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Recent advances in the regulation of testicular germ cell tumors by microRNAs

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1. ABSTRACT

Testicular germ cell tumors (TGCTs) are generally rare but represent the most common solid tumors in young men. They are classified broadly into seminoma, which resemble primordial germ cells (PGCs), and non-seminoma, which are either undifferentiated (embryonic carcinoma) or differentiated (teratoma, yolk sac tumor, choriocarcinomas) patterning. A widespread role for microRNAs (miRNAs), in diverse molecular processes driving initiation and progression of various types of TGCTs has been recently studied. We discuss the involvement of different miRNAs in the development and progression of different types of TGCTs. Moreover, we highlight the aberrant expression of miRNAs in TGCTs and several targets, which may define miRNAs as oncomiRs or tumor suppressors. A better understanding of miRNA biology may ultimately yield further insight into the molecular mechanisms of tumorigenesis and new therapeutic strategies against TGCTs.

2. INTRODUCTION

Testicular cancers are generally grouped into three broad categories with type I testicular germ cell tumors (TGCTs) being observed primarily in neonatal boys and young children (1). Type III TGCTs, also called spermatocytic seminomas, affect older men above 50 years of age and are derived from a slowly growing expansion of type B spermatogonia (2). In clinical practice, type II TGCTs are classified as seminomas and non-seminomatous tumors. Non- seminomatous tumors often contain multiple varieties of cell types and can be further sub-divided according to the histological and cellular phenotype in embryonic carcinomas (EC), choriocarcinomas (CC), yolk sac tumors (YST) and teratomas (TT). All type II TGCTs develop from a pre-invasive lesion termed carcinoma *in situ* (CIS), which has been identified as a dysfunctional fetal germ cell (Figure 1) (2, 3). In the developed world, the incidence of type II TGCTs, but not type I or III, has increased significantly over the last century to become

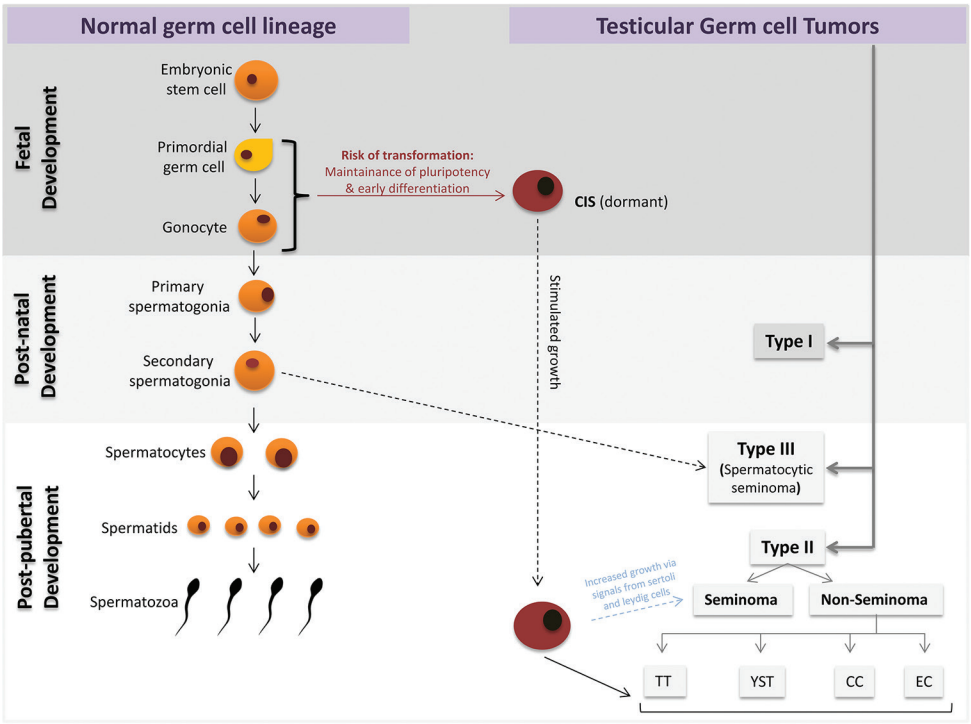

Figure 1. Histologically TGCTs are classified as Type I (neonatal boys and young children), Type II (20~40 years old men) & Type III (>50 years old men). Recent studies indicate maintained pluripotency combined with incomplete premature differentiation of gonocytes causes the specification of carcinoma *in situ* (CIS) cells. Signals caused by puberty make these cells to proliferate and once additional mutations accumulate CIS cells differentiate into type II TGCTs (56). Type II TGCTs are further classified as Seminoma and Non-Seminoma include: Embryonic carcinoma (EC), Choriocarcinoma (CC), yolk-sac tumors (YST) and teratoma (TT).

