

## MicroRNAs in Testicular Cancer: Implications for Pathogenesis, Diagnosis, Prognosis and Therapy

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**Abstract.** Testicular germ cell tumors (TGCTs) represent the most common type of solid tumors among men aged 15 to 40 years. An increasing incidence has been recorded in developed countries. In clinical practice, TGCTs are classified as seminomas and non-seminomatous tumors. Non-seminomatous tumors often contain multiple different cell types and can be further sub-divided according to the histological and cellular phenotype in embryonal carcinomas, choriocarcinomas, yolk sac tumors and teratomas. For the clinical management of TGCTs, blood-based markers such as lactate dehydrogenase, alpha-fetoprotein and human chorionic gonadotropin are essential tools for diagnosis, risk assessment and patient's prognosis. However, only 60% of patients with TGCTs show increased serum levels of these tumor markers. This proportion of patients is even lower for those with seminomas or pure embryonal carcinomas as alpha-fetoprotein is predominantly related to yolk sac tumor and human chorionic gonadotropin to choriocarcinoma.

Recently, small non-coding RNAs (microRNAs) were found to play a pivotal role in the process of malignant transformation in several types of cancers. By direct interaction with the larger messenger RNA molecules, microRNAs regulate the level of gene expression on a post-transcriptional level. MicroRNAs show great potential as novel biomarkers because they reveal high stability in body fluids, and they are easily and relatively inexpensive to detect by standard quantitative reverse transcriptase polymerase chain reaction assays. In the present

review, we summarize the latest research findings regarding the role of microRNAs in the pathogenesis of TGCTs. In addition, we indicate their potential as diagnostic, prognostic and therapeutic biomolecules.

### MicroRNAs

With growing knowledge about the human genome, it has become clear that less than 2% of the human genome consists of coding regions (exons) that are able to serve as a matrix for a respective messenger RNA (mRNA). This process of gene transcription in the nucleus is followed by the process of translation into a protein at the cytoplasmic level (1). Until the early 1990s, the rest of the genome was thought to function as a buffer against genetic damage, without having any specific role (some researchers called these pieces of DNA 'junk DNA') (1). Within the last 20 years, more and more of the enigmatic role of this non-protein-coding genomic region has been resolved. New classes of RNA molecules, so called non-coding RNAs, have been identified as 'new kids on the block' (19).

In this context, a new class of non-coding RNAs, small regulatory RNAs (microRNAs, or miRNAs) were discovered (2). miRNAs have since been investigated in several thousands of studies and were found to play an important role as post-transcriptional regulators. Notably, they are not only involved in many physiological processes, such as cell differentiation, but also influence pathological processes, including carcinogenesis (7).

Considering some general aspects, miRNAs are normally transcribed by RNA polymerase II as primary transcripts i-miRNA. Primary miRNAs form specific stem-loop structures and are cleaved in the nucleus by the RNase DROSHA into isolated hairpin loops (pre-miRNA) that are transported into the cytoplasm by the EXPORTIN 5 pore protein. In the cytoplasm, the loop is cleaved by the endoribonuclease DICER into mature miRNA. This molecule is single-stranded with a length of 19-22 nucleotides. Its specific sequence is known as the seed sequence (6, 7).

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miRNAs bind to complementary sequences in the 3'-untranslated region (UTR) or other regions of their target mRNAs, usually resulting in gene silencing through the induction of mRNA degradation or inhibition of protein translation.

In mammalian cells, the hybridization of miRNA and target mRNA is non-perfect. It is believed that mRNAs are recognized by multiple miRNAs and the total number of interactions determines the inhibition of protein translation (22).

The non-perfect complementarity between miRNAs and protein-coding genes makes it difficult to pinpoint relevant downstream targets of individual miRNAs. Several algorithms were developed to predict putative miRNA targets such as miRanda, Pic Tar and Target Scans (35).

As miRNAs interfere with the translation of a given mRNA to a protein, they can act as tumor-suppressor genes or oncogenes. For example, in chronic lymphatic leukemia, miR-15 and miR-16 function as tumor suppressors, whereas the miR-17 to -92 cluster is found to be an oncogene in lung cancer (22).

