Expression of microRNAs of C19MC in Different Histological Types of Testicular Germ Cell Tumour

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Abstract. Background: Testicular germ cell tumours (TGCTs) are the most common tumours in men aged from 20 to 40 years, with a steadily increasing incidence. This study aimed to characterize the expression of the miRNA cluster C19MC in TGCT and to evaluate the suitability of a C19MC miRNA as a serum biomarker. Materials and Methods: By quantitative reverse transcription PCR, we measured the expression of miR-517a-3p, miR-519a-3p, and miR-519c-3p in tissue samples of 25 TGCTs and the level of miR-517a-3p in serum samples obtained pre- and postoperatively from the same patients. Results: We detected a significantly higher expression of C19MC miRNAs in non-seminomas than in seminomas and in clinical stages 2 and 3 than in stage 1 in both tissue and serum samples. Conclusion: miRNAs of C19MC are overexpressed in more aggressive types of TGCT, suggesting they contribute to malignancy. Furthermore, they might serve as serum biomarkers for these types of TGCT.

Testicular cancer is the most frequent malignancy amongst men between the ages of 20 and 40 years (1). Even though testicular cancer only represents between 1% and 1.5% of male neoplasms (2), its incidence has been steadily increasing over the past decades (1). Testicular germ cell tumours (TGCT) are divided into seminomas and non-seminomas, the latter being further subdivided into embryonal carcinomas (ECs), yolk sac tumours (YST), choriocarcinomas, and teratomas. It is assumed that all TGCTs stem from a common precursor lesion called intratubular germ cell neoplasia unclassified (ITGCNU or IGCNU; also referred to as testicular intraepithelial neoplasia or carcinoma in situ) which, in turn, develops from a primordial germ cell (PGC) or gonocyte whose maturation is delayed or blocked (3). Voorhoeve et al. were the first to show that microRNAs are involved in the pathogenesis of TGCTs (4).

MicroRNAs (miRNAs) are small non-coding RNA molecules of approximately 22-24 nucleotides in length involved in the post-transcriptional regulation of gene expression. Depending on their sequence, they are able to specifically target mRNAs and repress their translation. As mediators of post-transcriptional regulation, miRNAs play an important role in various cellular processes, such as proliferation, differentiation and apoptosis but have also been associated with tumourigenesis. Accordingly, miRNA expression studies have been performed for many human tumour tissues [reviewed in (5)], including TGCTs. Two miRNA clusters were found to be consistently overexpressed in all histologic types of TGCT except for teratomas (4, 6-11). One of these is the miR-302/367 cluster, consisting of five genes located on chromosome 4; the other cluster is miR-371-3, a cluster of three miRNA genes mapping to chromosomal sub-band 19q13.4. The miRNAs of these two clusters are normally expressed in human embryonic stem cells (12), and in addition, miR-371-3 is also expressed in placental tissue (13). Reflecting their high expression in TGCT tumour tissue, miR-371-3 and miR 302/367 miRNAs are detectable in serum samples from patients with TGCT and a strong postoperative decrease has been noted, especially for miR-371a-3p, making these miRNAs promising candidates for serum-based TGCT biomarkers (11, 14-20). The miR-371-3 cluster is located in close vicinity to another miRNA cluster, namely C19MC (chromosome 19 miRNA cluster). C19MC consists of 46 miRNA genes encoding for 62 mature miRNAs (21). All mature miRNAs of C19MC are apparently processed from one large non-protein-coding polymerase II transcript (22). The cluster’s expression is regulated by a CpG island located about 17.6 kb upstream
and occurs exclusively from the paternal allele (24).
Just like miR-371-3, C19MC is exclusively expressed in
the placenta (13, 21, 25) and embryonic stem cells (26-30),
whereas in adult somatic cells, no or only very low
expression levels were detected (13, 31).
C19MC and cluster miR-371-3 have been associated with
oncogenic functions in various types of cancer (4, 32-38);
they have been shown to become drastically up-regulated by
chromosomal translocations in benign thyroid tumours (39),
by genomic rearrangements and amplifications of the
chromosome band in rare childhood brain tumours (40-42),
and by amplifications in parathyroid carcinomas (43).
Since miR-371-3 miRNAs have been found to be
expressed in almost all types of TGCTs (4, 6-11), we
hypothesized that these tumours might also express C19MC
miRNAs as miR-371-3 and C19MC are coexpressed in many
cases, e.g. in embryonic stem cells, placenta and the above
mentioned tumour entities. Therefore, we performed
quantitative reverse transcription PCR (qRT-PCR) for three
different members of C19MC (miR-517a-3p, miR-519a-3p,
and miR-519c-3p) on 25 primary TGCT samples. The results
were compared to the expression of these miRNAs in normal
testis and other normal adult tissues.

