50 reads were further analysed on their ability to separate both groups using univariate logistic regression analysis (Baggerly et al, 2004). Combinations of small RNAs were examined using support vector machines (linear kernel).

RESULTS

Characteristics of seminoma groups. The average age at diagnosis was 39.1 (± 7.2) years for non-metastasised, higher (45.2 years, ± 10.8) for lymphogenic metastasised and lower (32.1 years, ± 5.4) for occult metastasised seminoma. Primary tumour size was comparable between lymphogenic and occult metastasised seminoma (37.8 and 38.6 mm, respectively), but smaller (23.8 mm) in non-metastasised seminoma (Table 1).

RNA isolation. On average, we isolated almost two times as much total RNA from 10 to 20 mg of our lymphogenic (44.8 μg)/occult (44.5 μg) metastasised biopsy samples in comparison with the non-metastasised seminoma (22.6 μg; Table 1). The average RIN ranged between 8.0 and 8.9 for all of our samples. No DNA contamination could be detected in our samples.

Analysis of small RNA NGS results. The average total number of reads for lymphogenic/occult metastasised and non-metastasised seminoma was 1.3×10^7, 1.4×10^7 and 1.7×10^7 and on average 30–32% remained for further analysis after trimming of the reads (Table 2). From these reads, 59–68% appeared annotated reads, with 8.6–11% (3.6–5.7×10^4) representing annotated small RNAs. Of them, 137 small RNAs showed >50 reads and a two-fold difference to the reference. In univariate analysis, we identified 35, 32 and 38 small RNA species, which significantly discriminated lymphogenic/occult metastasised and both metastasis subtypes combined from non-metastasised seminoma, respectively (Table 3). The overlap of small RNAs separating either lymphogenic or occult metastasis from non-metastasised seminoma was 51–56% and increased up to 88% when comparing lymphogenic and occult small RNAs candidates with the small RNAs based on the combined metastasis subtypes (Figure 1). We finally used support vector machines enabling us to separate completely lymphogenic, occult metastasis and the combined metastasis subtypes from non-metastasised seminoma using a combination of two small RNA species only (Table 4). Altogether, 125, 52 and 6 combinations allowed a complete separation of lymphogenic ($n = 5$), occult metastasis ($n = 5$) and the combined metastasis subtypes ($n = 10$) from non-metastasised seminoma ($n = 5$), respectively.

DISCUSSION

In our study, we aimed to better discriminate metastasised (either lymphogenic or occult subtypes or both combined) from non-metastasised seminoma based on small RNA copy number changes examined in the primary tumour. A whole genome screening on small RNA species was performed using NGS. We demonstrated each metastasis subtype and its combination to be completely

Table 4. Summary on combinations of two small RNAs, which together allow to discriminate completely metastasis subtypes (lymphogenic and occult) and their combination from non-metastasised seminoma

Non- vs metastasised (lymphogenic and occult, n = 6)		Non- vs lymphogenic metastasised (n = 125)		Non- vs occult metastasised (n = 52)	
Small RNA1	Small RNA2	Small RNA1	Small RNA2	Small RNA1	Small RNA2
Mir-29a	Enst00000387347	Let-7a-1	Mir-99a	Let-7b	ENST00000466665
Mir-150	Mir-3676	Let-7a-2	Mir-99a	Mir-15a	ENST00000387347
Mir-150	Enst00000516461	Let-7a-3	Mir-99a	Mir-16-1	Mir-128-1
Mir-150	Enst00000516775	Let-7b	Mir-331	Mir-16-1	Mir-128-2
Mir-29c	Enst00000387347	Mir-15a	Mir-331	Mir-16-1	Mir-361
Mir-342	Mir-326	Mir-16-1	Mir-331	Mir-16-1	Mir-652
		Mir-18a	Mir-99a	Mir-19b-2	Mir-25
		Mir-22	Mir-99a	Mir-24-1	ENST00000466665
		Mir-23a	Mir-99a	Mir-24-2	ENST00000466665
		Mir-24-1	Mir-326	Mir-25	Mir-126
		Mir-24-1	Mir-331	Mir-25	Mir-342
		Mir-24-2	Mir-326	Mir-26a-1	ENST00000466665
		Mir-24-2	Mir-331	Mir-29a	Mir-326
		Mir-25	Mir-29a	Mir-29a	Mir-4454
		Mir-25	Mir-29b-1	Mir-29a	ENST00000474885
		Mir-25	Mir-29b-2	Mir-29a	ENST00000459522
		Mir-25	Mir-222	Mir-29a	ENST00000459949
		Mir-25	Mir-29c	Mir-29a	ENST00000463796
		Mir-29a	Mir-148a	Mir-29a	ENST00000466665
		Mir-29a	Mir-183	Mir-92a-1	Mir-150
		Mir-29a	Mir-15b	Mir-92a-2	Mir-150
		Mir-29a	Mir-128-1	Mir-99a	Mir-320a
		Mir-29a	Mir-128-2	Mir-99a	ENST00000466665
		Mir-29a	Mir-296	Mir-29b-1	Mir-326
		Mir-29a	Mir-378a	Mir-29b-1	Mir-505
		Mir-29a	Mir-340	Mir-29b-1	ENST00000474885
		Mir-29a	Mir-331	Mir-29b-1	ENST00000459522
		Mir-29a	Mir-423	Mir-29b-2	Mir-326
		Mir-29a	Mir-425	Mir-29b-2	Mir-505
		Mir-29a	Mir-625	Mir-29b-2	ENST00000474885
		Mir-29a	Mir-421	Mir-29b-2	ENST00000459522
		Mir-29a	Mir-744	Mir-182	Mir-342
		Mir-29a	ENST00000387347	Mir-181a-1	Mir-106b
		Mir-92a-2	Mir-29b-1	Let-7g	Mir-128-2
		Mir-92a-2	Mir-29b-2	Let-7g	Mir-106b
		Mir-93	Mir-182	Let-7g	Mir-378a
		Mir-93	Mir-183	Let-7g	Mir-505
		Mir-93	Mir-340	Let-7g	ENST00000387347
		Mir-93	ENST00000488123	Mir-30b	Mir-30e
		Mir-93	ENST00000463796	Mir-150	Mir-320a
		Mir-93	ENST00000466665	Mir-150	Mir-3676
		Mir-99a	Mir-107	Mir-150	ENST00000516461
		Mir-99a	Mir-30c-2	Mir-150	ENST00000516775
		Mir-99a	Mir-15b	Mir-106b	Mir-660
		Mir-99a	Mir-128-1	Mir-29c	Mir-505
		Mir-99a	Mir-128-2	Mir-29c	ENST00000474885
		Mir-99a	Mir-30c-1	Mir-29c	ENST00000459522

