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Real-World Application of Pre-Orchiectomy miR-371a-3p Test in Testicular Germ Cell Tumor Management

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Purpose: Current serum tumor markers for testicular germ cell tumor are limited by low sensitivity. Growing evidence supports the use of circulating miR-371a-3p as a superior marker for malignant (viable) germ cell tumor management. We evaluated the real-world application of serum miR-371a-3p levels in detecting viable germ cell tumor among patients undergoing partial or radical orchiectomy.

Materials and Methods: Serum samples were collected from 69 consecutive patients before orchiectomy. Performance characteristics of serum miR-371a-3p were compared with conventional serum tumor markers (α-fetoprotein/β-human chorionic gonadotropin/lactate dehydrogenase) between patients with viable germ cell tumor and those without viable germ cell tumor on orchiectomy pathology. Relative miR-371a-3p levels were correlated with clinical course. The Kruskal-Wallis test and linear and ordinal regression models were used for analysis.

Results: For detecting viable germ cell tumor, combined conventional serum tumor markers had a specificity of 100%, sensitivity of 58% and AUC of 0.79. The miR-371a-3p test showed a specificity of 100%, sensitivity of 93% and AUC of 0.978. Median relative expression of miR-371a-3p in viable germ cell tumor cases was more than 6,800-fold higher than in those lacking viable germ cell tumor. miR-371a-3p levels correlated with composite stage ($p=0.006$) and, among composite stage I cases, independently associated with embryonal carcinoma percentage ($p=0.0012$) and tumor diameter ($p<0.0001$). Six patients underwent orchiectomy after chemotherapy and were correctly predicted to have presence or absence of viable germ cell tumor by the miR-371a-3p test.

Abbreviations and Acronyms

β-hCG = β-human chorionic gonadotropin
AFP = α-fetoprotein
CS = composite stage
GCNIS = germ cell neoplasia in situ
GCT = germ cell tumor
LDH = lactate dehydrogenase
NPV = negative predictive value
NSGCT = nonseminomatous germ cell tumor
PC = post-chemotherapy
PPV = positive predictive value
RPLND = retroperitoneal lymph node dissection
STM = serum tumor marker

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Conclusions: If validated, the miR-371a-3p test can be used in conjunction with conventional serum tumor markers to aid clinical decision making. A positive miR-371a-3p test in patients after preoperative chemotherapy or with solitary testes could potentially guide subsequent orchiectomy or observation.

Key Words: biomarkers; neoplasms, germ cell and embryonal; microRNAs; testicular neoplasms

Germ cell tumors are the most common solid malignancy in men 20 to 40 years old.[1] Serum tumor markers are a critical factor in the diagnosis and management of GCT patients. Three conventional STMs have classically been used for GCT management, namely β-human chorionic gonadotropin, α-fetoprotein and lactate dehydrogenase.[1] Malignant nonseminomatous GCTs (namely yolk sac tumor, embryonal carcinoma and choriocarcinoma) have detectable STMs in 60% of cases, while seminomas have detectable markers in a modest 10% to 15% of cases.[2] The utility of these markers is further hampered by other factors, such as nonGCT specific elevation and poor sensitivity in the case of low tumor burden.[3] In the setting of recurrence STMs are the first sign of recurrence in only 40% of cases.[4]

MicroRNA (miRNA) have recently emerged as potential STMs for GCT monitoring.[5–7] miRNAs are short noncoding RNA involved in the post-transcriptional regulation of gene expression.[8] Malignant (viable) GCTs express 2 specific clusters of miRNAs, namely miR-371-373 and miR-302-367, which are stable and can be detected in patients.[9,10] Of these putative markers, miR-371a-3p has demonstrated greater than 90% sensitivity and specificity for detecting viable GCT.[11–13] Serum levels of miR-371a-3p were able to predict residual GCT following chemotherapy, even when conventional STMs failed to do so.[13] These characteristics also hold true in the context of chemotherapy naïve minimal residual disease.[12,14] Given the poor performance of traditional STMs, serum miRNA levels could potentially guide treatment with enhanced diagnosis and monitoring for recurrence.[15]

For malignant GCT cases a highly sensitive preamplified quantitative polymerase chain reaction assay is necessary to measure the sparse miRNA levels released into serum.[16] To this end, Murray et al developed a pipeline for which standardized high fidelity measurement could be achieved.[5] We report our experience using this protocol to measure circulating pre-orchiectomy miR-371a-3p at our institution and the utility of this marker in the management of patients with solitary testes and those who receive pre-orchiectomy chemotherapy.

