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## Review

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# MicroRNA-371a-3p as a blood-based biomarker in testis cancer

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**Abstract** MicroRNAs (miRNAs) are small noncoding RNAs involved in the regulation of mRNA transcription and translation, and possess all desirable features of an ideal tumor marker. Of almost 31 different miRNA clusters identified in germ cell tumors (GCTs), miR-371a-3p has shown exceptionally high sensitivity and specificity for both seminomatous and nonseminomatous GCTs. It is easily obtainable and correlates well with tumor burden. Recent multi-institutional prospective studies have shown promising test characteristics for miR-371a-3p as a diagnostic blood-based biomarker for GCT prior to orchiectomy including 80%–100% sensitivity and 90%–100% specificity. This accuracy may address other unmet needs in the management of patients with GCT. Early studies have suggested the utility of miR-371a-3p in detecting occult nodal metastasis in high-risk clinical stage I and early stage II disease. Ongoing clinical trials including SWOG 1823 and AGCT1531 are specifically designed to confirm the utility of miR-371a-3p in clinical stage I GCT. Despite its strong association with viable GCT after treatment with chemotherapy, miR-371a-3p does not seem to accurately predict the presence of teratoma in residual lesions. Also, standardization of extraction and interpretation methods is a necessary step to assure uniform results across different institutions.

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## 1. Introduction

Despite a dynamic modification in therapeutic approaches, especially chemotherapy regimens, there has been very minimal change in the diagnostic algorithm of testicular germ cell tumors (GCT) in the past two decades. Traditional serum tumor markers (STMs) including alpha fetoprotein (AFP), beta-human chorionic gonadotropin ( $\beta$ hCG), and lactate dehydrogenase (LDH) alongside cross sectional imaging with computed tomography (CT) are still considered the gold standard for diagnosis, staging, and surveillance of GCT, especially in early stage disease. However, STMs have low sensitivity for GCT and equivocal low elevations in various unrelated clinical scenarios. For instance, hypogonadism causes a compensatory increase in the pituitary production of hCG and luteinizing hormone. False-positive elevations of hCG can be associated with the presence of heterophile antibodies and potentially marijuana use [1]. Mild elevation of AFP levels in the range of 15–30 ng/mL can be a normal finding in stage I disease, often due to other sources of AFP or a long half-life [2]. LDH is considered a nonspecific marker for GCT and carries less value in decision making compared to AFP and hCG [1]. Also, STMs are only elevated in approximately 50% of GCTs [3]. CT has also been shown to be a suboptimal modality to accurately predict the presence of viable disease, particularly in stage I and early stage II disease (less than 3 cm retroperitoneal node) with low or normal STMs as a significant proportion of these lesions are not malignant [4]. The lack of an efficient diagnostic system places patients at risk of over- or under-treatment and both could have significant, sometimes irreversible consequences. Though patients with clinical stage I (CSI) disease are at risk of relapse, consensus guidelines recommend surveillance as overtreatment with chemotherapy and radiation are both associated with significant short- and long-term side effects [5]. The other challenging scenario where STMs and currently available imaging modalities fall short is in the accurate prediction of histology in post-chemotherapy residual lesions. Surgery in the post-chemotherapy setting is technically challenging and may subject patients to vascular reconstruction and other adjunct procedures such as nephrectomy to assure complete surgical resection [6,7]. Surgical series indicate that residual masses after chemotherapy are composed of fibrosis/scar tissue in approximately 50% of cases [8]. Therefore, there is an essential need for additional reliable diagnostic tools that can predict the presence of viable tumor, including teratoma, in early-stage disease and in the post-chemotherapy setting. This review summarizes the current evidence on the utility of serum miRNA as a diagnostic tool in GCTs, especially in the above mentioned equivocal clinical scenarios.

