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Translation research on testicular germ cell tumors

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Abstract Testicular germ cell tumors are the most common malignancy in men aged 14 to 44 years. Although exceptional cisplatin sensitivity results in cure rates of >90%, resistance can occur for which there are currently no alternative treatment options. Translational research in recent years has led to various breakthroughs in detection and classification of these tumors. The identification of miRNA-371 expression as a marker of malignant testicular germ cell tumors (with the exception of teratomas) enables significantly more sensitive and specific detection of these cancers in various clinical contexts (primary tumor, residual disease, relapse etc.). Moreover, the identification of several genetic aberrations that are associated with treatment resistance and poor outcome, such as *TP53* mutations or copy number gain on chromosome 3p, underlines the necessity of genetic screening for improved risk classification of testicular cancer patients.

Keywords Germ cell tumor · Biomarkers · miRNA · *TP53* · 3p25.3 gain

Translationeel onderzoek naar testiculaire kiemceltumoren

Samenvatting Testiculaire kiemceltumoren zijn de meest voorkomende maligniteit bij mannen tussen de 14 en 44 jaar. Hoewel uitzonderlijke gevoeligheid voor cisplatin resulteert in genezingspercentages van >90%, kan er resistentie optreden, waarvoor momenteel geen alternatieve behandelingsopties beschikbaar zijn. Translationeel onderzoek heeft de afgelopen jaren geleid tot meerdere doorbraken in de detectie

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en classificatie van deze tumoren. De identificatie van miRNA-371-expressie als marker van testiculaire tumoren (met uitzondering van teratomen) maakt het mogelijk om deze tumoren te detecteren op een significant gevoeligere en specifiekere wijze in verschillende klinische contexten (primaire tumor, resterende ziekte, terugval, etc.). Bovendien benadrukt de identificatie van verschillende genetische afwijkingen die verband houden met behandelingsresistentie en een slechte prognose, zoals *TP53*-mutaties en toename van het aantal kopieën op chromosoom 3p, de noodzaak van genetische screening voor een betere risicoclassificatie van zaadbalkankerpatiënten.

Trefwoorden kiemceltumor · biomarkers · miRNA · *TP53* · 3p25.3 gain

Introduction

Germ cell tumors (GCTs) are neoplasms that can be diagnosed in the gonads (testes, ovaries and dysgenetic gonads). They originate from early embryonal stem cells or germ cells in various stages of their maturation. In addition, they can also be identified in extra-gonadal locations, like the mediastinum and the brain, that correspond to the migration route of primordial germ cells, i.e., the stem cell of gametogenesis, during embryogenesis [1]. Various subtypes of GCTs can be recognized. These show striking similarities within the defined subtypes, both in clinical behavior, pathogenesis, and molecular constitution, being irrespective of sex of the patient (male, female or indeterminate) as well as anatomical localization (gonadal or extragonadal). The focus of this paper will be on testicular GCTs. Related to translational research, this paper will relate to three topics, being: 1) classification, 2) defined microRNAs as liquid biopsy biomarker, and 3) the underlying mechanism

of treatment resistance. These are selected based on recently reported promising results, with potential significant impact on clinical management of this population of relatively young patients. The overall aim is to have the optimal tools for (early) primary diagnosis, informative follow up as well as (more) effective treatment, specifically in case of presence or development of refractory disease.

In fact, testicular GCTs can be divided in three subtypes [1, 2]. Type II GCTs are the most frequently subtype and occur in post-pubertal men with a mean age in the 3rd and 4th decade of life. These, by definition, malignant tumors can present themselves as two clinically relevant types of GCTs: seminomas (mean age 35 years) and non-seminomas (mean age 25 years), based on their histological appearance. The latter can consist of various histological subtypes: embryonal carcinoma, yolk sac tumor, choriocarcinoma and teratoma, either as one pure subtype or mixed within one tumor (schematically illustrated in Fig. 1). Moreover, a seminoma component can be mixed with the various types of non-seminomas (mean age 30 years). These different histological components mirror cell types found during intra-uterine embryonal development. All histological elements of type II GCTs develop from a common precursor lesion called Germ Cell Neoplasia *In Situ* (GCNIS), previously referred to as CIS, IGCNU and TIN ([3] for a historical overview). The invasive elements almost universally show increased chromosomal copy numbers of the short arm of chromosome 12 (12p), which is therefore not related to initiation but progression [4]. Without further specification, this paper will deal with the type II testicular GCTs (TGCTs).