METHODS

Study Design and Participants

With institutional review board approval (STU 092016-001) we prospectively collected serum samples from consecutive patients undergoing orchiectomy for suspected or known testicular cancer from 2016 to 2019, immediately prior to surgery. The protocol for serum miRNA isolation and quantification is detailed in the supplementary Appendix (https://www.jurology.com).

Baseline clinicopathological data including age, composite stage, conventional STM levels immediately prior to orchiectomy, pathologic stage, receipt of pre-orchiectomy chemotherapy, and orchiectomy histology were collected. Composite staging was determined using the American Joint Committee on Cancer staging guides based on TNMS staging. For analysis, seminoma and NSGCT (excluding pure teratoma) were considered viable GCT. Benign pathology, ypT0 status in patients who received pre-orchiectomy chemotherapy, nonGCT malignancy (Leydig cell tumor and metastases to testis), and pure teratoma were grouped as absence of viable GCT (control group). Pure teratomas have shown low circulating miR-371a-3p expression in previous studies, and thus, were categorized under the control group.[12] All specimens were reviewed by a genitourinary pathologist. Patients underwent surveillance based on guideline recommendations. Recurrence status following orchiectomy was also documented.

Statistical Analysis

Intergroup differences of clinicopathological data were compared with the Kruskal-Wallis test and Dunn's post hoc test. A ROC curve was generated to graphically display the discriminative ability of miR-371a-3p to predict viable GCT over control group pathology. Accuracy, sensitivity, specificity, PPV and NPV were calculated for miR-371a-3p and conventional STMs. Conventional STMs were considered positive if they were above the upper normal limit provided by the laboratory (AFP 0-9 ng/ml, β-hCG 0-3 U/L, LDH 105-230 U/L). miR-371a-3p was considered positive if the Rq value was above a threshold determined by Youden index.[17]

Linear regression models and their Spearman correlation coefficients were calculated using tumor diameter, % embryonal carcinoma, % seminoma, % yolk sac tumor and % choriocarcinoma to identify predictors of high serum miR-371a-3p in chemonaïve CS I patients. An ordinal logistic regression model was used to examine the relationship between CS and serum miR-371a-3p. Statistical significance of models was determined by ANOVA. Control group and post-chemotherapy cases were excluded from all regression analyses. Two-tailed $p < 0.05$ was considered statistically significant. Data analysis was performed on R 3.2.2 software (R Foundation, Vienna, Austria) with the pROC[18] and MASS[19] packages. Graphical analysis was done on GraphPad Prism 8 Version 8.4.0.

RESULTS

Patient Characteristics

From 2016 to 2019, pre-orchiectomy serum for 69 patients was obtained and analyzed for miR-371a-3p expression. Ultimately, 58 patients had a confirmed diagnosis of testicular cancer, including those who received post-chemotherapy orchiectomy. Of the 69 patients, 58 patients had either viable seminoma (29 patients) or NSGCT (29 patients) on final pathology and 11 patients were considered controls. A description of the pathology found in NSGCT and control patients is listed in the supplementary Appendix (https://www.jurology.com).

As expected, viable GCT patients were younger than control patients with a median age of 30 years compared with 54 years, respectively.[20] Among viable GCT cases 45 were CS I, 6 were CS II and 7 were CS III. In the control group 1 patient with pure teratoma was CS I, 1 post-chemotherapy patient was CS II, 3 post-chemotherapy patients were CS III and 6 patients without testicular cancer were not assigned a composite stage. Most GCT patients (84%) had localized disease. In addition, 36 GCT (13 seminoma and 23 NSGCT) patients (62%) had at least 1 of the conventional STMs elevated and none of the control patients had elevated STMs. The median tumor diameter was greater for viable GCT patients than for control patients ($p=0.0298$), at 4.1 (IQR 2.4–6.7) centimeters compared with 1 (IQR 0.25–2.6) centimeter, respectively. A summary of relevant clinical details is reported in table 1. Staging for patients after receipt of chemotherapy is noted in the supplementary table (https://www.jurology.com).