the most common malignancy found in men aged between 20 and 40 years (4–6). Such findings have led to speculation that environmental factors impact on the tumorigenesis of this cancer (7). The apparent association of TGCTs with infertility raises the possibility that TGCTs may act as indicators of a general reduction in male reproductive health and rising fertility problems within the male population. Moreover, pre-existing sub-fertility increases in patients with type II tumors (8–9). Type II TGCTs are known to develop from dysfunctional gonocytes located within the seminiferous tubules (10), indicating that the risk factors predisposing an individual to TGCTs stays active in the early male fetal development, necessitating the investigation of early germ cell development in an effort to identify causative factors for type II TGCTs (Figure 1).

For the clinical management of TGCTs, increased levels of blood-based markers such as lactate dehydrogenase, alpha-fetoprotein and human chorionic gonadotropin are essential tools for diagnosis, risk assessment and patient's prognosis (11, 12). However, only 60% of patients with TGCTs show increased serum levels of these tumor markers (13). This proportion of patients is even lower for those with seminomas or pure EC as alpha-fetoprotein is predominantly related to YST and human chorionic gonadotropin to CC. Consequently, alternative and more operative markers should be investigated and introduced into TGCTs' prognostic and diagnostic field.

Accordingly recent evidence indicates that small non-coding RNA molecules include miRNAs, might also function as tumor suppressors and oncomiRs (oncogenes).