## 2. MicroRNA (miRNA)

Out of more than 900 different miRNAs expressed in the human genome, Voorhoeve et al. [9], for the first time, showed that two main clusters of miRNAs, including miR-302a–302d and miR-371–373 (a total of eight miRNAs), are over expressed in human GCT cell lines. As opposed to

circulating DNA, circulating miRNAs are relatively stable and resistant to degradation by serum RNAses as they are contained within membrane-bound particles, known as exosomes [10]. Murray et al. [11], in a proof-of-concept study, showed elevation of all eight members of miR-371–373 and miR-302–367 clusters in pediatric extragonadal GCT. They then tested their findings in a larger clinical cohort of patients with mostly negative classic STMs. They demonstrated universal elevation of miR-372-3p and miR-367-3p across several GCT scenarios regardless of age, tumor location (gonadal vs. extragonadal), histologic subtype (seminoma vs. non seminoma), or age [12]. Belge et al. [13] then reported similar findings in adult GCT patients for the first time. These microRNAs in addition to miR-371a-3p and miR-373-3p also possess other desired features of an ideal tumor marker such as a strong correlation with disease burden, stage and response to treatment [11,14]. Of all eight GCT-specific miRNAs, miR-371a-3p has the most notable test performance characteristics with sensitivity and accuracy of approximately 90% and 94%, respectively [12,15]. However, a panel of all four miRNAs (371a-3p, 372-3p, 373-3p, and 367-3p) has the highest sensitivity and specificity for detecting GCTs, especially in the post orchiectomy situation where an early (less than 1 day) miR-371a-3p measurement may lead to false negative results given its short half-life [16].

## 3. Clinical applications

### 3.1. Early stage GCT

CSI is the most common stage at presentation for both seminoma GCT (SGCT) and nonseminomatous GCT (NSGCT). The risk of relapse is estimated at about 15% for SGCT and 15%–50% for NSGCT [17]. Surveillance is considered the preferable option for CSI disease. Radiation (for Stage I seminoma) and retroperitoneal lymph node dissection (RPLND) (in Stage I non-seminoma) are alternatives. Consideration for adjuvant chemotherapy is mostly based on the presence of risk factors such as tumor size and lymphovascular invasion (LVI) [18]. The concern, however, is that even in the presence of LVI, 50% of patients with NSGCT are being overtreated and even one cycle of BEP could be associated with undesirable side effects in this patient population with a long life expectancy [19]. Therefore, most consensus guidelines favor active surveillance in CSI, even in the presence of risk factors for recurrence such as LVI [20]. The management of patients with CSI disease is one area where circulating miRNA could theoretically improve decision making, wherein patients with persistently positive miRNA following orchiectomy could be offered adjuvant treatment while patients with negative post-orchiectomy miRNA could be placed on surveillance.

The evidence to support the utility of miRNAs in monitoring CSI GCT was initially presented in a case series of two patients who had elevated serum miR-371a-3p levels prior to and days after orchiectomy, but months before any radiologic finding of retroperitoneal lymphadenopathy or metastasis [21]. van Aghtoven and

Looijenga [22] in a study of three different miRNAs in 250 patients with GCT, showed an area under the receiver operating characteristic curve (AUC) of 0.951 with a sensitivity of 90%, and a specificity of 86% (positive predictive value of 94% and negative predictive value of 79%) for miR-371a-3p. Inclusion of two other miRNAs, miR-373-3p and miR-367-3p, to the predictive model did not significantly change test characteristics. In a prospective multicenter study, Dieckmann et al. [23] reported elevated levels of miR-371a-3p in 38/46 patients who had recurrent disease, which corresponded to a sensitivity of 82.6%, a specificity of 96.1%, and an AUC of 0.921 for relapse detection. A lower sensitivity of miRNA in detecting relapse compared to its diagnostic sensitivity (about 90%) is presumably due to the presence of teratoma or other somatic type histologies at recurrence [24]. In the study by Dieckmann et al. [23], the authors did not specify the percentage of patients with CSI GCT who had a relapse on active surveillance. Nappi et al. [25] also measured serum miR-371a-3p levels in 111 patients with newly diagnosed GCT. Patients were assigned to three different risk groups (low, intermediate, and high) based on their clinical stage, STM status, and imaging findings. A total of 25 patients with CSIA and CSIB SGCT (20 patients) and CSIA NSGCT with normal STM and either no or sub-centimeter retroperitoneal adenopathy on imaging (five patients) were assigned to low-risk group. With a median follow-up of 14.5 months, elevated post orchiectomy miR-371a-3p was detected in only one patient. This was the only patient who developed recurrence. Thus, the specificity and positive predictive value of miR-371a-3p in the low-risk group was 100% and 100%, respectively. An uncharacteristically low recurrence rate (1/25) in this group was most likely due to the short follow-up period. Five patients with CSIB NSGCT with normal STM were assigned to intermediate risk group. One patient in this group experienced recurrence, which was also associated with elevated miR-371a-3p levels.