Table 1. *Patient characteristics at presentation*

	Viable GCT		Control		p Value
No.	58		11		
Median age (IQR)	30	(26–40)	54	(43–56)	<0.0001
% Race (No.):					
White	48	(28)	36	(4)	
Hispanic	48	(28)	36	(4)	
Black	2	(1)		-	
Asian	2	(1)	28	(3)	
% Histology (No.):					
Seminoma	50	(29)		-	
NSGCT	50	(29)		-	
Pure teratoma		-	9	(1)	
Benign		-	55	(6)	
Leydig cell tumor		-	18	(2)	
Secondary metastasis		-	18	(2)	
% Composite stage (No.):					
I	78	(45)	9	(1)	
II	10	(6)	9	(1)	
III	12	(7)	27	(3)	
N/A		-	55	(6)	
% Stage T (No.):					
0		-	9	(1)	
1	59	(34)	9	(1)	
2	31	(18)		-	
3	3	(2)		-	
4	3	(2)		-	
N/A	3	(2)	82	(9)	
% Stage N (No.):					
0	75	(43)	9	(1)	
1	7	(4)		-	
2	3	(2)		-	
3	12	(7)	36	(4)	
N/A	3	(2)	55	(6)	
% Stage M (No.):					
0	84	(49)	27	(3)	
1	12	(7)	18	(2)	
N/A	3	(2)	55	(6)	
Median cm tumor diameter (IQR)	4.1	(2.4–6.7)	1	(0.25–2.6)	0.0298
% Elevated conventional STMs (No.):	62	(36)		-	
AFP	36	(21)		-	
β-hCG	43	(25)		-	
LDH	34	(20)		-	
% PC (No.)	3	(2)	36	(4)	

Overall Performance

The median relative expression of serum miR-371a-3p in patients with viable GCT was >6,800x higher than in controls (fig. 1, *A*). The origin of circulating miR-371a-3p is from viable GCT. Thus, we attribute the high expression of serum miR-371a-3p in viable GCT due to presence of malignancy rather than larger tumor size.[21] With a relative expression threshold of 23.5, sensitivity and specificity were 93% and 100%, respectively. ROC analysis revealed an AUC of 0.978 (fig. 1, *B*). In contrast, conventional STMs combined for an AUC of 0.79 with a sensitivity and specificity of 58% and 100%, respectively. miR-371a-3p had a PPV of 100% and NPV of 73% (table 2). Five out of 6 patients with NSGCT with normal conventional STMs had a positive miR-371a-3p test. The other NSGCT patient had a 90% teratoma, and thus likely had a negative miR-371a-3p test. Fourteen out of 16 patients with seminoma with normal conventional STMs had a positive miR-371a-3p test. The other 2 seminoma cases likely had a negative miR-371a-3p test due to the small diameters. Of the seminoma patients, conventional STMs exhibited a sensitivity of 45%, while miR-371a-3p had a sensitivity of 93%. All viable GCT patients with positive conventional STMs had a positive miR-371a-3p test. We also coded individual STMs as either positive or negative using their normal ranges and found the sensitivity of the single marker miR-371a-3p test to be higher than the combination of all 3 conventional STMs (fig. 2).

CS II and III cases were excluded from all models due to the confounding presence of extratesticular viable GCT. Patients who received pre-orchiectomy chemotherapy were also excluded because chemotherapy may confound serum miR-371a-3p level. Tumor diameter (Spearman $r=0.6722$, $p<0.0001$) and percentage of embryonal carcinoma (Spearman $r=0.3133$, $p=0.0361$) correlated with miR-371a-3p expression (fig. 3). In a multiple linear regression model with diameter ($p<0.0001$) and percentage embryonal carcinoma ($p=0.0012$), both independently

