Universität Bremen

microRNAs as novel biomarkers for patients with testicular germ cell tumours

„microRNAs als neuartige Biomarker für Patienten mit testikulären Keimzelltumoren“

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1 Introduction

1.1 Testicular cancer

Testicular cancer (TC) is the most common solid malignancy in young adult men between the ages of 14 and 44 years and its incidence has risen worldwide in the past two decades (Trabert *et al.*, 2015; Siegel *et al.*, 2018). Worldwide TC accounts for ~1 % of newly diagnosed cancer and the incidence varies with ethnic origin with the highest in Scandinavian countries and the lowest in African and Asian countries (Ferlay *et al.*, 2015). In Germany, there are 4070 new cases per year (Robert Koch-Institut, 2017).

The risk factor that is most consistently associated with TC is cryptorchidism, which increases the risk nearly 5-fold (Purdue *et al.*, 2005; Bray *et al.*, 2006). Further risk factors are hypospadias (Trabert *et al.*, 2013), previous testicular cancer (developing cancer in the contralateral testis) (Fossa *et al.*, 2005; Kier *et al.*, 2016), low sperm count (Rud *et al.*, 2013), Klinefelter syndrome (Aguirre *et al.*, 2006) and a family history of TC with an 8-fold to 10-fold higher risk (Hemminki and Li, 2004). Testicular tumours are now highly treatable and the overall 5-year survival rate of men with TC exceeds 95 % (Stang *et al.*, 2013).

Most testicular tumours (about 90 - 95 %) arise from germ cells to generate the “GCT”, followed by gonadal stromal tumour (5 - 10 %), mixed GCT and secondary tumours (Boccellino *et al.*, 2017). Testicular tumours are classified in the World Health Organisation (WHO) classification. In the prior version of 2004 the classification was purely morphologically based and divided the germ cell tumours into those of a single or those of more than one histologic type (Eble *et al.*, 2004). The WHO published in 2016 a new classification system for GCTs with significant differences in comparison to the prior version of 2004. Now the WHO-recommended term for precursor lesions of invasive germ cell tumours of the testis is germ cell neoplasia in situ (GCNIS) and the testicular germ cell tumours are separated into two fundamentally different groups: those derived from GCNIS and those unrelated to GCNIS (Moch *et al.*, 2016). The initial description of GCNIS in 1972 used the term carcinoma in situ (CIS) (Skakkebæk, 1972) and GCNIS were formerly also refereed as testicular intraepithelial neoplasia (TIN) (Dieckmann and Loy, 1993) or intratubular germ cell neoplasia unclassified (IGCNU) (Eble *et al.*, 2004). Furthermore, in the new 2016 WHO classification the spermatocytic seminoma has been designated as a spermatocytic tumour and placed within the group of non-GCNIS-related tumours (Moch *et al.*, 2016).

1.2 Testicular germ cell tumours

Testicular tumours that arise from GCNIS are labelled as type II GCT. They are classified separately from those of other origin (type I and III) and are always malignant. These tumours occur in adolescents and young adults and are histologically subdivided into seminoma and nonseminoma. Type I tumours usually occur in paediatric (prepubertal, usually < 14 years of age) patients, although they can occur rarely in adult patients. These tumours are histologically subdivided into teratoma tumours (which are benign) and yolk sac tumours (which are malignant). Type III tumours (previously known as spermatocytic seminoma) are histologically composed of polymorphous populations of three types of tumour cells: small, intermediate and giant tumour cells (Cheng *et al.*, 2018).

In adults (type II GCT), morphologically homogeneous seminoma account for about 60 % of all testicular GCTs and heterogeneous nonseminomatous GCTs account for 40 % (Rajpert-De Meyts *et al.*, 2016). GCTs show a 'bell-shaped' age distribution of cases with a peak around 30 years, with seminoma overall occurring ten years later than nonseminoma. Median age at diagnosis is 33 years-old (25 years for nonseminoma, 35 years for seminoma) and an intermediate of 30 years for nonseminoma with a seminoma component. The majority (68 % comprising > 80 % of seminoma and > 60 % of nonseminoma) is diagnosed with localized disease (clinical stage I). Metastases emerge in 15 % and 20 % of stage I seminoma and nonseminoma patients, respectively, within a period of two to three years (Lobo *et al.*, 2019). Nonseminomatous type II GCTs types include embryonal carcinoma, yolk sac tumour, trophoblastic tumours (mainly choriocarcinoma) and teratoma. Mixed malignant GCTs composed of different germ cell types are common, representing 30 % of all testicular cancers (Stewart and Wild, 2014). These GCTs are totipotent, which is most likely related to their common cell of origin, GCNIS (Cheng *et al.*, 2018). Type II GCTs are usually associated with anomalies of the short arm of chromosome 12 (12p), such as isochromosome 12p, a condition in which the long arm of chromosome 12 is lost and the short arm is duplicated (Looijenga and Oosterhuis, 1999).

During human foetal testicular development, a rapid transition from primordial germ cells (PGCs) (which in the testis are germ cells not yet enclosed in seminiferous cords) to gonocytes takes place, later followed by much slower differentiation of gonocytes into pre-spermatogonia (also called infantile spermatogonia). At that time, the cells gradually lose their embryonic characteristics while acquiring features of germ cells manifested by the expression of male specific genes (Rajpert-De Meyts, 2006). Studies have shown that GCNIS cells seem to arise from PGCs or gonocytes and reside dormant in the testis until they start proliferating after puberty and eventually develop into a testicular tumour. It remains to be determined why these gonocyte-like cells do not differentiate to spermatogonia, but persist in postnatal testes. Human

spermatogonia do not express the pluripotency genes characteristic of GCNIS (Sonne *et al.*, 2009). The confirmation of GCNIS cells as arrested gonocytes indicates that the transition from gonocytes into spermatogonia is an essential area of study in order to understand the aetiology of GCT (McIver *et al.*, 2012). GCNIS is present in the testis many years before the clinical manifestation of the GCT (Dieckmann and Skakkebaek, 1999). For patients with GCNIS there is a cumulative probability of 70 % for manifestation of a testicular tumour after seven years (Krege *et al.*, 2001).

The staging system of testicular cancer is the 2017 Tumour, Node, Metastasis (TNM) of the International Union Against Cancer (UICC) including a S category for serum tumour markers (O'Sullivan *et al.*, 2017). According to the 2009 TNM classification, testicular cancer includes different substages (stage 0, I, IA, IB, IS, II, IIA, IIB, III, IIIA, IIIB, IIIC). Patients with clinical stage (CS) I have only a primary tumour, but patients with stage IS show evidence for a metastatic disease because of increased tumour markers (Albers *et al.*, 2015). Since 1997 metastatic testis tumours are classified according to the International Germ Cell Cancer Collaborative Group (IGCCCG) prognostic criteria (IGCCCG, 1997). Treatment decisions of testicular GCTs are based on the histological classification and clinical staging (Murray *et al.*, 2016).

Patients with testicular GCTs usually present a unilateral painless testicular mass (Rajpert-De Meyts *et al.*, 2016). Diagnostic means for testicular GCTs include palpation and sonography of the testicles and determination of the tumour markers α-fetoprotein (AFP), β-subunit of human chorionic gonadotropin (β-HCG or bHCG) and lactate dehydrogenase (LDH) in serum samples (Krege *et al.*, 2001).

After ultrasound, surgery must be performed for every patient if a malignant tumour is found. In cases of life-threatening disseminated disease due to extensive metastases, lifesaving chemotherapy should be given up-front (Albers *et al.*, 2015). Testicular GCTs CS1 are initially treated with orchiectomy, although management after orchiectomy varies. Chemotherapy is routinely administered for testicular GCT patients with CS2-3 (Murray *et al.*, 2016). In patients with stage 2A/B seminoma, chemotherapy with three courses of BEP (cisplatin, etoposide, bleomycin) or four courses of etoposide and cisplatin (EP) is recommended. Alternative a radiotherapy is possible and radiotherapy and chemotherapy appeared to be similarly effective in both stages. For patients with stage 2A/B nonseminoma primary chemotherapy and primary ‘nerve-sparing’ retroperitoneal lymph node dissection (RPLND) are comparable options. For metastatic disease with CS2C and CS3, the chemotherapy treatment depends on the prognosis risk group (Albers *et al.*, 2015).

Serum tumour markers play a critical role in the diagnosis, staging, risk stratification, and surveillance of patients with GCT. The rate of tumour marker decay after radical orchiectomy

is an important index to monitor, as a slow decline might be indicative of metastatic disease and should prompt a thorough systemic survey. The rate of tumour marker decline is already being utilized in the setting of metastatic GCTs to determine response to chemotherapy. Compared to any other solid organ malignancy, the role of serum tumour markers in GCT is unprecedented and these markers are instrumental in the diagnosis and management of testicular GCT (Barlow *et al.*, 2010). However, these three markers are increased in only approximately 60 % of testicular cancer cases (Mir *et al.*, 2016). AFP and β-HCG are secreted by nonseminomatous tumours, yolk sac tumour and syncytiotrophoblast of choriocarcinoma, whereas LDH is also secreted by seminoma (Rajpert-De Meyts *et al.*, 2015). Nevertheless, the role of LDH in the follow-up is debatable. It has limited sensitivity and specificity and a high rate of false-positive tests are found (Cathomas *et al.*, 2010). In seminoma less than 30 % show elevated β-HCG level during the course of the disease (Mir *et al.*, 2016). The interpretation of serum levels of these markers in patients with seminoma, pure embryonal carcinoma and teratoma is sometimes difficult and many cases are marker-negative (Rajpert-De Meyts *et al.*, 2015). So far, no consistent marker for the stem cell components seminoma and embryonal carcinoma is available, which limits the use of the serum markers for diagnosis and follow-up in a large proportion of germ cell cancers (Gillis *et al.*, 2013). Thus, because of the limited sensitivity additional markers are needed and several researchers are working on improved non-invasive biomarkers (Syring *et al.*, 2015). Recently, microRNAs (miRNAs) have been suggested to be a novel class of serum biomarkers (Cortez *et al.*, 2011). Their expression is frequently altered in urologic cancer and can be measured in body fluids (Catto *et al.*, 2011). Furthermore, they have the potential to qualify as biomarkers in various malignancies because they mostly reveal a high stability in body fluids (Mitchell *et al.*, 2008).

1.3 microRNAs (miRNAs)

In 1993, Rosalind C. Lee *et al.* discovered the *lin-4* gene, which is essential for the control of postembryonic development in Caenorhabditis elegans (C. elegans). This gene encoded for two small lin-4 transcripts instead of proteins. The larger transcript had a length of 61 nucleotides and the smaller transcript of 22 nucleotides. They suggested that lin-4 regulates the *lin-14* translation by binding the 3' untranslated region (UTR) of lin-14 mRNA (Lee *et al.*, 1993). This was the first finding of a small RNA. The shorter lin-4 RNA is now recognized as the founding member of an abundant class of tiny regulatory RNAs called microRNAs or miRNAs (Lee and Ambros, 2001).

Reinhart and his colleagues could show the existence of a second miRNA in C. elegans called let-7 only seven years later. This 21 nucleotide long miRNA controls genes also by binding to

the UTR. A dysregulation of let-7 leads to a loss of function of the *lin-4* gene (Reinhart *et al.*, 2000).

Since these findings, the number of known miRNAs is increasing rapidly. The latest release of the miRNA database miRBase (v22) in 2018 contains miRNA sequences from 271 organisms: 38589 hairpin precursors and 48860 mature miRNAs. This represents an increase in sequences of more than a third over the previous release. The human genome contains 1917 annotated hairpin precursors and 2654 mature sequences (Kozomara *et al.*, 2019).

miRNAs are small single stranded non-coding RNA molecules with approximately 22 nucleotides and they play an important regulatory role by targeting mRNAs for cleavage or translational repression (Bartel, 2004). They have been shown to control cell growth, differentiation, apoptosis and their expression has been implicated in tumourigenesis (Esquela-Kerscher and Slack, 2006; Farazi *et al.*, 2011). A single miRNA usually concurrently regulates a large number of target genes, and one gene might be regulated by multiple miRNAs (Lewis *et al.*, 2003; Rajewsky, 2006). More than 60 % of human protein-coding genes are regulated by miRNAs (Friedman *et al.*, 2009) and miRNA regulation seems to be the most abundant mode of posttranscriptional regulation (Jansson and Lund, 2012).

miRNA genes are evolutionarily conserved and may be located either within the introns or exons of protein-coding genes or in intergenic areas (Rodriguez *et al.*, 2004). miRNAs are significantly enriched in clusters in discrete genomic regions (Lagos-Quintana *et al.*, 2001; Ruby *et al.*, 2007; Marco *et al.*, 2013) and miRNAs from the same family share a high degree of sequence homology (Wang *et al.*, 2016). Findings suggest that clusters of proximal miRNAs are typically expressed as polycistronic, co-regulated units and that intronic miRNAs are generally co-expressed with their host genes (Baskerville and Bartel, 2005). Recent studies showed that nearly half of miRNA genes are located in the introns of protein coding or long non-coding RNA genes while the rest are distinct transcriptional units with their own promoters (Kim *et al.*, 2009). It seems to be that miRNAs of the same cluster regulate functionally related genes (Ventura *et al.*, 2008; Yuan *et al.*, 2009).

1.4 microRNAs and cancer

In the past decade, the role of miRNAs in cancer and in cell proliferation has gained significance given their critical role in regulating target genes (Andres-Leon *et al.*, 2017). The losses and gains of miRNA function have been shown to contribute to cancer development through a range of mechanisms (Croce, 2009). Mutations related to miRNA dysregulation often lead to developmental defects and pathological events (Kim and Nam, 2006; Bartel, 2009).

The first indication for the important role of miRNAs in the pathogenesis of cancer came from a report by Calin *et al.* (2002) that showed that patients diagnosed with chronic lymphocytic leukaemia (CLL), often have deletions or downregulation of two clustered miRNA genes, miR-15a and miR-16-1. These genes are located at chromosome 13q14 within a region of loss in CLL and these miRNAs function as tumour suppressors (Calin *et al.*, 2002). Calin *et al.* could show 2004 that over the half of all miRNA genes are located in cancer-associated genomic regions or in fragile sites of the genome and that the full complement of miRNAs in a genome may be extensively involved in cancers (Calin *et al.*, 2004). More detailed analyses confirmed the finding of fragile sites and showed also a positive correlation between fragility, cancer-specific translocation breakpoints and repeats, and between miRNAs and CpG islands (Lagana *et al.*, 2010). Another study could show that about half of the analysed human miRNA genes are associated with CpG islands and thus represent candidate targets of the DNA methylation machinery and an expanded analysis of several miRNA-associated CpG islands indicated that miRNA gene methylation is detectable at high frequencies, both in normal and malignant cells (Weber *et al.*, 2007).

Many regulatory factors switch on or off genes that direct cellular proliferation and differentiation. Damage to these genes, which are referred to as tumour-suppressor genes and oncogenes, could lead to cancer. Most tumour suppressor genes and oncogenes are first transcribed from DNA into RNA, and are then translated into protein to exert their effects (Esquela-Kerscher and Slack, 2006). miRNAs can also function as oncogenes and tumour suppressor genes (Garofalo and Croce, 2011). The specific miRNAs that are capable of transforming normal cells into tumour cells are known as oncomiRs (Andres-Leon *et al.*, 2017). Like a protein-coding gene, a miRNA can act as a tumour suppressor when its loss of function can initiate or contribute to the malignant transformation of a normal cell. The loss of function of a miRNA could be due to several mechanisms, including genomic deletion, mutation, epigenetic silencing, and/or miRNA processing alterations (Calin *et al.*, 2002; Calin *et al.*, 2005; Nakamura *et al.*, 2007). miRNAs are thought to act mainly as tumour suppressor genes additional data indicated that the expression of miRNAs is mainly downregulated in tumour tissues, as compared to corresponding healthy tissues, which supported the role of miRNAs as primarily tumour suppressors. There is evidence that many tumour suppressor miRNAs are downregulated even in many types of cancer e.g. miRNAs of the let-7 family (Yu *et al.*, 2007; Tokumaru *et al.*, 2008), miR-223 (Pulikkan *et al.*, 2010), miR-145 (Iorio *et al.*, 2005), and miR-200 family and miR-205 (Gregory *et al.*, 2008; Piovan *et al.*, 2012).

The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumour formation. In this situation, increased amounts of a miRNA, which might be produced at inappropriate times or in the wrong tissues, would eliminate the translation of a miRNA-

target tumour-suppressor mRNA and lead to cancer progression. Increased levels of mature miRNA might occur because of amplification of the miRNA gene, a constitutively active promoter, increased efficiency in miRNA processing or increased stability of the miRNA (Esquela-Kerscher and Slack, 2006). Many studies have shown that miR-21 has an anti-apoptotic role and is significantly upregulated in tumours e. g. breast cancer compared with normal tissues (Asangani *et al.*, 2008; Yan *et al.*, 2008). Another miRNA which acts as a powerful oncomiR in several types of solid tumours is miR-155 (Tili *et al.*, 2011; Babar *et al.*, 2012) and miRNAs of the cluster miR-17-92 are also transcriptionally upregulated in several different malignancies (He *et al.*, 2005; Mogilyansky and Rigoutsos, 2013). Many studies have shown a dysregulation of miRNAs for different tumour types e. g. breast, prostate, lung-, pancreatic, colon and testicular cancer (Iorio *et al.*, 2005; Johnson *et al.*, 2005; Voorhoeve *et al.*, 2006; Bloomston *et al.*, 2007; Ambs *et al.*, 2008; Schetter *et al.*, 2008).

Studying miRNA links to cancer is furthermore complicated by the genetic diversity of tumours and cancer cell lines and by that fact that most often many miRNAs are found dysregulated in the same tumour. Furthermore, due to the many transcripts regulated by individual miRNAs, their overall function in oncogenesis may be context dependent. Accordingly, a particular miRNA may be found upregulated in some cancer types, and thus supposedly seems to be oncogenic, but downregulated in other cancers, indicative of tumour suppressor function (Jansson and Lund, 2012). In addition, several specific miRNAs can act either as a tumour suppressor or an oncogene, depending on the context (Svoronos *et al.*, 2016). miRNA expression profiles may become useful biomarkers for cancer diagnostics and in addition, miRNA therapy could be a powerful tool for cancer prevention and therapeutics (Zhang *et al.*, 2007).

1.5 microRNAs and testicular cancer

Voorhoeve *et al.* showed in 2006 the first evidence that miRNAs function as oncogenes in GCTs. They showed that the miRNAs miR-372 and miR-373, which are permitting proliferation and tumourigenesis of primary human cells, are highly expressed in adult testicular disease and that these miRNAs neutralize p53 function, by directly inhibiting the expression of the tumour suppressor LATS2 (Voorhoeve *et al.*, 2006). In 2007, Gillis *et al.* performed a high throughput screen with miRNAs in GCT tissues and cell lines and confirmed the specific overexpression of the miR-371-3 cluster in testicular GCTs (Gillis *et al.*, 2007). These findings were extended by another study from Palmer *et al.* in 2010. They profiled 615 miRNAs in GCTs and demonstrated that miRNAs of the miR-371-3 cluster and, in addition, the miR-302/367 cluster were overexpressed in all malignant GCTs independent of patient age (paediatric or

adult), tumour histological subtype (yolk sac tumour, seminoma or embryonal carcinoma) or anatomical site (gonadal or extragonadal). They suggested that expression of these clusters in malignant GCTs either represents the persistence of an embryonic pattern of miRNA expression that is not present in normal tissues and teratoma (the latter having undergone somatic differentiation), or acquired re-expression, regulated by an as yet undetermined mechanism (Palmer *et al.*, 2010).

The first report that the miRNA clusters miR-371-3 and miR-302/367 are highly expressed also in serum was published by Murray *et al.* in 2011. They observed an overexpression of eight members of the two clusters in comparison to normal serum from healthy subjects using quantitative real-time PCR. Subsequent to these findings, they selected the most overexpressed one (miR-302) to analyse the serum levels of a 4-year-old boy with a yolk sac tumour from diagnosis to day 410 after diagnosis to track the treatment. The results showed that the levels declined after chemotherapy. This study demonstrated that these miRNAs are promising candidate biomarkers for improving disease monitoring (and potentially diagnosis) in malignant GCTs (Murray *et al.*, 2011). In addition to this study with respect to one paediatric GCT case, Belge *et al.* investigated in 2012 serum levels of miR-371, miR-372 and miR-373 in eleven adult GCT patients with CS1 and twelve healthy males. Postoperative measurements using real-time PCR revealed a significant decline of miRNA levels in all GCT patients (Belge *et al.*, 2012). These findings were confirmed in a more extensive study performed by Dieckmann *et al.* in 2012. They analysed serum levels of miR-371, miR-372 and miR-373 in serum of 20 GCT patients with CS1 and four patients with advanced disease before and after treatment, respectively. In six patients testicular vein blood (TVB) was examined additionally and miRNA expression was measured in fifteen matching tumour tissue specimens. In comparison to controls, the serum levels were much higher in GCT patients. TVB samples exceeded the levels in cubital vein blood (CVB) and the levels dropped down in GCT patients after treatment for all stages (Dieckmann *et al.*, 2012). Furthermore, another study published in the same year, showed that these miRNA changes occur also in GCNIS (Novotny *et al.*, 2012). In 2013 Gillis *et al.* used a panel consisting of four miRNAs (miR-371, -372, -373, -367) based on magnetic bead-based purification and qPCR quantification. They demonstrated an overexpression of these miRNAs in GCTs in comparison to controls and a trend for higher expression levels of these miRNAs in patients with metastases, pointing towards an association with tumour burden (Gillis *et al.*, 2013). Additionally, a report with a high-throughput miRNA profiling approach with 750 miRNAs was published. The previously identified miRNAs miR-371 and miR-372 were confirmed to be specifically elevated in serum from GCT patients and several novel miRNAs including miR-511, -26b, -769, -23a, -106b, -365, -598, -340, and let-7a were identified to discriminate germ cell cancer and controls (Rijlaarsdam *et al.*, 2015).

