Quantitative RT-PCR-based miRNA profiling of blastemal Wilms' tumors from formalin-fixed paraffin-embedded samples

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Abstract

Blastemal Wilms' tumors are associated with poor chemo-responsiveness and an adverse prognosis. Our aim was to contribute to the miRNA profiling of the disease, while demonstrating the value of archived formalin-fixed, paraffin-embedded (FFPE) samples as miRNA sources. MiRNA was extracted from tumor and normal tissues of 8 patients diagnosed with blastemal Wilms' tumor in Hungary. A quantitative real-time PCR-based protocol was used to identify miRNAs of interest and study the expression of selected miRNAs in all samples. Profiling of miRNA expression from FFPE samples turned out to be cost-effective in Wilms' tumor, as most miRNAs (including miRNA-194-5p, which was studied in all patients) showed expression alterations similar to the ones reported in the literature. MiR-184 expression was found to be lower than in previous studies, while the downregulation of miR-203a is a novel finding. MiR-184 may be downregulated in a subset of blastemal and other Wilms' tumors. A loss of miR-203a may or may not be specific to blastemal cells, but available evidence hints at its importance in the pathogenesis of Wilms' tumor. It should be considered for inclusion in future studies of miRNA expression.

Keywords: Wilms' tumor, miR-184, miR-203a, miR-194-5p, miR-34c-5p, quantitative real-time PCR

1. Introduction

Wilms' tumor (or nephroblastoma) is the most frequent renal malignancy in children under the age of 10. In Europe, treatment protocol consists of four to six weeks of pre-operative chemotherapy followed by surgical resection and post-operative adjuvant chemotherapy (based on histological assessment) (Bhatnagar, 2009). This is in accordance with recommendations by International Society of Paediatric Oncology (SIOP WT 2001). When histology is assessed, responsiveness to pre-operative chemotherapy is evaluated. Cases with chemotherapy-induced changes in more than two-thirds of the tumor mass are considered regressive, while other cases are classified according to the predominant cell type: blastemal, epithelial, stromal or mixed. Anaplastic changes seen in the context of the above tumor types predict a poorer outcome, and may occur focally or diffusely. Most histological subtypes without anaplasia are associated with a good prognosis: long-term survival is over 90 percent in localized cases and around 75 percent in metastatic disease (Szychot et al., 2014). However, the subtype showing blastemal predominance is less responsive to chemotherapy and associated with a higher risk (regardless of treatment protocol) with a 5-year overall survival of only 65 percent (Bhatnagar, 2009; Kinoshita et al., 2012). In patients treated according to the SIOP protocol, the two most frequently seen subtypes are regressive (showing appropriate response to chemotherapy, regardless of histological type) and blastemal (the most common histology of unresponsive tumors). Understanding differences between the two at the molecular level is key to identify factors underlying chemo-responsiveness (Watson et al., 2013).

Deregulation of miRNAs (a group of endogenous, approximately 22 nucleotides long, non-coding RNAs) has been observed in many types of malignant disease, including Wilms' tumor (Krutovskikh and Herceg, 2010). In our study, we extracted RNA from formalin-fixed, paraffin-embedded (FFPE) samples of 8 blastemal Wilms' tumors. Quantitative reverse transcription PCR (qRT-PCR) was performed to reveal deregulated miRNAs. Our aims were to establish a cost-effective protocol showing that archived FFPE samples are very useful miRNA sources in expression studies, and to contribute to the profiling of blastemal Wilms' tumors characterized by adverse prognosis.

2. Materials and methods

2.1. Study population

Criteria for inclusion in our study were the presence of Wilms' tumor with a prominent blastemal component (Fig. 1) and an available archived FFPE sample younger than 6 years (Table 1). Our clinical sample consisted of 8 Hungarian patients aged between 1 and 8 years (median age: 2.5 years) at the time of the diagnosis. Most cases were diagnosed at routine screening or from observation of common features. In one case (Patient ID: 7), the tumor was discovered during the follow-up of polycystic kidneys, while in another patient (ID: 5), abdominal ultrasonography was performed upon noting obesity and Cushingoid features. The patient was found to have cortisol-producing islets in the Wilms' tumor tissue.

All patients had unilateral Wilms' tumor. Chemo- and surgical therapy was performed according to the SIOP WT 2001 protocol. Tumor regression was judged using MRI after completing pre-operative chemotherapy in cases where the MRI preceded surgical therapy. Patient 7 was initially misdiagnosed for renal cell carcinoma, so pre-operative chemotherapy was not applied in this case.

Most patients remain in remission without any relapses during 2-5 years of follow-up. Only Patient 4 showed a relapse 1 year after the initial diagnosis, and received an autologous bone marrow transplant after completing relapse protocol. Currently (one year after the administration of the last regimen) she is in remission.

2.2. Laboratory methods

We used two FFPE samples per patient: one from the tumor tissue and one from the same surgical sample, but a tumor-free region. (Tumor samples did not contain anaplastic foci or nephrogenic rests, care was taken to include only blastemal tissue in analyzed FFPE sections.) From each sample, we extracted miRNA using a miRNeasy FFPE Kit by Qiagen (Cat No.: 217504), reported as the best performing kit on the market (Howe, 2017). We produced cDNA using a miScript II RT Kits (also from Qiagen, Cat No.: 218160) to be stored at -20°C. MiRNA yields were adequate from all FFPE samples (Table 1).