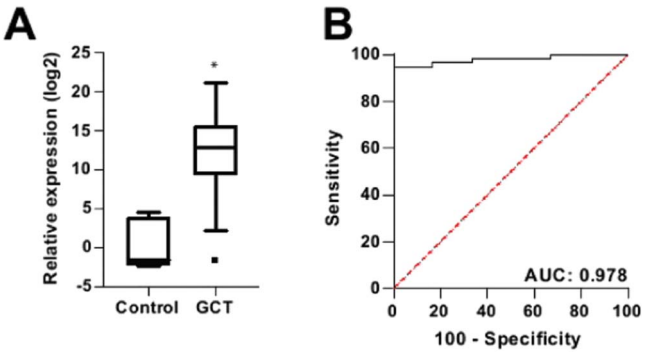

Figure 1. *A,* box plot showing relative expression of miR-371a-3p in controls and GCT. *B,* ROC shows performance of miR-371a-3p in GCT. Asterisk indicates $p < 0.05$.

correlated with miR-371a-3p expression. Furthermore, an ordinal logistic regression revealed a significant relationship between CS and miR-371a-3p expression ($p = 0.006$). A difference in serum miR-371a-3p expression was observed between CS I and CS III ($p = 0.0054$), but no significant difference was noted between CS II and CS III ($p = 0.7662$) and by GCT subtype ($p = 0.4385$) (fig. 4). Since only embryonal carcinoma was weakly correlated with miR-371a-3p expression and there was no correlation with other subtypes, we likely did not find a difference in expression between GCT subtypes.

Chemotherapy

Six patients received chemotherapy before orchiectomy due to widespread metastases. Five of these patients had biopsy proven seminoma with negative AFP at presentation, and 1 patient had biopsy proven NSGCT with elevation of AFP and β-hCG. Due to the potential for reduced miR-371a-3p expression in response to chemotherapy, an alternate threshold of 5.3 was calculated for this population. Four cases were ypT0 and correctly identified as such based on miR-371a-3p expression. Both patients with residual viable GCT had a positive miR-371a-3p test. In 1 patient with stage IIIC NSGCT and persistent residual retroperitoneal and liver masses after chemotherapy, subsequent orchiectomy revealed a small focus of embryonal carcinoma (2 mm). However, the RPLND and liver resection pathology were negative. In 1 patient with metastatic seminoma, imaging revealed ^{18}F-fluorodeoxyglucose, positron emission tomography-avid large residual masses in the left supraclavicular and para-aortic retroperitoneal lymph nodes. Orchiectomy was performed, and a repeat positron emission tomography scan showed residual viable GCT. Subsequently, the patient received high dose chemotherapy with stem cell transplant. miRNA before post-salvage chemotherapy RPLND was negative as was RPLND pathology, and the patient remains well in followup.

Solitary Testicles

Two patients with solitary testis underwent partial orchiectomy. One patient originally underwent left-sided orchiectomy at age 3 years after trauma. Upon presentation of a right testicular mass at age 25, he underwent partial orchiectomy, which revealed pure seminoma with a preoperative relative expression of 4.4 (negative miR-371a-3p test).

The other patient had a relative serum miR-371a-3p expression prior to radical orchiectomy, which revealed a yolk sac tumor, of 3,358. He was later diagnosed with germ cell neoplasia in situ by biopsy and had a relative expression of 571 (positive miR-371a-3p test) prior to the partial orchiectomy. Both cases had negative conventional STMs.

DISCUSSION

In this series of pre-orchiectomy serum samples, we show that the circulating miR-371a-3p test can reliably distinguish viable GCT from controls (lack of viable GCT). Our results have similar performance characteristics compared with other studies of miR-371a-3p in malignant GCT.[6,12,15] MiR-371a-3p sensitivity and specificity outperformed the conventional STMs, even in combination. Though the characteristics of miR-371a-3p have been explored in a variety of settings, we validate these in a cohort of patients undergoing orchiectomy for suspicion of GCT while exploring unique clinical scenarios encountered using miRNA testing.

Management of patients who received chemotherapy prior to orchiectomy for widely disseminated disease is an area where miRNAs may play a significant role. Residual viable GCT will be present within the testes after chemotherapy for up to 50% of patients.[22,23] Many of these patients will have residual masses in the retroperitoneum or extraretroperitoneal sites post-chemotherapy. The current standard of care is to perform PC-RPLND along with orchiectomy for patients with NSGCT and residual disease. Post-chemotherapy orchiectomy is standard for patients

Table 2. *Statistical parameters of serum tumor markers*

	AUC	Threshold	Sensitivity	Specificity	NPV	PPV	Accuracy
miR-371a-3p	0.978	23.5	0.931	1	0.733	1	0.942
Conventional serum tumor markers	0.79	NL*	0.579	1	0.314	1	0.647

* Upper normal limit used in diagnosis.