Type I GCTs of the testis occur in prepubertal boys, rarely above 6 years of age [5]. They can consist of only two histological subtypes, i.e., yolk sac tumor and teratoma, with most tumors only presenting with teratoma, and as such showing an overall benign course. There is no association with GCNIS. In fact, the cell or origin is likely a cell in the transition between embryonal stem cell and primordial germ cell [6], although this is still not proven so far. These tumors also show no gain of 12p, nor any other universal genetic feature. Lastly, Type III GCTs, also known as spermatocytic tumors, historically spermatocytic seminoma, happen in older men and are benign, and are characterized by gain of chromosome 9 [7]. The cell of origin is most likely either a spermatogonium or spermatocyte [8, 9].

## Developmental biology related histology- and liquid biopsy-based biomarkers

As indicated, TGCTs are derived from in principle pluripotent cells and therefore also express certain marker genes corresponding to this cell type, like *OCT3/4* (also known as *POU5F1*), *SOX2* and *NANOG*1. These markers are very specific and have proven to be useful in histological TGCT diagnosis, especially OCT3/4 [10]. Interestingly, several genes related to pluripotency, including NANOG, are located on the short arm of chromosome 12, suggesting that gain of this region could contribute to retaining pluripotency in TGCTs that is normally lost during development.

Retention of embryonal characteristics is also the source of some of the main clinically informative biomarkers used for detection, follow-up as well as risk classification. Serum levels of Alpha Feto Protein (AFP, an embryonic serum protein) and β-Human Chorionic Gonadotropin (β-HCG, a placental hormone) are used together with levels of LDH to screen for patients with TGCTs. Based on the origin, non-seminomas show overall higher levels of these markers. In addition, levels above a pre-defined threshold are informative to identify patients with a poor prognosis, forming the basis for the IGCCCG risk classification which is widely used to classify metastatic (T)GCT patients into low, intermediate or poor risk categories [11]. However, these markers have several significant limitations, including the observations that their levels are elevated in only 30–40% of patients at initial diagnosis and that they are not specific for (T)GCTs but can also be elevated in different cancer types and other conditions [12], indicating the need for more accurate biomarkers. One of the expected targets is a defined set of so-called microRNAs (miRNA). This refers to a family of non-coding RNAs, relevant for normal development and cellular maintenance by messenger RNA translation regulation (i.e., protein formation), which might become mis-regulated during the processes of disease development, including cancer (including (T)GCTs).

Unbiased miRNA profiling has shown that there are several miRNAs that show differential expression between healthy tissue and TGCTs [13]. This group of miRNAs also shows different expression levels within the different TGCT histologies, with the highest expression in embryonal carcinoma, intermediate expression in seminoma and no expression in teratoma. Follow-up studies suggest that miRNA 371-3p is the most sensitive and specific target to identify patients with malignant GCTs (Type II as well as yolk sac tumor part of Type I). This miRNA is part of a cluster with miRNA-372 and miRNA-373, which are also highly expressed in (T)GCTs, but whose levels are less specific in identifying patients with this tumor type [14]. MiRNA-371-3p has a sensitivity of 90% and a specificity of 94% [15], respectively, consistently outperforming the serum protein markers described above that reach a sensitivity of only ~60%12. In summary, it is informative both in type I and type II GCT non-teratomatous elements. Moreover, GCNIS can be identified using miRNA371-3p expression in approximately 50% of cases, while classic protein markers are not informative for this lesion. The most significant downside is that miRNA371-3p detection is not informative for pure teratomas because this