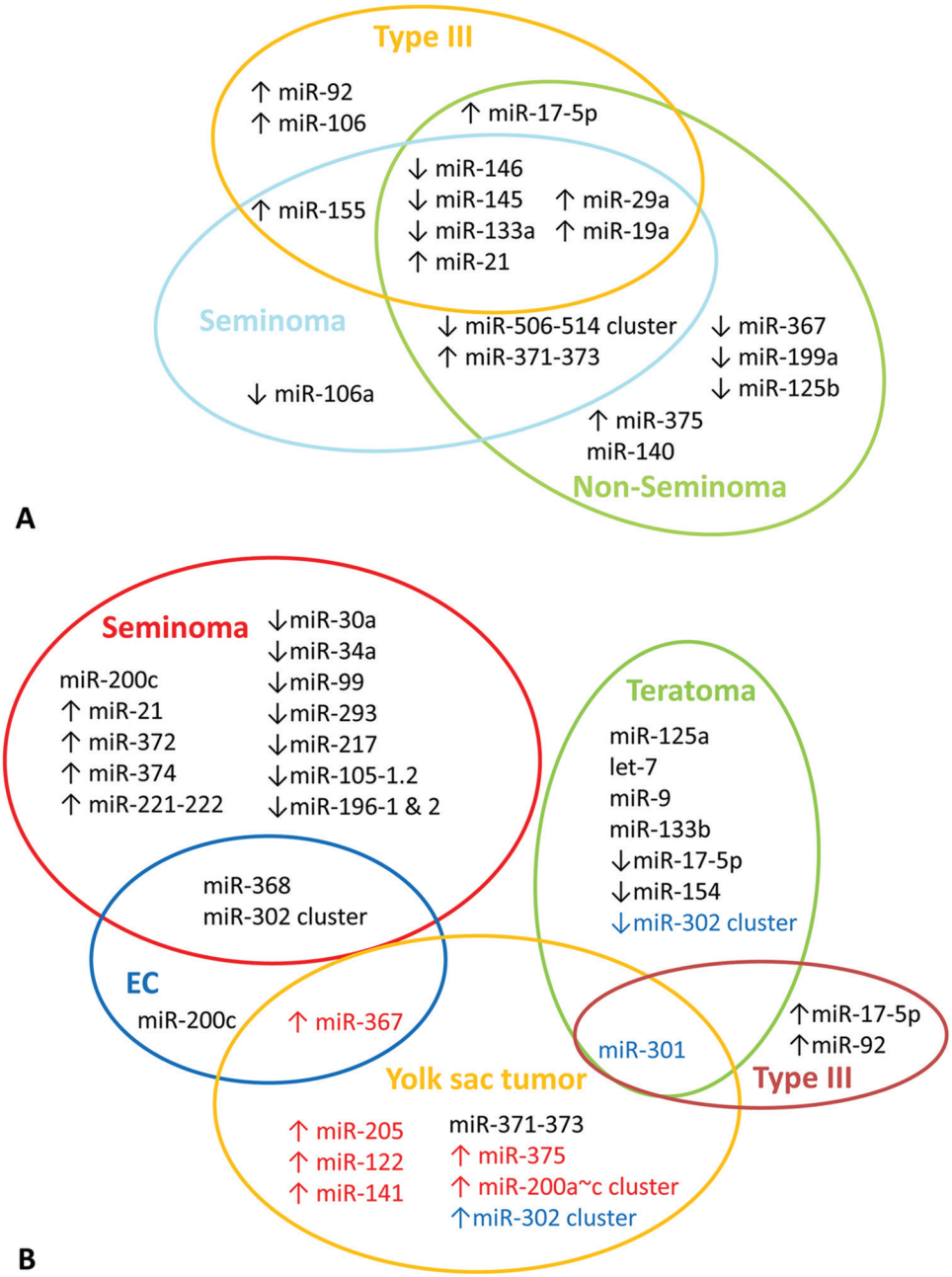

Figure 2. (A) Schematic diagram showing aberrantly expressed miRNAs in TGCTs as compare to normal testes. miRNAs up-regulated (↑) or down-regulated (↓) in type III TGCTs are encircled by yellow, in non-seminoma are encircled by green and in seminoma are encircled by blue circle. (B) Expression pattern in different types and sub-types of TGCTs compare with each other. Red font shows miRNAs expression as compare to seminoma (red circle), blue font shows miRNAs expression as compare to embryonic carcinoma (EC) (blue circle) and black font shows other aberrantly expressed miRNAs in these types and subtypes of TGCTs.

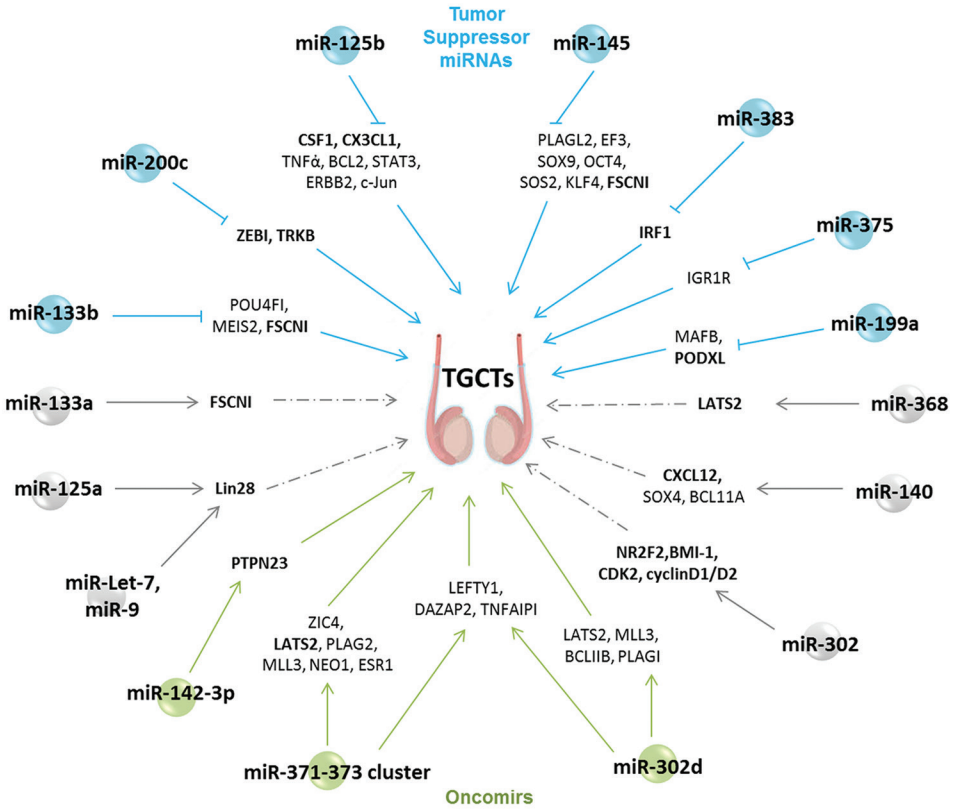

Figure 3. MiRNA molecules implicated in the development of testicular cancer including their proposed functions and; predicted (normal font) and confirmed (bold font) targets. Blue color indicates tumor suppressor miRNAs, green color indicates oncomiRs while grey color indicates those miRNAs which are find to be aberrantly expressed in TGCTs, targets are predicted or confirmed but functions are not reported yet. * (57).

miRNAs are an abundant class of endogenous small RNA molecules, 20–25 nucleotides in length (14–16), which regulate protein expression by mRNA cleavage or translation repression (15). A large quantity of miRNAs has been identified and subsequent studies indicated that they serve crucial roles in various biological processes and regulate the expression up to 30% of human genes (17, 18). In addition, expression profiling of miRNAs can also be used to distinguish as well as for differentiation of the major histological subtypes of TGCTs (19, 20, 21). As a result of their high sensitivity and the relatively easy method of detection, miRNAs have been shown to exhibit great potential as novel biomarkers for diagnosis, prognosis and therapy in cancer (20).