The expression profiling of miRNAs can be informative regarding cancer prognosis and has been extensively shown in many cancer types, including chronic lymphatic leukemia, colorectal cancer, breast and kidney cancer (1, 30-33).

Furthermore, there have been attempts to use miRNAs as predictive factors for pharmacological response, medical treatment approaches and adverse side-effects of drugs (19), (3).

The aim of the present short review is to discuss the current insights of the involvement of miRNAs in the pathogenesis of testicular cancer. Furthermore, we highlight and describe their potential as novel diagnostic and prognostic biomarkers for future purposes in patients with testicular cancer.

## MicroRNAs and the Pathophysiology of Testicular Cancer

**Testicular cancer and p53.** In most types of solid cancer, an inactivating mutation of the tumor suppressor gene *p53* is found. One explanation is that one of the physiological functions of *p53* is induction of senescence after oncogenic stress by inducing cellular arrest or apoptosis (35). In testicular germ cell tumors (TGCTs), inactivating mutations of *p53* are rare events. Instead of a *p53* mutation or deletion, *miR-372* and *miR-373* have been identified to be up-regulated in TGCTs (4, 5, 12, 29). These miRNAs have been shown to mimic the effects of mutated *p53*. In the rare cases of seminomas with a proven mutation of *p53*, a lack of up-regulation of this specific set of miRNAs was reported (22, 35).

Vorhoeve *et al.* have shown that cells transduced with miR-372 or miR-373 continue to proliferate after rat sarcoma viral oncogene homolog (RAS)-induced oncogenic stress, whereas control cells ceased proliferation. In the control cell

group, following RAS stimulation, *p53* is activated and its target gene *p21* is induced. Increased levels of *p21* inhibit cyclin-dependent kinase (CDK) activity, causing cells to arrest in G<sub>1</sub> phase (10, 35).

miR-372 and miR-373 do not directly inhibit *p53* activity. Indeed, their direct target is the large tumor suppressor homolog 2 (*LATS2*) gene. miR-372 and miR-373 bind to two sites in the 3'UTR of *LATS2* and thereby suppress the translation of *LATS2* mRNA. A reduction of *LATS2* protein level seems to be responsible for the sustained activity of CDK, allowing uncontrolled cell proliferation (35).

**Testicular cancer and E2F Transcription factor.** The retinoblastoma tumor-suppressor protein exerts its tumor suppressive function by regulating the E2F Transcription factor (E2F) family of transcription factors.

*miR-449a* and *miR-449b* have been found to be up-regulated by E2F1 expression after DNA damage. miR-449a and -b structurally resemble miR-34, a well-known *p53*-responsive and pro-apoptotic miRNA. miR-449a and -b suppress cell proliferation by activation of a *p53*-induced mechanisms and probably also by *p53*-independent mechanisms. High miR-449 levels were found in normal testes, lung and trachea but were not detectable in testicular and other cancer cells (21).

**Epigenetic modulation in testicular cancer.** Epigenetic alteration is another proposed mechanism of carcinogenesis. In TGCTs, hypermethylation of the intronic region dynamine 3 (*DNM3*) at chromosome 1q24.3 leads to down-regulation of miR-199a(6). The target of miR-199a is the anti-adhesive transmembrane protein podocalyxin-like protein 1 (*PODXL*). *PODXL* is an anti-adhesion transmembrane protein that inhibits cell-cell interaction, which is causative for developing an invasive phenotype and metastasis (6). *PODXL* is highly expressed in malignant testicular tumor and negatively correlated with miR-199a expression. Hypermethylation of *DNM3* leads to *miR-199a* depression and up-regulation of *PODXL*. miR-199a was also linked to other malignancies, including gastric cancer, bladder cancer, uveal melanoma and ovarian cancer (34).

In a xenograft mouse model, expression of miR-199a in cancer cells led to suppression of cancer migration, invasion and cell growth.

When comparing the different histological subtypes of testicular cancer it was found that *miR-199a* has a higher degree of methylation in seminomas than in non-seminomatous tumors (6).