Fig. 1 Schematic representation of the histological subtypes in type II germ cell tumors and their characteristics regarding miRNA-371 expression (**a**) and their cisplatin resistance (**b**)
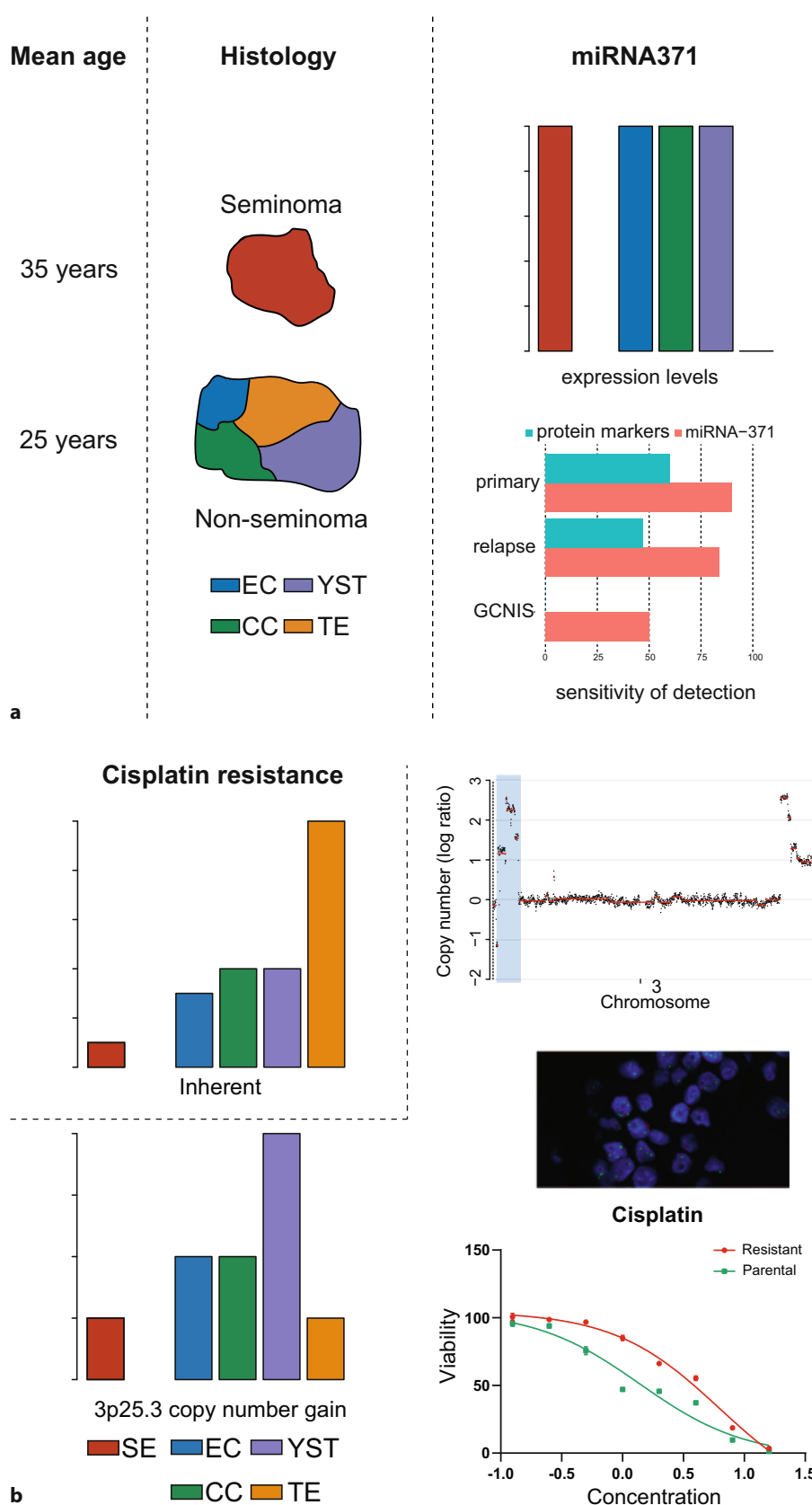
miRNA is not expressed in this histology (i.e., due to loss of the embryonic characteristics). However, the sensitivity for teratoma detection using the forementioned classical serum markers is also <25%, stressing the need for sensitive biomarkers for the identification of patients with mature teratoma (both during the process of initial diagnosis as well as follow up). Levels of miRNA375-3p have been suggested to be an informative marker for the detection of teratoma [16], however, other studies have not been able to reproduce these results [17, 18]. An interesting alternative biomarker for teratoma could be the methylated fragment of the *RASSF1A* promotor [19], although this requires confirmatory studies, specifically focusing on the more modern detection methods, like digital droplet based polymerase chain reaction (ddPCR).