The data reported in these publications are promising that the measurement of miRNAs in serum may open up a new dimension of biomarkers combining the prospects of possibly high specificity and sensitivity in GCT patients. However, there is still much work to be done to develop the test method, technically, and to define, clinically, what kind of patients benefit from it most and best.

1.6 Aim of the thesis

Based on the new findings of previous studies, it is the aim of this thesis to validate miRNAs of the miR-371-3 cluster (miRNAs: miR-371, miR-372, and miR-373) and miR-302/367 cluster (miRNA: miR-367) as biomarkers for patients with GCTs. For this reason, it was planned to analyse serum levels of these miRNAs in cubital vein blood (CVB) of GCTs in comparison to non-testicular malignancies (NTMs), Leydig cell tumours (LCTs) and controls and in testicular vein blood (TVB) to prove whether GCTs are the source of these miRNAs and how specific they are. Furthermore, the miRNA expression in other body fluids like urine, seminal plasma and hydrocele was to be analysed and it was to be demonstrated which of these miRNAs has the highest discriminatory power and thus the highest sensitivity and specificity for GCTs.

In detail, the aims of the publications included in this thesis were the following:

Publication I

To compare the miRNA miR-371 expression in serum of GCT patients, GCNIS patients, controls and cases with NTMs, to analyse the decline of expression after surgery in CS1 patients, and to determine the expression in other body fluids (seminal plasma, urine, and pleural effusion fluid).

Publication II

To compare the miRNA miR-371 expression in TVB, CVB and hydrocele fluid (HY) in GCT patients and controls and in addition to analyse the association of RQ values with clinical factors.

Publication III

To test in a preliminary study the utility of the miRNAs miR-371, miR-372, miR-373, and miR-367 as GCT serum biomarkers and to show in the main study the use of the most promising one as a biomarker for GCT patients. To define a cut-off value to discriminate tumours and controls and to calculate the diagnostic sensitivity and specificity.

2 Materials and methods

2.1 Sample materials

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (ethics approval was given by Ärztekammer Bremen ref. 301, 2011) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. All patients and control persons were adult and had given informed consent.

2.1.1 Serum samples

The serum samples were provided by the Albertinen-Krankenhaus Hamburg, the Bundeswehrkrankenhaus Hamburg, the Universitätsklinikum Hamburg-Eppendorf, and the Klinikum Bremen-Mitte, Germany.

The majority of the serum samples were taken from patients with testicular germ cell tumours (GCTs). Serum samples of patients with germ cell neoplasia in situ (GCNIS), the uniform precursor of all adult GCTs, were analysed, too. Healthy men or individuals with non-malignant scrotal diseases (hydrocele, spermatocele and epididymitis) served as controls.

In addition to the cubital vein blood (CVB) samples, testicular vein blood (TVB) samples were taken from GCT patients and from controls without malignant disease.

Furthermore, serum samples were also collected from patients with non-testicular malignancies (NTMs): prostatic carcinoma, renal cell carcinoma, bladder carcinoma, thyroid carcinoma, oesophageal carcinoma, hepatocellular carcinoma, rectal carcinoma and colonic carcinoma.

The CVB samples were collected in serum separation tubes (Sarstedt, Nümbrecht, Germany) from patients during routine blood examinations and the TVB samples were obtained during surgery from veins of the spermatic cord. They were kept at room temperature for approximately 60 min to allow for complete coagulation. After centrifugation (10 min, 2,500 x g) serum was stored deep frozen at −80 °C until further processing.

2.1.2 Other body fluids (pleura effusion, seminal plasma, urine)

In one GCT patient with widespread metastases, additional to the serum sample, pleura effusion fluid was used for analysis. Furthermore, urine specimens from GCT patients and seminal plasma samples from healthy men were investigated. All kind of body fluids were provided by the Albertinen-Krankenhaus Hamburg.

Pleural effusion aspirate, seminal plasma and urine specimens were obtained during routine clinical examinations and stored deep frozen at -80 °C until further processing.

2.1.3 Hydrocele fluid

Some of the GCT cases had in addition to the tumour a tumour surrounding hydrocele large enough for harvesting 2 ml of the fluid for analysis. Three patients with idiopathic hydrocele served as controls. The samples were stored deep-frozen at -80 °C until processing. The hydrocele fluids were provided by the Albertinen-Krankenhaus Hamburg.

2.2 Methods

All studies presented in this thesis were performed at the University of Bremen.

2.2.1 RNA isolation

Total RNA was extracted from 200 µl of each sample of all kinds of body fluids (**Publication I**: serum, pleura effusion fluid, urine, seminal plasma; **Publication II**: serum, hydrocele fluid; **Publication III**: serum) using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). In Publication III the eluation step was performed twice as the only modification relative to the manufacturer´s instructions. Concentration and purity of the isolated RNA were determined by spectrophotometry using a BioPhotometer (Eppendorf, Hamburg, Germany).

2.2.2 cDNA synthesis

For all samples, reverse transcription (RT) was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). RT primers represented an equal mixture of specific stem-loop primers from the relevant miRNA assays (Applied Biosystems, Darmstadt, Germany) of one target and one control miRNA.
The following primers were used:
Publication I: hsa-miR-371a-3p (Assay ID 002124) and hsa-miR-20a-5p (Assay ID 000580)
Publication II: hsa-miR-371a-3p, (Assay ID 002124) and hsa-miR-93-5p (Assay ID 000432)
Publication III: hsa-miR-371a-3p (Assay ID 002124), hsa-miR-372-3p (Assay ID 000560), hsa-miR-373-3p (Assay ID 000561) and hsa-miR-367-3p (Assay ID 000555) and hsa-miR-93-5p (Assay ID 000432)
6 µl total RNA were added to 9 µl of the RT reaction mix. The reactions with a final volume of 15 µl were incubated in the GeneAmp PCR-System 2700 (Applied Biosystems, Darmstadt, Germany) at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min, respectively. The cDNA was stored at -20 °C.

2.2.3 Preamplification

Because of the low amount of miRNAs in body fluids, a preamplification was performed to amplify the miRNAs before the quantitative real-time PCR.

Publication I

For preamplification, 0.75 µl of each miRNA assay (Applied Biosystems, Darmstadt, Germany) were diluted in 13.5 µl nuclease-free water. The PCR with a final volume of 50 µl (12.5 µl prepared solution, 12.5 µl RT product, 25 µl TaqMan Universal PCR Master Mix (Applied Biosystems Darmstadt, Germany)) was performed at 95 °C for 10 min, followed by 14 cycles of 95 °C for 15 sec and at 60 °C for 4 min using the GeneAmp PCR-System 2700 (Applied Biosystems, Darmstadt, Germany). The preamplification product was diluted 1:5 in nuclease-free water and used for qPCR.

Publication II and III

For preamplification, miRNA assays (Applied Biosystems, Darmstadt, Germany) represented an equal mixture of the two miRNAs (target and control). The PCR with a final volume of 20 µl (12 µl assays diluted in nuclease-free water, 4 µl 5x RealTime ready cDNA Pre-Amp Master (Roche, Mannheim, Germany) and 4 µl cDNA) was performed at 95 °C for 1 min, followed by 14 cycles of 95 °C for 15 s and at 60 °C for 4 min using the GeneAmp PCR-System 2700 (Applied Biosystems, Darmstadt, Germany). The preamplification product was diluted 1:2 in nuclease-free water and used for qPCR.

2.2.4 Quantitative real-time PCR

Publication I

For the quantitative real-time PCR, 9 µl of the preamplification product was added to 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany) and 1 µl 20x TaqMan microRNA assay (Applied Biosystems, Darmstadt, Germany) using the Applied Biosystems 7300 real-time PCR System (Applied Biosystems, Darmstadt, Germany).

The relative quantification was performed with miR-20a as endogenous control. For each sample, the reaction was performed in triplicate. A negative control without reverse transcriptase was added to detect contamination with genomic DNA. Non-template negative controls for each miRNA were included in every plate. PCR conditions were 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. Data were analysed using the 7300 Software version 1.2.3 (Applied Biosystems, Darmstadt, Germany). The relative quantity (RQ) was calculated using the ΔΔCT method (Livak and Schmittgen, 2001).

Publication II and III

For the quantitative real-time PCR, 5 µl of the preamplification product was added to 10 µl FASTStart Universal Probe Master (Roche, Mannheim, Germany), 4 µl nuclease-free water, and 1 µl of 20x TaqMan microRNA assay (Applied Biosystems, Darmstadt, Germany) using the Applied Biosystems 7500 real-time PCR System (Applied Biosystems, Darmstadt, Germany). The relative quantification was performed with miR-93 as endogenous control. All PCR experiments were carried out in triplicate. A negative control without reverse transcriptase was added to detect contamination with genomic DNA. Non-template negative controls were included in every plate. PCR conditions were 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Data were analysed using the 7500 Software version 2.0.6 (Applied Biosystems, Darmstadt, Germany). The relative quantity (RQ) was calculated using the ΔΔCT method (Livak and Schmittgen, 2001).

2.2.5 Statistical analysis

Publication I

Statistical analysis was done with Microsoft Excel (MS Excel, Microsoft Corp., Redmond, USA). The mean RQ was compared with the two-sided Mann-Whitney U-test for independent comparisons and with the Wilcoxon signed-rank test for dependent variables using InStat 3 (GraphPad Software, Inc., San Diego, USA). A p value of < 0.05 was considered being significant.

Publication II

Individual RQ values measured in CVB and TVB were tabulated along with clinical data using Microsoft Excel (MS Excel, Microsoft Corp., Redmond, USA). Correlation of RQ values in TVB with those in preoperative CVB was analysed by employing the Pearson product-moment correlation coefficient. Cross comparisons of mean RQ levels of CVB, TVB and hydrocele fluid of various groups were performed with the Wilcoxon signed-rank test and with the two-sided Mann-Whitney U-test using InStat 3 (GraphPad Software, Inc., San Diego, USA).

The ratio of RQ values of TVB and preoperative CVB (TVB/CVB) was calculated in patients and controls. The mean results of TVB/CVB ratios found in the CS1 and CS2-3 were compared to each other and to controls. The associations of clinical factors were analysed for TVB/CVB ratio and for miRNA expression in TVB and CVB, respectively. Statistical evaluations were performed with univariate and multivariate analyses using the R version 3.01 (R Foundation for Statistical Computing, Vienna, Austria) (R Core Team, 2015). A p value of < 0.05 was considered being significant.

Publication III

Median RQ values for independent subgroups were compared using the Mann-Whitney U test, whereas related groups were compared using the Wilcoxon signed rank test.

Bonferroni correction was applied in the preliminary study to adjust for multiple testing. In addition to empirical calculations, the distribution of RQ values was modelled using Kernel density estimation to obtain a more realistic assessment of the distribution in a larger sample size.

Receiver operating characteristic (ROC) analysis was performed to evaluate the discriminatory power of the markers analysed with RQ = 5 as the cu-toff value to evaluate sensitivity and specificity in the main study.

The frequency of categorical data was compared using the Pearson χ^2 test. Multiple regression analysis was performed to analyse the association between marker expression and tumour diameter or pT stage. Exact 95 % confidence intervals (CI) were calculated. For values based on Kernel density estimation, 95 % CIs were calculated by bootstrapping with n = 2500 simulations.

All tests were two-sided, and significance was assumed at $p < 0.05$. Statistical analysis was performed using SPSS version 22 (IBM, Armonk, NY, USA) and R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria) (R Core Team, 2015).

3 Results

In the framework of this doctoral thesis, the following results were achieved:

3.1 Publication I: MicroRNA miR-371a-3p in serum of patients with germ cell tumours: evaluations for establishing a serum biomarker

Previous studies have shown that miRNAs of the miR-371-3 cluster have a strong association with testicular GCTs and that the expression can be measured in serum of patients with GCT (Murray *et al.*, 2011; Belge *et al.*, 2012; Dieckmann *et al.*, 2012). Of the three miRNAs of the miR-371-3 cluster, miR-371 performed most favourably, because it revealed a considerably high expression in seminoma and nonseminoma and it also showed the highest postoperative decrease (Belge *et al.*, 2012; Dieckmann *et al.*, 2012). Based on these findings, this publication deals with the miRNA miR-371 (termed miR-371a-3p in the publication) to show the potential as a novel biomarker for patients with GCT. miR-371 levels were measured by quantitative real-time PCR in serum samples of 25 GCT patients, six GCNIS patients (termed TIN in the publication), 20 healthy males, and 24 non-testicular malignancies (NTMs).

Serum measurements of GCT patients and controls were compared and the results showed that GCT patients had significantly higher miR-371 serum levels than controls ($p < 0.0001$). This demonstrates that miR-371 expression could principally distinguish between healthy men and GCT patients.

There is evidence for the expression of miR-371 in GCNIS cells (Novotny *et al.*, 2012). However, no serum studies of patients with GCNIS have been published so far. In this study, GCNIS patients had slightly higher serum levels than controls, but this difference was not statistically significant. The mean RQ value of GCT patients was significantly higher than that of GCNIS patients ($p < 0.05$). This suggests that GCNIS cells release a lower concentration of miRNAs into the blood stream than GCT cells.

The analysis of other types of cancer was essential to exclude that miR-371 is released by them into the bloodstream, too. For all NTMs investigated in this study, the miR-371 expression in serum was in the range of the controls. Furthermore, a comparison of miR-371 levels of GCTs and NTMs showed a significant difference ($p < 0.0001$). That demonstrates that miR-371 is not overexpressed in other cancer types and thus specific for GCTs.

To identify the testicular tumour as the source of the miRNA, TVB was analysed. TVB samples of GCT patients had 65.4-fold higher mean serum levels than the corresponding peripheral blood samples ($p < 0.0001$) and this indicates that the testicular tumour is the primary source of this miRNA in serum of GCTs.

Based on these results, the velocity of decay of miR-371 was analysed after elimination of the source of circulating miRNAs in five CS1 patients. The measurements showed a very rapid decline to lower than 5 % of the initial value within 24 h after surgery. The clearance of miR-371 completed after another 1-5 days. A rapid decline of the miRNA after surgical removal of the primary tumour represents a feature of a clinically valuable biomarker.

Until now, it is not known whether miRNAs are cleared from the urine during the filtration processes of the kidney. In this study, there was no miR-371 expression in the three urine specimens of GCT patients detectable in contrast to the high miRNA expression levels in the corresponding serum.

In comparison to this, the other body fluids measured in this study (seminal plasma and pleural fluid) had high concentrations of this miRNA. The malignant pleural effusion fluid had a 6.5-fold higher miRNA expression level than the simultaneously examined corresponding CVB of the patient. Both, the serum level and the pleural fluid level of this patient with large metastatic load were much higher than the mean serum level of CS1 patients. The mean miRNA level in seminal plasma in healthy individuals was 177.2-fold higher than serum levels of controls.

This study demonstrated that GCT patients have high miR-371 serum levels and that there is an association of serum levels with tumour bulk. Furthermore, miR-371 serum levels are much higher in TVB than in corresponding CVB samples and the levels dropped down to normal range after treatment. The non-expression in non-testicular malignancies showed the high specificity of miR-371 for GCTs. These results support that this miRNA could serve as a novel serum biomarker for GCTs, but further exploration in a large-scale clinical study is needed.

-I-

MicroRNA miR-371a-3p in serum of patients with germ cell tumours: evaluations for establishing a serum biomarker

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Own contribution

- Carrying out the molecular genetic study
- Analysis of the data together with Nina Winter, Jörn Bullerdiek and Gazanfer Belge
- Interpretation of the data together with Klaus-Peter Dieckmann, Jörn Bullerdiek and Gazanfer Belge
- Drafting the manuscript together with Klaus-Peter Dieckmann, Jörn Bullerdiek and Gazanfer Belge
- Critical revision and final approval of the manuscript together with all other authors

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ORIGINAL ARTICLE

MicroRNA miR-371a-3p in serum of patients with germ cell tumours: evaluations for establishing a serum biomarker

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SUMMARY

As only 60% of the patients with germ cell tumour (GCT) express the classical markers, new markers as for example microRNAs (miRNAs) are required. One promising candidate is miR-371a-3p, but data are sparse to date. We measured serum levels of miR-371a-3p in GCT patients, in controls, and in cases with other malignancies. We also assessed the expression in other body fluids and we looked to the decline of serum miR-371a-3p levels after treatment. miR-371a-3p levels were measured by quantitative polymerase chain reaction in serum samples of 25 GCT patients, 6 testicular intraepithelial neoplasia (TIN) patients, 20 healthy males and 24 non-testicular malignancies (NTMs). Testicular vein blood (TVB) was examined in five GCT patients and five controls. Five GCT patients had serial daily measurements after orchiectomy. Five seminal plasma samples, three urine specimens and one pleural effusion fluid were processed likewise. GCT patients had significantly higher miR-371a-3p serum levels than controls and NTMs. Serum levels of controls, TINs and NTMs were not significantly different. TVB samples of GCT patients had 65.4-fold higher serum levels than peripheral blood. Malignant pleural effusion fluid had extremely high levels of miR-371a-3p, seminal plasma had strongly elevated levels by comparison with serum levels of controls. In urine of GCT patients, no miR-371a-3p expression was detected. Daily measurements after orchiectomy in stage 1 patients revealed a decline by 95% within 24 h. Serum levels of miR-371a-3p appear to be a promising specific biomarker of GCTs as is suggested by high serum levels in GCT patients, the rapid return of elevated levels to normal range after treatment, the association of serum levels with tumour bulk, the non-expression in NTMs and the much higher levels of miR-371a-3p in TVB. This potential marker deserves further exploration in a large-scale clinical study.

INTRODUCTION

microRNAs (miRNAs) are small, non-coding RNA molecules, which are involved in post-transcriptional gene regulation thus playing an essential role in many biological processes as, for example cell differentiation, apoptosis and tumour development (Bartel, 2004; Esquela-Kerscher & Slack, 2006; Farazi *et al.*, 2011). Some miRNAs are abundantly expressed in cancer tissues (Catto *et al.*, 2011). In general, they are characterized by a high stability in body fluids once being released from tumour cells (Reis *et al.*, 2010; Weber *et al.*, 2010). Three members of the cluster miR-371-3 (miR-371a-3p, miR-372 and miR-373-3p) have shown a strong association with testicular germ cell tumours (GCTs) (Gillis *et al.*, 2007; Palmer *et al.*, 2010; Murray *et al.*, 2011; Ruf *et al.*, 2014; Syring *et al.*, 2014). These characteristics fuelled the hope that these miRNAs could serve as serum biomarkers of GCTs, particularly in light of the clinical need for more sensitive markers in this disease (Bezan *et al.*, 2014). Accordingly, in a pilot study, we found the serum levels of all three miRNAs to be significantly higher in GCT patients than in controls and to drop to the level of controls immediately after orchiectomy (Belge *et al.*, 2012; Dieckmann *et al.*, 2012). Of the three miRNAs evaluated, miR-371a-3p performed most favourably, because it revealed a considerably high expression in seminoma and non-seminoma and it also showed the highest post-operative decrease (Belge *et al.*, 2012; Dieckmann *et al.*, 2012). Thus, miR-371a-3p serum levels seem to be a promising biomarker of GCTs. Notwithstanding, before making its way into clinical practice more biological and biochemical characteristics of this putative serum biomarker need to be elaborated. If, in fact, miR-371a-3p originates from the testicular GCT, one must assume higher serum levels in testicular vein blood (TVB) than in peripheral blood. Also, if this marker is specific for GCT, then a slight elevation should also be expected in patients who are only afflicted with testicular intraepithelial neoplasia (TIN), the

uniform progenitor of GCTs (Dieckmann & Skakkebaek, 1999). From a clinical point of view it is important to know, how fast the elevated serum levels of the presumed marker will decrease subsequent to treatment. Another important question with respect to the specificity of this miRNA is its expression in other malignancies. Accordingly, we performed measurements of serum levels of miR-371a-3p in TVB and compared these levels with those found in peripheral blood. We also looked to miR-371a-3p serum levels in patients with TIN only and in a variety of non-testicular malignancies (NTMs). Finally, we looked to the velocity of serum level decrease after treatment by performing repeat measurements in GCT patients clinical stage 1 (CS1).

MATERIAL AND METHODS

Ethics statement

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

Sample collection

All patients and control persons enrolled in this study were adult. Cubital vein blood samples (CVB) were obtained from patients during routine blood examinations. Blood samples were collected in serum separation tubes (Sarstedt, Nümbrecht, Germany) that were kept at room temperature for approximately 60 min to allow for complete coagulation after blood aspiration. The samples were then centrifuged to separate serum and aliquots were frozen at −80°C until further processing. In one patient with widespread metastases, the expression of miR-371a-3p was analysed in CVB and additionally in aspirated fluid from pleura effusion of this patient. To prove the expression level of miR-371a-3p in other body fluids, five seminal plasma samples from healthy individuals and three urine specimens from GCT patients were investigated additionally.

The patient sample consisted of six patients with TIN only, 24 patients with the following NTMs: 7 prostatic carcinoma, 4 renal cell carcinomas, 8 bladder carcinomas, 1 thyroid carcinoma, 1 oesophageal carcinoma, 1 hepatocellular carcinoma, 1 rectal carcinoma and 1 colonic carcinoma. For comparison, 25 patients with GCT all with CS1 (Lugano classification) were included. Twenty male patients with non-malignant scrotal diseases (hydrocele, spermatocele and epididymitis) served as controls. Twenty of the GCT patients and 17 of the controls had been reported previously (Dieckmann *et al.*, 2012). All of the GCT patients had measurements prior to surgery and again 2–6 days post-operatively. Five of the patients had serial daily measurements post-operatively for 3–6 days to study the decay of serum levels after treatment. In five GCT patients and in five patients without malignant disease, TVB samples were analysed. Pleural effusion aspirate, seminal plasma and urine specimens were obtained during routine clinical examinations.

