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Prediction of desmoid tumor progression using miRNA expression profiling

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Desmoid tumor is a rare connective tissue tumor with locoregional aggressiveness but unpredictable behavior. The miRNA profile was ascertained for 26 patients included in the Desminib phase II trial and an independent validation cohort of 15 patients. Predictive and prognostic supervised analysis on the Desminib cohort failed to identify miRNAs differentially expressed between progressive and non-progressive patients under imatinib treatment or between progressive and non-progressive patients after discontinuation of imatinib. However, an unsupervised hierarchical clustering of the Desminib cohort identified two groups (A and B) of 13 patients each, where only the number of previous lines of treatment before inclusion in the study differed significantly between the two groups. Time to progression after discontinuation of imatinib was longer in group B than in group A. Fifteen miRNAs were highly statistically differentially expressed between groups A and B, targeting more than 3000 genes, including *AGO1*, *BCL2*, *CDK6*, *SMAD4*, *PTEN*, *CCND1*, *VEGFA*, and *RB1*. These results were confirmed in the independent validation cohort: hierarchical clustering of these 15 miRNAs identified two groups, in which time to recurrence was statistically different (28.8 months *vs* 68.8 months). These results provide the first indication of the prognostic value of miRNA expression profiling with a possible direct impact on patient management. A more precise miRNA signature must now be determined to select patients who would not benefit from surgical resection of their tumor and who ought to be monitored without treatment.

esmoid tumor (DT), also known as "aggressive fibromatosis", is a rare fibroblastic proliferative disease, affecting between 2 and 4 people per million per year.[1] The majority of DTs arise sporadically due to *CTNNB1* (β-catenin gene) mutation.[2] Desmoid tumors may also arise from a predisposing genetic condition called Gardner syndrome, characterized by germline mutations in the *APC* gene and associated with familial adenomatous polyposis (FAP), or also as rare forms of familial FAP.[3] Approximately 50% of DT occurs in the extremities, other main locations being the trunk wall and mesentery.[4] These tumors never metastasize and are considered as locally malignant tumors, with unpredictable behavior; although the condition remains static in most patients, it also may involve nervous, vascular, or muscular structures leading to serious symptoms or even patient death, particularly in the case of intra-abdominal location.

Treatment normally involves surgery, unless it is anticipated that this will result in serious functional and/or aesthetic sequelae. However, spontaneous stabilization or regressions are regularly seen. Furthermore, despite successful surgical removal (R0 or R1 resection), recurrences are common (>40%), questioning the effectiveness of surgery as a treatment for these tumors. A retrospective study, evaluating a conservative approach (no surgery and no radiotherapy) by a watch-and-wait policy or use of systemic treatment, has resulted in prolonged progression-free survival in a substantial proportion of patients (~50%).[5] Unfortunately, in this study, the authors failed to determine prognostic factors to select patients who would benefit from this strategy. Recently, the National Comprehensive Cancer Network (NCCN) updated its guidelines on soft tissue sarcoma including "observation as an option for patients with resectable desmoid tumors that are small and asymptomatic, not causing morbidity, pain, or functional limitation".[6]

MicroRNAs are non-coding small RNA molecules synthesized from intronic regions ranging in size from 16 to 35 nucleotides. They are processed by specific complexes of proteins containing Drosha and Dicer and integrated into RNA-induced silencing complexes. The mature miRNAs direct the RNA-induced silencing complex (RISC) to the complementary sequences in mRNA, resulting in translation inhibition and accelerated mRNA degradation.[7] MicroRNA expression is deregulated in many cancers, resulting in a tumor miRNA signature, which provides the means to classify tumors with respect to their tissue origin and molecular alterations. Thus, miRNAs constitute potent diagnosis and/or prognosis biomarkers through their tissue specificities and their involvement in oncogenic processes.

This study was undertaken to define the potential predictive and/or prognostic value of an miRNA expression profile of DT, to identify rapidly progressing and non-progressing patients.

