The Past and Future of Biomarkers in Testicular Germ Cell Tumors

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Abstract

Testicular germ cell tumor (GCT) is the most common malignancy in 18- to 40-year-old men. Unlike most other cancers, GCT is frequently curable even when metastatic. These tumors can be classified histologically into seminoma and non-seminoma, which determines treatment. Therefore, successful treatment requires accurate diagnosis, classification, and monitoring. Serum tumor markers, including lactate dehydrogenase, α-fetoprotein, and β-human chorionic gonadotropin, aid in the classification and staging of GCTs. These markers therefore play a critical role in the decision-making process when managing GCT patients. However, there exist many scenarios in which these markers fail to perform adequately. This is particularly true in the case of seminoma, where only 10% to 15% will have elevated serum tumor markers. Non-specific elevation of these markers is also a common occurrence, complicating the interpretation of borderline positive results, particularly in follow-up. To bridge this gap in performance, next generation biomarkers are being investigated. In this review, we consider the role of conventional serum tumor markers in GCT management and discuss recent advances in the next generation of biomarkers, with a focus on circulating microRNAs. We discuss the value that circulating microRNAs could bring as an addition to currently used markers, as well as potential weaknesses, in GCT management.

Introduction

Testicular germ cell tumor (GCT) is the most common solid tumor in 18- to 40-year-old men, and accounts for more life-years lost than any other non-pediatric malignancy [1]. GCTs are histologically classified as seminoma or non-seminomatous GCT (NSGCT). Seminomas retain pluripotency markers and genotypically resemble primordial germ cells, their presumed cell of origin. NSGCT can be further subdivided based on differentiation into embryonic germ layers (teratoma), toward extraembryonic elements (choriocarcinoma, yolk sac tumor) or early embryonic elements (embryonal carcinoma) [2]. These classifications are not mutually exclusive: approximately 15% of NSGCT also contain seminomatous elements [3]. Identification of these elements is critical to determining optimal clinical management.

Serum tumor markers (STMs) can help to identify the presence of GCT, as well as the presence of particular components. Conventionally, 3 STMs have been used to monitor GCT: lactate dehydrogenase (LDH), α-fetoprotein (AFP), and β-human chorionic gonadotropin (β-hCG). STMs are particularly useful in NSGCT, 60% to 85% of which secrete detectable STMs (**Table 1**). However, these markers are less helpful in seminoma, in which only 10% to

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Abbreviations

AFP	α-fetoprotein
β-hCG	β-human chorionic gonadotropin
GCNIS	germ cell neoplasia in situ
GCT	germ cell tumor
IGCCCG	International Germ Cell Cancer Collaborative Group
LDH	lactate dehydrogenase
miRNA, miR-	microRNA
NSGCT	non-seminomatous germ cell tumor
RPLND	retroperitoneal lymph node dissection
STM	serum tumor marker

15% of cases have elevated markers, and AFP is absent by definition [4]. Further, markers often are normal in the case of early recurrence or low tumor burden, and non-specific processes may cause them to be elevated. Despite these limitations, STMs have proven integral in the diagnosis and monitoring of GCTs. This is evidenced by the current staging system including an S-stage classification, indicating the presence of these markers [5].

Although current markers play a critical role in the management of GCTs, there are numerous scenarios where they fail to provide adequate information. Perhaps most glaringly, 10% to 50% of men presenting with clinical stage I disease (any T, N0M0S0) with normal STMs post-orchiectomy will harbor occult disease [6]. Up to 30% of patients with clinical stage II disease (T any N1M0S0-1) will ultimately be found to have pathologic N0 disease [7]. There is currently no way to identify these patients at point of care.

In this review, we discuss currently used conventional STMs in the context of GCT diagnosis and management. We also examine the next generation of GCT markers, particularly circulating microRNAs (miRNAs, miR), which hold promise to fill the critical gaps left by currently conventional STMs [6].