In addition, detection of miRNA371-3p has also been shown to be superior to protein markers in relapse detection (83% vs <50%) [15, 20]. Moreover, several cases have been described where increased miRNA371-3p expression was detected months to years before clinical (including radiological) detection, indicating its potential application in surveillance as well [21, 22]. Interestingly, a health economic analysis has shown implementation of miRNA371-3p detection could lead to a reduction of the cost of surveillance of TGCTs of up to 44% due to the reduced need for imaging procedures [23], suggesting that this method could have clinical as well as economic benefits.

In spite of all the effort to determine their clinical value, not much is known about the functional role of miRNAs in (T)GCTs, however, it has been established that miRNA372 and miRNA373 can have an oncogenic role in primary (i.e., non-cancerous) cells. When an oncogenic RAS mutant is introduced into these cells, they normally enter senescence, however, expression of miRNA372 or miRNA373 prevents this and enables the cells to keep growing. This effect is functionally similar to P53 inactivation, which led the authors to investigate the link between these miRNAs and P53. They found that miRNA372-373 overexpression inhibits LATS2 expression leading to increased CDK2 activity and cell cycle progression, similar to observed when *TP53* is mutated [24]. Interestingly, TGCTs show a very low frequency of *TP53* mutations compared to most other types of cancer, suggesting that mir372-373 expression obviates the need for P53 inactivation. Concordantly, *TP53* mutations were only discovered in TGCTs that show lower levels of miRNA-372-373. However, this is likely not the only mechanism involved, because lack of functional P53 does not result in absence of expression of this set of miRNAs. In addition, cisplatin refractory GCTs (with the exception of teratoma) still express this cluster, which is relevant in de context of the use of this liquid biopsy biomarker in this clinical setting.

Besides the functional similarities between over-expression of the microRNA371-373 cluster *TP53*

mutations there is also one clear difference. *TP53* mutations are associated with poor therapy response and outcome in patients and were shown to induce cisplatin resistance when induced in GCT cell lines [25, 26]. For the miRNA371-3 cluster it was reported that higher expression was correlated with higher tumor mass, however, no association with outcome and therapy response was reported. This suggests that mutation of *TP53* has a broader impact in GCTs than only the re-activation of the cell cycle described before. Possibly related to this, *TP53* mutations are preferentially observed in mediastinal (Type II) GCTs [25], which could suggest that the difference in microenvironment (mediastinum versus testis) requires a more potent oncogenic driver (*TP53* mutation versus miRNA371-373 overexpression) [27].