Based on these preliminary data, two clinical trials have been designed to address the role of miRNAs in predicting recurrence in early stage GCT (Table 1):

- 1) SWOG S1823 is a prospective observational cohort study designed to assess the predictive role of miR-371a-3p in patients with newly diagnosed GCT, primarily focusing on CSI disease. The primary outcome of the trial is the positive predictive value within each of the early stage SGCT and NSGCT groups using plasma miR-371a-3p expression at relapse. Patients will be assessed prior to orchiectomy, post-orchiectomy, and every 3 months for 2 years (NCT04435756).
- 2) AGCT 1531 is a multi-arm phase III interventional study comparing active surveillance, carboplatin-based chemotherapy, and cisplatin-based chemotherapy in low and standard risk pediatric, adolescent, and young adult GCT patients. A secondary outcome in patients with CSIA/B SGCT or NSGCT is to assess the utility of miRNA-371-373 and miRNA-302. Samples are collected pre-orchiectomy and then monthly for 3 months, every 3 months for 1 year, and then every 6 months for another year (NCT03067181).

The other area of uncertainty in the management of early stage GCT is STM negative early clinical stage II (less than 3 cm retroperitoneal node) disease. This scenario is mostly encountered in stage IIA SGCT, but occasionally seen in NSGCT. Prior studies have shown that up to 40 percent of patients with CSIIA disease and normal STM have pathologic stage I disease after surgery [26]. Stable and low levels of elevated STMs in CSIIA NSGCT could also pose the same therapeutic challenge. A commonly applied, anecdotal approach is to repeat imaging in 6–8 weeks and offer intervention if there is any evidence of growth in nodal size [27]. This is another area where miRNA could have utility in identifying ideal candidates for intervention versus surveillance. In the previously mentioned case control study by Nappi et al. [25], 34 patients with CSIIA and low volume (less than 3 cm retroperitoneal node) CSIIB SGCT (21 patients) and NSGCT (13 patients) were included in an intermediate risk group. All patients had negative or only mildly elevated STMs. In the SGCT subgroup, five patients had pathologically confirmed nodal disease and only one did not have an elevated miR-371a-3p. In the NSGCT group, five patients had pathologically confirmed nodal disease and all of them had elevated miR-371a-3p. Overall, miRNA-371 showed specificity and PPV of 100%, sensitivity of 90% and a negative predictive value of 96% in detecting viable disease in CSIIA/low volume IIB GCT [28]. In a recent study of 24 patients with stage I and II GCT who underwent primary RPLND, Lafin et al. [29] reported an AUC of 0.965, sensitivity of 100%, and specificity of 92% for miR-371a-3p in differentiating between viable GCT and fibrosis/teratoma.

## 3.2. Advanced GCT

### 3.2.1. Monitoring response to chemotherapy

Currently, the number of cycles and type of chemotherapy regimen for advanced GCT is determined based on risk categories determined by the IGCCCG classification. In good risk disease, patients will receive three or four cycles of chemotherapy depending on the regimen while intermediate and poor risk categories mandate four cycles of treatment, regardless of regimen [30]. Dieckmann et al. [12] reported that in the majority of patients with CSII GCT who received systemic therapy, levels of miR-371a-3p declined to normal levels after only one cycle of treatment and there were insignificant changes in miRNA levels with additional cycles. They also observed a significant decrease in miRNA levels in patients with CSIII and metastatic disease after the first course of systemic therapy. However, in this group with more advanced disease, miRNA levels did not normalize after one cycle of treatment. Although classic STMs seem to have a strong correlation with response to chemotherapy [31], the findings by Dieckmann et al. [12] could potentially be used to justify a role for miRNA in an adaptive approach to systemic treatment where treatment stops when miRNA levels are converted to normal, especially in patients with low volume good risk disease.