miRNAs are being increasingly recognized as non-invasive
biomarkers of cancer. The miRNAs of miR-371-3 have been
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miRNAs have been demonstrated to be detectable in serum
and plasma, particularly of pregnant women (44-46), where
they have been linked to a number of pregnancy-related
conditions such as pre-eclampsia [reviewed in (47)]. In order

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Table I. Samples used in this study.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histology</th>
<th>Clinical stage (Lugano)</th>
<th>Examined sample type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tissue</td>
</tr>
<tr>
<td>1</td>
<td>80-90% Cystic teratoma, 5-10% EC, 5% YST, single CC cells</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Seminoma, &lt;5% yolk sac tumour</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>50% Teratoma, 45% EC, 5% YST</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Seminoma: including ITGCNU</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Seminoma</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>EC; including ITGCNU</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>EC</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Seminoma</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>60% EC, 35% YST, 5% seminoma; including ITGCNU</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>70% Teratoma, 30% CC</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Seminoma</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Seminoma</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>60% Seminoma, 40% EC</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>EC; including ITGCNU</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>Seminoma</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>60% YST, 30% EC, 10% choriocarcinoma; including ITGCNU</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>Seminoma</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>Seminoma</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>50% EC, 50% seminoma</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>60% EC, 40% seminoma; extensive ITGCNU</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>&gt;95% Teratoma, &lt;5% EC</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>EC</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>Seminoma; including ITGCNU</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>Seminoma; including ITGCNU</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>25</td>
<td>Seminoma</td>
<td>1</td>
<td>+</td>
</tr>
</tbody>
</table>

CC: Choriocarcinoma; EC: embryonal carcinoma; YST: yolk sac tumour; ITGCNU: intratubular germ cell neoplasia unclassified.

Table II. Control samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Histology/diagnosis</th>
<th>Examined sample type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tissue</td>
</tr>
<tr>
<td>NT 1</td>
<td>Testis</td>
<td>+</td>
</tr>
<tr>
<td>NT 2</td>
<td>Testis</td>
<td>+</td>
</tr>
<tr>
<td>Duodenum</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Endometrium</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Thyroid</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Control 1</td>
<td>Non-TGCT related</td>
<td>-</td>
</tr>
<tr>
<td>Control 2</td>
<td>Non-TGCT related</td>
<td>-</td>
</tr>
<tr>
<td>Control 3</td>
<td>Non-TGCT related</td>
<td>-</td>
</tr>
</tbody>
</table>

NT: Normal testis; TGCT: testicular germ cell tumour.
to explore if C19MC miRNAs are also suitable as serum biomarkers for TGCTs, we measured the levels of miR-517a-3p in pre- and postoperative serum samples obtained from the same patients as the tumor tissue samples.

**Materials and Methods**

**Testicular germ cell tumor and normal tissue samples.** Formalin-fixed paraffin-embedded (FFPE) samples of primary TGCTs from 25 patients were used in the study. The samples included 12 seminomas and 13 non-seminomas (four ECs and nine mixed tumors of various compositions) of tumor stages 1-3 (Table I). Additionally, two samples of apparently normal testicular tissue and three samples of other normal adult tissues (endometrium, duodenum, and thyroid) were investigated (Table II).

**Serum samples from patients with TGCT and without TGCT.** Cubital vein blood was obtained from 22 out of the 25 patients undergoing orchiectomy pre- and postoperatively during routine blood sampling (Table I). Additionally, three serum samples were obtained from men undergoing medical treatment for non-TGCT-related reasons. The study was approved by the local Ethics Committee (Arztekammer Bremen, approval number: 301, 2011) and informed consent was given by all patients. Serum was prepared within 2 h of blood draw and stored at –80°C until used for RNA isolation.