Materials and Methods

Patients. This study was carried out as a retrospective translational research program on tumor samples of patients included in the Desminib trial.[8] Forty patients with progressive or recurrent DT that could not be treated with curative surgery or radiotherapy were included in the Desminib phase I /II trial to evaluate the efficacy and toxicity of imatinib. Patients with adequate end organ function were treated with 400 mg imatinib daily, increasing to 800 mg in the case of progressive disease. Best clinical response to imatinib was defined according to the Response Evaluation Criteria in Solid Tumors, and evaluated every 3 months. All the tumoral evaluations under treatment except two were reviewed by a radiological independent validation committee. Informed consent was obtained from each patient for enrolment in the study and archival of pathology specimens.

A second independent cohort was established from 15 patients with relapsing and non-relapsing DT, treated at Center Leon Berard (Lyon, France) between 1990 and 2005.

Tissue samples. Formalin-fixed paraffin-embedded (FFPE) tissue sections from tumors at initial diagnosis of patients included in the study were obtained from pathology centers. It has been established that FFPE samples are of high enough quality for miRNA profiling.[9,10]

RNA extraction and quantitative real-time PCR. The FFPE tumors were washed with toluene, ethanol, and Tris/EDTA and then lysed for 24 h in ATL buffer (Qiagen, Courtaboeuf, France) supplemented with proteinase K (Qiagen) at 60°C in rotative agitation. Total RNA was extracted from tumors using a single phenol/chloroform extraction protocol with TRIzol, according to the manufacturer's instructions (Life Technologies, Saint Aubin, France). Five hundred nanograms of total RNA were subjected to microfluidic PCR carried out by Applied Biosystems (Life Technologies). In brief, RNA was reversed transcribed using multiplexed specific looped miRNA primers from the TaqMan MicroRNA Reverse Transcription kit. The resulting cDNA was then subjected to real-time quantitative PCR on a TaqMan Low Density Array card, whereby RT products are introduced through microchannels into miniature wells that are preloaded with dehydrated specific primers and probes. Analyses were carried out on 384 miRNAs.

Statistical analysis. For each miRNA, the threshold cycle (C_t) was calculated by the ABI 7900 Sequence Detection System software. A cut-off of 37 was applied to discard late C_t values according to the manufacturer's instructions.[11] The median method was used for normalization[12,13] and each miRNA was expressed by relative quantification (Rq = $2^{-\Delta\Delta C_t}$).

Supervised and unsupervised hierarchical clusterings were carried out using TIBCO Spotfire Decision Site software (Palo Alto, California, USA). Supervised analysis was carried out on several groups of patients: responsive versus stable and progressive disease during 1 year of treatment with imatinib to search for a predictive value; and progressive versus non-progressive disease following termination of imatinib treatment to search for a prognostic value.

Univariate logistic regression analysis was carried out on patients grouped according to unsupervised hierarchical clustering with the following variables: sex (male *vs* female); age at diagnosis (continuous variable); tumor size (at inclusion in Desminib trial, continuous variable); tumor location (mesenteric *vs* abdominal wall *vs* extra-abdominal), FAP (yes *vs* no), number of previous lines of treatment before inclusion in the study (patients treated by radiotherapy and/or chemotherapy

and/or hormone therapy and/or non-steroidal anti-inflammatory drugs as an ordinal variable with four categories); lymphocyte count (at inclusion in the trial, continuous variable); presence of the KIT M541L exon 10 variant (yes *vs* no); and level of immunohistochemical expression of β-catenin, cyclin D1, and platelet-derived growth factor-β (0 *vs* 1 *vs* 2 *vs* 3).