**Male infertility and testicular cancer.** Infertile men are nearly three-times more likely to develop TGCTs than are those who are fertile (36). A possible connection between male infertility and TGCTs might be through a pathway which is

regulated by *miR-383* (18). *miR-383* expression is down-regulated in the testes of infertile men with maturation arrest. Down-regulation of *miR-383* results in enhanced proliferative activity of germ cells. The direct target of *miR-383* is the interferon regulatory factor-1 (*IRF1*) which has been identified as a tumor-suppressor gene. However, in spermatogonia and early spermatocytes, it seems to have a pro-mitogenic role. *IRF1* is a downstream target of *miR-383* and is negatively correlated with *miR-383* expression. The inhibition of *IRF1* leads to reduced levels of cyclin D1, CDK2 and p21. Cyclin D1 seems to be the major gene responsible for *miR-383* mediated antiproliferative effects. Furthermore, ectopic expression of *miR-383* leads to a decrease in CDK4 expression. The cyclin D1-CDK4-p21 complex leads to phosphorylation and inactivation of the retinoblastoma tumor-suppressor protein (pRb).

Thus, *miR-383* is a negative regulator of proliferation by targeting *IRF1* and through inactivation of the pRb pathway (18).

## Diagnosis

TGCTs arise from carcinoma *in situ* cells which resemble malignant primordial germ cells of fetal origin (15, 24). They persist in the testis until puberty or early adulthood and will then progress in either seminoma (which generally do not differentiate) or non-seminomatous tumors.

Carcinoma *in situ* cells exhibit a gene expression pattern similar to that of embryonic stem cells, which explains their pluripotency. They can form the germ cell lineage (seminoma) or embryonal stem cell components (embryonal carcinoma), with possible further differentiation to choriocarcinoma, yolk sac tumors or teratoma (16, 27, 28). As miRNAs regulate cell differentiation and other physiological processes, it has been supposed that in cases of malignant transformation, the miRNA profile changes. Several studies indicate that miRNAs are involved in this critical step of carcinogenesis (11, 12).

For instance, in seminoma a significant difference in miRNA expression was found when compared with normal testicular tissue. *miR-221*, *miR-222*, *miR-372* and *miR-374* are up-regulated in seminoma whereas other miRs including *miR-30a*, *miR-34a*, *miR-99*, *miR-105-1.2*, *miR-106a*, *miR-293*, *miR-217*, *miR-196-1*, and *miR-196-2* are down-regulated.

In addition, expression profiling of miRNAs can also be used for differentiation of the major histological subtypes of TGCTs (12, 22, 23).

The *miR-302* cluster is expressed in human embryonic stem cells and therefore in embryonal carcinoma and seminoma also (11, 12). With differentiation of embryonal carcinomas to teratomas, the expression of *miR-302* cluster is down-regulated. The same expression pattern is found for *miR-17-5p* and *miR-154*. They are expressed in embryonal

carcinoma and down-regulated upon differentiation to teratomas (12). *miR-301* is predominantly found in the more differentiated tissues, such as spermatocytic seminomas, yolk sac tumors and teratomas, whereas *miR-301* is absent from embryonic stem cells and embryonal carcinoma (12).

As mentioned in the chapter of "MicroRNAs and the pathophysiology of Testicular cancer", the *miR-371* to *miR-373* cluster is expressed in seminomas, embryonal carcinomas, yolk sac tumors and is related to wild-type p53 status (22, 23, 12).

## Monitoring

After tumor tissue-specific miRNAs were identified in the serum of patients, the idea was born that miRNAs could qualify as circulating biomarkers, a concept that is well-known for other molecular components such as circulating tumor cells or circulating free DNA (14, 25, 26, 37), (13).

In a prospective study by Dieckmann *et al.*, serum levels of *miRNA-371* to *-373* from 24 patients with TGCTs (20 seminoma, four non-seminomatous tumors) were quantified by reverse transcriptase polymerase chain reaction (8). Out of the three miRNAs quantified, *miR-371a-3p* showed the most impressive result as considerably high expression was revealed in treatment-naïve patients, with a strong decline after surgical removal of the tumor. In this study, 20 patients presented with clinical stage I disease. Only 25% showed elevation of the classical markers (alpha-fetoprotein, human chorionic gonadotropin), whereas 85% of patients had a higher level of *miRNA-371a-3p* when compared with the mean value of healthy controls. Moreover, in stage I disease, the serum level of *miRNA 371a-3p* decreased significantly after orchiectomy (a 336.7-fold reduction). In four patients with advanced disease serum levels of *miRNA-371a-3p* dropped into normal range of the control population after completion of chemotherapy treatment. Interestingly, neither a correlation between miRNA expression level in tumor tissue and serum, nor a correlation with the extent of tumor volume was observed (8).