RNA Isolation

For RNA isolation from serum, pleura, urine and seminal plasma samples, 200-μL frozen body fluid was thawed on ice, total RNA extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and RNA was quantified by spectrophotometry (Eppendorf, Hamburg, Germany).

cDNA synthesis

For all samples, reverse transcription (RT) was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). 55 ng total RNA from each sample was used.

RT primers represented an equal mixture of two miRNAs (miR-371a-3p, Assay ID 002124 and miR-20a, Assay ID 000580)-specific stem-loop primers from the relevant miRNA assays (Applied Biosystems). The reactions with a final volume of 15 μL were incubated in the GeneAmp PCR-System 2700 (Applied Biosystems) at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min respectively.

Pre-amplification of RT products

For pre-amplification, 0.75 μL of each miRNA assay was diluted in 13.5 μL nuclease-free water. The PCR with a final volume of 50 μL (12.5 μL of this solution, 12.5 μL of RT product, 25 μL TaqMan Universal PCR Master Mix (Applied Biosystems) was performed at 95°C for 10 min, followed by 14 cycles of 95°C for 15 sec and 60°C for 4 min using the GeneAmp PCR-System 2700 (Applied Biosystems). The pre-amplification product was diluted 1:5 in nuclease-free water.

Quantitative real-time PCR

For the quantitative real-time PCR, 9 μL of the pre-amplification product was added to 10 μL TaqMan Universal PCR Master Mix and 1 μL of 20× TaqMan microRNA assay using the Applied Biosystems 7300 real-time PCR System (Applied Biosystems). The relative quantification was performed with miR-20a as endogenous control. For each sample, the reaction was performed in triplicate. A negative control of amplification was performed for each sample without reverse transcriptase. Non-template negative controls for each miRNA were included in every plate. PCR conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Data were analysed using the 7300 system software (Applied Biosystems). Cycle threshold (Ct) values were normalized to the internal control, miR-20a (Livak & Schmittgen, 2001). Ct values later than 39 were noted as not detectable.

Statistical analysis

Descriptive statistical analysis was performed using Excel software (Microsoft Corp., Redmond, USA). The two-sided Mann–Whitney *U*-test was employed for statistical comparisons of mean serum levels of various groups. A p-value of less than 0.05 was considered being significant.

RESULTS

The mean RQ-value of NTMs was in the range of the mean RQ-value of controls (Fig. 1). The difference was statistically not significant. Moreover, the individual RQ-values of patients with NTM varied similarly to the controls from 0 (not detectable) to 14 (Fig. 2). GCT patients CS1 had significantly higher mean miR-371a-3p serum levels than controls ($p < 0.0001$) and NTMs ($p < 0.0001$), respectively. TIN patients had slightly higher serum levels than controls and NTMs; however, this difference was not statistically significant (Fig. 1). The mean RQ-value of GCTs was significantly higher than that of TIN patients ($p < 0.05$). Individual data are provided in Table S1 (A, B, E and H) and standard deviation in Figure S1.

Figure 1 Synopsis of mean levels of miR371a-3p in various groups. Comparison of mean RQ-values in GCTs, TIN patients, other malignancies and controls. In addition, results of miR-371a-3p expression in serum and pleural effusion of one CS3 GCT patient is shown. C, controls; CS1, clinical stage 1; CS3, clinical stage 3; NTM, non-testicular malignancy; pt, patient; TIN, testicular intraepithelial neoplasia; [1], RQ-value; *, significant; ***, extremely significant. The *y* axis is plotted on a $\log_{10}$ scale.

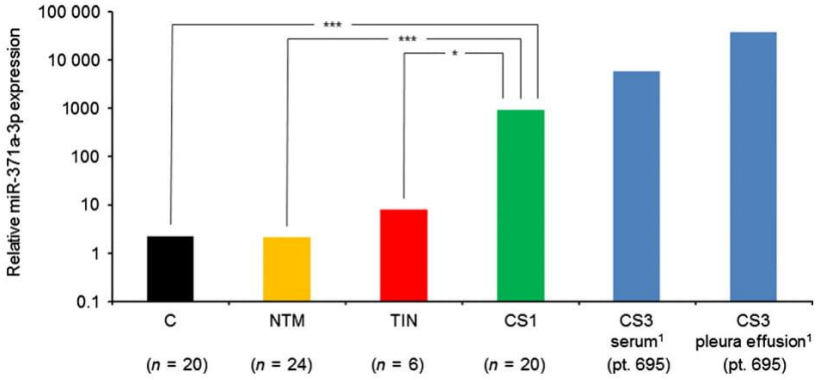
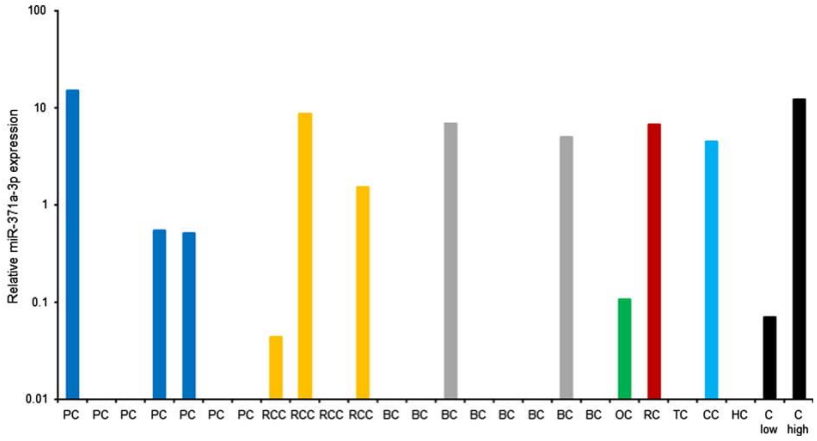


Figure 2 Individual results in non-testicular malignancies. Each column represents the measurement of the relative miR-371a-3p value in an individual patient/person. BC, bladder cancer; CC, colon cancer; C, controls (showing the lowest and highest detectable expression among 20 controls); HC, hepatocellular carcinoma; OC, oesophageal carcinoma; PC, prostatic cancer; RC, rectal cancer; RCC, renal cell carcinoma; TC, thyroid carcinoma;. The *y* axis is plotted on a $\log_{10}$ scale.



The pleural effusion fluid had a 6.5-fold higher miRNA expression level than the simultaneously examined corresponding CVB of the patient. Both, the miR-371a-3p serum level and the pleural fluid level of this patient with large metastatic load are much higher than the mean serum level of GCT CS1 patients (Fig. 1). Individual data are provided in Table S1 (D).

In the urine specimens of three GCT patients, no miR-371a-3p was identified, while miR-20a was detected within Ct value 22 and 24.

The mean seminal plasma expression of miR-371a-3p in healthy individuals was 177.2-fold higher than the mean level found in serum of controls ($p < 0.01$) but it was 2.3-fold lower than the mean serum expression of GCT CS1 patients (Fig. 3).

In TVB, miR-371a-3p levels were higher than in corresponding CVB, both in controls and in patients with GCT. The difference was 27.3-fold in GCT patients ($p < 0.0001$) and 3.4-fold in the controls (Fig. 3). Comparing mean CVB levels of CS1 patients with TVB levels of controls, the difference was 118.3-fold. Individual data are provided in Table S1 (A, C, E, F and G) and standard deviation in Figure S2.

The miR-371a-3p expression in each group relative to the control group for Figs 1 & 3 is shown in Table 1.

The serial daily measurements in five patients after orchiectomy revealed a rapid decay of serum miR-371a-3p levels. All the patients showed a decrease to less than 5% of pre-operative levels within 24 h. Further measurements revealed a complete clearance within 1–6 days in all of the patients. The quantitative decay curves of miR-371a-3p expression for all of the five CS1 GCT patients are given in Fig. 4(A–E) showing the RQ-values from before surgery to 72 h (A–D) and to 144 h (E) thereafter. The mean RQ-values of the five patients at the different time points after surgery are shown in Fig. 4(F).

DISCUSSION

The central result of this study is the accumulation of further evidence for the value of circulating serum miR-371a-3p as a biomarker of GCTs.

Figure 3 Comparison of miR-371a-3p measurements in testicular vein blood, cubital vein blood and seminal plasma. Comparison of mean RQ-values of CVB and TVB in controls and CS1 GCT patients. In addition, mean miR-371a-3p expression in five seminal plasmas is given. For better understanding, the mean serum levels of CS1 patients are shown (same as in Fig. 1). C, controls; CS1, clinical stage 1; CVB, cubital vein blood; TVB, testicular vein blood; *, significant; **, highly significant; ***, extremely significant. The *y* axis is plotted on a $\log_{10}$ scale.

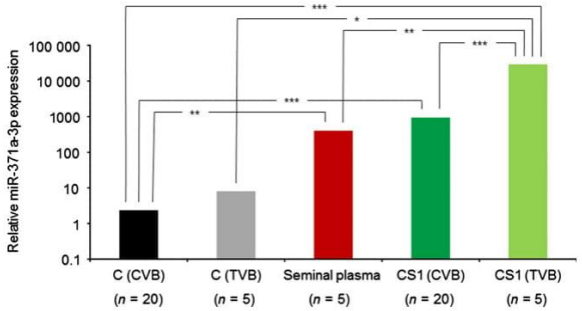


Table 1 Mean expression of miR 371a-3p in the groups examined

Samples	*n*	Relative miR-371a-3p expression	
C (CVB)	20	Mean RQ value	1.00
NTM	24	Mean RQ value	1.75
TIN	6	Mean RQ value	1.95
C (TVB)	5	Mean RQ value	3.42
Seminal plasma	5	Mean RQ value	177.23
CS1 (CVB)	20	Mean RQ value	404.14
pt. 695 CS3 serum	1	RQ value	2590.11
pt. 695 CS3 pleural effusion	1	RQ value	16 806.81
CS1 (TVB)	5	Mean RQ value	26 415.28

miR-371a-3p expression in each group relative to the control group for Figs 1 & 3. C, controls; CS1, clinical stage 1; CS3, clinical stage 3; CVB, cubital vein blood; n, number of samples; NTM, non-testicular malignancy; pt, patient; TIN, testicular intraepithelial neoplasia; TVB, testicular vein blood.

Recently, a method for analysing this miRNA in serum of GCT patients using real-time PCR was developed (Murray *et al.*, 2011; Belge *et al.*, 2012). miR-371a-3p has the potential of becoming a serum biomarker of malignant GCTs (Murray & Coleman, 2012) because, as a rule, substantially increased levels of miR-371a-3p have been detected in serum of patients with GCTs compared to controls. Also, after orchiectomy there was a significant decline into the range of controls (Belge *et al.*, 2012; Dieckmann *et al.*, 2012; Murray & Coleman, 2012; Gillis *et al.*, 2013).

If serum levels of miR-371a-3p expression are to be used as specific biomarkers of GCT, the potential of false-positive results needs to be explored. As a great number of circulating serum miRNAs are released by various types of cancer, for example prostate cancer, bladder cancer and renal cell carcinoma (Mahn *et al.*, 2011; Wulfken *et al.*, 2011; Sanders *et al.*, 2012; Scheffer *et al.*, 2012; Bezan *et al.*, 2014), it was mandatory to exclude that miR-371a-3p is released from malignancies other than GCT. Accordingly, in all 24 malignancies investigated herein, the miR-371a-3p expression in serum was in the range of the 20 controls. The NTM mean RQ-value does not significantly differ from the mean RQ-value of controls and both of the mean RQ-values (NTMs and controls) are extremely different from the mean RQ-value of GCTs CS1. In all, this result clearly accords with the view of miR-371a-3p being specific for GCTs.

The finding of significantly higher levels of miR-371a-3p in TVB than in peripheral blood indicates that the primary source causing the elevation of this miRNA in serum of GCT patients must be the testicular tumour itself. A slight difference between miR-371a-3p levels in TVB and CVB was also found in controls; however, this difference was only 3.4-fold, whereas the difference was 27.3-fold in GCT patients. Thus in all, the present results further underscore the specificity of miR-371a-3p for GCTs.

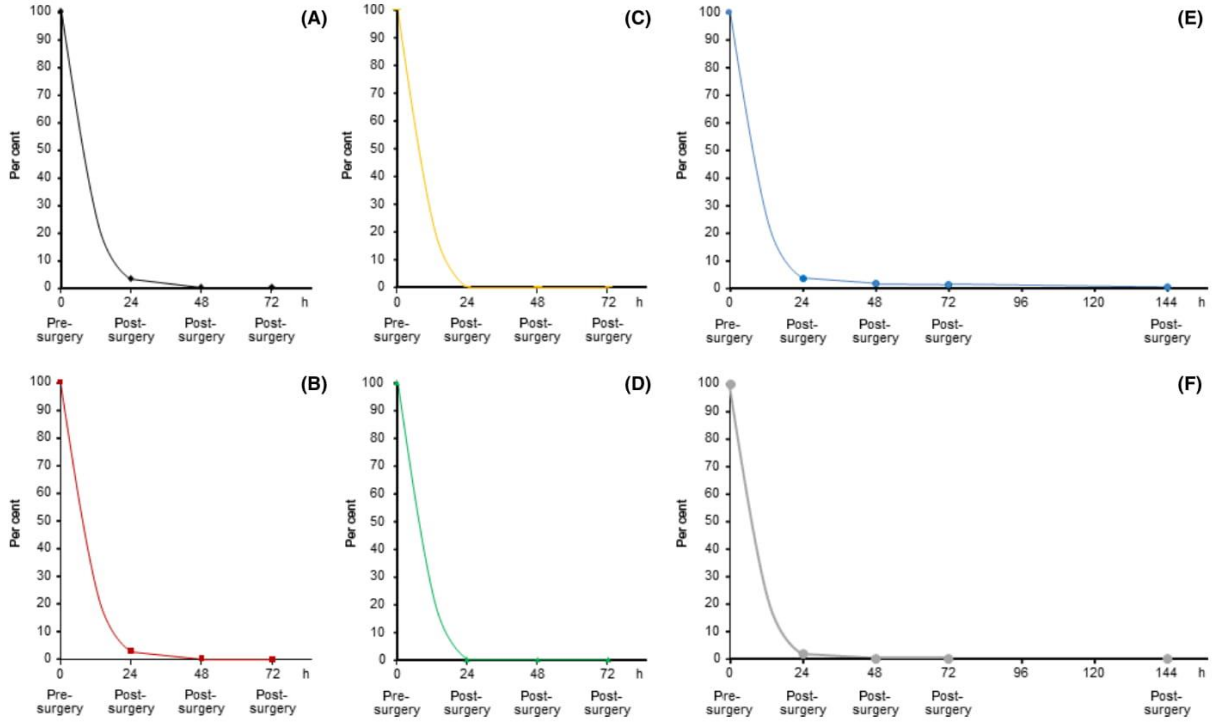
Reportedly, miR-367 is another promising biomarker for testicular GCTs (Murray *et al.*, 2011; Murray & Coleman, 2012; Gillis *et al.*, 2013; Syring *et al.*, 2014). Simultaneous measurement of both miR-367 and miR 371a-3p could further increase the specificity of the test.

A very high miR-371a-3p expression was measured in the serum of one patient with systemic dissemination of non-seminoma. This result closely accords with miR-371a-3p measurements of four patients with GCT clinical stages 2 and 3, as reported previously (Dieckmann *et al.*, 2012). The miR-371a-3p expression of these five patients with advanced disease is obviously higher than the mean RQ-value of CS1 patients (Dieckmann *et al.*, 2012). These findings suggest that the miRNA level is associated with tumour load and with clinical stage, accordingly. This assumption is supported by the extremely high level of miR-371a-3p found in the pleural effusion fluid of the same GCT patient. As large-volume metastatic deposits were located at the pleural walls of this patient, the aspirated fluid from the effusion had been in direct contact with the miRNA releasing tumour cell population. So, a higher expression of miR-371a-3p in the aspirated fluid is an analogous finding to the higher levels found in TVB than in CVB, and is again another piece of evidence for germ cell cancer being the origin of miR-371a-3p in body fluids.

TIN is the uniform precursor of GCTs and it may be present in a testicle many years before the GCT becomes invasive (Dieckmann & Skakkebaek, 1999). There is evidence for the expression of miR-371a-3p (and others) in TIN cells (Novotny *et al.*, 2012; McIver *et al.*, 2013), however so far, no serum studies of patients with TIN have been documented. This study is the first to report measurements of serum levels of miR-371a-3p in six such patients. There appears to be a slight trend towards a higher mean RQ-value in TIN patients than in controls; however, this difference is not significantly different, statistically. The mean RQ-value of TIN patients is significantly lower than the mean RQ-value of GCTs CS1. As the expression of miR-371a-3p in body fluids is apparently associated with tumour bulk (vide supra), one must assume that the number of cells in TIN-bearing testicles secreting miR-371a-3p is not sufficient to achieve high (measurable) levels of circulating miRNAs in the peripheral blood. Also, the present negative result could be explained by the assumption that TIN cells release lesser amounts of miRNAs into body fluids than full-blown germ cell tumour cells do. Thus, it would be interesting to look to testicular vein blood of a patient with TIN only. However, this would be a very rare clinical situation and can hardly be expected to be available for investigation.

The velocity of decay of miR-371a-3p after elimination of the source of circulating molecules has been shown in five GCT patients CS1. In fact, there is a very rapid decline to lower than 5% of the initial value within 24 h after orchiectomy. After

Figure 4 Decay of serum levels of miR 371a-3p after treatment. Daily measurements of miR-371a-3p expression in five individual CS1 GCT patients. The starting value was always measured before orchiectomy. A–D: RQ-values up to 72 h after surgery. E: RQ-values up to 144 h after surgery. F: mean RQ-values of all five patients are shown up to 72 h after surgery (summary).



another 1–5 days, clearance of miR-371a-3p has been completed. The rapid decline of miR-371a-3p levels as a response to curative treatment is an indispensable feature of any clinically valuable biomarker. So, this feature may substantially aid qualifying miR-371a-3p as a new biomarker of GCTs.

miR-371a-3p is predominantly expressed in embryonic stem cells, GCTs, and in the placenta (Bar *et al.*, 2008; Laurent *et al.*, 2008; Ren *et al.*, 2009; Bullerdiek & Flor, 2012; Gillis *et al.*, 2013). We analysed the miRNA expression in five seminal plasma of healthy young men and found an increased expression akin to the extent found in serum of GCT patients CS1. The increased expression of miR-371a-3p in seminal plasma may be explained by the fact that the germ cells directly release the miRNAs into the seminal plasma.

In three GCT patients, we analysed miR-371a-3p expression concomitantly in serum and urine. While serum levels were found expectedly high, no expression was detectable in urine. This finding is somehow surprising at first glance, because other body fluids, for example seminal plasma and pleural fluid evidently harbour these miRNAs (vide supra). Putatively, miRNAs at least miR-371a-3p are cleared from the urine during the filtration processes of the kidney.

There are certainly limitations of our study relating to methodological-technical and to statistical problems. With respect to our laboratory technology, we used only miR 20a for normalization although the employment of more than a single microRNA has been widely adopted for serum studies. With regard to statistical analysis, we acknowledge that larger sample sizes are clearly needed for more meaningful conclusions. However, the results obtained so far are very much promising and thus worth to be reported.

CONCLUSION

miR-371a-3p appears to be an auspicious biomarker of GCTs as is suggested by high serum levels in GCT patients, the association of serum levels with tumour bulk, the rapid return of elevated levels to normal range after treatment, the non-expression in non-testicular malignancies and the much higher levels of miR-371a-3p in testicular vein blood. This presumed marker clearly deserves further exploration in a large-scale clinical study.

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AUTHOR CONTRIBUTIONS

MS carried out the molecular genetic study, analysis and interpretation of the data, drafting of manuscript; GB involved in study concept, analysis and interpretation of data, drafting of manuscript.;TB contributed to patient enrolment, collection of samples for analysis, compilation of clinical data; RI contributed to patient enrolment, collection of samples for analysis, compilation of clinical data; NW carried out the kinetic profiles and

analysis of data; JB provided study concept, analysis and interpretation of data, drafting of manuscript; KPD involved in study concept, patient enrolment, interpretation of data, drafting of manuscript; All authors critically revised and finally approved the manuscript.

MEETING COMMENTS*

Hector Chemes (Buenos Aires, Argentina)

Have you measured serum levels of microRNA 371-3 in young boys with yolk sac tumours?

Klaus-Peter Dieckmann (Hamburg, Germany)

That is an interesting question but we have no young children in our study.

Leendert Looijenga (Rotterdam, Netherlands)

The preliminary data from our studies in Rotterdam, and Matthew Murray's data from Cambridge, UK, include paediatric patients with germ cell tumours. Serum levels of microRNAs are as informative in children as in adults. Childhood yolk sac tumour is associated with raised serum levels, but mature teratomas are not.

William Boellaard (Rotterdam, Netherlands)

If patients have metastatic disease at the time of orchidectomy is there a partial decline in serum microRNA levels following orchidectomy?

Klaus-Peter Dieckmann

Patients with metastatic disease have a small decline in microRNA serum levels after orchidectomy followed by a greater sharp fall in serum levels after the first course of chemotherapy.

Niels E Skakkebæk (Copenhagen, Denmark)

You measured microRNA in the seminal plasma of five patients. What were their diagnoses?

Klaus-Peter Dieckmann

These were five healthy males without testicular cancer.

Hubert Schorle (Bonn, Germany)

MicroRNA 371-3 serum levels are the same in patients with CIS/TIN as in healthy controls but you did not show error bars in your cohort. Serum levels rise with tumour stage for seminomas and non-seminomas. Have you performed statistical analyses and are your data robust?

Klaus-Peter Dieckmann

Our data are still preliminary and have to be analysed further. We shall have statistical data with error bars when more samples have been tested. I am convinced that the test is very robust.