Time to progression was compared by log–rank test following Kaplan–Meier analysis. To detect differential expression of miRNAs between different groups of interest, we used a one-way ANOVA with a *P*-value threshold of 5%. To identify genes targeted by miRNAs of interest, we only considered the validated target in a database combining the two validated datasets (TarBase version 6.0: last release August 1, 2012[14]; miRTarbase: last release November 1, 2013).[15]

Results

Patients. Forty patients were included in the Desminib phase II trial. Detailed results of the study were published in 2010.[8] Two patients included in the clinical study refused to sign consent for biological research on their tumor sample. Paraffin-embedded tissues samples of 34 of these patients, taken at initial diagnosis, were obtained from pathology centers. Six tissue samples were excluded from the study as they had been fixed in Bouin's solution. RNA was extracted from 28 paraffin-embedded tissues and obtained in sufficient quantity (at least 500 ng in 3 μL) for 26 patients. The 15 patients included in the validation cohort from Center Leon Berard had been surgically treated. Table 1 presents the characteristics of the 41 patients analyzed (26 patients in the Desminib cohort and 15 patients in the validation cohort).

The characteristics of the patients corresponded to those described in the literature.[1,4] Among the 41 patients analyzed, the majority were women with a median age at diagnosis of 40 years. The tumors were mainly localized in an extra-abdominal location (the majority in the trunk and, in a few cases, in upper and lower limbs or in head and neck). Median tumor size was 88 mm at inclusion into the Desminib study and slightly smaller (67.5 mm) in the validation cohort. This was the only criteria differing between the two cohorts. More than half of patients received no drug-based treatment or only one line of treatment, and 37% of them received two, three, or four lines of treatment.

In the Desminib cohort, after a median follow-up of 68 months, median time to progression (TTP) was 2 years and median overall survival is not reached. The majority of patients had stable (19 patients) or responsive (3 patients) disease under imatinib. After discontinuation of imatinib, 10 patients were progressive within 23.5 months of follow-up and 14 never progressed. In the validation cohort, after a median follow-up of 56.8 months, 6 patients had recurrence and 9 were recurrence-free.

Supervised analysis. Predictive and prognostic supervised analysis on the Desminib cohort failed to identify miRNAs differentially expressed according to the Bonferroni correction between patients responsive versus non-responsive to imatinib, and between progressive versus non-progressive following discontinuation of imatinib.

Unsupervised analysis. The unsupervised hierarchical clustering of the Desminib cohort identified two groups of 13 patients each, called group A and group B. To identify clinical and/or biological factors that could explain the partition of patients in the two clusters, univariate analysis by logistic regression was

Table 1. Characteristics of the 41 patients with desmoid tumors analyzed in this study

Characteristic	Desminib cohort n = 26 (%)	Validation cohort n = 15 (%)
Gender		
Male	8 (31)	6 (40)
Female	18 (69)	9 (60)
Median age at diagnosis [range], years	40 [20–72]	35.4 [0.4–71.3]
Location		
Intra-abdominal	4 (15)	1 (7)
Abdominal wall	3 (12)	1 (7)
Extra-abdominal	19 (73)	13 (86)
Median tumor size [range], mm	88 [25–220]	67.5 [35–150]
FAP		
Yes	3 (12)	1 (7)
No	23 (88)	14 (93)
Performance status		
0	18 (69)	NA
1	6 (23)	NA
Unknown	2 (8)	NA
Number of treatment lines (RT/CT/hormone/NSAID)		
0	10 (38)	5 (33)
1	8 (30)	6 (40)
2	3 (12)	1 (7)
3	3 (12)	2 (13)
4	2 (8)	1 (7)
Median lymphocytes count [range] G/l	2.2 [0.7–3.2]	NA
Median TTP [range], months	24.6 [0.9–42.3]	24.7 [5.6–82.1]
Response under imatinib		
CR, PR	3 (12)	NA
SD, PD	23 (88)	NA
Median follow-up [range], months	68 [31–210]	56.8 [7.1–233.6]

Desminib cohort, treated with imatinib; validation cohort, treated surgically. CR, complete response; CT, chemotherapy; FAP, familial adenomatous polyposis; G/l, Giga/liter; NA, not available; NSAID, non-steroidal anti-inflammatory drug; PD, progressive disease; RT, radiotherapy; SD, stable disease.