### 3.2.2. Post chemotherapy residual mass

In patients with NSGCT who have normal STMs following chemotherapy, all residual masses greater than 1 cm are recommended to be surgically removed [32]. However, 45%

**Table 1** Current trials on the role of microRNA-371 in early stage GCT.

Trial detail	SWOG S1823 (NCT04435756)	AGCT 1531 (NCT03067181)
Start date	- June 1, 2020	- May 8, 2017
Completion date	- April 1, 2025 (expected)	- June 30, 2027 (expected)
Enrollment, <i>n</i>	- 956 (estimated)	- 2059 (estimated)
Design	- Prospective observational cohort	- Multicenter randomized open label clinical trial
Arms	- Not applicable	- Arm I (bleomycin, carboplatin, and etoposide ×4). - Arm II (bleomycin, etoposide, and cisplatin ×4). - Arm III (bleomycin, carboplatin, and etoposide ×3). - Arm IV (bleomycin, etoposide, and cisplatin ×3). - Arm V (active surveillance for stage I GCT).
Stratification variables	- None defined	- None defined
Primary outcome	- Positive predictive value of microRNA-371	- 2-year overall survival. - 2-year event free survival.
Secondary outcome	- Biobank of serial liquid biospecimens	- Percentage of hearing loss; - Percentage of peripheral neuropathy.
Inclusion criteria	- New diagnosis of a GCT confirmed pathologically or serologically. - All primary sites, stages, and histological subtypes of GCT are eligible; - metachronous second primary GCTs are eligible. - If surgery is planned, male patients with clinical stage I testicular cancer must have orchiectomy completed within 42 days prior to registration. - Registered within 42 days after diagnosis and prior to initiation of a management plan or treatment for the disease.	- There is no age limit for the low risk stratum. - Newly diagnosed stage I GCT for low risk stratum; - Risk 1 group <11 years of age at enrollment. - Risk 2 group >11 and < 25 years of age at enrollment. - Stage II or higher for standard risk groups. - Histologic confirmation required. - All primary sites, and stages, histological subtypes of GCT are eligible.
Exclusion criteria	- None defined	- Pure teratoma; - Prior RPLND or chemotherapy.
Locations	- US and Canada	- US, Canada, Japan, New Zealand, Puerto Rico, and Saudi Arabia

GCT, germ cell tumor; RPLND, retroperitoneal lymph node dissection; US, the United States.

of tumors show necrosis/fibrosis. In patients with SGCT, the indications for post-chemotherapy surgery include residual masses greater than 3 cm with positive positron emission tomography (PET) scan [33]. However, PET has a high false positive rate due to inflammatory or granulomatous reactions associated with SGCT [34]. Therefore, there is a lack of tools to accurately predict the histology of post chemotherapy residual masses and identify appropriate surgical candidates. Leao et al. [35] studied a group of 43 patients with NSGCT who had bio-samples available before and after retroperitoneal lymph node dissection (RPLND). All these patients had normalized classic STMs after chemotherapy. They measured three different miRNAs including miR-371, -373, and -367 and assessed the correlation between miRNA levels prior to RPLND and the

presence of viable disease on final pathology. They demonstrated that miR-371a-3p had the highest predictive value of the three different miRNAs (AUC 0.84). The addition of the other two miRNAs to the model did not significantly improve accuracy. When they considered only residual masses measured 3 cm or less in largest diameter, miR-371a-3p showed a sensitivity and negative predictive value of 100% in detecting viable GCT. However, approximately 45% of post-chemotherapy residual masses harbor teratoma, which if left unresected can lead to growing teratoma syndrome or rarely contain somatically transformed components, namely sarcoma [36]. Unfortunately, teratomas do not express miR-371a-3p and this marker has not been able to identify teratoma components [37]. Although some evidence has pointed towards miR-375 as a

potential biomarker for teratoma [38], follow-up studies have failed to show any significant association between this marker and teratoma [39]. This includes a recent study by Lafin et al. [40] that reported poor test characteristics for both serum miR-375-3p and miR-375-5p for predicting the presence of teratoma in post-chemotherapy residual mass (miR-375-3p: 86% sensitivity, 32% specificity, AUC: 0.506; miR-375-5p: 55% sensitivity, 67% specificity, AUC: 0.556).