Mark Greene (Rockville, USA)

Biomarker projects often go astray because we focus on patients who already have the disease. It is important to discover if the marker is detectable before the disease is clinically evident, and this might be possible by testing serum collected from large cohort studies. There are hundreds of cohort studies in which individuals are enrolled when they are healthy, blood and serum samples are banked, and they embark on long term follow up. Testicular cancer is a rare tumour therefore only a small number of cases will be detected in a large number of enrolled individuals. We should be collecting samples now in order to discover prediagnostic serum elevations, and to assess the potential for a screening test. Testicular germ cell tumours grow relatively rapidly which might make it difficult to use biomarkers as a screening tool.

Klaus-Peter Dieckmann:

We had 4 patients with CIS/TIN but no invasive tumour at the time of serum sampling. They had only a minimal increase in serum levels of microRNA. Such a marginal increase would be of little value as a screening test.

Carsten Rusner (Halle, Germany)

From a clinical point of view, how expensive is a serum microRNA test compared to the standard serum markers of AFP and hCG? MicroRNA is very sensitive and specific, and would be a useful routine test for testicular tumour patients.

Klaus-Peter Dieckmann

The microRNA test is at the experimental stage carried out in the research laboratory with several steps being performed by hand. Each examination costs about €60 plus lab technician's time. We are improving the test and will produce a "kit" for rapid measurement which should be available at the end of 2015. An AFP test costs about €10. At present, serum tests for AFP and βhCG have a sensitivity of about 50-60% for detecting active germ cell tumour, whereas the microRNA test has a sensitivity of 90%.

*[Correction added on 22 December 2014, after first online publication: Meeting Comments added.]

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. CT values of miR-371a-3p and miR-20a of the samples with miR-371a-3p detection.
Figure S1. Boxplot for Figure 1.
Figure S2. Boxplot for Figure 3.

3.2 Publication II: MicroRNA miR-371a-3p – A Novel Serum Biomarker of Testicular Germ Cell Tumors: Evidence for Specificity from Measurements in Testicular Vein Blood and in Neoplastic Hydrocele Fluid

The previous study by Spiekermann *et al.* showed evidence that serum levels miR-371 could be a promising biomarker for testicular GCT. It was demonstrated that serum levels of this miRNA are very high in the majority of GCT patients and that the levels are very low in healthy men and in men suffering from other malignancies. The data verified a high concentration of miR-371 in TVB samples and a significant decrease of CVB serum levels after cure from GCT in CS1 patients (Spiekermann *et al.*, 2015).

This publication expands the measurements of miR-371 (termed miR-371a-3p in the publication) in CVB and TVB samples as well as in tumour surrounding hydrocele fluid using quantitative real-time PCR. In total, 66 patients were analysed divided into 51 patients with clinical stage 1 disease (CS1) and 15 with systemic disease (CS2-3). All patients provided CVB and corresponding TVB samples and six patients had been reported earlier (Dieckmann *et al.*, 2012; Spiekermann *et al.*, 2015).

The peripheral miR-371 serum levels and the TVB samples were much higher in GCT patients than in controls. Postoperatively, the mean miR-371 serum levels of 33 CS1 patients dropped to RQ < 9 in the range of the controls. For comparison of TVB miRNA levels of GCT patients with those of healthy males, ten patients with non-malignant testicular pathology provided TVB and CVB samples. miR-371 levels were higher in TVB samples than in corresponding CVB samples, both in controls and in patients. The difference of the mean RQ values was 294-fold in CS1 patients, 80.3-fold in CS2-3 patients and 4.6-fold in controls. These results strongly suggest that circulating miR-371 molecules in serum do specifically originate from testis. The correlation of TVB and CVB levels was analysed and the results show a weak correlation of individual miRNA levels in CVB with those of TVB. The Pearson product-moment correlation coefficient was $R^2 = 0.62$ in the group of CS1 patients and $R^2 = 0.63$ in the group of metastasized GCT patients. Nine GCT cases had a tumour surrounding hydrocele and the fluid was used for analysis in comparison to three patients with idiopathic hydrocele, which served as controls. In all cases, the miR-371 levels were much higher in hydrocele fluid than in the corresponding serum. The mean RQ value in hydrocele fluid was 627,384 in the seven CS1 patients and 6,816,157 in the two metastasized patients. In hydrocele controls, no miR-371 expression was detected. This leads to the assumption that there is direct leakage of miRNAs of the tumour into the hydrocele fluid.

Associations with clinical factors were analysed by descriptive statistical methods. Upon multivariate statistical analysis of the entire group, two significant associations were noted for

CVB miRNA levels: testis length (p = 0.0493) and tumour size (p = 0.0211) and for TVB levels a significant association with testis length (p = 0.0129) was observed.

Furthermore, miR-371 expression was compared with serum levels of the classical markers. The classical markers AFP and β-HCG (any or both) were expressed by 14 CS1 patients (27.5 %; 95 % exact CI: 15.9 – 41.7 %) whereas miR-371 was expressed by 42 (82.3 %; 95 % CI: 69.1 – 91.6 %). Three of the nine patients with no miRNA expression had teratoma. In the CS2-3 patients with metastases nine had elevated classical markers (60.0 %; 95 % CI: 32.3 – 83.7 %), while 14 had increased miRNA levels (93.3 %; 95 % CI: 68.1 – 99.8 %). The one patient who did not express miR-371 had teratoma. The lacking expression of miR-371 in teratoma had been noted earlier (Spiekermann *et al.*, 2015). However, no other molecule has the potential as a biomarker for teratoma to date.

The present study provides a great deal of evidence that miR-371 in blood specifically derive from GCT cells. This is supported by the findings of much higher levels of this miRNA in TVB and in tumour surrounding hydrocele fluid than in peripheral serum. Additionally, this study shows an association of miRNA levels with testicular length and with higher pT stage.

In summary, these results confirm previous findings that miR-371 has the potential as a GCT biomarker, but further clinical studies are warranted to evaluate the usefulness of this novel marker a larger cohort and in particular for treatment monitoring and long-term follow up of metastasized patients.

-II-

MicroRNA miR-371a-3p – A Novel Serum Biomarker of Testicular Germ Cell Tumors: Evidence for Specificity from Measurements in Testicular Vein Blood and in Neoplastic Hydrocele Fluid

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Own contribution

- Designing the study together with Klaus-Peter Dieckmann, Jörn Bullerdiek and Gazanfer Belge
- Performing the measurements together with Gazanfer Belge
- Writing the manuscript together with Klaus-Peter Dieckmann, Jörn Bullerdiek and Gazanfer Belge
- Interpreting the data together with Klaus-Peter Dieckmann, Jörn Bullerdiek and Gazanfer Belge
- Critical reading and approval of the final version of the manuscript together with all other authors

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MicroRNA miR-371a-3p – A Novel Serum Biomarker of Testicular Germ Cell Tumors: Evidence for Specificity from Measurements in Testicular Vein Blood and in Neoplastic Hydrocele Fluid

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Key Words
microRNA · Testicular neoplasms · Serum · Biomarker · Hydrocele

Abstract
Background: microRNAs (miRs)-371-3 are suggested to be novel biomarkers of germ cell tumors (GCTs), but their specificity is unresolved. We aimed at clarifying the origin of miR 371a-3p by measuring this miR in peripheral vein blood, and in fluids present in the vicinity of GCTs. ***Methods:*** miR-371a-3p levels were measured by quantitative PCR in 9 tumor surrounding hydroceles and in cubital vein blood (CVB) and testicular vein blood (TVB) of 64 GCT patients, 51 with clinical stage (CS) 1, 13 with CS2–3. Thirty three CS1 cases had also postoperative CVB measurement. TVB miR levels were compared with those of CVB. Associations with clinical factors were analyzed statistically. ***Results:*** TVB miR levels were 294-fold, 80-fold and 4.6-fold higher than those in CVB of CS1 patients, CS2–3 patients and controls, respectively. Neoplastic hydrocele fluid comprised of very high miR levels. In CS1, miR levels dropped to normal postoperatively. Statistically, CVB miR levels are significantly associated with tumor size ($p = 0.0211$) and testis length ($p = 0.0493$). TVB miR levels are associated with testis length ($p = 0.0129$). ***Conclusions:*** This study provides evidence for the origin of circulating miR 371a-3p molecules from GCT cells. miR-371a-3p represents a specific serum biomarker for germ cell cancer.

Background

Clinical management of testicular germ cell tumors (GCTs) is largely based on the monitoring of serum tumor markers [1–3]. However, the markers beta human chorionic gonadotropin (bHCG), alpha fetoprotein (AFP) and lactate dehydrogenase (LDH) are expressed only by 60% of GCTs [4–6]. Particularly, seminoma expresses bHCG in less than 20% of cases and AFP in none [7]. Therefore, more sensitive markers are needed. So far,

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none of the suggested new markers could qualify for clinical employment [8, 9]. Recently, microRNAs (miRs) have been suggested to be a novel class of serum biomarkers [10–12]. Regarding testicular GCT, miRs-371-3 as well as miR-302 and miR-367 represent promising candidates [13–20].

miRs are small molecules of ribonucleic acid consisting of about 20 base pairs. They are released from the cellular nucleus and remain stable in body fluids. They can be measured there by quantitative real time polymerase chain reaction (qPCR). miRs-371-3 and miR-302 have been detected in GCT tissue [14, 21–23] and elevated serum levels have been documented in several pilot studies [15, 16, 24]. miR-371a-3p appears to be the most sensitive marker because it showed the greatest decrease of levels in response to treatment [16, 17].

Evidence for the specificity of serum levels of miRs-371-3 for testicular GCT is still equivocal despite the following observations: (1) high serum levels of these miRs in the majority of patients, (2) very low levels in healthy men, (3) in men suffering from non-testicular malignancies and (4) a significant decrease of levels after cure [25]. Another way of confirming the specificity of miRs-371-3 for testicular GCT would be to show particularly high levels of these miRs in body fluids being in close contact to the testicular neoplasm.

Blood in the venous drainage of testicular tumors comprises higher concentrations of bHCG than peripheral blood [26] and the origin of these high levels from the tumor represents settled knowledge [27]. Likewise, bHCG and AFP have been documented in tumor surrounding hydrocele fluid (HY) in higher concentrations than in the peripheral circulation [28]. The difference was explained by the direct leakage of marker molecules into surrounding compartments [29].

This study aimed at ascertaining the specificity of miR-371a-3p for GCT. We measured peripheral serum (cubital vein blood; CVB) levels of this miR preoperatively and compared these levels with postoperative findings and with those found in testicular vein blood (TVB). We also assessed the fluid of tumor surrounding hydroceles of testicular GCTs.

Methods

Patients and Samples

From June 2011 to December 2014, all consecutive patients undergoing surgery for suspected testicular malignancy provided CVB samples preoperatively. TVB samples were obtained during surgery from veins of the spermatic cord by puncture with a gauge 18 needle. Usually, around 2 ml TVB was aspirated; however, some cases had vessels inaccessible for aspiration. CVB and TVB samples were collected in serum separation tubes (Sarstedt, Nümbrecht, Germany) that were maintained at room temperature for approximately 60 min to allow for complete coagulation after blood aspiration. After that the samples were centrifuged (10 min, 2,500 *g*) to separate serum, and aliquots were frozen at –80°C until further processing. Sixty-four patients were eligible, 51 with clinical stage (CS) 1 disease and 13 with systemic disease (CS2–3). All these patients provided both, preoperative CVB and TVB samples (clinical details in online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000444303); 6 had been reported earlier [24, 25]. In 33 CS1 patients, postoperative CVB samples were available, additionally.

For comparison of TVB miR levels of patients with those of healthy males, 10 patients undergoing scrotal surgery for non-malignant diseases but who were otherwise healthy provided TVB and CVB samples (online suppl. table 2).

Nine GCT cases had a tumor surrounding hydrocele large enough for harvesting 2 ml of the fluid for analysis. Three patients with idiopathic hydrocele served as controls (online suppl. table 3). All patients had given informed consent. Ethical approval was given by Ärztekammer Bremen (ref. 301, 2011).

For this study on specificity, we restricted all laboratory analyses to miR-371a-3p of the miR-371-3 cluster because clinically, this miR appears to be the most promising marker. All of the candidate miRs are located in the clusters miR-371-3 and miR 302/367 on closely related chromosomal regions [30] and accordingly, the miRs of these 2 clusters are biologically interrelated [22]. The expression of these 2 clusters represents an embryonic pattern of miRs expression. It is therefore rational to assume that if one of these miRs is verified to specifically derive from GCT, then very probably the others do so too.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from 200 µl serum or HY using the miRNeasy Mini kit (Qiagen, Hilden, Germany) and RNA was quantified by spectrophotometry (Eppendorf, Hamburg, Germany). Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Darmstadt, Germany). RT primers represented an equal mixture of 2 miRNAs (miR-371a-3p, assay ID 002124 and miR-93, assay ID 000432)-specific stem-loop-primers from the relevant miRNA assays (Applied Biosystems). The reactions with a final volume of 15 µl were incubated in the GeneAmp PCR-System 2700 (Applied Biosystems) at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min, respectively.

Preamplification and qPCR

For preamplification, miRNA assays represented an equal mixture of the 2 miRNAs, and RealTime ready cDNA Pre-Amp Master (Roche, Mannheim, Germany) was used. The PCR was performed at 95°C for 1 min, followed by 14 cycles of 95°C for 15 s and 60°C for 4 min using the GeneAmp PCR-System 2700 (Applied Biosystems). The preamplification product was diluted in the ratio 1:2 in nuclease-free water and used for qPCR.

Serum levels of miR-371a-3p were measured by qPCR with the TaqMan miRNA assay using the Applied Biosystems 7500 real-time PCR System (Applied Biosystems). All PCR experiments were carried out in triplicate using the FastStart Universal Probe

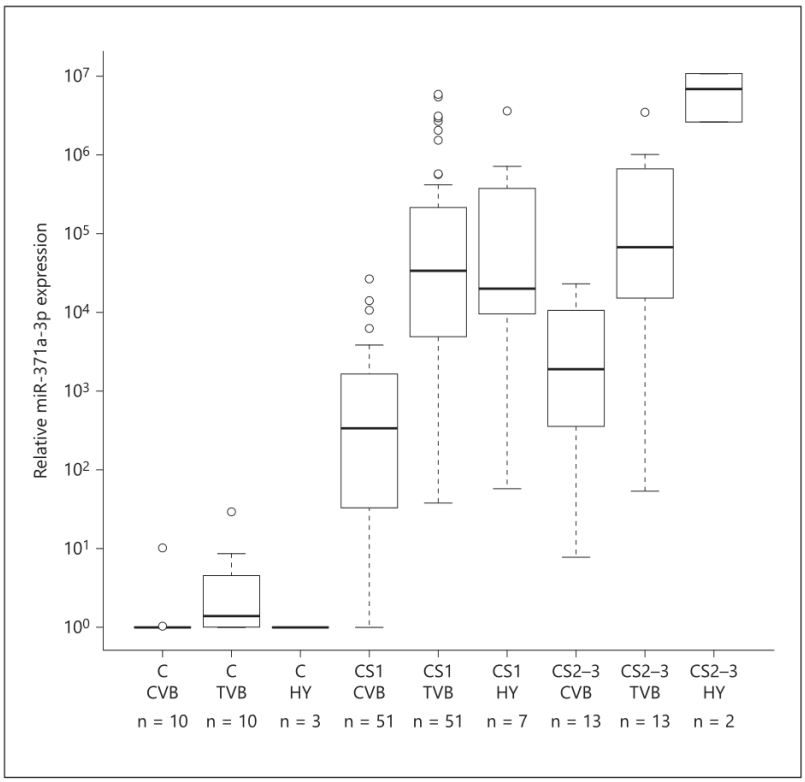

Fig. 1. miR-371a-3p expression in CVB, TVB and HY in CS1 and CS2–3 patients and controls. Boxes show the median miR expression (bold bar) and quartile ranges (extension of box) of the patient group, whiskers denote variation within 1.5-fold of interquartile range, outliers are documented as dots. C = Controls. The y axis is plotted on a $\log_{10}$ scale.

Master (Roche, Mannheim, Germany). A negative control of amplification was performed for each sample without reverse transcriptase. Non-template negative controls were included in every plate. PCR conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. Cycle threshold (CT) values were normalized to miR-93 as an internal control. Data were analyzed using the 7500 software version 2.0.6 (Applied Biosystems). Normalized miR-371a-3p expression levels (relative quantification (RQ) values) were calculated using the $2^{-\Delta\Delta CT}$ method ($\Delta\Delta CT = \Delta CT - \Delta CT_{calibrator}$, where $\Delta CT = CT_{miR\text{-}371a\text{-}3p} - CT_{miR\text{-}93}$) [31].

The upper limit of the normal range of peripheral serum levels was considered to be RQ = 10 because controls ranged up to this value.

Statistical Analysis

Individual RQ values measured in CVB and TVB were tabulated along with clinical data using commercially available database software (MS Excel, Microsoft Corp., Redmond, USA). Correlation of RQ values found in TVB with those in preoperative CVB was analyzed by employing the Pearson product-moment correlation coefficient. Comparison of mean RQ values of the various groups was performed with Wilcoxon and Mann–Whitney U tests using InStat software (GraphPad Software, Inc., San Diego, USA). A p value of <0.05 was considered significant.

The relation of RQ values of TVB and preoperative CVB (TVB/CVB) was calculated in patients and controls. The mean results of TVB/CVB relations found in the CS1 and CS2–3 were compared to each other and to controls. The following factors were analyzed for potential associations with miR levels: histology (seminoma vs. non-seminoma), age, tumor size, testis length, pathological tumor (pT) stage and localization of the tumor (left vs. right). Associations of these factors were analyzed for TVB/CVB relations and for miR expression in TVB and CVB, respectively. Statistical evaluations were performed with univariate and multivariate analyses using the R software version 3.01 [32].

Results

Mean miR-371a-3p Expression in Various Groups

miR-371a-3p levels were significantly higher in TVB than in corresponding CVB, both in controls and in patients (fig. 1; online suppl. tables 1, 2). The difference was 294-fold in CS1 patients, 80-fold in CS2–3 patients and 4.6-fold in controls (table 1).

Median RQ values of the various groups with quartile ranges are presented in figure 1. Results of statistical cross

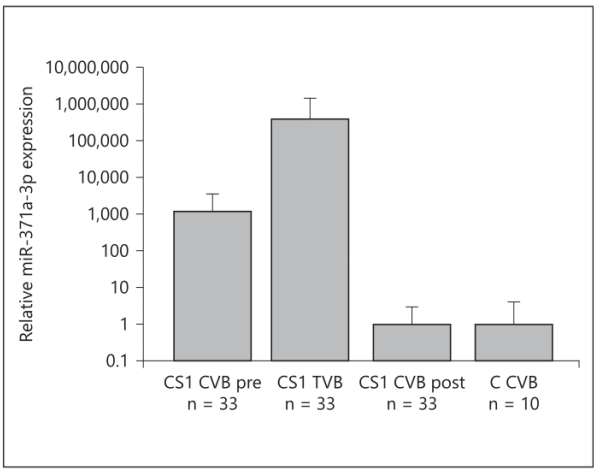

Fig. 2. Postoperative decrease of miR-371a-3p levels in CS1 patients. Columns show mean miR 371a-3p levels prior to surgery and corresponding postoperative levels in 33 CS1 patients. For comparison, mean values of controls and testicular vein measurements are given additionally. C = Controls; pre = preoperative; post = postoperative. The y axis is plotted on a $\log_{10}$ scale.

Table 1. Mean miR-371a-3p expression in CVB and in corresponding TVB in patients and controls

Group	Source of serum	n	Mean RQ values	Relation TVB/CVB
C	CVB	10	0.9	4.58
C	TVB	10	4.3	
CS1	CVB	51	1,843.6	294.02
CS1	TVB	51	542,064.6	
CS2–3	CVB	13	6,186.9	80.29
CS2–3	TVB	13	496,766.6	
All patients	CVB	64	2,725.8	195.49
All patients	TVB	64	532,863.4	

C = Controls.

Table 2. Statistical cross comparisons (p values) of miR-371a-3p levels of the groups documented in figure 2. Groups in the first column are compared to those in the second column

Patient sample/fluid examined	p value
C/CVB	
CS1/CVB	<0.0001
CS2–3/CVB	<0.0001
C/TVB	
CS1/TVB	<0.0001
CS2–3/TVB	<0.0001
CS1/CVB	
CS1/TVB	<0.0001
CS2–3/CVB	0.0277
CS1/HY	
CS1/CVB	0.0313
C/CVB	0.0004
C/TVB	0.0007
CS2–3/CVB	0.0456
CS2–3/CVB	
CS2–3/TVB	0.0002

C = Controls.

comparisons of the various groups are summarized in table 2. In brief, mean peripheral serum miR levels of GCT patients are significantly higher than those of controls. The same is true for TVB miR levels. Postoperatively, the mean serum miR level of 33 CS1 patients dropped to RQ <9 (fig. 2 and online suppl. table 1).

There was a weak correlation of individual CVB miR levels with those of TVB. The Pearson product-moment correlation coefficient was $R^2 = 0.62$ in the CS1 group and $R^2 = 0.63$ in the metastasized group (fig. 3a, b). For each group, one outlier was omitted.

Hydrocele miR levels were much higher than corresponding levels in peripheral blood. In hydrocele controls, no miR-371a-3p expression was detected (fig. 1 and online suppl. table 3).

Association with Clinico-Pathological Factors

The multivariate (table 3) and univariate (table 4) analyses of possible associations of RQ values in CVB and TVB with clinical factors revealed a complex pattern of results. Because of the wide variation of miR levels in the TVB samples, logarithmical RQ values were employed to look for associations.

Upon multivariate analysis, the relation of RQ values TVB/CVB was not associated with any of the factors tested in any of the 3 patient groups analyzed (CS1, CS2–3, and entire GCT group). However, peripheral serum miR levels were found to be significantly associated with tumor size and testis length in the entire group of patients. Testicular vein miR levels were significantly associated only with the testis length.