Table 2. Univariate logistic regression analysis on clinical and biological factors in Desminib cohort of patients with desmoid tumors treated with imatinib

	P-value	OR	IC 95%
Age	0.8020	0.99	0.93; 1.05
Gender (M/F)	1.0000	1.00	0.18; 5.48
Location			
Extra-abdominal			
Intra-abdominal	0.2550	0.24	0.01; 2.3
Abdominal wall	0.4400	0.36	0.01; 4.45
Tumor size	0.4970	1.01	0.99; 1.02
Number of previous treatment lines	*0.0387*	*2.44*	*1.17; 6.78*
KIT541 (M/WT)	0.4070	2.36	0.31; 21.61
β-Catenin			
1			
2	0.3630	0.29	0.01; 3.93
3	0.7080	0.60	0.02; 8.25
Cyclin D1			
0			
1	0.2940	0.27	0.01; 2.56
PDGFR β			
1			
2	0.5300	0.57	0.09; 3.26
3	0.2680	0.24	0.01; 2.51
Lymphocyte count	0.5310	1.53	0.41; 6.29

F, female; M, male; M/WT, mutant/wild type; OR, odds ratio; PDGFRβ, platelet-derived growth factor receptor β.

carried out. The results are presented in Table 2. The only significant association between grouped members was the number of previous lines of treatment before inclusion in the Desminib trial, with an odds ratio of 2.44 (1.17–6.78) and a P-value of 0.0387. Group B patients received fewer lines of treatment prior to inclusion in the Desminib trial.

Time to progression after termination of imatinib treatment was compared between groups A and B by log–rank analysis. Three patients were excluded from the study due to of lack of follow-up. The median follow-up after discontinuation of imatinib was 23.6 months (12.4–39.0). Six out of 11 patients in group A, and 3/12 patients in group B relapsed. Although not statistically significant, the median TTP in group B was longer than that of group A and justified the validation cohort (Fig. 1).

By ANOVA, 15 miRNAs were found differentially expressed with statistical significance between groups A and B. Table 3 lists these 15 miRNAs and their P-values. All of these miRNA were underexpressed in group A, which is associated with poor prognosis. After database interrogation, more than 3000 genes were found to be targeted by these 15 miRNAs. Among them, 50 genes were targeted by at least three miRNAs, including

AGO1, *BCL2*, *CDK6*, *SMAD4*, *PTEN*, *CCND1*, *VEGFA*, and *RB1*.

To validate these results, the 15 miRNAs were used to carry out a hierarchical clustering of the validation cohort. Two groups of 6 and 9 patients were identified, called group A' and group B', respectively. Univariate analysis by logistic regression failed to find any significant clinical parameters to explain the distribution of patients in the two groups. Parameters tested were age and sex of the patient, location and size of the tumor, and number of treatment lines. The results are presented in Table 4. After a median follow-up of 53 months (range, 3–230 months), 4 patients out of 6 in group B' and 5 patients out of 9 in group A' recurred. Time to recurrence after initial surgery was compared by log–rank analysis between groups A' and B' and found to be significantly longer in group B' than in group A' (Fig. 2).