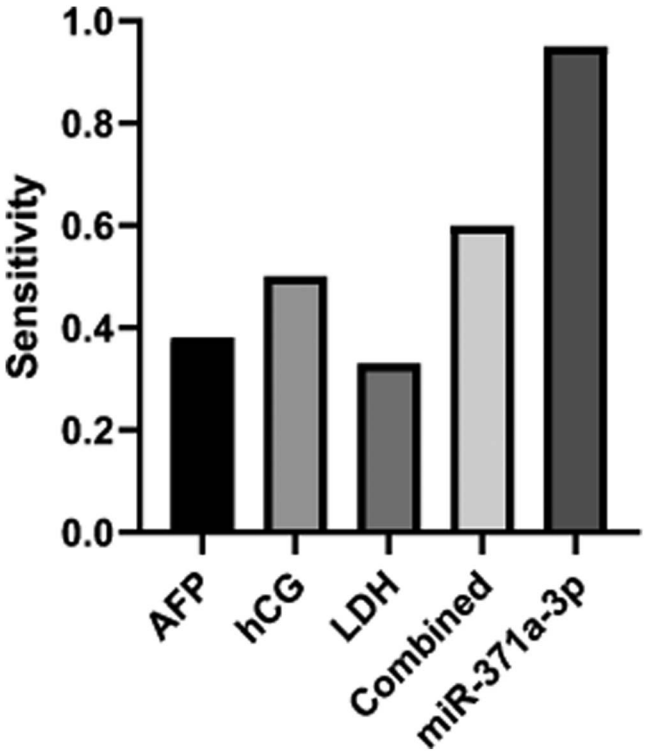

Figure 2. Sensitivity of miR-371a-3p test as compared to conventional STMs in chemonaïve cases.

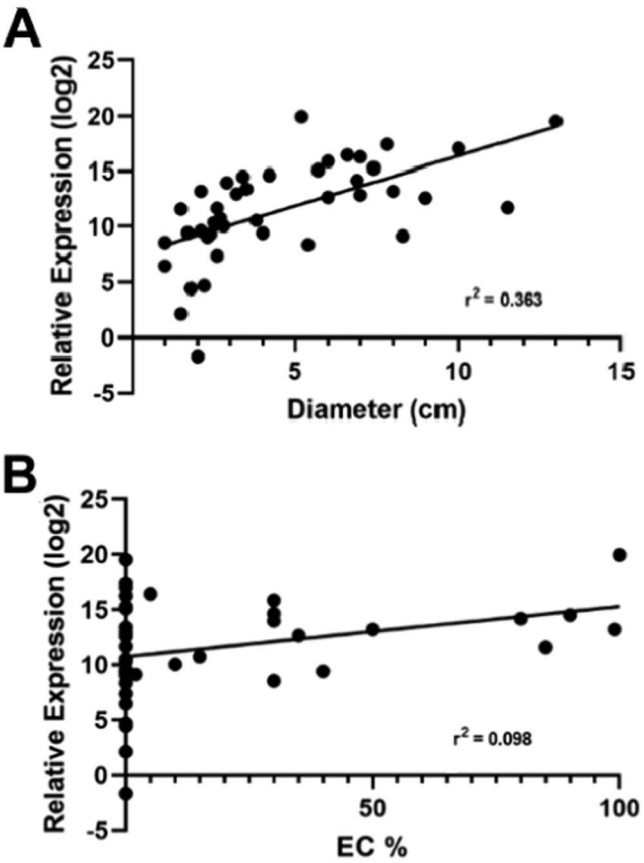

Figure 3. *A,* scatter plot of relative expression of miR-371a-3p when plotted against tumor diameter in CS I chemonaïve cases (45). *B,* expression of miR-371a-3p as function of percentage of embryonal carcinoma found in primary tumor in CS I chemonaïve cases (45). r^2 Values for linear regression models are listed.