**RNA isolation.** Total RNA was isolated from FFPE samples with the innuPREP Micro RNA Kit (Analytik Jena AG, Jena, Germany) according to the manufacturer’s instructions with the following modifications: Lysis of the paraffin sections preceding RNA isolation was conducted using TLS-Lysis Solution and Proteinase K from the innuPREP DNA Micro Kit (Analytik Jena AG, Jena, Germany) without prior deparaffinization. Sections were incubated for 1 h at 60°C and 15 min at 80°C. From serum samples, total RNA was isolated using the QIAGEN miRNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions with minor modifications for serum samples: for 200 μl serum, 1 ml of QIAzol and 200 μl chloroform were used.

**Quantitative reverse transcription PCR (qRT-PCR).**

**FFPE samples.** For FFPE tissue samples, reverse transcription of miR-517a-3p, miR-519a-3p, miR-519c-3p, and RNA, U6B small nuclear (RNU6B) (for normalization) was performed using the TaqMan microRNA RT Kit (Life Technologies Corporation, Carlsbad, CA, USA) and specific stem-loop primers supplied with the respective TaqMan microRNA assays (assay IDs: miR-517a-3p: 002402, miR-519a-3p: 002415, miR-519c-3p: 001163, RNU6B: 001093; Life Technologies Corporation) as described by the manufacturer with an input of 200 ng of total RNA. qRT-PCR reactions were carried out on a 7300 Real-Time PCR System (Life Technologies Corporation) using Universal PCR Master Mix (2×) (Life Technologies Corporation) and the specific TaqMan microRNA assays mentioned above according to the manufacturer’s instructions. Relative quantity (RQ) was calculated using the ΔΔCt method (48).

**Serum samples.** For quantification of miR-517a-3p in serum samples, 6 μl of total RNA were reverse transcribed using the TaqMan microRNA RT Kit (Life Technologies Corporation) and a primer pool consisting of 1 μl each of the stem loop primers for miR-517a-3p and miR-93-5p (for normalization) as specified above.
Because of the low concentration of RNA in serum, a pre-amplification step was performed prior to qPCR. The pre-amplification reaction consisted of 4 μl of RT product, 1.12 μl assay (diluted 1:100 with nuclease-free water) each of miR-517a-3p and miR-93-5p, 4 μl 5x Real-Time ready cDNA Pre-Amp Master (Roche, Mannheim, Germany) and nuclease-free water to make a total reaction volume of 20 μl. Pre-amplification was performed at 95˚C for 1 min, followed by 14 cycles of 95˚C for 15 s and 60˚C for 4 min. The pre-amplification product was then diluted 1:2 in nuclease-free water and 5 μl of the diluted pre-amplification product were used for qPCR. The qPCR reaction consisted of 10 μl of the FASTstart Universal Probe Master (Roche), 1 μl of the specific assay, and nuclease-free water in a total reaction volume of 20 μl. qPCR was performed on a 7500 Fast Real-Time PCR System (Life Technologies Corporation) with the following cycling conditions: 10 min at 95˚C, then 40 cycles of 15 s at 95˚C and 1 min at 60˚C. RQ was calculated using the ΔΔCt method (48).

Statistical analysis. The Mann–Whitney U-test was used to determine differences in expression between different tumour types and clinical stages. The Pearson correlation coefficient was calculated to determine correlations. Statistical analysis was carried out with GraphPad PRISM software (Version 6.07) (GraphPad Software, Inc., La Jolla, CA, USA). A p-value of less than 0.05 was considered significant.

Results

C19MC is highly expressed in normal testicular tissue.

Before focusing on TGCTs, we were interested to determine the level of C19MC miRNAs in normal testicular tissue in comparison to other normal adult tissues. Therefore, we quantified the expression of miR-517a-3p, miR-519a-3p, and miR-519c-3p in two samples of normal testis and in three samples of normal somatic adult tissues (duodenum, endometrium, and thyroid). For all miRNAs tested, the expression in testicular tissue clearly exceeded that observed in any of the somatic adult tissues (by between about 53- and 1086-fold) (Figure 1).

Expression of C19MC differs depending on histological type and clinical stage.

Next, we quantified the expression of miR-517a-3p, miR-519a-3p, and miR-519c-3p (Figure 2) in 25 TGCT samples.