Discussion

This study presents the first results of an miRNA profile expression analysis in DT in a cohort of patients included in a clinical phase II trial (Desminib trial) that ensures a good quality follow-up and exhaustiveness of the data recorded. The main limitation of our study is linked to the small cohort sizes, which is unavoidable with such rare mesenchymal tumors. The characteristics of patients in the studied cohorts (Desminib and validation cohorts) are similar to those described in the largest retrospective study.[4]

As miRNAs have not yet been studied in DT, we used an explanatory approach of the analysis with no assumption. First, predictive and prognostic supervised analyses were carried out. It was difficult to accurately split the groups into responsive versus non-responsive patients because the response evaluation to medical treatment of DT is not clearly established: the

Fig. 1. Right panel, heat map clustering of miRNA expression in desmoid tumors of the Desminib cohort, treated with imatinib (26 samples; groups A and B, 13 patients each). Each column represents the relative levels of expression for each miRNA and each row shows the expression levels for each patient sample. The red or green color indicates relatively high or low expression, respectively; gray squares indicate absent data points. Left panel, log–rank analysis comparison of time to progression (TTP) after discontinuation of imatinib of groups identified by unsupervised analysis in the Desminib cohort. NA, not available.
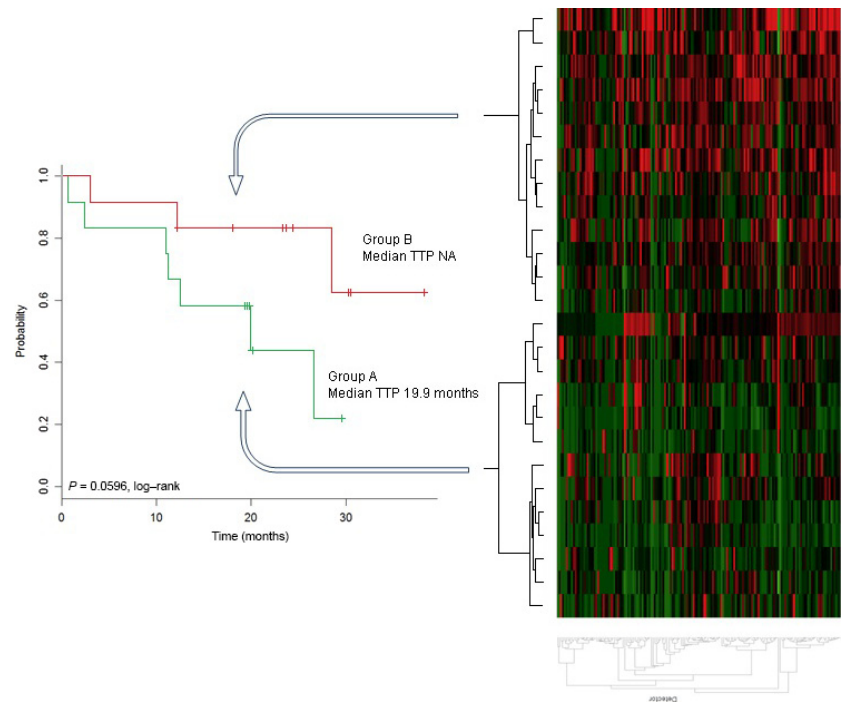
Table 3. MicroRNAs differentially expressed between two groups identified by unsupervised hierarchical clustering of Desminib cohort or patients with desmoid tumors treated with imatinib

Detector ANOVA	P-value
hsa-miR-26a	6,51259E-08
hsa-miR-199a-3p	8,93264E-07
hsa-miR-374b	1,83335E-06
hsa-let-7g	2,46322E-06
hsa-miR-374a	2,52505E-06
hsa-miR-708	1,9259E-05
hsa-miR-103	3,07889E-05
hsa-miR-195	3,65417E-05
hsa-miR-410	4,77677E-05
hsa-miR-93	8,39891E-05
hsa-miR-19b	8,7169E-05
hsa-miR-100	0,000105825
hsa-miR-411	0,000107016
hsa-miR-31	0,000114059
hsa-miR-26b	0,000121591

Table 4. Univariate logistic regression analysis on clinical and biological factors in validation cohort of patients with desmoid tumors treated surgically

	P-value	OR	IC 95%
Age	0.636	1.06	0.95; 1.09
Gender (M/F)	0.668	1.60	0.19; 16.31
Location			
Extra-abdominal			
Intra-abdominal	0.997	–	–
Abdominal wall	0.996	–	–
Tumor size	0.928	0.99	0.97; 1.03
Number of treatment lines	0.293	2.21	0.77; 18.04

–, not enough cases in each category to have reliable data; F, female; M, male; OR, odds ratio.