Conventional Serum Tumor Markers

Lactate dehydrogenase

Lactate dehydrogenase (LDH) is a ubiquitously expressed enzyme in all cells that reversibly converts lactate to pyruvate [8]. Circulating LDH levels are associated with high cell turnover, and the level may be elevated in non-GCT malignancies, including lymphoma and renal cell carcinoma. Elevated LDH has also been reported in the case of cell lysis or injury, such as rheumatologic disorders, myocardial infarction, and other muscular disease [8]. Therefore, LDH is the least GCT-specific of the 3 conventional STMs. This problem is exacerbated by the fact that the enzymatic activity assay used to detect LDH varies considerably across laboratories. In part because of this variation, the precise half-life of LDH is unknown, but it is on the order of days, not hours [9]. Despite low specificity, LDH has relatively high sensitivity compared with other STMs: it is elevated in 40% to 60% of GCTs and may be the only positive marker in the case of seminoma, where as few as 30% of patients will have LDH elevation [8].

α-Fetoprotein

AFP is composed of an α-globulin molecule and carbohydrate moiety [9]. AFP is normally produced by the fetal yolk sac and liver, and therefore post-pubertal serum levels are generally low (<12 ng/mL) [9,10]. A chemiluminescent sandwich enzyme assay is typically used to detect it, which may have different upper limits of normal parameters across laboratories [9]. The half-life of AFP is estimated at 5 to 7 days [8].

AFP is produced in most yolk sac tumors and can also be detected in some patients with embryonal carcinoma or teratoma elements. Composition of the teratomatous element may play a role in AFP levels; in pure teratoma specimens, AFP may be produced by gastrointestinal or hepatic elements in the tumor [9]. AFP level is related to clinical stage in NSGCT, with patients with stage I disease showing AFP elevation in 10% to 20% of cases before orchiectomy, and patients with metastatic disease showing elevation in 40% to 60% of cases [8].

Elevated AFP levels preclude a diagnosis of seminoma by definition [8]. Even if pathologic examination of the orchiectomy specimen reveals seminoma exclusively, very high levels of AFP indicate the presence also of an element of NSGCT. In exceptional cases AFP may be elevated in patients with seminoma with hepatic metastases when liver regeneration is occurring [11]. Minimal or non-specific AFP elevations must be considered with caution when attempting to classify such cases. Several reports exist of marginal AFP elevation (generally < 20 ng/mL) in cases of pure seminoma [12]. False positive AFP levels are often associated with certain liver conditions, including chronic liver disease, hereditary ataxia telangiectasia, hepatocellular carcinoma, or with a history of gastric or hepatic surgery [13]. Other potential scenarios include lung or gastrointestinal cancers, including colon, stomach, and pancreatic cancers [8]. Additionally, liver damage due to systemic therapy, alcoholism, or viral infection may lead to erroneously high AFP levels [14]. Therefore, absent other indications of GCT, it is recommended that patients with mildly elevated but stable AFP be managed by surveillance [10].

TABLE 1.

Histology-specific serum AFP and β-hCG levels [9]

Histologic subtype	AFP	β-hCG
Seminoma	–	±
Embryonal carcinoma	±	±
Choriocarcinoma	–	++
Yolk sac tumor	++	–
Teratoma	±	–

++: strongly positive levels; – : negative levels; ± : marker may be negative or moderately positive.
Adapted from Murray MJ, Huddart RA, Coleman N.[9] with permission of Springer Nature.

β-Human chorionic gonadotropin

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein, composed of an α- and a β-subunit. Generally, both the α-β heterodimer and the free β-subunit are measured and combined into total hCG, referred to as β-hCG [9]. β-hCG is generally measured with a double antibody immunometric assay, with normal levels determined as < 2 IU/L [8]. Elevated β-hCG is found in both seminoma and NSGCT and is the most commonly elevated STM in adult GCT patients.

Approximately 15% to 20% of seminoma cases will have elevated β-hCG due to the presence of syncytiotrophoblast cells [8]. Although elevated β-hCG levels are associated with tumor bulk, there is no known association between β-hCG levels and risk of metastasis following orchiectomy in patients with stage I disease. β-hCG is also not incorporated into International Germ Cell Cancer Collaborative Group (IGCCCG) risk stratification for patients with pure seminoma [5].