In another study, Gillis *et al.* compared serum levels of *miR-371* to *-373*, *miR-302* and *miR-367* from 80 patients with TGCTs with those of 47 healthy controls. *miR-371* to *-373* and *miR-367* showed the most promising results. By combining these miRNAs, a clear separation of tumor from control samples was possible. The miRNAs had an overall sensitivity of 98%, whereas the traditional serum markers alpha-fetoprotein/human chorionic gonadotropin revealed only a sensitivity of 36%/57%, which was even lower when applied for seminomas alone (11).

These results underline the superiority of miRNAs for diagnosis and monitoring of stem cell components when compared to traditional markers.

Further studies with larger patient cohorts are warranted to confirm these promising first results.

## Prognosis and Treatment

TGCTs respond well to chemotherapy and more than 80% of patients with metastatic TGCTs are curable using a cisplatin-based first-line chemotherapy (9). It is supposed that this high cure rate might be related to elevated wild-type p53 function in TGCTs or high levels of the pro-apoptotic BCL2-associated X protein (BAX) and low levels of the anti-apoptotic BCL2 protein (high BAX:BCL2 ratio).

When comparing TGCT cell lines with somatic cell lines after cisplatin exposure, differential gene expression was identified (9). For miR-372 to -373, which have frequently been reported to be overexpressed in TGCTs, around 40 target genes were identified. Neogenin homolog 1 (*NEO1*), *LATS2* and estrogen receptor 1 (*ESR1*) were the only genes that have the predicted binding sites for both p53 and miR-372 to -373. *NEO1* and *LATS2* were down-regulated in GCT cells after cisplatin exposure, while *ESR1* was up-regulated (9).

Another reason for an altered expression of miR-372 to -373 targeted genes might be the down-regulation of the endoribonuclease *DICER1* gene after cisplatin exposure. Loss of *DICER1* was reported to cause embryonic lethality and loss of stem cell population. Cisplatin-induced down-regulation of *DICER1* may repress the oncogenic functions of miR-372 to -373 (9).

Several studies have shown that aberrant miRNA expression is related to overall survival, disease stage and the development of metastases and disease recurrences (1, 32).

Moreover, expression levels of miRNA-302a have been associated with increased sensitivity of TGCTs to cisplatin. Up-regulation of miRNA-302 enhances cisplatin-induced G<sub>2</sub>/M phase arrest and subsequent apoptosis (20).

On the other hand, cisplatin resistance has been associated with high levels of cytoplasmatic p21. miR-106b seed family members regulate p21 expression levels. Octamer binding transcription factor 4 (OCT4) regulates the expression level of miR-106b. OCT3/4 are markers of pluripotency and are therefore expressed in embryonic stem cells and TGCTs but not in mature teratomas (17, 22).

Cisplatin-resistant cells have lower levels of OCT4 and miR-106b family members, and high amounts of cytoplasmic p21, resulting in CDK2 inhibition and prevention of cisplatin-induced apoptosis. Cytoplasmatic localization of p21 can be caused by phosphorylation of p21. Phosphorylation of p21 is mediated through protein kinase B (pAKT). Deactivation of AKT via the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 or with the specific AKT inhibitor triciribine leads to nuclear localization of p21, which in turn is no longer capable of blocking cisplatin-induced apoptosis (17).

Germ cell tumors often represent curative disease, including advanced disease stages, when treated with chemotherapy. Nevertheless, short- and long-term side-effects

such as infertility, renal impairment, lung toxicity and others, can occur from chemotherapy. Improved prognostic tools are needed to categorize the risk of the individual patient in order to minimize such effects of treatment. miRNAs seem to have great potential as diagnostic, prognostic and, ultimately, therapeutic biomolecules in this future perspective.

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