Response Evaluation Criteria in Solid Tumors are not adapted to these slow growing tumors. Predictive analysis failed to identify specific imatinib targets that could explain the sensitivity of DT to this drug, but proposed new targets to further explore (*PARP*, *IGF1R*, and *HSP90B*). Prognosis supervised analysis was difficult to interpret for the Desminib cohort as the majority of patients had recurrent and/or progressive disease at inclusion in the trial.

We applied hierarchal clustering to our patient cohort, an increasingly routine method for processing "high-dimensional data". A study upstream on the robustness of clustering has shown that the Euclidean distance and Ward's method gave the best results for small cohorts of sarcoma tumors. Beneficially, this method does not require any prior knowledge of

the groups. This analysis of the Desminib cohort led to the classification of two groups of patients.

The results of logistic regression showed a significant association between classification into group B and fewer lines of treatment administered prior to inclusion in the Desminib trial, likely reflecting the aggressiveness of the tumor. In clinical practice, it is known that systemic treatments (chemotherapy, hormone therapy, and non-steroidal anti-inflammatory drugs) are of little effect and have unwanted side-effects. When several medical therapeutics are tried for one patient, it usually reflects an aggressive tumor, uncontrolled and/or symptomatic, and available therapies are successively attempted to relieve recurrent patient symptoms. The number of treatment lines has previously been used as a parameter in clinical trials evaluating other low-grade tumors, such as T-cell lymphomas.[16] These data suggested a prognostic value of the miRNA profile on tumor aggressiveness. This was confirmed by the comparison of TTP after discontinuation of imatinib in the two groups: although not statistically significant ($P = 0.0596$), the TTP curves of the two groups were clearly distinct. The lack of sta-
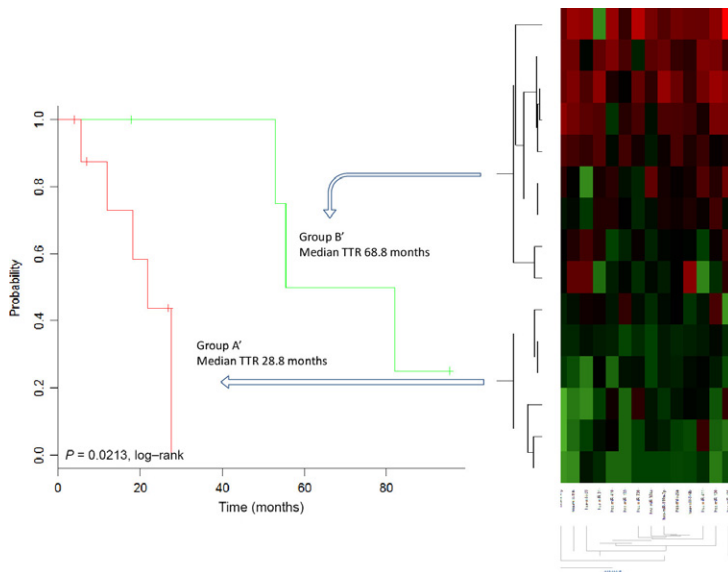
Fig. 2. Right panel, heat map clustering of miRNA expression in desmoid tumors of the validation cohort, treated surgically (15 samples: group A', 6 patients; group B', 9 patients), for 15 miRNAs found to be significant in the Desminib analysis. Left panel, log–rank analysis comparison of time to recurrence (TTR).

tistical significance may be due to the limited size of the cohort and to the fact that all patients included in the Desminib trial had a recurrent, aggressive tumor.