There was a strong correlation between the expression of the three C19MC miRNAs (miR-517a-3p vs. miR-519a-3p: r=0.93, p<0.01; miR-517a-3p vs. miR-519c-3p: r=0.904, p<0.01; miR-519a-3p vs. miR-519c-3p: r=0.971, p<0.01) but strong variation in the expression of these miRNAs among the samples. Low levels, i.e. similar or even lower than those observed in normal testicular tissues, characterized seminomas of clinical stage 1 as well as mixed tumours with a predominant teratoma component of clinical stage 1. Higher expression levels were found in non-seminomas (except the aforementioned tumours with a high teratoma portion) regardless of clinical stage. Furthermore, seminomas
and tumours with a predominant teratoma component of clinical stage 3 were also characterized by higher expression. The expression levels of miR-517a-3p \((p<0.01)\), miR-519a-3p \((p<0.01)\), and miR-519c-3p \((p<0.05)\) were significantly different between seminoma and non-seminoma samples. The results presented above suggest a different expression pattern in teratoma tissues than in other non-seminoma samples. However, the number of teratoma samples was limited.

Figure 3. Level of miR-517a-3p in serum samples obtained pre- and postoperatively from patients with disease at clinical stage 1 (A) and clinical stages 2 and 3 (B). Sample IDs are also indicated.
samples in the sample set is too small for them to be evaluated separately. There were also significant differences in the expression levels of miR-517a-3p ($p<0.01$), miR-519a-3p ($p<0.01$), and miR-519c-3p ($p<0.01$) between the samples of clinical stage 1 and the samples of stages 2 and 3 (combined). Overall, the data show that C19MC expression levels depend on tumour type and clinical stage.

miR-517a-3p was detectable in serum of patients with TGCT, with higher levels expressed in advanced clinical stages and non-seminomas. The expression of miR-517a-3p was quantified in pre- and postoperatively obtained serum samples from 22 out of the 25 patients with TGCT. No serum samples were available for cases 1, 3, and 5 (Table I). The expression levels were compared to those of three male individuals hospitalized for non-TGCT-related reasons. In line with our previous results, elevated levels of miR-517a-3p were found in most serum samples of patients with non-seminomatous tumours with clinical stage 1 (Figure 3A) and in seminomas and non-seminomas from patients with clinical stage 2 or 3 disease (Figure 3B). However, there were two exceptions: the serum samples of non-seminomatous cases 13 and 16 (both clinical stage 1) had preoperative serum miR-517a-3p levels in the range of the controls. Interestingly, the serum sample of patient 21 (>95% teratoma) also had an elevated miR-517a-3p level, even though no overexpression was observed in the corresponding tissue.

A significantly higher level of miR-517a-3p was observed in serum samples of patients with clinical stages 2 or 3 as compared to clinical stage 1 samples ($p<0.01$; Figure 4A) and in seminoma vs. non-seminoma serum samples ($p<0.01$; Figure 4B). miR-517a-3p was also measured after surgical removal of the tumour. If the level of miR-517a-3p indeed indicates the presence of a tumour, then the levels should drop to the level of the controls when the patient is tumour-free. In patients with clinical stage 1 disease this should be the case after surgical removal of the tumour, in metastasized cases at the latest after completion of chemotherapy.

In all cases with clinical stage 1 disease that had elevated miR-517a-3p levels preoperatively, a decline to the level of the controls was observed after surgery (Figure 3A). Of the seven serum samples of patients with metastatic disease (clinical stage 2 and 3) a postoperative decline below the level of the controls was observed in four cases (patients 2, 8, 14, and 19), in two cases the level decreased but was still above that of the controls (patients 10 and 22). One case (patient 7) even displayed a higher serum level after surgery (Figure 3B).

Discussion

In this study, we demonstrated that miRNAs of C19MC are highly expressed in normal testicular tissue compared to other normal adult tissues. Most interestingly, we found that C19MC is also expressed in TGCTs, but in contrast to miR-371-3 that is overexpressed in all TGCTs except teratomas (4, 6-11), we found C19MC to be differentially expressed depending on histological type and clinical stage. Higher expression of C19MC characterized non-seminomas (except teratomas) and advanced clinical stages regardless of histology. These results support and complement previously reported results: In paediatric germ cell tumours of male and female origin, Palmer
et al. analyzed miRNA expression by microarray expression profiling and found that members of C19MC belonged to the top-ranked overexpressed miRNAs in paediatric ECs compared to non-malignant tissues (7). Novotny et al. also used microarrays to examine miRNA expression in adult seminomas, ECs and ITGNCU cells (10). They observed up-regulation of many miRNAs of C19MC (including miR-519c-3p) in ECs in comparison to normal testis, seminomas and ITGNCU cells. However, in their study, other members of C19MC (including miR-519a-3p) displayed similarly high expression in EC, seminomas, ITGNCU and normal testis. This discrepancy might be due to the different methods used in the two studies. Novotny et al. examined only seminomas and EC (and no other types of non-seminomas), their focus being on ITGNCU cells, but provided no information on clinical stage of the samples (10). To the best of our knowledge, we are the first to provide evidence that tumours of other histological types also overexpress C19MC miRNAs and that the expression is higher in those with advanced clinical stage. It is possible, however, that the overexpression in mixed tumours containing EC tissue is significant due to this component. Three out of the four samples displaying the highest C19MC expression in our study were pure ECs, the fourth comprised 50% EC tissue. However, sample 10, that did not contain any EC tissue, also displayed an elevated expression level. In order to determine which components in mixed tumours in fact contribute to overexpression of C19MC (and to what extent), in situ hybridization experiments for miRNAs could be performed.