Approximately 10% to 20% of patients with stage I NSGCT will have elevations in β-hCG before orchiectomy [8]. Up to 40% of patients with disseminated disease will display elevated β-hCG levels. However, β-hCG is detectable in a subtype-specific manner; although β-hCG can be present in patients with embryonal carcinoma, β-hCG levels are greatest in patients with significant portions of choriocarcinoma. In contrast to seminoma, the IGCCCG risk stratification criteria include β-hCG as an important prognostic factor for NSGCT [5].

Although β-hCG is the most frequently elevated STM in GCT, its specificity remains a concern. Hypogonadism is a particular confounding factor, as it can occur following orchiectomy [8]. Hypogonadism leads to a compensatory increase in hCG from the pituitary gland. Additionally, hypogonadism can cause an increase in levels of luteinizing hormone (LH), which has an identical α- subunit and similar β-subunit to hCG. This can lead to a cross-reaction in the β-hCG assay and erroneous results, although most β-hCG assays no longer cross-react with LH. Additional potentially confounding factors include marijuana use, tumor lysis pursuant to chemotherapy, and the presence of heterophile antibodies [15,16]. Other cancer types, including lymphoma, leukemia, and neuroendocrine tumors, are also known to produce detectable β-hCG, but generally not to the high levels that can occur in some patients with GCTs (>10 000 IU/L) [15].

Serum microRNAs as Biomarkers in Testicular GCTs

Conventional STMs play an essential role in the diagnosis and monitoring of patients with GCTs. However, as outlined above, they are hampered in part by histological considerations and limited performance characteristics [6]. GCT-specific serum microRNAs (miRNA, miR-) [17–20] have emerged over the past decade as highly accurate tools for monitoring GCT patients, outperforming conventional STMs [9,21]. The measurement of these miRNAs may significantly change the way that GCT patients are diagnosed and treated.

MicroRNAs are short, non-coding RNAs that control gene expression in a target-specific manner [6]. Although longer RNA strands are notorious for being very labile, miRNA are remarkably stable, and have been proposed as markers in other cancer types [22,23]. In 2006, Voorhoeve et al. described overexpression of miR-372 and -373 in GCT tissues and cell lines, and

hypothesized that these miRNAs acted as oncogenes by inhibition of p53 signaling via LATS2 suppression [24]. A body of work following this study has since demonstrated overexpression of 8 miRNAs in malignant GCT tissues, all from the miR-371~373 and miR-302/367 clusters [25,26]. This expression pattern was absent in teratoma, but otherwise occurred regardless of histology, site of origin, or sex or age of the patient. Critically, these results were the first to demonstrate a universal molecular abnormality of this disease.

In the next seminal study in this arena, Murray and colleagues demonstrated that through the use of a highly sensitive qPCR-based assay, including a pre-amplification step, these malignant GCT-specific miRNAs were elevated in the serum of a GCT patient compared with a pool of normal controls [27]. The levels were also found to be informative of treatment response and disease status on follow-up. These results triggered an avalanche of investigations concerning the performance and potential inclusion of the serum miRNA test in routine clinical diagnosis and management for patients with GCTs.

Screening

There are few data available to assess whether GCT-associated miRNAs are useful in screening for the development of invasive GCT. However, there may be some utility of serum miRNAs in the case of screening at-risk patients for the presence of germ cell neoplasia in situ (GCNIS), the presumed precursor lesion of post-pubertal GCTs. Novotny et al. reported overexpression of GCT-associated miRNAs in GCNIS tissue compared with normal adult testis controls [28]. An initial study suggested that serum miRNAs do not detect GCNIS, but a subsequent study indicated that up to 50% of patients with GCNIS lacking frank GCT have elevated serum miRNAs [29,30]. Importantly, these studies were conducted in a small number of patients and further validation is required in this setting.

Pre-orchiectomy

A solid testicular mass detected by physical examination or sonography is initially considered a malignancy. These techniques can overlook non-malignant causes of a testicular mass, and therefore STMs may play an important role here. Conventional STMs may not be informative in this context, particularly in seminoma, where only 10% to 15% of cases show elevated markers [4]. The superior sensitivity of circulating miRNAs over conventional STMs could therefore be valuable in the pre-orchiectomy setting.