In CS1 patients, peripheral serum levels were not associated with any factor while TVB levels proved associated with testis length. In metastasized patients, peripheral miR levels were associated with tumor size, whereas TVB levels had no association with any factor.

Upon univariate analysis of the entire group of patients, there were significant associations of peripheral

Table 3. Significant associations of miR levels with clinical factors in TVB and CVB stratified for CS1 and CS2–3: multivariate analyses

Patient group	Source of serum	Clinical parameter with significant association	p value
All patients	Relation CVB/TVB	None	
All patients	CVB	Tumor size Testis length	0.0211 0.0493
All patients	TVB	Testis length	0.0129
CS1	Relation CVB/TVB	None	
CS1	CVB	None	
CS1	TVB	Testis length	0.0068
CS2–3	Relation CVB/TVB	None	
CS2–3	CVB	Tumor size	0.012
CS2–3	TVB	None (log values)	

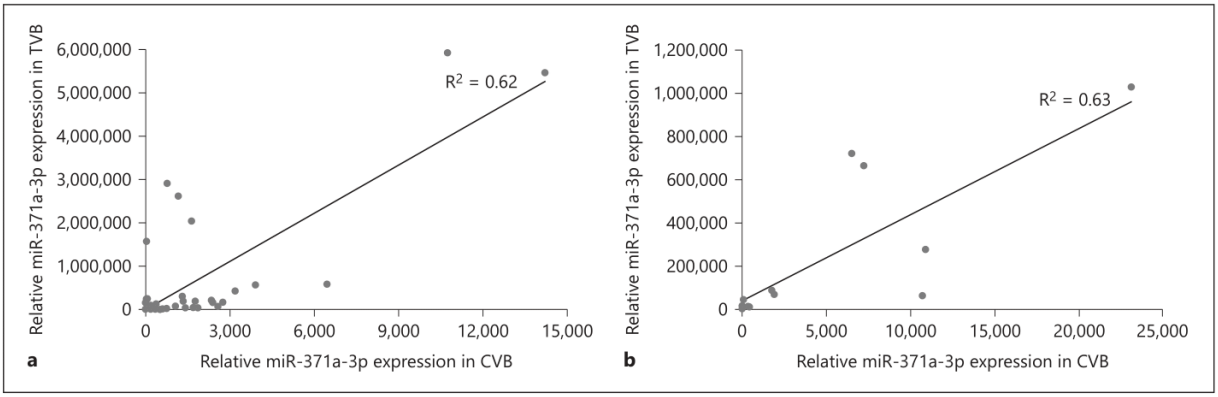

Fig. 3. a Statistical correlation of testicular vein miR levels with corresponding peripheral vein miR levels: CS1 patients. **b** Statistical correlation of testicular vein miR levels with corresponding peripheral vein miR levels: patients with metastases (CS2–3). R^2 = Pearson product-moment correlation coefficient.

miR levels with all parameters tested except for age. In the entire group, TVB miR levels were associated with tumor size, testis length and pT stage (table 4).

Discussion

The key results of this study are the findings of significantly higher levels of miR-371a-3p in TVB than in the peripheral circulation, the drop of miR levels into the normal range after treatment, and finally, the high expression of miR-371a-3p in neoplastic HY. These results strongly suggest that circulating miR-371a-3p molecules in serum do specifically originate from GCT cells.

Hydrocele formation is a common benign anomaly caused by excess production of the peritesticular fluid from the layers of the tunica vaginalis. Some testicular neoplasms are surrounded by a neoplastic hydrocele. As early as in 1932, Zondek detected endocrine products of testicular tumors in the neighboring HY of 2 patients [33]. We found high levels of miR-371a-3p in the tumor surrounding HY in all of our cases, and hydrocele miR levels were much higher than those in the peripheral circulation. These results mirror the findings of elevated bHCG and LDH in tumor surrounding hydroceles in 20 GCT patients [29]. The rational explanation for the great

Table 4. Significant associations of miR levels in TVB and CVB with clinical parameters in CS1 and CS2–3 patients: univariate analyses

Patient group	Source of serum	Significant associations	p value
All patients	CVB	Testis length	<0.0001
		Tumor size	<0.0001
		pT stage	0.0005
		Localization	0.0327
		Histology	0.0415
All patients	TVB	Testis length	0.0005
		Tumor size	0.0406
		pT stage	0.0312
CS1	CVB	Testis length	0.0010
		Tumor size	0.0028
		pT stage	0.0046
		Localization	0.0262
CS1	TVB	Testis length	0.0006
		pT stage	0.028
CS2–3	CVB	Tumor size	0.0001
		Testis length	0.0080
CS2–3	TVB	Tumor size (log values)	0.0300
		pT stage (log values)	0.0165

difference between the levels in HY and peripheral circulation, respectively, is direct penetration of miR molecules from the tumor into the neighboring compartments.

The spermatic vein plexus represents the only venous effluent from the testis. This drainage system contains all metabolic products of the testicle in much higher concentrations than the peripheral circulation where the testicular output is diluted with the total blood volume of the body. In all cases, we found a much higher expression of miR-371a-3p in TVB than in CVB. In healthy males, there was also a small difference between TVB and CVB levels, suggesting a limited release of miR-371a-3p also in healthy testicles. However, in GCT patients, the difference between TVB and CVB is manifold higher. In view of the vascular anatomy of the scrotum, the rational explanation for the high miR-371a-3p levels in TVB of patients is that these molecules are released from testicular GCT cells. Analogous experience has been reported with the classical markers where higher concentrations of bHCG and AFP were documented in TVB than in peripheral blood [27, 34–36].

The relation of miR-371a-3p levels in TVB vs. CVB was higher in CS1 patients (TVB/CVB = 294) than in metastasized cases (TVB/CVB = 80). This difference in the relations cannot be explained by different statistical correlations in the stage groups because the correlation coefficients are identical in both groups ($R^2 = 0.62$ in CS1 and 0.63 in CS2–3 (fig. 3a, b)). Also, the TVB/CVB relation is not influenced by any clinical factor. However, there is a simple biological explanation for the different relations: in CS1, the primary GCT is the only source releasing miR-371a-3p molecules, whereas in systemic disease, the metastatic deposits represent additional sources increasing the peripheral miR expression. So, the denominator of the TVB/CVB relation is greater in metastasized than in localized disease reducing the relation in these cases. These findings underscore the perception of GCT being the origin of circulating miR-371a-3p.

A possible confounding factor of measurements of TVB is compression or mechanical manipulation of the tumor-bearing testicle upon surgery. However, this factor would only increase miR levels in TVB to a certain degree in some cases and most probably, it cannot account for the large differences between TVB and CVB that were found in the entire patient cohort.

In CS1 patients, we noted a distinct drop of serum miR levels after surgery. Again, this observation is in accordance with the understanding that these miRs are specifically released from the tumor. Once the source of production is eliminated, serum levels are supposed to clear. Similar results have been reported previously [15, 16, 24], and the present report is a confirmation based on a larger patient number.

The search for associations of serum miR levels with clinical parameters revealed a complex array of results upon univariate analysis (table 4), and the biological mechanisms resulting in this intricate pattern of statistical findings remain elusive. The results might be confounded by multiple testing because many factors were tested in many different groups. Also, due to small patient numbers in several subgroups, some results might have come by chance. However, 2 significant associations were noted upon multivariate analysis of the entire group of patients and in various subgroups alike, and these factors appear to be important clinically: testis length and tumor size.

Both factors are proxies for the number of tumor cells. So, the association of serum miR expression with testis length and tumor size does probably reflect the specific production of these miR molecules in GCT cells. The well-recognized association of tumor size with metastatic risk in seminoma [37] might contribute to the association observed because seminoma cases clearly outnumber non-seminoma in this series. Surprisingly, miR levels of

TVB in localized disease are not associated with tumor size. This negative finding could relate to the heterogeneous composition of many GCTs [38] and apparently, not all of the compartments of GCTs do equally express miR-371a-3p. Necrotic zones, cystic areas or teratoma may represent areas with lacking miR expression. Also, as reported earlier, the extent of miR expression in tumor tissue does not seem to correlate with corresponding serum levels [24]. Obviously, miR production is confined to specific areas or cell populations of the neoplasm and thus, tumor size does only indirectly affect serum levels. Moreover, the biological pathways regarding the release of miR molecules from the tumor into serum appear to be different from the mechanisms governing the release of the classical markers AFP and bHCG where rather close correlations of tissue expression and serum levels are recognized [39].

The only association revealed by univariate analysis that is worth highlighting is the possible effect of higher pT stage on miR levels. This association was observed for TVB in all stages and for CVB in all patients together and in CS1 cases alone (table 4). As pT stage 2 denotes vascular invasion of the tumor [40], direct drainage of released miR molecules into serum is probably facilitated in this setting. In light of this rational biological explanation, the association of miR levels with local tumor stage deserves credit, although statistical evidence is achieved only on the univariate level.

Conclusions

This study provides evidence for the understanding that circulating miR-371a-3p molecules specifically derive from GCT cells. The strongest support comes from the findings of much higher levels of this miR in TVB and in tumor surrounding HY than in peripheral serum. Further evidence comes from the distinct drop of miR levels after surgery in CS1 patients and from the associations of miR levels with testicular length and with higher pT stage.

Although our data exclusively relate to miR-371a-3p, it is rational to assume that the other candidate miRs (No. 372, 373, 367, 302) are likewise specific for GCT because they are closely related genetically and their clinical features are similar to the miR evaluated here. With regard to sensitivity, it is probably useful to employ a panel of these miRs in a clinical test rather than miR-371a-3p alone.

In all, circulating miR-371a-3p molecules in serum represent a highly specific biomarker of GCT. Further clinical studies are warranted to evaluate the usefulness of this novel marker in daily practice.

Disclosure Statement

The authors declare that they have no competing interests.

Authors' Contributions

K.-P.D., M.S., J.B. and G.B. designed the study, interpreted the data, and wrote the manuscript. M.S. and G.B. performed the miR measurements. K.-P.D., T.B., R.I. and P.A. collected the human samples and ascertained the clinical data. W.W. carried out the statistical analyses. T.L. performed the histological examinations of orchiectomy specimens. All authors read and approved the final version of this manuscript.

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3.3 Publication III: Serum Levels of MicroRNA miR-371a-3p: A Sensitive and Specific New Biomarker for Germ Cell Tumours

In the two previous studies, the expression of the miRNA miR-371 was analysed in CVB and TVB as well as in other body fluids like urine, seminal plasma and hydrocele fluid. The measurements were also performed for GCNIS patients and patients with NTM (Spiekermann *et al.*, 2015; Dieckmann *et al.*, 2016). In this publication, a preliminary study was performed to analyse the usefulness of the miRNAs miR-371 (termed miR-371a-3p in the publication), miR-372 (termed miR-372-3p in the publication), miR-373 (termed miR-373-3p in the publication), and miR-367 (termed miR-367-3p in the publication) of the two miRNA clusters miR-371-3 and miR-302/367 in an unselected patient cohort consisting of 50 GCT patients (40 CS1, and ten patients with metastatic disease CS2-3) and 20 non-tumour controls.

In the preliminary study, each of the four miRNAs revealed significantly higher expression levels in GCT patients than in controls with a significance after correction for multiple testing with $p < 0.0001$ for all miRNAs. Metastasized patients had higher expression levels than CS1 patients, but after Bonferroni correction this difference was only significant for miR-371 and miR-367 ($p = 0.001$ and $p = 0.006$, respectively). Of the four miRNAs, miR-371 had the highest ability to discriminate patients and controls and had the greatest difference in median RQ values between controls and patients, while the interquartile ranges (IQR) of miR-372 and miR-373 in patients overlapped considerably with those for controls. The results showed that miR-367 performed second best, whereas miR-372 and miR-373 had considerably lower discriminatory power. miR-371 revealed the highest sensitivity and specificity (AUC: 0.9432; 95 % CI: 0.874 - 0.982) according to the ROC analyses with density estimation. All comparisons for miR-371 between GCT patients and controls revealed highly significant p values ($p < 0.001$).

In addition, the four miRNAs were tested together as one marker panel and one increased miRNA led to an overall positive score. For this panel the maximum efficiency was obtained with a sensitivity of 92 % and a specificity of 80 %. With the same sensitivity of 92 %. miR-371 reached a specificity of 84.7 % The discriminatory power of miR-371 alone was thus better than that of the miRNA panel and therefore, miR-371 was selected for more extensive expression analyses.

In the main study miR-371 expression was analysed in serum samples from a cohort of 166 GCT patients with CS1-3, ten patients with relapse, twelve patients with Leydig cell tumours and serum samples from 106 male controls (94 with non-malignant scrotal disease and twelve healthy men).

First, the median expression of miR-371 of controls was compared to patients with LCTs, and to patients of all three clinical stages. In patients with CS1 and CS2 preoperative expression levels were compared with postoperative measurements. A significantly higher expression

($p < 0.001$ for nearly all comparisons) was observed in preoperative median GCT subgroups than in controls or patients with LCTs. In addition, significant differences were also detected between preoperative and postoperative measurements in CS1 and CS2 patients. A comparison of the different histologic GCT subgroups (S, NS, and T) revealed that teratoma had very low median expression in CS1 and CS2 patients, in the range of controls. A significant difference of miRNA levels between seminoma and nonseminoma was shown for CS1 patients.

For miR-371 the diagnostic sensitivity was 88.7 % (95 % CI: 82.5 - 93.3 %) using empirical data for all 150 preoperative samples and 106 controls, and the specificity was 94 % (95 % CI: 86.9 - 97.3 %), with an AUC of 0.945 (asymptotic 95 % CI: 0.916 - 0.974). Using the density estimation model, sensitivity was 86.3 % (95 % CI: 79.7 - 90.4 %) and specificity was 92.5 % (95 % CI: 89.0 - 95.9 %), with an AUC of 0.939 (95 % CI: 0.907 - 0.965). There was also a different miRNA expression between CS1 patients and metastasized patients, with sensitivity of 81.4 % (95 % CI: 72.1 - 87.0 %) and specificity of 92.5 % (95 % CI: 89.0 - 96.1 %) in CS1 ($n = 107$), and sensitivity of 98.6 % (95 % CI: 94.8 - 99.9 %) and specificity of 92.5 % (95 % CI: 88.9 - 96.3 %) in CS2-3 ($n = 43$).

Multiple regression analysis revealed in CS1 seminoma a highly significant association between miR-371 expression and tumour diameter ($p < 0.001$), but no association with pT stage.

Furthermore, the sensitivity of miR-371 was compared to the sensitivity of the classical GCT markers in seminoma and nonseminoma (all comparisons $p < 0.001$). The results for the entire group showed that the diagnostic sensitivity of miR-371 (88.7 %) was much higher than those of AFP, β-HCG, and LDH (50.4 %) together.

miR-371 levels were also measured in individual patients with metastases (18 CS2 patients, nine CS3 patients and nine with relapsing disease) during the course of chemotherapy. In twelve CS2 patients, the levels dropped to the normal range after the first cycle of chemotherapy and remained low during the later course. After completion of the therapy, miR-371 expression was almost in the range of controls. In the nine CS3 patients, miR-371 expression decreased markedly for all except one after the first cycle of chemotherapy, but for this patient no further information were available. The other patients revealed low miR-371 expression level until completion of treatment. All nine patients with relapse had elevated expression levels and, like the other metastasized patients, levels decreased after the first cycle of chemotherapy with one exception. The levels remained also low in all cases except one, who showed miR-371 level as well as the AFP values rising to over 300 % of the starting value. This indicates that the tumour is growing despite the treatment.

-III-

Serum Levels of MicroRNA miR-371a-3p: A Sensitive and Specific New Biomarker for Germ Cell Tumours

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Own contribution

- Study concept and design: together with Klaus-Peter Dieckmann, Arlo Radtke and Gazanfer Belge
- Analysis and interpretation of the data: together with Klaus-Peter Dieckmann, Arlo Radtke, Werner Wosniok and Gazanfer Belge
- Drafting of the manuscript: together with Klaus-Peter Dieckmann, Arlo Radtke and Gazanfer Belge
- Statistical analysis: together with Arlo Radtke, Werner Wosniok and Gazanfer Belge
- Administrative, technical or material support: together with Thomas Balks, Arlo Radtke and Klaus-Peter Dieckmann

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Platinum Priority – Testis Cancer

Editorial by Rui Henrique and Carmen Jerónimo on pp. 221–222 of this issue

Serum Levels of MicroRNA miR-371a-3p: A Sensitive and Specific New Biomarker for Germ Cell Tumours

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Abstract

Background: Clinical management of germ cell tumours (GCTs) relies on monitoring of serum tumour markers. However, the markers α-fetoprotein (AFP), the β-subunit of human chorionic gonadotropin (bHCG), and lactate dehydrogenase (LDH) are expressed in <60% of GCT cases.
Objective: To test the utility of the microRNAs (miRNAs) miR-371a-3p, miR-372-3p, miR-373-3p, and miR-367-3p as sensitive and specific GCT serum biomarkers.
Design, setting, and participants: Serum levels of miRNAs were measured in 166 consecutive patients with GCT before and after treatment and in 106 male controls. In the first 50 consecutive patients, all four miRNAs were measured. In the main study, only the most sensitive miRNA was further analysed.
Outcome measurements and statistical analysis: The specificity and sensitivity of the four miRNAs were studied using receiver operating characteristic curves. miRNA sensitivities were compared to those of classical markers. Statistical cross-comparisons of miRNA levels for GCT subgroups and controls were performed at various time points during treatment.
Results and limitations: Overall, miR-371a-3p performed best, with 88.7% sensitivity (95% confidence interval [CI] 82.5–93.3%) and 93.4% specificity (95% CI 86.9–97.3%) and an area under the curve of 0.94, outperforming AFP, bHCG, and LDH (combined sensitivity 50%). According to Kernel density estimation, the sensitivity and specificity were 86.3% and 92.5%, respectively. miR-371a-3p levels dropped to normal after completion of treatment. The miRNA levels correlated with treatment failure and relapse. Teratoma did not express miR-371a-3p.
Conclusions: The miRNA miR-371a-3p is a specific and sensitive novel serum GCT biomarker that accurately correlates with disease activity. Validation of this test in a large-scale prospective study is needed.
Patient summary: miR-371a-3p is a novel serum marker for germ cell tumours that is expressed by 88.7% of patients and thus is far more sensitive and specific than classical serum markers. It correlates with tumour burden and treatment results. Validation in a large patient cohort is needed.

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1. Introduction

Monitoring of the serum biomarkers α-fetoprotein (AFP), the β-subunit of human chorionic gonadotropin (bHCG), and lactate dehydrogenase (LDH) is a cornerstone of clinical management of testicular germ cell tumours (GCTs) [1]. However, these markers are expressed in <60% of cases, so novel sensitive markers are needed [2]. Although many substances have been suggested as biomarkers for GCT, none have qualified for clinical use [3].

In 2011, microRNAs (miRNAs) of the clusters miR-371-3 and miR-302/367 were suggested as new serum biomarkers [4]. The miRNA molecules represent a particular class of small RNAs consisting of approximately 20 base pairs [5]. After release from the cell, these molecules remain stable in extracellular fluids [6] and can be measured by quantitative polymerase chain reaction (qPCR).

The miRNAs of the miR-371-3 and miR-302/367 clusters were originally detected in GCT tissue [7–10] and four independent pilot studies confirmed elevated serum levels [11–15]. Furthermore, circulating miRNAs of the two clusters are clearly specific for GCT because it was demonstrated that they are absent in other malignancies [16], and much higher levels of these miRNAs were found in testicular vein blood than in the peripheral circulation [17]. The goal of the present study was to further evaluate the usefulness of miR-371a-3p, miR-372-3p, miR-373-3p, and miR-367-3p as serum biomarkers of GCT in an unselected large patient sample. To determine whether the four miRNAs would be equally appropriate as serum biomarkers, all were tested in a preliminary study consisting of 50 GCT patients. The miRNA with the highest discriminatory power was then further evaluated in a cohort of 166 patients. We explored the utility of that miRNA as a serum biomarker by comparing its sensitivity to that of classical markers and by monitoring the response of miRNA levels to treatment.

2. Patients and methods

2.1. Patients

From June 2011 to September 2015, a total of 166 patients with GCT and 12 patients with Leydig cell tumour (LCT) who were aged 18–60 yr were prospectively enrolled from four institutions (Albertinen-Krankenhaus Hamburg, Bundeswehrkrankenhaus Hamburg, Universitätsklinikum Hamburg-Eppendorf, Klinikum Bremen-Mitte). Sixty-four participants were briefly mentioned previously in relation to a specific analysis of miRNA levels in testicular vein blood (Table 1) [12,17].

As controls, 106 male participants from the same age group were recruited (12 healthy men and 94 patients with benign scrotal conditions such as hydrocele, spermatocele, epididymitis, and varicocele) (Table 1). The first consecutive 50 GCT patients and 20 controls were participants in a preliminary study that was conducted separately. In the main study, serum samples before orchiectomy were available for 150 of the 166 GCT patients. To monitor changes in miRNA levels secondary to chemotherapy, serum samples were repeatedly collected (once per cycle) from 18 patients with clinical stage 2 (CS2) disease, nine patients with CS3 disease, and ten patients experiencing relapse. Serum aliquots were frozen and stored at −80 °C before further processing (Supplementary methods). All patients gave informed consent. Ethics approval was given by Ärztekammer Bremen (reference 301, 2011). Further clinical details are shown in Supplementary Tables 1–7.

2.2. Laboratory methods

For RNA isolation, an miRNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract total RNA from 200 μl of serum. Reverse transcription (RT) was performed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). The RT product was preamplified, and levels of miR-371a-3p (assay 002124), miR-372-3p (assay 000560), miR-373-3p (assay 000561), and miR-367-3p (assay 000555) were measured by qPCR using a TaqMan miRNA assay (Applied Biosystems). Cycle threshold (C_t) values were normalised to miR-93-5p (assay 000432) as an internal control, and the relative quantity (RQ) was calculated using the $2^{-\Delta\Delta Ct}$ method [18]. Details of the measurement methods are described in the Supplementary methods [19].