with seminoma, with management of residual extragonadal masses on a case-by-case basis. We assessed the utility of circulating miR-371a-3p for detecting residual intratesticular viable GCT and potentially informing retroperitoneal histology. Of 6 cases where pre-orchiectomy chemotherapy was used in our series, orchiectomy pathology revealed benign tissue in 4 cases. Previously, Leão et al reported that circulating miR-371a-3p expression accurately correlated with viable GCT at post-chemotherapy surgery.[13] Similar to our findings, they identified 2 patients that had viable GCT on orchiectomy pathology only and fibrosis/necrosis on RPLND pathology. As our understanding of the role of circulating miRNA testing develops further, it is tempting to consider orchiectomy after chemotherapy in patients with widely disseminated disease prior to RPLND. Surveillance may be recommended over PC-RPLND in patients that convert from miR-371a-3p positive to negative following orchiectomy, potentially sparing the patient unnecessary surgery.

However, this must be weighed against the risk of harboring pure teratoma, which is generally chemotherapy resistant and undetectable by serum miR-371a-3p.[12] No patients in our cohort with pure teratoma received pre-orchiectomy chemotherapy. Therefore, we cannot assess miR-371a-3p performance in this setting. However, Leão et al and others have demonstrated that serum miR-371a-3p is insensitive to teratoma following chemotherapy.[13] Despite initial hope for miR-375 being a possible biomarker for teratoma, preliminary investigations are disappointing.[12,24] Further work to identify microRNA or other circulating markers of teratoma is warranted.[12,24,25] Additionally, a negative miR-371a-3p test before orchiectomy without prior chemotherapy does not rule out GCT, as the tumor may harbor pure teratoma or a seminoma smaller than 2 cm (which may have low expression of miR-371a-3p in different laboratory conditions).[11] Importantly, miR-371a-3p must be considered as an adjunct to conventional STMs as the latter still have discriminative value. An example would be a patient with pure seminoma on orchiectomy histology but elevated diagnostic serum AFP. The AFP informs that the patient should be treated as a NSGCT, which would not have been captured by the miR-371a-3p test only.

Ultrasound and conventional STM quantification are the cornerstones of testicular cancer diagnosis.[1] However, there may be situations in which these 2

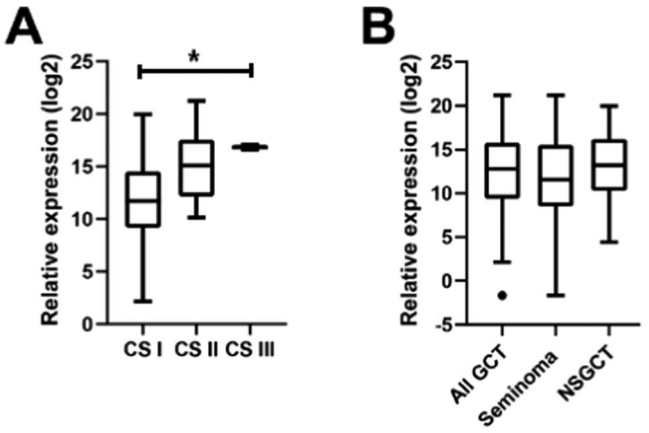

Figure 4. *A*, comparison of miR-371a-3p expression across different composite stages in chemonaïve GCT cases. Patient counts for CS I, II and III are 45, 6 and 7, respectively. *B*, comparison of miR-371a-3p expression in CS I GCT subtypes (24 seminoma and 21 NSGCT). Asterisk indicates $p < 0.05$.

tests are indeterminate. The miR-371a-3p test may be particularly informative in situations where testis sparing surgery is a priority, such as in patients with solitary testes. In these cases, making the distinction between viable and benign tumor is imperative. Recent investigation has yet to confirm whether circulating miR-371a-3p can distinguish GCNIS from healthy testes. However, if a patient has a carcinoma that is truly in situ, then we may expect to see little circulating miR-371a-3p due to a lack of invasion into surrounding blood vessels. We report 2 cases in which serum was drawn before orchiectomy in the setting of a solitary testicle. Final pathology identified seminoma in 1 case and GCNIS without evident viable tumor in the other, with a negative and positive miR-371a-3p test, respectively.