This setting is where the utility of circulating miRNAs has so far been best described (**Table 2**). In 2013, Gillis et al. examined serum miRNA expression in 80 GCT patients, in non-cancer controls, and in patients with non-GCT testicular masses [31]. Using magnetic bead purification but no pre-amplification step, this study identified that a 4-member panel (miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p) outperformed a full panel that included miR-302a~d from the miR-302/367 cluster, refining the necessary targets for an informative test. The test performance was acceptable, with 98% sensitivity and 48.3% specificity, although without the pre-amplification step [28], specificity was necessarily low in order to retain high sensitivity. The test was negative for non-GCT testicular masses and returned to normal in post-orchiectomy stage I GCT patients. This revised 4-member panel would set the basis for future studies.

These results were confirmed in an expanded cohort by van Agthoven and Looijenga in 2017 [29]. Magnetic bead purification was used in combination with the standard pre-amplification step to examine a 3-member panel lacking miR-372-3p in 250 GCT patients, 60 non-GCT patients, and 104 healthy male controls. Performance improved as expected, with a 90% sensitivity and 91% specificity in the combined panel. Importantly, this study aligned with previous and subsequent reports that serum miRNA does not detect pure teratoma [32–34].

More recently, attempts have been made to reduce the 4-member panel further, while maintaining sensitivity. In 2017, Dieckmann et al. compared the performance of circulating miR-371a-3p to the full 4-member panel, using a column-based extraction and standard pre-amplification. This study reported comparable performance between miR-371a-3p alone and the full panel (92% sensitivity, 85% specificity) [34]. Following this report, Dieckmann et al. used the same method to examine miR-371a-3p exclusively in 616 GCT patients and 258 controls, the largest cohort yet [35]. This study reported 90% sensitivity and 94% specificity in viable GCT, a return to normal levels following orchiectomy in the majority of stage I patients, and no detectable circulating miR-371a-3p in pure teratoma. No long-term follow-up data were available to determine the clinical significance of persistently elevated serum miR-371a-3p levels post-orchiectomy in clinical stage I patients, which is an outcome measure in current clinical trials (see below) [20].

The refinement of target selection and methods in the context of pre-orchiectomy has resulted in a miRNA-based test that significantly outperforms conventional STMs. Although these methods have begun to converge to a single standard of normalization and quantification, further work is required. These refined methods are now being used in other scenarios, some examples of which follow.

TABLE 2.

Performance characteristics of serum pre-orchiectomy GCT-associated miRNAs for prediction of GCT on final orchiectomy histopathology.

Reference	Sensitivity, %	Specificity, %	PPV, %	NPV, %	AUC
Gillis *et al.* [27]	98	48.3	NR	NP	0.96
Van Agthoven and Looijenga [26]	90	91	94	7	0.962
Dieckmann *et al.* [31]	90.1	94	97.2	82.7	0.966

AUC: area under the curve; GCT: germ cell tumor; NPV: negative predictive value; PPV: positive predictive value.

Identification of occult metastases in patients with early stage I/stage I disease

From 10% to 50% of patients with clinical stage I viable GCT, normal conventional STMs, and no radiographic evidence of metastasis will ultimately relapse [6]. It is extremely challenging to identify which of these patients harbor occult disease, as diagnostic tools are limited. Further, 85% to 90% of stage IA NSGCT patients will be cured by orchiectomy alone; surveillance is preferred in these patients to prevent overtreatment, but the potential for metastases currently cannot easily be ruled out [36]. Patients with occult disease would be better served if they were to receive a single cycle of BEP or primary retroperitoneal lymph node dissection (RPLND), as both are associated with excellent outcomes [37]. Additionally, early identification of occult metastatic seminoma could permit earlier, less intensive treatment.

Circulating miRNA levels are associated with tumor mass, raising potential concerns about whether they reach detectable levels in the case of radiographically occult metastases [34]. Recent studies provide evidence that circulating miRNA levels are detectable even when current identification strategies fail. Nappi et al. reported that miR-371a-3p levels in plasma accurately predicted relapse in all cases in their small cohort [38]. Lafin et al. demonstrated that serum miR-371a-3p accurately detected minimal residual pathologically confirmed viable GCT at primary RPLND [39]. In this 24-patient cohort with normal conventional STMs, miR-371a-3p showed a 100% sensitivity and 92% specificity, demonstrating the value of circulating miRNAs in this setting.