The classical serum tumour markers AFP, bHCG, and LDH were measured according to laboratory guidelines [20]. Preoperative values were available for 139 patients.

Table 1 – Clinical data for patients with germ cell tumours, patients with Leydig cell tumours, and control subjects for the preliminary and main studies

Group	Preliminary study			Main study		
	n	Age (yr)	Tumour diameter (mm)	*n*	Age (yr)	Tumour diameter (mm)
Total GCT patients	50	37.0 (28.0–46.0)	27.0 (15.3–40.0)	166	38.5 (30.3–46.0)	29.0 (18.0–45.0)
CS1, total	40	39.0 (32.5–46.0)	25.5 (15.0–40.0)	107	40.0 (32.0–46.5)	25.0 (15.0–38.0)
CS1, seminoma	24	44.5 (38.8–48.3)	26.5 (15.0–40.0)	78 [a]	43.0 (35.0–47.8)	25.0 (15.0–38.0)
CS1, nonseminoma	16	31.0 (23.0–35.3)	25.5 (17.5–35.8)	29	30.0 (23.0–35.0)	26.5 (19.5–39.3)
CS2, total	6	27.0 (26.3–28.5)	19.5 (16.0–26.0)	38	39.5 (31.0–47.0)	33.5 (20.0–61.3)
CS2, seminoma	–	–	–	17	36.0 (31.0–46.0)	40.0 (24.0–65.8)
CS2, nonseminoma	6	27.0 (26.3–28.5)	19.5 (16.0–26.0)	21	41.0 (31.0–48.0)	30.0 (17.3–52.5)
CS3, total	4	38.5 (28.8–46.5)	52.5 (41.3–61.3)	11	36.0 (25.0–44.5)	60.0 (45.0–78.0)
CS3, seminoma	1	48.0	–	1	48.0	–
CS3, nonseminoma	3	31.0 (26.5–38.5)	60.0 (52.5–62.5)	10	33.5 (25.0–41.5)	62.5 (52.5–82.3)
Patients with relapse	–	–	–	10 [a]	29.0 (26.8–39.3)	–
Leydig cell tumours	–	–	–	12	46.0 (33.3–50.3)	–
Control subjects	20	36.0 (28.3–48.3)	–	106	38.0 (26.0–48.0)	–

GCT = germ cell tumour; CS = clinical stage. Data are presented as median (interquartile range).
[a] One patient is included in the CS1 seminoma group and in the group of relapsing patients because of relapse 2 yr after carboplatin therapy.

2.3. Statistical analysis

Median RQ values for independent subgroups were compared using the Mann-Whitney *U* test, whereas related groups were compared using the Wilcoxon signed rank test. Bonferroni correction was applied in the preliminary study to adjust for multiple testing. In addition to empirical calculations, the distribution of RQ values was modelled using Kernel density estimation to obtain a more realistic assessment of the distribution in a larger sample size. Receiver operating characteristic (ROC) analysis was performed to evaluate the discriminatory power of the markers analysed. We chose RQ = 5 as the cutoff value to evaluate sensitivity and specificity in the main study. The frequency of categorical data was compared using the Pearson χ^2 test. Multiple regression analysis was performed to analyse the association between marker expression and tumour diameter or pT stage. Exact 95% confidence intervals (CIs) were calculated. For values based on Kernel density estimation, 95% CIs were calculated by bootstrapping with $n = 2500$ simulations. All tests were two-sided, and significance was assumed at $p < 0.05$. Statistical analysis was performed using SPSS version 22 (IBM, Armonk, NY, USA) and R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria) [21].

3. Results

3.1. Preliminary study

The preliminary study revealed a significantly higher level of expression for each of the four miRNAs in GCT patients compared to controls (Fig. 1A–D), with significance retained after correction for multiple testing ($p < 0.001$ for all miRNAs). Patients with metastases had higher expression levels than those with CS1 disease, but after Bonferroni correction this difference was only significant for miR-371a-3p ($p = 0.001$) and miR-367-3p ($p = 0.006$). Of the four miRNAs, miR-371a-3p had the highest ability to discriminate between patients and controls. The difference in median RQ values for controls and patients was greatest for miR-371a-3p, whereas the interquartile ranges for miR-372-3p and miR-373-3p in patients overlapped considerably with those for controls. Thus, of the four miRNAs tested, miR-371a-3p had the highest sensitivity and specificity (area under the curve [AUC] 0.943; 95% CI 0.874–0.982) according to ROC analyses with density estimation (Fig. 1E–H).

When the four miRNAs were tested together as one marker panel with the assumption that one increased miRNA constituted an overall positive score, maximum efficiency (highest Youden index) was obtained, with sensitivity of 92% and specificity of 80%. miR-371a-3p reached specificity of 84.7% when set to the same sensitivity of 92%. Because the discriminatory power of miR-371a-3p alone was at least similar to that of the miRNA panel, only miR-371a-3p was selected for further analysis in the extended patient sample.

3.2. Main study

Figure 2A shows the median expression of miR-371a-3p in controls, in patients with LCTs, and in the three clinical GCT stages in conjunction with postoperative measurements in patients with CS1 and CS2. We observed highly significant differences in preoperative median values between GCT subgroups and controls or patients with LCTs. Significant differences were also noted between preoperative and postoperative values in CS1 and CS2 patients.

Stratifying GCT patients into various histologic subgroups (Fig. 2B) revealed that teratoma had very low median expression, close to the normal range, in patients with CS1 and CS2 disease. Among CS1 patients, miRNA levels in seminoma and nonseminoma were significantly different from each other and from teratoma. The median miRNA levels for the subgroups featured in Figure 2A,B are listed in Supplementary Table 8 with *p* values related to the various cross-comparisons shown in Supplementary Table 9.

The diagnostic sensitivity of miR-371a-3p was calculated to as 88.7% (95% CI 82.5–93.3%) using empirical data for all 150 preoperative samples and 106 controls, and the specificity was 93.4% (95% CI 86.9–97.3%), with an AUC of 0.945 (asymptotic 95% CI 0.916–0.974). Using the density estimation model, sensitivity was 86.3% (95% CI 79.7–90.4%) and specificity was 92.5% (95% CI 89.0–95.9%), with an AUC of 0.939 (95% CI 0.907–0.965) (Fig. 2C, Supplementary Fig. 1). We also found dissimilar miR expression between localized and disseminated disease, with sensitivity of 81.4% (95% CI 72.1–87.0%) and specificity of 92.5% (95% CI 89.0–96.1%) in CS1 ($n = 107$), and sensitivity of 98.6% (95% CI 94.8–99.9%) and specificity of 92.5% (95% CI 88.9–96.3%) in CS2–3 ($n = 43$).

In CS1 seminoma, multiple regression analysis revealed a highly significant association between miR-371a-3p expression and tumour diameter ($p < 0.001$), but no association with pT stage (Fig. 3).

Figure 4 shows the superior sensitivity of miR-371a-3p compared to the classical GCT markers in both histologic subgroups (all comparisons $p < 0.001$). For the entire group of 139 GCT patients for whom all values were available, the overall sensitivity of miR-371a-3p was considerably higher than that of the combined AFP, bHCG, and LDH markers (87.8% vs 50.4%). Further details are given in Supplementary Table 10.

The individual repeated measurements of miR-371a-3p among 18 CS2 patients during chemotherapy are shown in Figure 5A. Expression of miR-371a-3p decreased in 12 patients after orchiectomy and dropped to the normal range in the majority of patients after the first cycle of chemotherapy, remaining there during the later course. In one patient (case #118) miR-371a-3p expression was approximately 20% of the starting value until cycle 3, but then dropped to zero. On completion of therapy, most patients had miR-371a-3p expression of almost zero; two patients had expression of 1.5% and 2.2%, respectively, of the starting value.

In the nine CS3 patients, miR-371a-3p expression decreased markedly for all except one after the first cycle of chemotherapy (Fig. 5B). No further information is available for this patient. miR-371a-3p expression levels in the other patients remained low until completion of treatment.

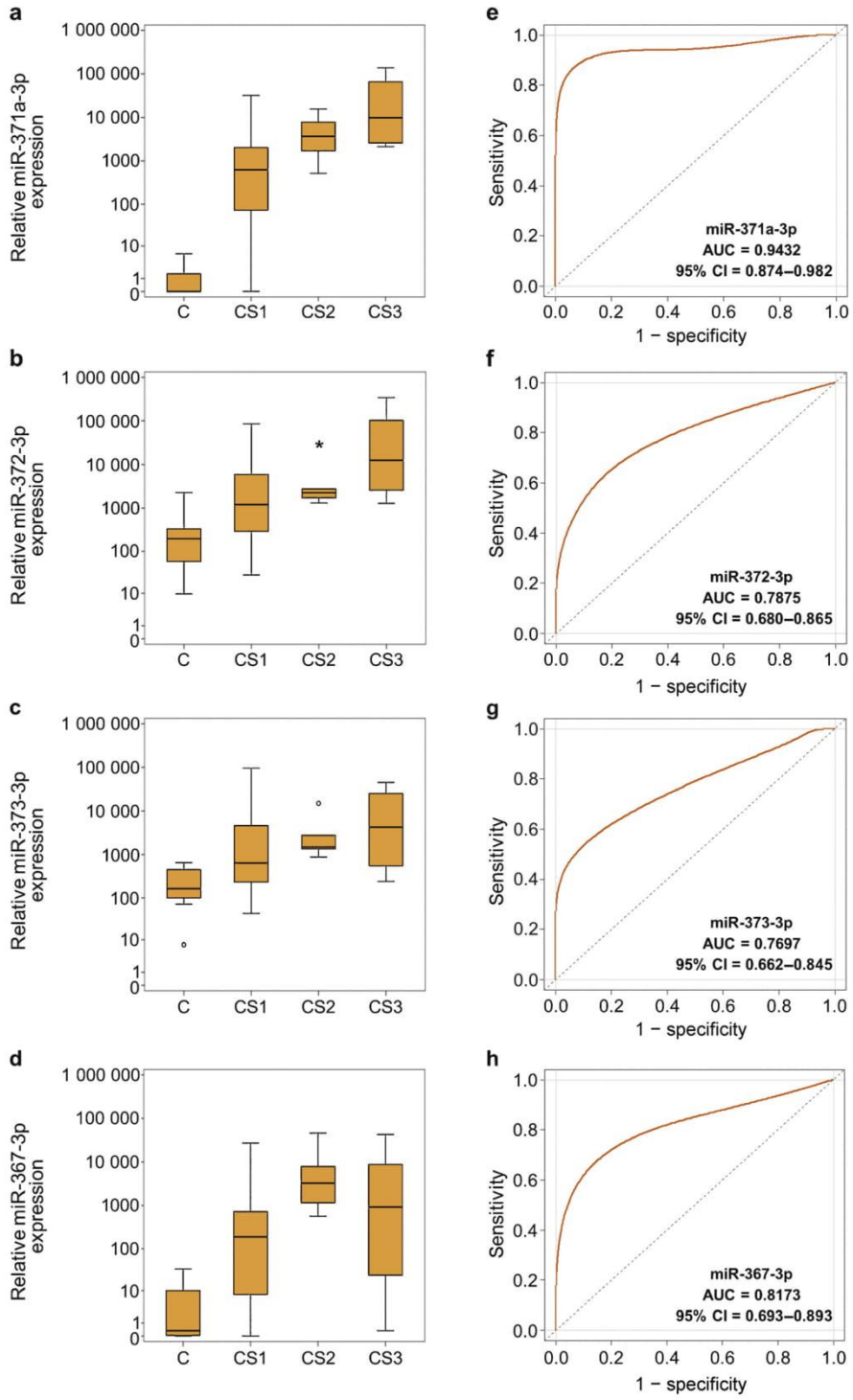

Fig. 1 – Box plots of relative microRNA (miRNA) expression in 50 patients with germ cell tumours and 20 controls (preliminary study) for (A) miR-371a-3p, (B) miR-372-3p, (C) miR-373-3p, and (D) miR-367-3p. The bold lines within the boxes show the median miRNA expression in patients with CS1, CS2, and CS3 disease and controls, with box size indicating the interquartile range. The *y*-axis is plotted on a $\log_{10}$ scale. (E–H) Receiver operating characteristic curve with value for area under the curve (AUC) and 95% confidence interval (CI) for the corresponding box plot. C = controls; CS = clinical stage.

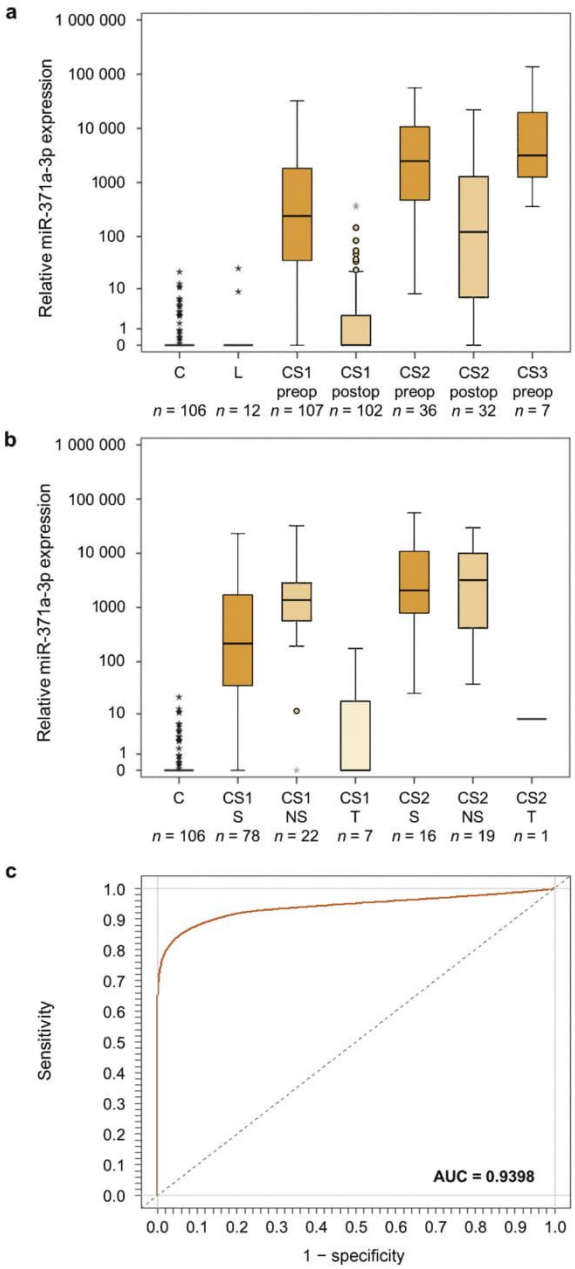

Fig. 2 – Box plots and receiver operating characteristic (ROC) curves of miR-371a-3p expression in patients with germ cell tumours and controls (main study). (A) Box plot of miR-371a-3p expression in controls, in patients with Leydig cell tumours, and in patients with clinical stage 1 (CS1), CS2, or CS3 disease. Data for preoperative patient cohorts are shown in orange, and for postoperative cohorts in light orange. (B) Box plot of miR-371a-3p expression in patients with tumours of different histologic subtypes and in controls. Data for seminomas are shown in orange, for nonseminomas in light orange, and for teratomas in light light orange. (C) ROC curve with area under the curve (AUC) for miR-371a-3p expression in 150 preoperative GCT serum samples and 106 controls. The *y*-axis in A and B is plotted on a $\log_{10}$ scale. C = controls; L = Leydig cell tumours; NS = nonseminoma; postop = postoperative; preop = preoperative; S = seminoma; T = teratoma.

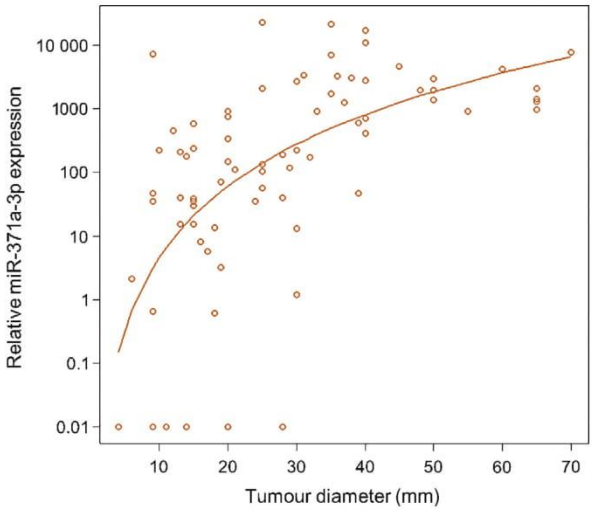

Fig. 3 – Association between miR-371a-3p expression and tumour diameter among 77 patients with clinical stage 1 seminoma. The association is highly significant ($p < 0.001$). The adjusted R^2 is 0.386. The *y*-axis is plotted on a $\log_{10}$ scale.

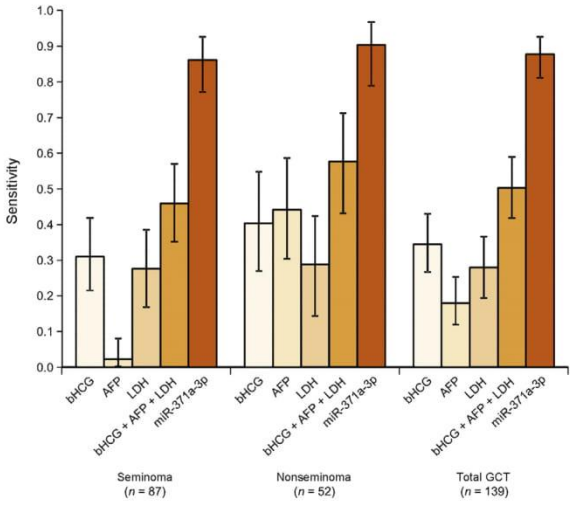

Fig. 4 – Comparison of the classical markers α-fetoprotein (AFP), β-subunit of human chorionic gonadotropin (bHCG), and lactate dehydrogenase (LDH) with miR-371a-3p according to preoperative measurements. The bars represent the sensitivity of bHCG (Pale orange bars), AFP (light light orange bars), LDH (light orange bars), combined bHCG + AFP + LDH (orange bars), and miR-371a-3p (dark orange bars) among patients with seminoma and nonseminoma and in the entire germ cell tumour (GCT) cohort. Error bars represent 95% confidence intervals.

Figure 5C shows individual miR-371a-3p expression in nine patients with relapse, all of whom had elevated expression levels. As with the other patients with metastases, miR-371a-3p levels decreased after the first cycle of chemotherapy, with one exception (case #159). Levels remained low for all patients except one (case #163), in whom miR-371a-3p expression increased to >300% of the starting value. In this case, the course of miR-371a-3p

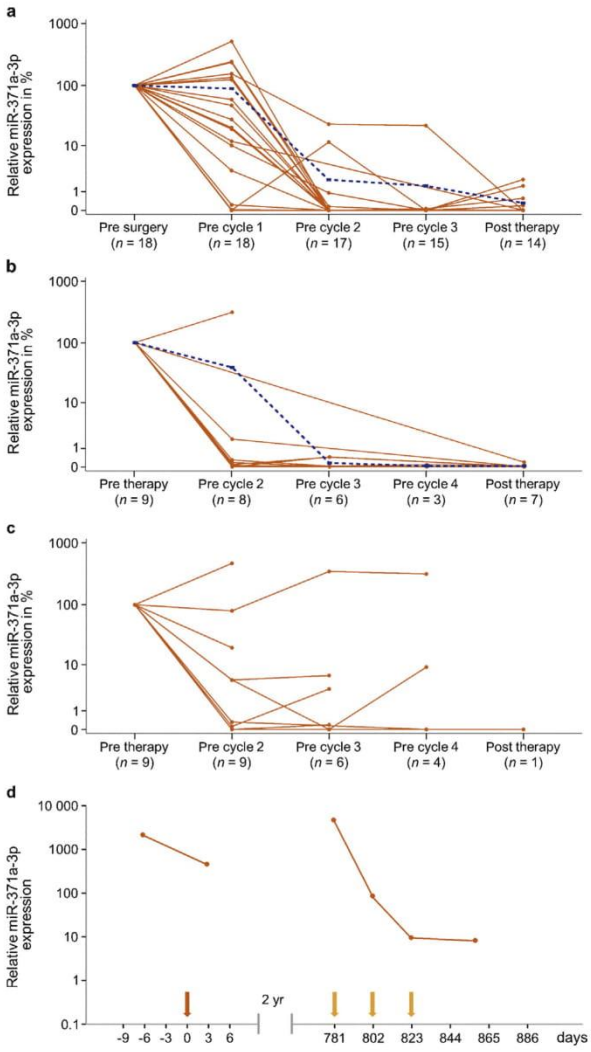

Fig. 5 – Changes in miR-371a-3p levels in individual patients with systemic disease during the course of chemotherapy. Each line denotes an individual patient. Dotted lines represent mean values for the cohort. The values represent percentages of the starting value. The *y*-axis shows values for relative quantity and is plotted on a $\log_{10}$ scale in all diagrams. (A) Patients with clinical stage 2 (CS2) disease, (B) CS3 disease, (C) relapsing disease, and (D) an individual patient with CS1 seminoma who relapsed 2 yrs after adjuvant carboplatin therapy. The orange arrow indicates the time point of orchiectomy, and the light orange arrows the chemotherapy courses for relapse.

expression mirrored that of AFP levels, which likewise rose to >300%, indicating tumour progression.

One patient with CS1 seminoma relapsed 2 yr after adjuvant carboplatin treatment (Fig. 5D). Notably, this patient had persistently high miRNA expression after orchiectomy. The level had further increased at relapse, but dropped to normal on remission after chemotherapy.