These results were validated in an independent cohort that included patients with recurrent and non-recurrent tumors following surgical excision. This cohort was better suited to searching for a prognostic value. Indeed, unsupervised clustering identified two groups of patients with significantly different rates of time to recurrence, confirming the prognostic value of miRNA profile expression. This is in line with previous studies in various other forms of cancer that have indicated the prognostic value of miRNA.[17–20]

The next step of this analysis was to identify discriminating miRNAs between the clusters in order to: (i) define a prognosis signature; and (ii) determine which genes were targeted by the miRNAs. Using the ANOVA test and Bonferroni correction, 15 miRNAs were found to be significantly differentially expressed in the unsupervised analysis of the Desminib cohort. These 15 miRNAs target more than 3000 genes. We selected several genes known to be involved in signaling pathways in cancer that could represent interesting new targets to explore the biological mechanisms of DT and develop new therapeutics. We decided to focus on genes targeted by several miRNAs, to increase their biological relevance. *PTEN* is a key cell cycle regulator and a tumor suppressor gene. Genetic inactivation or reduced expression of *PTEN* is frequently associated with cancers. Mutations in *SMAD4* and *PTEN* are known to be involved in the development of familial juvenile polyposis.[21] *VEGFA* is known to be involved in angiogenesis. *Rb* is also considered to be a tumor suppressor as it inhibits cell cycle progression. Both CDK6 and cyclin D1 phosphorylate *Rb*, inhibiting its activity. A lack of *Rb* nuclear staining was shown by immunohistochemistry in 13 cases of DT (interestingly, the staining was present in palmar fibromatosis as well as in the vascular tissue adjacent to the fibroproliferative lesion).[22] *Bcl2* is classified as an oncogene as long as it acts as an anti-apoptotic. In a previous study looking for predictive response factors to imatinib in the Desminib trial, we found inconsistent expression of cyclin D1 by immunohistochemistry analysis on tissue microarray. Interestingly, all patients (5/31 tested) with histological staining for cyclin D1 were classified into group A, which included those with the most aggressive disease.

It may be considered inconsistent that some oncogenes and tumor suppressor genes are targeted by miRNAs overexpressed in the same prognostic group of patients. But we have to keep in mind that DTs are very special tumors, on the border between malignancy and benign tumors, whose development is sustained by unclear biological mechanisms. It has already been established that some genes could act as oncogenes in some tumors, and as tumor suppressors in others. Complementary data (not shown) confirmed that miRNA levels are not directly and perfectly correlated to protein expression and that other genetic and epigenetic phenomena may interfere. This miRNA study should be first considered as an exploratory approach to help to identify interesting candidates that could be involved in the biology of these tumors. Their precise roles have to be defined in complementary protein expression studies.

These 15 miRNAs provide interesting leads for prognostic study, but validation in a larger and prospective cohort of DT is required before they can be used clinically. These results are the first indication of the prognostic value of miRNA expression profiling in DT. The implications of such a discovery will be important in the management of patients with these tumors that are particularly over-treated. It is now evident that a substantial proportion of patients (~50%) do not benefit from surgical resection of their tumor, instead often suffering from functional and/or esthetic sequelae for a tumor that may not have progressed. This is now so well established that a prospective phase II clinical trial is ongoing to systematically offer DT patients active surveillance, with surgical management only in the case of progressive disease. There is a deep need for clinical and/or biological surrogate markers of aggressiveness for these tumors to select patients who may benefit from such a watch-and-wait policy. The results of our study need to be confirmed in larger patient cohorts, and we plan to analyze tumor samples from patients, including those enrolled in the ongoing phase II trial, to establish their miRNA profile. The ultimate goal will be to define a robust prognostic signature based on a few miRNAs to predict the behavior of a newly diagnosed tumor and adapt the treatment accordingly. We will also evaluate the miRNA profile in the serum of patients in order to develop a non-invasive diagnostic tool.

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Disclosure Statement

The authors have no conflict of interest.

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