There have been attempts to use miRNAs as predictive factors for pharmacological response, medical treatment approaches and adverse side-effects of drugs (22, 23). The aim of the present review is to discuss the emerging field of oncomiRs and tumor suppressor miRNAs and current insights of the involvement of these miRNAs in the pathogenesis of TGCTs. Furthermore, we describe and highlight their potential as novel diagnostic and prognostic biomarkers for future purposes in patients with TGCTs.

3. ABERRANTLY EXPRESSED miRNAs IN TGCTs

Aberrant expression of miRNAs correlates with various cancers, as well as with various types of TGCTs. Previous studies revealed that some miRNAs exhibit abnormal expressions in TGCTs samples as compare to

normal testis. For example, down-regulation of miR-506~514 cluster was previously reported in seminomas and EC as compared to CIS and normal testis, suggesting its important role in TGCTs development (24). A former study has shown significantly lower miR-199a expression in TGCTs compared with normal testicular germ cells (25, 26). Likewise high miR-449 levels were found in normal testis, lung and trachea but were not detectable in testicular and other cancer cells (27). Furthermore, relatively higher expressions of miR-142-3p are found in TGCTs compared with normal testis (28). Other miRNAs such as miR-99a, miR-100 and miR-145 are reported to be down-regulated and; miR-512-3p, miR-515, miR-517~518 and miR-525 are up-regulated in TGCTs (29).

Some of the miRNAs are differentially expressed in histological subtypes of TGCTs. Such as miR-302 cluster is expressed both in EC and seminoma (30, 31) while its expression is down-regulated with differentiation of EC to teratomas but up-regulated in YST (32). The same expression pattern is found for miR-17-5p and miR-154 that is they are expressed in EC but down-regulated upon differentiation to teratomas (Figure 2) (31). Conversely another study found that miR-371~373 and miR-302 clusters are overexpressed in all malignant TGCTs regardless of histological subtypes and patient age (33) but they didn't measured miR-302 cluster expressions in teratoma. Moreover, miR-301 is predominantly found in the more differentiated tissues, such as spermatocytic seminomas, YST and teratomas, whereas miR-301 is absent in EC (Figure 2) (31, 34). MiR-146 expression is lower in seminomas, spermatocytic seminomas, and even different types of non-seminomatous tumors such as EC and teratomas compared with normal testis (31).

3.1. miRNAs as tumor suppressors

Like a coding gene, a miRNA can act as a tumor suppressor with the loss of its function and 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 (35, 36–38). Advances in recent research suggest that some miRNAs can act as tumor suppressors via regulating tumor cell proliferation and invasion such as miR-133a, miR-133b and miR-145. Both miR-133b and miR-145 can target an oncogenic gene FSCNI concordantly and other target genes individually to control carcinogenesis (31, 39, 40). Anti-proliferation and anti-invasion effects of miR-199a were demonstrated and identified through its direct targets, PODXL and MAFB that mediates the tumor suppressor activity of miR-199a (Figure 3) (25, 26). Likewise, miR-449a and -b suppresses cell proliferation by activation of p53-induced mechanisms along with probable contribution by p53-independent mechanisms (27). MiR-125b is another tumor suppressor miRNA recently reported in TGCTs which suppresses tumor growth significantly via targeting tumor cell-derived chemokines CSF1 and CX3CL1, which are known to control the recruitment of tumor associated macrophages to tumor site (41).

A possible connection between TGCTs and male infertility might be through a pathway which is regulated by miRNAs. Infertile men are nearly three-times more likely to develop TGCTs than are those who are fertile (42). Previous study reveals that miR-383 expression is down-regulated in the testis of infertile men with maturation arrest. And this down-regulation of miR-383 results in enhanced proliferation and reduced apoptosis of germ cells via targeting one of the tumor suppressor genes, IRF1 (43). Other miRNAs with a clear tumor suppressor role have also been reported, although the evidence supporting this claim is merely correlative. Substantial experimental data are lacking, and miRNA knockout mice that develop or are predisposed to TGCTs have not yet been reported.