4. Discussion

The study results reveal that serum levels of miR-371a-3p apparently fulfil all prerequisites for a valuable biomarker. First, miR-371a-3p is biologically specific for GCT, although, theoretically, an immune response reaction cannot be completely excluded. Healthy control subjects and patients with nonmalignant scrotal disease, as well as those with non-GCT of the testis (LCT), did not express the marker [13,14]. Second, the marker expression correlated with clinical stage. Patients with metastases had significantly higher miRNA levels than CS1 cases. In CS1 seminoma, miR-371a-3p levels correlated with tumour size. Third, miRNA levels correlated with treatment effects. In CS1 disease, elevated miR-371a-3p levels dropped to normal postoperatively, mirroring the tumour-free situation. In patients with metastases, miR-371a-3p expression decreased with chemotherapy to reach normal levels on completion of therapy. Notably, patients with relapsing disease also expressed the marker, as observed in nine cases. In addition, failure of therapy can be highlighted by the marker, as observed in one relapsing patient who had increasing miRNA levels on disease progression despite chemotherapy. It is also noteworthy that one patient with CS1 disease that relapsed after 2 yrs had supranormal postoperative miR-371a-3p expression. Persistent miRNA elevation might have heralded pending clinical relapse. Overall, serum miR-371a-3p levels adequately correlated with the actual state of disease and treatment response.

The association between miRNA expression levels and histologic subtype is unexplained. However, it could be hypothesised that correlation between the degree of morphologic tumour differentiation and miRNA serum levels exists because the miR-371–3 cluster is expressed primarily in undifferentiated stem cells [22]. This would explain the low expression in teratoma, which represents the GCT subtype with the highest degree of morphologic differentiation [23]. Conversely, the higher expression of miR-371a-3p in nonseminoma could be related to the high proportion of embryonal carcinoma in our nonseminoma group. This histologic subtype is biologically close to stem cells [24]. By contrast, seminoma cells have a somewhat higher degree of differentiation, with morphologic similarity to spermatogonia [25], and thus feature lower miR-371a-3p expression than embryonal carcinoma.

Since 2012, pilot studies have documented the applicability of miRNAs of the 302/367 and 371–3 clusters as serum markers of GCT. Accordingly, it was proposed that all miRNAs of the two clusters be used as one diagnostic panel in clinical practice. The present study confirms that miR-371a-3p is the miRNA with the highest ability to discriminate GCT patients from control subjects [14]. MiR-367-3p performed second best, whereas miR-372-3p and miR-373-3p had considerably lower discriminatory power.

The optimised quantification methods used in this study revealed that miR-371a-3p has specificity at least identical to the panel of four miRNAs when the sensitivity was set to an equal value. In practice, it is probably not necessary to use the panel of all miRNAs, as previously suggested [13,15,26,27]. In light of the economic constraints in clinical practice and the results of the present study, it seems rational to use miR-371a-3p exclusively, adding only miR-367-3p for unresolved cases.

The diagnostic specificity of miR-371a-3p is extraordinarily high compared to the classical AFP, bHCG, and LDH markers. More than 86% of GCT patients express this novel marker. The expression is most valuable in seminoma, in which <20% of patients express bHCG [28]. Because miRNA expression is associated with tumour size, small CS1 seminomas may have only slightly increased levels. A minor limitation of the utility of miR-371a-3p is its lack of expression in teratoma. However, this finding is in accordance with the lack of expression of classical markers in this histologic subtype [29].

Limitations of our study include the comparatively low number of patients with metastases. Owing to the somewhat dissimilar expression of the new marker in localized and systemic disease, confounding of the results by selection is conceivable, so the clinical applicability of the test remains uncertain so far. Long-term observational data are missing, and it has still not been formally proven that low miRNA levels on completion of treatment remain low. Nevertheless, preliminary data indicate that levels remain low during follow-up unless the disease recurs.

5. Conclusions

The promising results from pilot studies were confirmed. To the best of our knowledge, this study is the first to use density estimation for a large sample size to gauge the real distribution of RQ values for GCTs and controls. The study also provides evidence of changes in miR-371a-3p expression during chemotherapy in a fairly sizeable patient sample and documents miR expression in relapsing patients. miR371a-3p appears to be a highly sensitive and specific serum GCT biomarker that can aid during monitoring of GCT and could possibly help in sparing radiologic examinations [30]. A prospective large-scale validation study is under way with a view to implementing the test in clinical practice.

Author contributions: Klaus-Peter Dieckmann and Gazanfer Belge had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data: Balks, Oing, Becker, Ruf, Bokemeyer, Matthies, Oechsle, Melchior, Hammel, Dieckmann.
Analysis and interpretation of data: Belge, Radtke, Spiekermann, Dieckmann, Wosniok.
Drafting of the manuscript: Belge, Dieckmann, Radtke, Spiekermann.
Critical revision of the manuscript for important intellectual content: Melchior, Matthies, Ruf, Oechsle, Bokemeyer.
Statistical analysis: Wosniok, Radtke, Spiekermann, Belge.
Obtaining funding: Belge, Dieckmann.
Administrative, technical, or material support: Balks, Dieckmann, Spiekermann, Radtke.
Supervision: Dieckmann, Belge.
Other: None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2016.07.029.

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4 Discussion

microRNAs (miRNAs) are small non-coding RNA molecules (typically 21-23 nucleotides in length), which are involved in several essential biological processes that cover embryogenic development, cell differentiation, apoptosis, and tumourigenesis (Bartel, 2004; Lu *et al.*, 2005; Farazi *et al.*, 2011). Other fields where miRNAs also play key roles are stem cells and stem cell differentiation (Heinrich and Dimmeler, 2012) and regarding induced pluripotency it seems that miRNAs of the cluster miR-302/367 are able to produce induced pluripotent stem cells (iPSCs) from both human and mouse fibroblasts (Anokye-Danso *et al.*, 2011). miRNAs have the potential to be a novel class of serum biomarkers in various malignancies (Cortez *et al.*, 2011; Ralla *et al.*, 2014). Some miRNAs are abundantly expressed in cancer tissue (Catto *et al.*, 2011) and they are characterised by a high stability in body fluids once released from tumour cells (Reis *et al.*, 2010; Weber *et al.*, 2010).

The established serum tumour markers for testicular germ cell tumours AFP, β-HCG, and LDH play an important role in the clinical management of GCT patients. But because of their limited sensitivity, additional markers are needed (Syring *et al.*, 2014) and, so far, no consistent markers for the stem cell components SE and EC are available (Gillis *et al.*, 2013). Particularly seminoma express β-HCG in less than 20 % of cases and AFP in none (Weissbach *et al.*, 1997) and because in summary only 60 % of all patients with GCT have increased levels of the classical tumour markers β-HCG, AFP and LDH, there is an ongoing need for new biomarkers (Belge *et al.*, 2012; Dieckmann *et al.*, 2012). Circulating miRNAs are now starting to be studied in prospective clinical trials in patients with GCT, with the ultimate aim of embedding miRNA quantification in routine clinical practice (Murray *et al.*, 2016).

miRNAs of the miR-371-3 and miR-302/367 clusters were originally detected in GCT tissue (Voorhoeve *et al.*, 2006; Looijenga *et al.*, 2007; Palmer *et al.*, 2010; Bing *et al.*, 2012) and elevated serum levels of miRNAs of these clusters have been documented in several pilot studies (Murray *et al.*, 2011; Belge *et al.*, 2012; Dieckmann *et al.*, 2012; Gillis *et al.*, 2013).

In 2004, Suh *et al.* demonstrated that the miRNAs miR-371, miR-372, miR-373 are located at 19q.13.4 and sequence comparison of these miRNAs showed that the miRNAs are found in a cluster and that they are highly related (Suh *et al.*, 2004). This cluster is the human homologue of mouse miR-290-295 expressed in mouse ES cells (Houbaviy *et al.*, 2003).

The human miRNAs miR-371, miR-372, and miR-373 are clustered within 1.1 kb on chromosome 19. The miR-371-3 cluster consists of eight mature miRNAs: miR-371a-3p, miR-371a-5p, miR-371b-3p, miR-371b-5p, miR-372-3p, miR-372-5p, miR-373-3p, and miR-373-5p (miRbase, 2019). In embryonic stem cells (ESCs) the miR-371-3 cluster is involved in the maintenance of the pluripotent state (Zovoilis *et al.*, 2008) and further studies verified that this cluster shows a high expression level in ESCs (Lakshmipathy *et al.*, 2007; Laurent *et al.*, 2008;

Stadler *et al.*, 2010). An increased expression was also detected in thyroid adenoma (Rippe *et al.*, 2010). Another study showed that there is a significant upregulation of miR-373 in breast cancer metastasis and that this miRNA stimulates cancer cell migration and invasion (Huang *et al.*, 2008). Rearrangements of 19q13.4 are also found in other human cancers, suggesting that activation of these miRNA clusters might be a more general characteristic of human tumours (Brown *et al.*, 2012). Since 2006 it is known that the miR-371-3 cluster is highly expressed in adult GCTs and a genetic screen of primary human cells led to the suggestion that the two members miR-372 and miR-373 act as oncogenes in GCTs, via inhibition of the tumour-suppressor gene LATS2 (Voorhoeve *et al.*, 2006).

The human miRNAs miR-302a, miR-302b, and miR-302c, miR-302d, miR-367 are clustered on chromosome 4 located at 4q25 (miRbase, 2019). The miR-302/367 cluster is highly conserved and vertebrate-specific (Houbaviy *et al.*, 2003; Suh *et al.*, 2004). The miRNAs miR-302a-c share a similar seed sequence which leads to identical target mRNAs (Kuo *et al.*, 2012). This cluster is highly expressed in ECSs (Ren *et al.*, 2009) and involved in the first steps of differentiation of ESCs (Zovoilis *et al.*, 2008). However, during the late differentiation and in adult cells, the expression of the cluster is downregulated (Ren *et al.*, 2009). Accumulating evidence demonstrates that the miR-302/367 cluster plays significant roles in regulation of cellular proliferation, differentiation and reprogramming and is specifically expressed in embryonic stem cells, induced pluripotent stem cells (iPSCs) or tumour cells (Gao *et al.*, 2015). It is noted that the miRNAs from the two gene clusters described in this thesis (for instance, miR-302 on chromosome 4 and miR-372 on chromosome 19) are similar to some extent, implicating that they may have originated from a common ancestral miRNA gene (Suh *et al.*, 2004).

Malignant GCTs, except teratoma, regardless of patient age, anatomical site or histological subtype, show coordinate overexpression of the miR-371-3 cluster and the miR-302/367 cluster. Most miRNAs from the two clusters share the same functional seed sequence ‘AAGUGC’ (at 5' nucleotide positions 2-7), which is responsible for binding and regulating mRNA targets (Palmer *et al.*, 2010). Potential applications for these miRNAs include diagnosis of malignant GCTs in relatively inaccessible sites without the need for surgery, disease monitoring during chemotherapy and detection of subclinical tumour recurrence without serial computed tomography imaging and its inherent secondary cancer risks (Murray and Coleman, 2012).

The first studies about miRNAs of the clusters miR-371-3 and miR-302/367 fuelled the hope that these miRNAs could serve as serum biomarkers for GCTs, particularly in light of the clinical need for more sensitive markers in this disease (Bezan *et al.*, 2014). Building on these previous studies until 2013, three studies were performed during this thesis to focus on the utility of these miRNAs as biomarkers for GCTs.

In the first study, the miRNA miR-371 expression in serum of GCT patients was compared to GCNIS patients, controls and cases with NTMs. In addition, the expression of miR-371 was analysed in TVB as well as in other body fluids and the velocity of the decay of miRNA levels in GCT patients was determined.

If miR-371 expression serum levels will be used as a biomarker for GCTs, the potential of false-positive results needs to be explored. Therefore, it was mandatory to exclude that miR-371 is released from malignancies other than GCT. The analysis of all 24 NTMs showed a miR-371 expression in serum in the range of the 20 controls. This clarifies that miR-371 is specific for testicular GCTs. The expression analysis in urine did not lead to a conclusive result. The level of the endogenous control was very low and miR-371 was not detectable. Maybe the concentration of these miRNAs is too low in urine. No other studies regarding this question are published to compare the results. Until now, it is not clear whether miRNAs, especially miR-371, are finally cleared from the urine during the filtration processes of the kidney.

Furthermore, other body fluids, like seminal plasma from healthy men and pleural effusion fluid from a GCT patient with systemic dissemination of nonseminoma were anylaysed. The miR-371 expression in seminal plasma from healthy men was > 177-fold higher than the mean level in controls, but 2.3-fold lower than the mean expression in serum of GCT patients. This is the first report about miR-371 expression in seminal plasma from healthy men. The analysed miRNA miR-371 is predominantly expressed in embryonic stem cells and in GCTs (Bar et al., 2008; Laurent et al., 2008; Ren et al., 2009; Gillis et al., 2013). The increased expression in seminal plasma of healthy young men in this study may be explained by the fact that the germ cells directly release the miRNAs into the seminal plasma. It would be helpful to analyse the miR-371 expression in seminal plasma from GCTs. Furthermore, it would be of great value to validate the results in a larger cohort to answer further questions regarding the role of this miRNA in the embryonic development. Two years after publication of the first study presented in this thesis, Pelloni *et al.* published a study regarding miRNA expression of the two clusters miR-371-3 and miR-302/367 in seminal plasma of testicular cancer patients in comparison to the expression in serum. They investigated serum and seminal plasma of 28 pre-orchiectomy patients subsequently diagnosed with testicular cancer, the seminal plasma of another 20 patients 30 days post-orchiectomy and a control group consisting of 28 cancer-free subjects. Serum miRNA expression was analysed using RT-qPCR and TaqMan Array Card 3.0 platform was used for miRNA profiling in the seminal plasma of cancer patients. The study showed that 37 miRNAs were differentially expressed in the seminal plasma of cancer patients. The miRNA miR-142 was upregulated in seminal plasma from GCT patients, but miR-371 and miR-372 (along with miR-34b) was downregulated compared to controls. In contrast, in serum expression of miR-371 and miR-372 was increased. This confirms the results of the studies presented in this thesis. The miRNA miR-373 and the miR-302/367 family showed no

significant differences and therefore, miR-371 and miR-372 are the best candidate serum markers for GCT patients, with miR-371 showing the greatest specificity, as it was not detected in the serum of any control subject (Pelloni *et al.*, 2017).

In the study presented in this thesis, a very high miR-371 expression was measured in the pleura effusion fluid, even higher than in the corresponding serum of the CS3 patient. This higher expression of miR-371 in the aspirated fluid is an analogous finding to the higher levels found in TVB than in corresponding CVB, and is again another piece of evidence that the testicular tumour builds the origin of miR-371 in the body fluids. In this study, the expression level in serum of the CS3 patient was higher than in serum of patients with localised disease. This result is in line with previous findings that the mean expression of five patients with advanced disease is higher than the mean expression in CS1 patients (Dieckmann *et al.*, 2012). These results suggest that the miRNA level is associated with tumour load and with clinical stage, accordingly. This assumption is supported by the extremely high level of miR-371 detected in the pleural effusion fluid of the same GCT patient. As large-volume metastatic deposits were located at the pleural walls of this patient, the aspirated fluid from the effusion had been in direct contact with the miRNA releasing tumour cell population.

Currently diagnosing GCNIS, the uniform precursor of GCTs, represents a method of early detection of GCT at the pre-invasive stage. Practically, the only way of diagnosing the lesion is testicular biopsy with immunohistochemical examination of the specimen (Hoei-Hansen *et al.*, 2006; Hoei-Hansen *et al.*, 2007). There is evidence for the expression of miR-371 (and others) in GCNIS cells (Novotny *et al.*, 2012). However so far, no serum studies of patients with GCNIS have been documented. This is the first report that published serum analyses of six patients with GCNIS. A slight trend towards a higher mean RQ value in GCNIS patients than in controls was demonstrated in this study. However, this difference was statistically not significant and has to be validated in a larger cohort. Maybe the number of GCNIS cells, which are secreting miR-371, is not sufficient to achieve high levels of circulating miRNAs in the peripheral blood or these cells release less miRNA molecules into the blood stream than GCT cells do. Two years after this study Radtke *et al.* measured serum levels of miR-371 and miR-367 in 27 patients with GCNIS and no concomitant GCT before treatment and after treatment (n = eleven) as well as in two corresponding TVB sample. In addition, four orchiectomy specimens of patients with GCT were examined immunohistochemically and by *in situ* hybridization (ISH) with a probe specific for miR-371 to look for the presence of this miRNA in GCNIS cells. The median serum level of miR-371 was significantly higher in patients with GCNIS than in controls, but miR-367 levels were not elevated. The highest levels were found in patients with bilateral GCNIS and expression was also elevated in TVB. After treatment, all elevated levels dropped to the normal range. In two orchiectomy specimens, miR-371 was detected by *in situ* hybridization (ISH) in GCNIS cells. These results show that

serum measurements of miR-371 can replace control biopsies after treatment of GCNIS and the test can guide clinical decision making regarding the need of testicular biopsy in cases suspicious of GCNIS, too (Radtke *et al.*, 2017).

To be sure that the tumour itself is the source of the high miR-371 expression levels in patients with GCT, corresponding TVB samples were analysed. The finding of significantly higher levels of miR-371 in TVB than in peripheral blood indicates that the primary source causing the elevation of this miRNA in serum of GCT patients must be the testicular tumour itself. This assumption was confirmed by the analysis of the miRNA level decrease in CS1 patients after removing the tumour by surgery. The serial daily measurements in five patients after surgery revealed a rapid decay of serum miR-371 levels and all patients showed a decrease to less than 5 % of preoperative levels within 24 h. A complete clearance was reached within 1-6 days in all of the patients. Thus in all, the presented results further underscore the usefulness of miR-371 for GCTs because of the high serum levels in GCT patients, the association of serum levels with tumour bulk, the rapid return in CS1 patients of elevated levels to normal range after surgical removal of the tumour, the expression in non-testicular malignancies in the range of controls, and because of the much higher levels of miR-371 in TVB than in corresponding CVB. In summary, the most important result of this study is the evidence, that there is a value of circulating serum miR-371 as a biomarker for GCTs, but this presumed marker clearly deserves further exploration in a large-scale clinical study.

In parallel to this study, a second German working group from Bonn focussed on the measurement of miRNAs of the miR-371-3 cluster and the miR-302/367 cluster. All three miRNAs of the miR-371-3 cluster (miR-371, miR-372, miR-373) but only one of the miR-302/367 cluster (miR-367) had increased expression levels in patients with testicular GCTs compared to healthy individuals and patients with non-malignant testicular disease. In particular, miR-371 allowed for the highest sensitivity (84.7 %) and specificity (99.0 %) of GCT patients. In addition, miR-367 was increased in nonseminoma compared to seminoma cases and serum miRNA levels were increased in patients with advanced local stage and metastases. In nine patients with localized GCT with CS1A serum levels of miR-371 decreased postoperatively, indicating tumour specific release (Syring *et al.*, 2015). These findings are in line with the results published in the first study presented in this thesis..

In the second study of this thesis, published one year after the first, the expression of the miRNA miR-371 was analysed again to clarify the potential as a biomarker for GCTs. A larger cohort of TVB and corresponding CVB samples in GCT patients and controls was investigated in comparison to the first study. In addition, hydrocele fluid of GCT patients and controls was analysed and univariate and multivariate analyses of possible associations of RQ values in TVB and CVB with clinical factors performed.

As a first result, the miR-371 expression was significantly higher in TVB than in corresponding CVB samples in all cases. In healthy males, the difference between TVB and CVB levels was small. This leads to the assumption that there is a limited release of miR-371 also in healthy testicles. In GCT patients, the difference between TVB and CVB was much higher. These results confirm the findings reported in the previous study by Spiekermann *et al.*, 2015. The TVB/CVB ratio of miR-371 levels was higher in CS1 patients than in metastasized patients, but the correlation coefficients were identical in both groups. The biological explanation for the different relations is that in CS1 patients, the primary GCT is the only source releasing miR-371, whereas in systemic disease the metastases represent additional sources increasing the peripheral amount. This leads to a greater denominator of the TVB/CVB ratio in metastasized than in localized disease reducing the ratio in these cases. These findings substantiate the assumption that the tumour is the origin of the circulating miR-371. This is supported also by the observation that the mean miR-371 level of CS1 patients dropped after surgery to a level near the mean RQ level of controls. These findings confirm also previous studies of other working groups (Dieckmann *et al.*, 2012; Gillis *et al.*, 2013; Syring *et al.*, 2015). The classical marker β-HCG is increased in hydrocele fluid of GCT patients in comparison to the corresponding CVB even in patients with seminoma who were negative for the marker in the periphery (Madersbacher *et al.*, 1994). Based on these findings, miR-371 was measured in nine GCT patients with a tumour surrounding hydrocele and three patients with idiopathic hydrocele served as controls. The results showed much higher miR-371 levels in hydrocele fluid than in the corresponding peripheral blood confirming the findings for the classical marker β-HCG. In the controls, no miR-371 expression could be detected and these results underline the suggestion that the miRNA molecules are released from the tumour into the neighbouring compartments.

In addition, possible associations of the miR-371 expression in TVB/CVB ratios and in TVB and CVB, respectively, were analysed with the following clinical factors: histology (seminoma vs. nonseminoma), age, tumour size, testis length, pathological tumour (pT) stage and localization of the tumour (left vs. right) by descriptive statistical methods. Upon univariate analysis, the search revealed a complex pattern of results and the only appreciable association is the possible effect of higher pT stage on miRNA levels. However, the biological mechanisms resulting in this intricate pattern of statistical findings are unclear. Upon multivariate analysis, two significant associations were noted of the entire group of patients and in various subgroups: testis length and tumour size. CVB levels in all patients are significantly associated with tumour size and testis length and TVB levels in all patients are associated with testis length. Both factors deal with the number of tumour cells and that does probably reflect the specific production of the miRNA molecules in the cells of the tumour. However, the biological pathways regarding the release of the miRNAs from the tumour into the blood are not clear.