Table 4. (Continued)					
Non- *vs* metastasised (lymphogenic and occult, *n* = 6)		Non- *vs* lymphogenic metastasised (*n* = 125)		Non- *vs* occult metastasised (*n* = 52)	
Small RNA1	Small RNA2	Small RNA1	Small RNA2	Small RNA1	Small RNA2
		Mir-99a	Mir-361	Mir-296	Mir-342
		Mir-99a	Mir-331	Mir-26a-2	ENST00000466665
		Mir-99a	Mir-339	Mir-342	Mir-151a
		Mir-99a	Mir-425	Mir-342	ENST00000474885
		Mir-99a	Mir-505	Mir-342	ENST00000459522
		Mir-99a	ENST00000516881		
		Mir-29b-1	Mir-30c-2		
		Mir-29b-1	Mir-30d		
		Mir-29b-1	Mir-15b		
		Mir-29b-1	Mir-128-1		
		Mir-29b-1	Mir-128-2		
		Mir-29b-1	Mir-30c-1		
		Mir-29b-1	Mir-331		
		Mir-29b-1	Mir-425		
		Mir-29b-1	Mir-505		
		Mir-29b-1	ENST00000387347		
		Mir-29b-2	Mir-30c-2		
		Mir-29b-2	Mir-30d		
		Mir-29b-2	Mir-15b		
		Mir-29b-2	Mir-128-1		
		Mir-29b-2	Mir-128-2		
		Mir-29b-2	Mir-30c-1		
		Mir-29b-2	Mir-331		
		Mir-29b-2	Mir-425		
		Mir-29b-2	Mir-505		
		Mir-29b-2	ENST00000387347		
		Mir-16-2	Mir-331		
		Mir-16-2	ENST00000387347		
		Mir-197	Mir-150		
		Mir-182	Mir-142		
		Mir-183	Mir-142		
		Mir-183	ENST00000365160		
		Mir-181a-1	Mir-331		
		Mir-221	Mir-331		
		Mir-222	Mir-128-2		
		Mir-222	Mir-106b		
		Mir-222	Mir-331		
		Mir-222	ENST00000387347		
		Let-7g	Mir-15b		
		Let-7g	Mir-128-1		
		Let-7g	Mir-331		
		Mir-23b	Mir-331		
		Mir-125b-1	Mir-331		
		Mir-128-1	Mir-29c		
		Mir-142	Mir-130b		
		Mir-142	Mir-326		
		Mir-142	Mir-421		
		Mir-142	Mir-1260b		