These studies and others suggest that circulating miRNAs may help avoid overtreatment of the 20% to 30% of stage IIA, marker negative patients that are ultimately devoid of occult disease on histology [7,40]. Additionally, because low circulating miRNA levels immediately before chemotherapy are associated with complete response, this tool may in the future aid clinicians deciding between observation and further treatment [36]. Similarly, this principle might be extended to patients who have received primary RPLND and who are being considered for surveillance or adjuvant chemotherapy.

Response to treatment in patients with disseminated disease

The utility of circulating miRNAs in the context of chemotherapy is an area of active study. Dieckmann et al. found in patients with stage II/III GCT that serum miRNA levels both tracked treatment response during chemotherapy and, in 2 cases, suggested resistance [35]. Seventy out of 118 patients with systemic disease exhibited a decrease in serum miR-371a-3p levels after the first cycle of chemotherapy. As a group, stage II patients exhibited no further statistically significant reduction after the first cycle, while stage III patients exhibited no further reduction after the second cycle. The 2 patients in this cohort who experienced disease progression and ultimately died showed rising circulating miR-371a-3p levels. Mego et al. examined plasma miRNA levels in 180 patients with metastatic GCT treated with systemic therapy and found that miRNA levels were associated with IGCCCG risk group and response [41].

Patients with primary metastatic or relapsed GCT will receive induction chemotherapy, and those without radiographically complete response will receive a post-chemotherapy RPLND [41]. This surgery is currently the only accurate method to determine if residual masses harbor teratoma or viable GCT. It is also the only way

to treat teratoma and can contribute to therapeutic management in patients with chemo-resistant viable GCT [42,43]. However, approximately 50% of patients receiving this operation will harbor only fibrosis/necrosis, meaning 50% of the patients who undergo this operation will have done so unnecessarily. Leão et al. examined circulating miRNA at post-chemotherapy RPLND and found that miR-371a-3p exhibited the best performance to detect residual viable GCT, but did not find any discriminatory capacity for the presence of pure teratoma [44]. A recent report from TCGA expression data suggested that teratoma and yolk sac tumor tissue express high levels of miR-375 [45]; however, Lafin et al. did not find any discriminatory capacity of this circulating miRNA in 3 teratoma-only patients at RPLND [39]. Additional studies have confirmed these negative results in larger cohorts [42,43].

Surveillance

Circulating miRNAs represent a powerful tool capable of changing the way GCT patients are monitored. Because this assay cannot detect pure teratoma, the likelihood of teratoma formation must always be kept in mind. The development of teratoma is an extremely rare occurrence in patients with pure seminoma, and in this arena, circulating miRNAs may in future supplant axial imaging and conventional STMs. For patients with NSGCT, teratoma formation must remain a consideration and warrants ongoing infrequent axial imaging. In this setting, circulating miRNAs may offer a complementary test, permitting a reduction in the number of axial scans required for surveillance [6].

Conclusions

Conventional STMs play a critical role in the current diagnosis, treatment, and monitoring of patients with GCT. Unfortunately, their performance is limited in many situations by false positives and low sensitivity. This limitation extends from diagnosis, through treatment and into surveillance, impacting decision-making across the spectrum of patient care. The promise of circulating miRNAs as an addition to these markers is supported by a growing body of literature [17–20, 27,29,35]. Inclusion of circulating miRNAs alongside conventional STMs could aid in identification of false positives, such as in a case report describing elevated AFP levels pursuant to liver regeneration [12]. Two large clinical trials (AGCT1531 [NCT03067181] and SWOG-S1823) are further studying the role of miRNA in patients with GCTs.

Despite generally excellent performance of circulating miRNAs, limitations remain. First, a standard method of collection, normalization, and quantification must be devised to reduce variation across laboratories and enable large-scale validation. Second, identification of pure teratoma by circulating biomarkers remains an elusive target. Differentiation of pure teratoma from viable GCT is an unmet clinical need; recommendation of surgery or chemotherapy will often depend on segregating the two.

Despite these modest limitations, circulating miRNAs have the potential to change the way that GCT cases are managed, aiding clinicians in decision-making to provide greater benefit to their patients.

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