Fifty percent of patients with GCNIS progress to invasive GCTs within 5 years.[26] This is particularly relevant as 5% of patients diagnosed with GCT will have GCNIS in the contralateral testis.[27] One patient from our series had pure GCNIS diagnosed after partial orchiectomy. He had a yolk sac tumor diagnosed from a contralateral orchiectomy 4 months prior. In this time, his relative miR-371a-3p expression decreased from 3358 before left radical orchiectomy to 571 before right partial orchiectomy. The seminoma sample was well below our relative expression threshold (likely due to its small diameter of 1 cm) and GCNIS (2.5 cm) is only anticipated to produce elevated serum miR-371a-3p in approximately 50% of cases.[28] In addition, our study did not assess miR-371a-3p level after partial orchiectomy. As a result, we would advise caution when interpreting miRNA expression in solitary testis and in diagnosing GCNIS until larger series are published.

Our study corroborates the findings from previous studies and builds upon the knowledge we have regarding using serum miR-371a-3p in clinical practice, especially in patients with solitary testes. Moreover, by using a control group of patients with benign or nonviable GCT (as opposed to only healthy patients) we describe a real-world demographic of patients who present to a urologist. The miR-371a-3p test is $50 more expensive compared to the quantification of conventional STMs. However, in a recent study it was shown that the miR-371a-3p test can save about $69 million per year in the long run.[29]

This study is limited by the lack of longitudinal follow up with post-orchiectomy miR-371a-3p. Thus, we are unable to correlate nadir miR-371a-3p levels with recurrence. However, a previous study showed that miR-371a-3p levels decline after orchiectomy and after chemotherapy in CS I, II and III patients.[11] Moreover, we use the upper limit of normal for conventional STMs and the Youden index for miR-371a-3p. This may have contributed to some bias since the upper limit of normal conventional STMs is a predetermined value and does not have optimal sensitivity and specificity, whereas the Youden index maximizes the sensitivity and specificity. However, this study demonstrates that the excellent performance of the serum miR-371a-3p test is reproducible and adds to a growing body of literature that establishes the clinical usage of targeted miRNA tests in the context of testicular malignant GCT.

CONCLUSION

We report that miR-371a-3p has a sensitivity and specificity of more than 90% for detecting viable GCT in the diagnostic pre-orchiectomy setting. Our findings validate the conclusion of previously published series in a cohort of patients undergoing orchiectomy for suspicion of GCT at a single large tertiary care center. Our results highlight the utility of a positive miR-371a-3p test in post-chemotherapy, solitary and GCNIS settings to possibly inform decisions regarding orchiectomy and resection of extratesticular residual disease. Further studies are necessary to corroborate these findings.

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EDITORIAL COMMENT

The advent of serum markers in the 1970s represented a milestone achievement in the clinical management of testicular tumors. However, the utility of these markers was hampered by low sensitivity and specificity. Serum levels of microRNA-371a-3p (the so-called M371 test) first suggested as novel marker in 2012 (reference 9 in article), are continuing their maturation process. Recent investigations show great promise for the test to close the diagnostic gap left by the classical biomarkers.

Badia et al confirm the greater than 90% sensitivity and specificity of the test for the diagnosis of germ cell tumors including seminoma. Their study design is noteworthy in that all of the control cases had testicular lesions other than active germ cell cancer. Even in this real-world scenario, the excellent performance of the test was confirmed. For the first time, the authors applied the test to patients awaiting post-chemotherapy orchiectomy and found the test correctly predicting ypTo cases. This result accords with findings in post-chemotherapy

retroperitoneal masses where elevated microRNA serum levels were found only in masses with viable cancer but not in those consisting of necrosis (reference 13 in article). Another noteworthy finding is the correlation of microRNA levels with percent embryonal carcinoma in the primary tumor supporting previous observations of graded expressions of microRNA-371a-3p across histological subtypes of germ cell cancer. The present article adds another crucial piece to the jigsaw puzzle of microRNA-371a-3p and it nourishes the hope that the M371 test will soon become a valuable diagnostic tool for the clinical management of testicular cancer.

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