There is a growing interest in using radiomics to differentiate the histology of post chemotherapy residual masses. Baessler et al. [41] demonstrated that CT radiomics-based machine learning classifiers were able to differentiate necrosis/fibrosis from viable GCT/teratoma with a sensitivity and negative predictive value of 88%. It seems plausible that the combination of radiomics and miRNA could collectively improve our ability to predict the histology of post-chemotherapy residual masses with a high degree of certainty.

## 4. Considerations and future directions

### 4.1. Standardizing methodology

There are several methodologies currently used for different aspects of miRNA assessment including collection, extraction, and normalization. Variations at each level potentially affect the quality of processing and alter the measured microRNA levels. The majority of retrospective studies have used serum samples collected in serum separator tubes, whereas in more recent prospective studies, sample collection has been done via Streck tubes which can be stored at room temperature and for up to 7 days [24]. This is particularly relevant since evidence suggests that the type of collection tube, preparation, handling, and storage of samples significantly affects miRNA levels [42]. Nappi et al. [28] also showed that miRNA extracted from plasma has slightly higher specificity compared to miRNA extracted from serum. Several reference miRNAs such as miR-30b-5p, miR-451a and miR-23a-3p are being used for normalization purposes and to reduce the variability of miRNA expression. However, there is no consensus for a single reference miRNA and the use of a combination of reference miRNAs is recommended [24]. Therefore, future efforts should be directed towards standardizing the measurement process to facilitate widespread use of miRNA.

## 5. Prognostic marker

All the evidence above suggests the superior performance of miRNA in “detecting” the presence of viable GCT at different disease stages. However, there are limited data about its prognostic ability. Recently, Lobo et al. [43] assessed the role of miRNA in predicting relapse in patients with CSI GCT (both SGCT and NSGCT). They did not find any association between miR-371a-3p levels following orchiectomy or percent decline in miR-371a-3p from before to after orchiectomy or at relapse. They also incorporated

miR-371a-3p into multivariate models with clinical variables known to be associated with relapse and showed that miR-371a-3p did not add any additional independent prognostic value for future relapse. However, they noticed that miR-371a-3p levels were consistently elevated at the time of relapse, while classic STMs were normal in almost two thirds of those patients, confirming miR-371a-3p's role as a reliable detector of viable GCT [43]. Based on these results, miR-371a-3p may have limited utility as a tool for risk stratification. There are, however, some other members of miRNA family such as miR-29c-5p, miR-506-3p, miR-1307-5p, and miR-371a-5p that have shown potential capability to act as a prognostic marker in SGCT [44]. The results of currently recruiting trials, where prospective and serial collection of miRNAs are performed at different stages, will provide more definitive evidence in this regard.

## 6. Conclusion

Recent data have shown the ability of miRNA to serve as a reliable and readily available blood-based biomarker in patients with GCT. Clinical applications include the utility of miRNA in various clinical scenarios including the diagnosis of GCT prior to orchiectomy, detection of occult metastases in early stage disease, and response to therapy in advanced disease. The results of ongoing clinical trials are eagerly anticipated as we work towards a future where standardized processing and interpretation may allow miRNA to direct personalized care in patients with GCT.

## Author contributions

*Study concept and design:* Hamed Ahmadi, Thomas L. Jang, Siamak Daneshmand, Saum Ghodoussipour.

*Data acquisition:* Hamed Ahmadi, Thomas L. Jang, Siamak Daneshmand, Saum Ghodoussipour.

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## Conflicts of interest

The authors declare no conflict of interest.

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