Table 4. (Continued)					
Non- *vs* metastasised (lymphogenic and occult, *n* = 6)		Non- *vs* lymphogenic metastasised (*n* = 125)		Non- *vs* occult metastasised (*n* = 52)	
Small RNA1	Small RNA2	Small RNA1	Small RNA2	Small RNA1	Small RNA2
		Mir-142	ENST00000387347		
		Mir-142	ENST00000516881		
		Mir-145	Mir-331		
		Mir-125b-2	Mir-331		
		Mir-126	Mir-331		
		Mir-150	Mir-130b		
		Mir-150	Mir-331		
		Mir-150	Mir-345		
		Mir-150	Mir-421		
		Mir-150	Mir-766		
		Mir-150	Mir-744		
		Mir-150	Mir-1260b		
		Mir-150	Mir-3676		
		Mir-150	ENST00000516461		
		Mir-150	ENST00000516775		
		Mir-150	ENST00000387347		
		Mir-150	ENST00000410361		
		Mir-150	ENST00000431311		
		Mir-128-2	Mir-29c		
		Mir-29c	Mir-378a		
		Mir-29c	Mir-326		
		Mir-29c	Mir-331		
		Mir-29c	Mir-425		
		Mir-29c	Mir-744		
		Mir-29c	ENST00000387347		
		Mir-328	Mir-331		
		Mir-148b	ENST00000365160		
		Mir-331	Mir-574		
		Mir-331	Mir-660		
		Mir-425	Mir-660		

The total number of small RNA combinations per group is shown within parentheses.

discriminated from non-metastasised seminoma using two small RNAs combined.

MiRNAs are previously described to be involved in different distant (e.g. migration, angiogenesis and colonisation of distant organs) and local (e.g. changes in tumour microenvironment) processes in metastatic cascade. Interestingly, the miRNAs identified in our study are involved in local tumour microenvironmental changes only. For instance, miRNAs 221 and 222 are involved in degradation of extracellular matrix via regulation of MMP-1 and associated with more aggressive lung cancer (Garofalo *et al*, 2009; Liu *et al*, 2009). Let-7 and miR-181 are involved in inflammatory processes as a part of the metastatic cascade. The Let-7 family targets oncogenes such as *HMGA2* and *KRAS* and seems to be a key component in the so-called epigenetic switch (Johnson *et al*, 2005; Mayr *et al*, 2007; Iliopoulos *et al*, 2009). MicroRNA-181 inhibits tumour suppressor genes *PTEN* and *CYLD* (Iliopoulos *et al*, 2010). Cancer-associated fibroblasts are involved in tumour formation and progression where miR-15 and miR-16 regulate *FGF2* and *FGFR1* in prostate cancer (Musumeci *et al*, 2011), miR-18 and miR-19 in breast cancer (Yu *et al*, 2010) and miR-320 is reprogramming the tumour microenvironment by PTEN regulation (Bronisz *et al*, 2012). Owing to the link of our miRNAs with metastatic processes identified in other tumour entities, it could be speculated about their significance for identification of metastasis in other tumour entities. However, separate studies such as ours are necessary to prove that.

We did also identify miRNA changes being associated with cisplatin resistance in germ cell tumours (Port *et al*, 2011a). Recently, other authors demonstrated that miRNA 371-73 cluster and miRNA 302 allowed discriminating between seminoma-bearing patients and healthy persons (Gillis *et al*, 2007; Palmer *et al*, 2010; Dieckmann *et al*, 2012). In the next step, we examined the suitability of these miRNA to predict the metastasis status, but these miRNAs in our analysis appeared not significantly associated with the metastasis status.

The complete separation of metastasis from non-metastasised seminoma using a combination of two small RNA species points to the significant diagnostic potential of these biological markers, which is in line with previous examinations on the transcriptional level showing the superiority of molecular marker over

epidemiological or clinical–histological parameter (Port *et al*, 2011b; Ruf *et al*, 2012).

Interestingly, the discrimination in our study occurred irrespective of the metastasis subtype, a finding that was expected, as we recently demonstrated lymphogenic and occult metastasised seminoma to be indistinguishable on the transcriptional level (Ruf *et al*, 2014).

The tumour size in this study appears different between metastasised and non-metastasised seminoma, indicating a potential for discriminating both groups. According to previous studies performed on larger groups ($n = 527$), the association of tumour size with metastasis status appeared significant, but the discriminatory capacity of this and other clinical–histological parameter (e.g. tumour/testicular volume, tumour length) did not exceed a concordance of 65% (Ruf *et al*, 2013b), indicating a limited discrimination ability of tumour size in contrast to the small RNAs examined in this study. Moreover, according to our experiences RNA examinations should be performed in cryopreserved tissues or using solvents to protect RNA. We cannot recommend performing RNA analysis on paraffin-embedded tissue because of rapid and tissue-specific RNA degradation taking place during the fixation process with alcohol (Port *et al*, 2007).

Our study has certain weaknesses such as the low number of cases examined (total $n = 15$). However, the molecular biological methodology applied provides a deepness that under financial considerations does not allow large-scale studies. Hence, this study provides hints towards certain small RNA species comprising a significant diagnostic potential for prediction of metastasis in seminoma, but certainly these candidate small RNA species have to be examined on a larger independent group for validation purposes using qRT–PCR as the gold standard for gene expression measurements.

CONCLUSION

In summary, metastasised and non-metastasised seminoma can be completely discriminated with a combination of two small RNAs irrespective of apparent lymphogenic or occult metastasis.

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