Cisplatin resistance

Besides *TP53* mutations and aberrations in associated genes, like *MDM2* amplifications [25], also aberrations in *WNT/CTNNB1* signaling, the absence of KIT mutations and increased tumor mutational burden have been associated with cisplatin resistance in TGCTs based on the Next Generation Sequencing (NGS) analysis of resistant tumor material [28]. Moreover, we recently established a relationship between copy number gain of chromosome 3p cytoband p25.3 and cisplatin resistance in a set of independently generated cell line models, as well as a relationship between this gain of this region and poor outcome in multiple publicly available datasets [29]. The inclusion of this biomarker improves significantly upon the IGCCCG classification and could therefore be used to better assess patients risk of poor treatment response. Interestingly, the 3p25.3 copy number gain described before occurs throughout all histologies but is only significantly associated with poor prognosis in non-seminomas. Moreover, it occurs significantly more often in yolk sac tumors then in other histological subtypes. Interestingly, yolk sac tumor histology is often enriched in refractory or relapsed tumors [30, 31], suggesting that certain histologies may be more amenable to acquiring genetic aberrations that induce cisplatin resistance. However, the driving mechanisms behind the association between 3p25.3 and cisplatin resistance remain to be determined and are the goal of currently ongoing studies. In this context the preliminary results will be extended regarding the role of 3p25.3 copy number gain in Type I and II GCTs beyond the testis including the ovary, mediastinal, and intracranial localizations. These results might facilitate a unique starting point to develop alternative treatment options for GCT patients with refractory disease, both intrinsic as well as acquired (with the exception of teratoma).

Apart from gain of chromosome 3p25.3 and genetic aberrations in the TP53/MDM2 axis also micro satellite instability and decreased expression of DNA mis-

match repair proteins have been associated with cisplatin resistance [30, 32], suggesting that DNA repair, possibly as a result from separate underlying mechanisms, could play a general role in (T)GCT cisplatin resistance. A recent in vitro study supports this hypothesis by showing that the balance between the activation of different DNA repair pathways contributes to cisplatin resistance in this cancer indeed [33].

Other than cisplatin resistance that is acquired through genetic aberrations or other molecular changes, also inherent cisplatin resistance can play an important role in the variation in clinical behavior of (T)GCTs. The different histological subtypes show very different sensitivities to cisplatin. Seminomas are very sensitive and are therefore classified as low risk, irrespective of other criteria [11]. Teratoma, on the other hand is completely insensitive, and therefore, residual teratoma often remains after treatment. The teratoma is not intrinsically malignant but can cause problems because of its localization in respect to vital organs and in some cases can grow post chemotherapy [34], and is, therefore, required to be surgically resected. Moreover, teratoma also can progress to non-germ cell malignancies that are generally more aggressive and therapy resistant than TGCTs [35]. There is currently no good biomarker to identify these teratomas, since the miRNA-371-3p is not expressed, and therefore not detectable in liquid biopsies, so it is difficult to identify (residual) teratoma. However, combining miRNA371-3p expression with methylation analysis of the tumor marker RASSF1AM does enable the detection of teratoma, enabling easier identification of patients that require surgical resection [19]. Although of significant interest, based on the forementioned improvements of detection methods, it requires further evaluation related to its clinical impact.

Conclusion and future perspectives

In recent years significant progress has been made in detection/monitoring and classification and of (T)GCTs. As described above miRNA371 expression seems to a universal biomarker of both Type I and Type II GCTs (except teratoma) and can be widely used for, among others, screening, monitoring of therapy response and relapse detection. Moreover, the IGCCCG classification combined with chromosome 3p25.3 copy number status and the presence of *TP53* mutations, identifies a group of patients that do very poorly and might benefit from other treatment options. However, the only option that is currently available is more intensive chemotherapy treatment. One of the remaining challenges is, therefore, to identify novel therapeutics that benefit this patient group. This will likely also require a broader understanding of the molecular mechanisms that drive (T)GCT formation as well as the development of cisplatin resistance in some patients. Elucidating I) the role

of 12p gain in Type II GCTs II) the functional effect of miRNA expression in (T)GCTs and III) the mechanism through which 3p25.3 causes cisplatin resistance are in our view some important milestones that can significantly contribute to this goal.

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