This study demonstrates and also confirms previous findings that the miRNA miR-371 is specific for GCTs and has the potential to be used as a biomarker for GCTs. However, validating studies in large-scale are needed and an agreed protocol for test procedure needs to be developed.

In the third study in this thesis, a preliminary study was performed to test the utility of four miRNAs (miR-371, miR-372, miR-373, and miR-367) of the miRNA clusters miR-371-3 and miR-302/367. In the main study, the miRNA with the highest discriminatory power (miR-371) was elevated for its use as a possible new biomarker for patients with GCT.

All four miRNAs had significantly higher expression levels in comparison to controls and patients with metastases had higher expression levels than those with localized disease. miR-371 showed the greatest difference of mean RQ values for patients and controls and the highest ability to discriminate these groups. Of the four tested miRNAs, miR-371 had the highest diagnostic sensitivity and specificity according to ROC analyses with density estimation. Previous publications from other working groups preferred a panel comprising various miRNAs of the miRNA clusters miR-371-3 and miR-302/367 (Gillis *et al.*, 2013; Murray *et al.*, 2016). In this preliminary study, four miRNAs were tested together as one marker panel. The maximum efficiency of the panel was obtained with a diagnostic sensitivity of 92 % and a specificity of 80.0 %. With the same sensitivity of 92.0 %, miR-371 reached a specificity of 84.7 %. This means that the discriminatory power of miR-371 alone was even better than the selectivity of the whole miRNA panel. For application in clinical practice, it is much more preferable to use a test including only one marker, because this is cost-saving and limits measurement errors as well as extensive interpretation of data. The miRNA miR-371 was used for the main study including 166 GCT patients, ten patients with relapse, twelve with LCT and 106 controls. This was the first published study including miRNA expression analyses in serum of more than 150 GCT patients and more than 100 controls. The diagnostic sensitivity and specificity was calculated for 150 preoperative GCT samples and samples of 106 controls. Using empirical data, the diagnostic sensitivity of miR-371 was 88.7 %, the specificity was 93.4 % and overall, more than 88 % of the analysed GCT patients revealed increased miR-371 expression level. The results for the entire group of 139 GCT patients showed for the combined classical markers AFP, β-HCG, and LDH a sensitivity of 50.4 %. These results make it clear that there is a high medical need for new and reliable GCT biomarkers.

The following criteria have to be fulfilled for a valuable tumour marker: the substance should be produced only by the malignancy itself, it is secreted into body fluids, the test procedure should be reproducible and safe, the marker levels correlate with the amount of the disease, the marker can be detected at an early stage of the disease, the half life is short, the presence correlates with response to the therapy and the majority of patients with the disease do express the marker (Bates and Longo, 1987; Lange and Winfield, 1987). The two previous studies and,

in particular, the third study presented in this thesis, demonstrate that the miRNA miR-371 fulfils all these requirements for a valuable biomarker. The miRNA is biologically specific for GCTs and not expressed by other types of cancer. miR-371 is released by the tumour itself into the bloodstream. In patients with systemic disease even after surgical removal of the primary tumour, the metastases represent additional sources for the increased miRNA levels. In addition, patients with metastases had significantly higher miRNA levels than CS1 patients covering the correlation of expression level correlates with clinical stages. The expression levels also correlate with response to therapy and highlight treatment failure as well. GCNIS patients had slightly higher serum levels than controls. This could offer an opportunity for the early diagnosis of GCNIS in the future.

Daily measurements of miR-371 expression showed a very rapid decline to lower than 5 % of the initial value within 24 h after surgery for CS1 patients. There are elevated level in nonseminoma as well as in seminoma. The expression correlates with clinical stages, tumour size and bulk and the miRNA is also elevated in relapsing disease.

In the second and third study presented in this thesis, a standardised method for miRNA quantification using serum samples was established.

Recently, Anheuser *et al.* published a report about five individual GCT patients with seminoma. They retrospectively correlated miR-371 levels with individual clinical features. The results demonstrated the exceptional usefulness of the new serum biomarker miR-371 in diagnosing and staging testicular GCT. The marker had indicated active germ cell cancer despite negative or inconclusive findings with the classical staging methods. Analysing miRNA levels in serum of GCT patients could have helped to assess correctly ambiguous radiological findings, to point to the presence of metastatic disease or to exclude metastatic disease and it would have influenced therapeutic decisions differently (Anheuser *et al.*, 2017).

However, further studies are required to validate the miR-371 expression in a larger cohort of patients with metastases during the long-term observation after treatment as well as to monitor surveillance therapy for patients with GCT.

5 Summary

Testicular cancer is the most common solid malignancy in young adult men between the ages of 14 and 44 years. Tumour marker are essential for diagnosis, staging and therapy monitoring as well as for the long-term monitoring of patients. The established serum tumour markers for testicular germ cell tumours (GCTs) are AFP, β-HCG, and LDH. However, in summary only 60 % of all patients have increased levels of these classical tumour markers and because of their limited sensitivity, additional markers are urgently needed. Recently, miRNAs have been suggested to be a novel class of serum biomarkers. These small non-coding RNA molecules are involved in several essential biological processes that cover embryogenic development, cell differentiation, apoptosis, and tumourigenesis. Many studies demonstrated a dysregulation of miRNAs in conjunction with different tumour types and miRNA expression profiles may become potential biomarkers for cancer diagnostics. In GCTs, miRNAs of the miR-371-3 cluster and miR-302/367 cluster are overexpressed. The aim of this thesis was to validate the application of miRNAs of these clusters as biomarkers for patients with GCT. The results of the three studies about miRNA expression analyses are presented within this thesis.

The first study showed significantly higher serum levels of miR-371 in 20 CS1 patients than in 20 controls and 24 NTMs. The mean RQ values of NTMs were in the range of controls. This underlines the specificity of this miRNA for GCTs. TVB samples of GCT patients had significantly higher mean serum levels than the corresponding CVB samples. This indicated that the tumour itself could be the primary source of the miRNA elevation in serum. The six GCNIS patients had slightly higher serum levels than controls and measuring expression levels of miR-371 could possibly be used to diagnose GCNIS in the future. Furthermore, this study demonstrated in six CS1 patients a rapid decline of miR-371 expression after surgery to the range of controls, which is an indispensable feature of a valuable biomarker.

The second study focussed on the measurements of miR-371 expression levels in tumour surrounding hydrocele fluid. The analysis of the hydrocele fluid and corresponding serum of nine GCT patients and three controls with idiopathic hydrocele showed much higher miR-371 levels in the fluid than in the serum. In hydrocele controls, no miR-371 expression was detected. These results underline the assumption that there is a direct penetration of miRNA molecules from the tumour into the neighbouring compartments. Based on the previous study, another aim of this study was the analysis of miR-371 expression in CVB and TVB samples in a larger extent than before. miRNA expression was significantly higher in TVB than in corresponding CVB samples in all 51 CS1 patients and in all 13 metastasized patients. TVB and CVB levels were significantly higher in GCT patients than in ten controls, respectively. In healthy males, the difference between miR-371 levels in TVB and CVB was small. These findings confirm the results of the first study and show that the vein of the spermatic cord

contains much higher miRNA concentrations than the peripheral circulation, which is diluted with the total blood volume. This verifies that miR-371 is released from testicular GCT cells. Another aim of this study was to measure the postoperative decrease of miR-371 expression in CS1 patients in a larger cohort as in the previous published study. The results achieved in this study confirmed a rapid decline of miR-371 level in 33 CS1 patients after surgery to the normal range.

In the third study, the utility of four miRNAs of two miRNA clusters miR-371-3 and miR-302/367 as GCT biomarkers was tested in a cohort of 50 GCT patients and 20 controls. Each of the four miRNAs revealed significantly higher expression levels in GCT patients than in controls. Metastasized patients had higher expression levels than CS1 patients. However, miR-371 showed the highest sensitivity and specificity and thus the discriminatory power of miR-371 alone was better than that of the miRNA panel consisting of all four miRNAs. miR-371 had the highest ability to discriminate GCT patients and controls and was selected for the main study with 166 GCT patients, ten patients with relapse, twelve with LCT and 106 controls. Statistical cross-comparisons of miRNA levels between GCT subgroups, controls and LCTs showed highly significant differences in preoperative median values. In addition, it was shown that patients with metastases had significantly higher miRNA expression levels than CS1 patients. In CS1 cases, elevated miR-371 levels dropped to the normal range postoperatively, mirroring the tumour-free situation. These results confirmed those of the two studies published previously. In patients with metastases, miR-371 expression levels decreased during the chemotherapy and reached the level of controls on completion of therapy. All patients with relapsing disease had elevated expression levels. Using empirical data for 150 preoperative GCT samples and samples of 106 controls, the diagnostic sensitivity of miR-371 was 88.7 % and the specificity was 93.4 %. Overall, it was proven that more than 86 % of GCT patients had increased miR-371 expression. These results are especially valuable for seminoma, in which only < 20 % of patients express β-HCG.

In summary, all three studies demonstrated that the miRNA miR-371 fulfils all prerequisites for a valuable biomarker for GCTs. However, further studies are needed to validate the miR-371 expression in a larger cohort of patients with metastases during the long-term observation after treatment.

6 Zusammenfassung

Hodenkrebs ist die häufigste maligne Erkrankung bei jungen Männern im Alter zwischen 14 und 44 Jahren. Tumormarker sind für die Diagnose, die klinische Stadieneinteilung, sowie das Therapie-Monitoring und für die Nachsorge von Patienten unverzichtbar. Die etablierten Serummarker für testikuläre Keimzelltumoren (GCTs) sind AFP, β-HCG und LDH. Sie sind jedoch insgesamt bei nur 60 % aller Patienten erhöht und aufgrund ihrer limitierten Sensitivität werden neue Marker dringend benötigt. In letzter Zeit hat sich gezeigt, dass miRNAs ein neue Klasse von Serum-Biomarkern darstellen. Diese kleinen nicht-codierenden RNA-Moleküle sind in diverse biologische Prozesse, wie die Embryonalentwicklung, Zelldifferenzierung, Apoptose und Tumorentstehung involviert. Viele Studien haben gezeigt, dass bei verschiedenen Tumortypen eine Dysregulierung von miRNAs vorliegt und miRNA-Expressionsprofile als mögliche Biomarker in der Krebsdiagnostik eingesetzt werden könnten. Bei GCTs sind miRNAs der beiden Cluster miR-371-3 und miR-302/367 überexprimiert. Das Ziel dieser Dissertation war es anhand von miRNA-Expressionsanalysen zu überprüfen, ob miRNAs dieser Cluster als Biomarker für Patienten mit GCT eingesetzt werden können.

Die erste Studie zeigte signifikant erhöhte Serumlevel von miR-371 bei 20 CS1-Patienten im Vergleich zu 20 Kontrollen und 24 Patienten mit nicht-testikulären malignen Erkrankungen (NTMs). Die mittleren RQ-Werte der NTMs lagen im Bereich der Kontrollen. Dies verdeutlicht die Spezifität dieser miRNA für GCTs. Des Weiteren ergab die Analyse der Expressionslevel im Hodenvenenblut (TVB) von fünf GCT-Patienten signifikant höhere mittlere Serumlevel als im korrespondierenden Kubitalvenenblut (CVB). Dies deutet darauf hin, dass der Tumor die primäre Quelle für die erhöhte miRNA-Expression im Blut der Patienten ist. Die sechs Patienten mit GCNIS hatten leicht erhöhte Serumlevel im Vergleich zu den Kontrollen und die Serum-Messung von miR-371 könnte eventuell zukünftig für die Diagnose von GCNIS eingesetzt werden. Außerdem konnte in dieser Studie bei fünf CS1-Patienten nach der Operation eine schnelle Abnahme der miR-371-Expression belegt werden, was ein unverzichtbares Leistungsmerkmal eines hochwertigen Biomarkers darstellt.

Der Fokus der zweiten Studie lag auf der Messung der miR-371-Expression in der, den Tumor umgebenden, Hydrozelenflüssigkeit (HY). Die Analyse der HY und korrespondierenden Serumproben von neun Patienten mit GCT und drei Kontrollen mit ideopathischer Hydrozele zeigte viel höhere Level der miR-371 in der Flüssigkeit als im Serum. In der HY der Kontrollen konnte keine Expression detektiert werden. Diese Ergebnisse verdeutlichen die Annahme, dass ein direktes Eindringen der miRNA-Moleküle aus dem Tumor in die umgebenden Kompartimente vorliegt. Basierend auf der vorherigen Studie, war ein weiteres Ziel dieser Studie die Expression von miR-371 in TVB- und korrespondierenden CVB-Proben in einem größeren Umfang zu analysieren. Die Expression war bei jedem der 51 CS1-Patienten und

13 metastasierten Patienten im TVB signifikant höher als im CVB. Die miRNA-Level in den TVB- und CVB-Proben waren bei den Patienten signifikant höher als bei den zehn Kontrollen, die nur einen geringen Unterschied zwischen der miRNA-Expression in TVB und CVB aufwiesen. Diese Resultate bestätigen die Ergebnisse der ersten Studie und zeigen, dass die miiRNA-Konzentration in der Vene des Samenstrangs viel höher ist als im peripheren Blut, welches durch das gesamte Blutvolumen verdünnt wird. Dies belegt, dass miR-371 von den Zellen des testikulären GCT freigegeben wird. Ein weiteres Ziel der Studie war es, im Vergleich zu der zuvor veröffentlichten Studie, den postoperativen Abfall der miR-371-Expression in einer größeren Kohorte von CS1-Patienten nachzuweisen. Die erzielten Ergebnisse konnten einen raschen Abfall der miR-371-Level nach der Operation auf ein normales Niveau belegen. In der dritten Studie wurde in einer Vorstudie der Nutzen von vier miRNAs der zwei miRNA-Cluster miR-371-3 und miR-302/367 als Biomarker für GCTs in einer Kohorte von 50 Patienten und 20 Kontrollen analysiert. Jede der vier miRNAs zeigte signifikant höhere Expressionslevel in GCT-Patienten als in Kontrollen. Bei den metastasierten Patienten wurden höhere Expressionslevel als bei den CS1-Patienten nachgewiesen. Jedoch wies miR-371 die höchste Sensitivität und Spezifität auf und die Trennschärfe dieser einen miRNA war besser als die des gesamten miRNA-Panels bestehend aus allen vier miRNAs. Da miR-371 am besten geeignet war, um GCT-Patienten von Kontrollen zu unterscheiden, wurde diese miRNA für die Hauptstudie mit einem Patientenkollektiv bestehend aus 166 Patienten mit GCT, zehn mit Rezidiv, zwölf mit Leydigzelltumoren (LCTs) und 106 Kontrollen eingesetzt. Statistische Quervergleiche zwischen den histologischen GCT-Untergruppen, Kontrollen und LCTs zeigten hoch signifikante Unterschiede der präoperativen Medianwerte. Außerdem konnte gezeigt werden, dass Patienten mit Metastasen signifikant höhere Expressionslevel als CS1-Patienten aufweisen. Bei den CS1-Patienten fiel das Level der miR-371 postoperativ in den Bereich der Kontrollen, was den tumorfreien Zustand widerspiegelt. Diese Ergebnisse bestätigen die der beiden zuvor veröffentlichten Studien. Bei den Patienten mit Metastasen nahm die miR-371-Expression während der Chemotherapie ab und erreichten nach Abschluss der Therapie den Bereich der Kontrollen. Auch die Patienten mit Rezidiv hatten erhöhte Expressionslevel. miR-371 hat eine diagnostische Sensitivität von 88,7 % und eine Spezifität von 93,4 %. Insgesamt war die Expression bei mehr als 88 % der Patienten erhöht. Die Ergebnisse sind besonders für Seminome wertvoll, da nur < 20 % dieser Patienten eine Erhöhung von β-HCG aufweisen.

Zusammenfassend konnte in den drei Studien gezeigt werden, dass die miRNA miR-371 alle Voraussetzungen für einen hochwertigen Biomarker für Patienten mit GCT erfüllt. Jedoch sind weitere Studien notwendig, um die Expression von miR-371 in einer größeren Kohorte von GCT-Patienten mit Metastasen nach der Behandlung während der Langzeitbeobachtung zu validieren.

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8 Abbreviations

°C	Degree Celsius
%	Percent
β-HCG	β-subunit of human chorionic gonadotropin
ΔΔCT	Delta delta CT
µl	Microliter
χ^2	Pearson's Chi-square
AFP	α-fetoprotein
AUC	Area under the curve
BEP	Chemotherapy with cisplatin, etoposide, and bleomycin
C	Control
cDNA	Complementary DNA
CI	Confidence interval
CIS	Carcinoma in situ
CLL	Chronic lymphocytic leukaemia
CS	Clinical stage
CT	Cycle threshold
CVB	Cubital vein blood
DNA	Deoxyribonucleic acid
EC	Embryonal carcinoma
ESCs	Embryonic stem cells
g	Gravitational acceleration g = 9.81 m/s^2
GCNIS	Germ cell neoplasia in situ
GCTs	Germ cell tumours
h	Hour
HY	Hydrocele fluid
IGCCCG	International Germ Cell Cancer Collaborative Group
IGCNU	Intratubular germ cell neoplasia unclassified
iPSCs	Induced pluripotent stem cells
IQR	Interquartile ranges
ISH	In situ hybridization
kb	Kilo base pairs
L	Leydig cell tumours
LATS2	Large tumour suppressor homologue 2
LCTs	Leydig cell tumours
LDH	Lactate dehydrogenase

min	Minute
miR	MicroRNA
miR-302/367	MicroRNA cluster on chromosome 4
miR-371-3	MicroRNA cluster on chromosome 19
miRNA	MicroRNA
mRNA	Messenger RNA
ml	Millilitre
NS	Nonseminoma
nt	Nucleotide
NTMs	Non-testicular malignancies
PCR	Polymerase chain reaction
PGCs	Primordial germ cells
PLAP	Placental alkaline phosphatase
pT stage	Pathological tumour stage
qPCR	Quantitative polymerase chain reaction
R^2	Pearson product-moment correlation coefficient
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RPLND	Retroperitoneal lymph node dissection
RQ	Relative quantity
RT	Reverse transcription
S	Seminoma
SE	Seminoma
sec	Second
T	Teratoma
TC	Testicular cancer
TGCTs	Testicular germ cell tumours
TIN	Testicular intraepithelial neoplasia
TNM	Tumour, Node, Metastasis
TVB	Testicular vein blood
UICC	International Union Against Cancer
UTR	Untranslated region
WHO	World Health Organisation
YST	Yolk sac tumour

9 Complete list of publications

9.1 Peer-reviewed paper

1. Belge G, Dieckmann KP, Spiekermann M, Balks T, Bullerdiek J. (2012). Serum levels of microRNAs miR-371-3: a novel class of serum biomarkers for testicular germ cell tumors? Eur Urol. 61 (5):1068-9.

2. Dieckmann KP, Spiekermann M, Balks T, Flor I, Löning T, Bullerdiek J, Belge G. (2012). MicroRNAs miR-371-3 in serum as diagnostic tools in the management of testicular germ cell tumours. Br J Cancer. 107 (10):1754-60.
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3. Spiekermann M, Belge G, Winter N, Ikogho R, Balks T, Bullerdiek J, Dieckmann KP. (2015). MicroRNA miR-371a-3p in serum of patients with germ cell tumours: evaluations for establishing a serum biomarker. Andrology. 3 (1):78-84.
   The first two authors contributed equally to this work.

4. Spiekermann M, Dieckmann KP, Balks T, Bullerdiek J, Belge G. (2015). Is relative quantification dispensable for the measurement of microRNAs as serum biomarkers in germ cell tumors? Anticancer Res. 35 (1):117-21.

5. Dieckmann KP, Spiekermann M, Balks T, Ikogho R, Anheuser P, Wosniok W, Loening T, Bullerdiek J, Belge G. (2016). MicroRNA miR-371a-3p - A Novel Serum Biomarker of Testicular Germ Cell Tumors: Evidence for Specificity from Measurements in Testicular Vein Blood and in Neoplastic Hydrocele Fluid. Urol Int. 97 (1):76-83.
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6. Flor I, Spiekermann M, Löning T, Dieckmann KP, Belge G, Bullerdiek J. (2016). Expression of microRNAs of C19MC in Different Histological Types of Testicular Germ Cell Tumour. Cancer Genomics Proteomics. 13 (4):281-289.

7. Dieckmann KP, Radtke A, Spiekermann M, Balks T, Matthies C, Becker P, Ruf C, Oing C, Oechsle K, Bokemeyer C, Hammel J, Melchior S, Wosniok W, Belge G. (2017). Serum Levels of MicroRNA miR-371a-3p: A Sensitive and Specific New Biomarker for Germ Cell Tumours. Eur Urol. 71 (2):213-220.
   The first three authors contributed equally to this work.

9.2 Oral presentations

1. Spiekermann M. microRNAs als potentielle neue Biomarker für testikuläre Keimzelltumoren. 31. Treffen Norddeutscher Humangenetiker in Bremen 2012

9.3 Poster presentations

1. Spiekermann M, Dieckmann KP, Balks T, Flor I, Löning T, Bullerdiek J, Belge G. Expression of microRNAs miR-371-3 in testicular tumors. 24. Jahrestagung der Deutschen Gesellschaft für Humangenetik (GfH) in Dresden 2013

2. Spiekermann M, Dieckmann KP, Bullerdiek J, Belge G. microRNAs: Neue Biomarker bei testikulären Keimzelltumoren. 13. Bremer Krebskongress in Bremen 2013
   (Award for the best scientific poster)

3. Spiekermann M, Radtke A, Oechsle K, Matthies C, Melchior S, Dieckmann KP, Belge G. microRNA miR-371a-3p – a novel biomarker for monitoring testicular germ cell tumors. 27. Jahrestagung der Deutschen Gesellschaft für Humangenetik (GfH) in Lübeck 2016

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11 Erklärung

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

„microRNAs als neuartige Biomarker für Patienten mit testikulären Keimzelltumoren“

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

Bremen, 23.04.2019

Meike Spiekermann