The reasons for the different expression of C19MC in the different histological subtypes of TGCTs are unknown. TGCTs are assumed to originate from PGCs. In early PGCs, imprinting genes are methylated according to their parental origin (49) but shortly after they enter the genital ridge, PGCs undergo global demethylation including the erasure of parental imprints (49-50). In line with this, CpG islands of imprinting genes are usually found to be unmethylated in both seminomas and non-seminomas (51). If this is in fact the case for the CpG island of C19MC in all histological types of TGCT, other factors must be responsible for the different expression. However, it is possible that some tumour types undergo de novo methylation of the CpG island during their development; particularly in teratomas, silencing of the C19MC CpG island would be expected upon differentiation. It would therefore be of great interest to examine the methylation status of the C19MC CpG island in different tumour subtypes and stages, as well as in PGCs and ITGNCU cells.

As to the functions of C19MC miRNAs, these miRNAs have been associated with oncogenic but also tumour-suppressive functions in various tumour entities [reviewed in (52)]. For example, the up-regulation of C19MC has been observed in hepatocellular carcinomas where miR-517a-3p, miR-519d-3p and miR-520c-3p were demonstrated to promote proliferation, invasion and migration, and inhibit apoptosis (35, 38). The up-regulation of C19MC (and particularly the overexpression of miR-519a-3p) is associated with poorer overall survival in oestrogen receptor-positive breast cancer and enhances cell viability (37). By contrast, miR-519a-3p, miR-519b-3p, and miR-519c-3p have been shown to act as tumour suppressors in cancer of the ovary, lung and kidney by reducing proliferation and tumour growth (53-54). miR-519a-3p was shown to inhibit tumour growth in laryngeal squamous cell carcinoma (55) and miR-517a-3p to reduce proliferation and induce apoptosis in bladder cancer (56). However, we found C19MC miRNAs to be significantly overexpressed in non-seminomas, which are generally more aggressive than seminomas, with a lower average age of disease onset (3), and in advanced tumour stages, suggesting that C19MC miRNAs might be involved in promoting malignant behaviour in TGCTs such as metastasis.

Over recent years, miRNAs have evoked increasing interest in the search for new biomarkers for diseases as they have been shown to be extremely stable in serum and plasma (57). For TGCTs, most studies have focused on miRNAs of the clusters miR-371-3 and miR-302/367 (11, 14-19). Of note, a recent study undertook a high-throughput approach to screen for other possible miRNA serum biomarkers (20). The authors confirmed the previously identified miR-371a-3p and miR-372-3p to be specifically elevated in serum of patients with TGCT. Additionally, nine other miRNAs were shown to be discriminative between germ cell cancer and controls. As our results obtained from tissue samples showed that C19MC miRNAs were elevated in a subset of TGCTs, we sought to evaluate the potential of C19MC miRNAs as serum biomarkers for those cases. A suitable marker should be elevated in patients and decrease to a level observed in controls after completion of therapy. Our data suggest that miR-517a-3p fulfils these requirements for patients with non-seminomatous tumours and those with advanced clinical stages. However, these data must be validated with a larger patient cohort and, in particular, it needs to be investigated whether the serum levels of patients with clinical stage 2 or 3 indeed decrease to the levels of controls after completion of therapy. Nevertheless, herein we provide initial evidence that miR-517a-3p (and possibly other C19MC members) might serve useful as serum biomarkers for use in surveillance and therapy monitoring of these patients.

References