3.2. miRNAs as oncomiRs

The list of miRNAs that function as oncomiRs is short, but the evidence for their role in TGCTs is very strong. Genome-wide miRNA

profiling studies have provided evidence of miRNA up-regulations in TGCTs. It has been reported recently that oncogenic miRNAs suppress tumor suppressor genes and promote tumorigenesis (44). PTPN23 is one of the important tumor suppressor candidates and is involved in the tumorigenesis of various organs. In the absence of PTPN23 protein expression in human TGCTs relatively higher miR142-3p expression are observed, suggesting that miR142-3p plays an important role in the pathogenesis of TGCTs by repressing PTPN23 expression (28). Similarly miR-372 and miR-373 have been shown to play oncogenic roles in human TGCTs by targeting the tumor suppressor LATS2 (45). These two miRNAs promote cell proliferation and tumor development by neutralizing p53-mediated CDK inhibition, possibly through direct inhibition of expression of the tumor suppressor gene LATS2 (21, 45). To date, very few miRNAs have been functionally characterized in TGCTs. However, the functional roles of other differentially expressed miRNAs in TGCTs have yet to be characterized.

To study the remarkable responsiveness of TGCTs to miRNA dependent therapeutics, genetically engineered mouse models of TGCTs can be designed in future by conditionally activating oncomiRs and inactivating tumor suppressor miRNAs. Previously TGCTs mouse model has shown rapid germ cell tumorigenesis by activating oncogene Kras and inactivating tumor suppressor gene Pten. TGCTs in these mice were characterized histologically as teratoma grew bilaterally in some and unilaterally in other mice (46). Identification of the molecular features that make TGCTs responsive to miRNA therapeutics may yield broadly applicable biomarkers for chemosensitivity or chemoresistance and provide avenues for the development of more effective therapies for other cancers as well.

4. miRNA PROFILING TO IMPROVE DIAGNOSIS AND OUTCOME PREDICTION

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 (47–51). In a research by Dieckmann *et al*. serum levels of miRNA-371~373 from 24 patients with TGCTs (20 seminoma and 4 non-seminomatous tumors) were quantified by reverse transcriptase polymerase chain reaction (52). Out of the three miRNAs quantified, miR-371a-3p showed impressive result as considerably high expression was revealed in untreated 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. 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 with the extent of tumor volume was observed (53). Another study, compared serum levels of miR-371~373, miR-302 and miR-367 from 80 patients with TGCTs with those of 47 healthy controls. MiR-371~373 and miR-367 showed the most promising results. By combining these miRNAs, a clear separation of tumor from control samples was made 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% to 57%, which was even lower when applied for seminomas alone (30). 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 suggested to confirm these promising results.

miRNAs may increase sensitivity of TGCTs to clinically established chemotherapeutic drugs. TGCTs 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. Moreover, expression levels of miRNA-302a have been associated with increased sensitivity of TGCTs to cisplatin. Up-regulation of miRNA-302 enhances cisplatin-induced G2/M phase arrest and subsequent apoptosis (53). On the other hand, cisplatin resistance has been associated with high levels of cytoplasmic p21. MiR-106b seed family members regulate p21 expression levels. OCT4 regulates the expression level of miR-106b. OCT3 and 4 are markers of pluripotency and are therefore expressed in embryonic stem cells and TGCTs but not in mature teratomas (54, 55). 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 for future perspective.

5. FUTURE PERSPECTIVES

The rapidly progressing field of miRNAs continues to reveal the diversity and complexity of the RNA world. However, despite remarkable recent progress, the connection between TGCTs and miRNAs remains unclear with vague explanations to numerous unanswered questions. More sophisticated *in vivo* models are needed to identify and define miRNA functions because miRNAs can have significant effects on the transcriptome, while their full biological properties are unlikely to be explained by the suppression of a single or few proteins.

Although miRNAs only moderately suppresses their targets, miRNAs could exert both strong and broad effects, largely because they suppress many genes as they impact multiple feedback loops with other regulators of gene expression. Since modulating the level of a single miRNA could eventually affect many pathways at the same time, therefore, targets should be experimentally validated rather than predicted. Another potential challenge for future studies relates to the probabilistic tissue-specific functions of some miRNAs. The function of a given miRNA is dictated by numerous targets that are expressed in a given cell type.

6. CONCLUSION

In this review, we discussed miRNA regulation of target gene expression in relation to the establishment of TGCTs and discussed how miRNAs can act as oncomiRs and tumor suppressors. The characterization of the roles of the regulatory miRNAs in TGCTs and its prognosis requires further investigation and consolidation in both animal models and humans.

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