In a pilot experiment, we ran two 96-well miScript miRNA PCR Arrays (by Qiagen, Cat No.: 331221 MIHS-112ZF), originally designed for human prostate cancer but suitable for other types of genitourinary malignancy. We used primers for 80 mature miRNAs and 4 miRNA precursors suggested as relevant in tumorigenesis, along with 6 endogenous and 6 exogenous controls. Using a Roche LightCycler 96 PCR instrument, we performed expression analysis of appropriately diluted tumor and control cDNA samples from the same patient (Patient ID: 1). We applied the $\Delta\Delta$Ct method and calculated fold changes as described in the literature (Livak and Schmittgen, 2001), using a single endogenous control gene to ensure that results are comparable between the pilot arrays and later PCR experiments. Our chosen control gene was small nucleolar RNA U61 (*SNORD61*), as it showed median expression out of all suggested endogenous controls and its reliability was supported by literature data across different tissues (Sperveslage et al., 2014; Zehentmayr et al., 2016).

Based on initial results (see section 3.1), four miRNAs were selected for further investigation in the remaining patients: miR-34c-5p, miR-184, miR-194-5p, and miR-203a. (Regarding the rationale for selection, please see section 4.) In the following experiments, we used Qiagen's miScript Primer Assays (Cat Nos.: MS00003332, MS00003640, MS00006727, MS00003766, and MS00033705) for each of the chosen miRNAs, and an assay for *SNORD61* as control. Median Ct values of technical triplicates were used. In all other aspects, fold changes in miRNA expression were calculated the same way as above.

3. Results

3.1. Pilot experiment: PCR Array

Observed fold changes for 80 mature miRNAs and 4 precursors between the Wilms' tumor and the control sample from Patient 1 are demonstrated in Table 2. Although no technical replicates were available on the 96-well array, results were satisfactory with the exception of a few instances: we failed to detect miR-93-5p and miR-96-5p in the control sample, and an overexpression of miR-26a with an unrealistic magnitude was considered an artifact (according to the literature, it should be underexpressed (Akpa et al., 2016)).

3.2. Quantitative RT-PCR experiments using individual primers

Expression analysis in the other patients (IDs: 2 to 8) showed reliable results with low variance among triplicates. Table 3 shows a summary of fold changes found in the expression of miR-184, miR-203a, miR-34c-5p and miR-194-5p in all patients, as well as the average fold change for each miRNA. As Patient 7 did not receive pre-operative chemotherapy, an average was also calculated with the omission of this case for easier comparability.

4. Discussion

A recent large-scale study has shed some light on the genetic background of Wilms' tumors, with the conclusion that diverse somatic or germ-line mutations may lead to a more or less common downstream deregulation pattern (including miRNA expression signature) in early renal progenitor cells (Gadd et al., 2017). Still, in spite of common features due to the underexpression of miRNAs belonging to certain families (e.g. let-7 (Wegert et al., 2015)), profiling efforts carried out so far show some considerable differences between the expression patterns of certain individual miRNAs (Liu et al., 2013; Ludwig et al., 2016). Further studies on miRNA expression may be needed to achieve a consensus. Apart from fresh-frozen samples, peripheral blood has also been shown to be a reliable miRNA source in Wilms' tumor (Schmitt et al., 2012), but these may only be acquired from newly diagnosed patients. In contrast, using FFPE samples allows older cases to be studied – to our knowledge, ours is the first paper to report such results in Wilms' tumor.

Methodological studies on miRNA extraction from FFPE samples are available: they suggest that the short length and high stability of miRNA makes it resistant to degradation, allowing for efficient extraction from FFPE samples aged up to 7-10 years with results comparable to fresh-frozen tissue (Howe, 2017; Liu and Xu, 2011; Dijkstra et al., 2012). Still, such methods have not become widespread despite the availability of FFPE samples in pathological archives.

Among the miRNAs tested by the PCR Array (Table 2), there were some that had scarcely been studied or reported in the literature, but showed notable expression changes in our pilot experiment – such as miR-184, which was massively downregulated, while in literature reports it showed no change (Liu et al., 2013) or a slight underexpression especially in blastemal tumors (Ludwig et al., 2016). The downregulation of miR-203a also seemed to be a new finding as it was previously reported to be somewhat elevated in unspecified Wilms' tumors and not yet studied in the blastemal subtype (Liu et al., 2013). An extreme upregulation of miR-34c-5p (while possibly an artifact) also caught our attention. When selecting miRNAs to study individually in the remaining patients, we aimed to confirm these (apparently novel) findings, and also included miR-194-5p, which was already known to be one the most downregulated miRNAs in blastemal and other Wilms' tumors, so it served as a positive control allowing for a comparison between our FFPE-based data and results available in the literature (Liu et al. 2013; Ludwig et al., 2016).

As demonstrated in Table 3, expression alterations of miR-34c-5p did not show a universal direction across different samples, making results inconclusive. (The unlikely level of upregulation observed in Patient 1 was indeed confirmed to be an artifact.) Results obtained with primers for the other three miRNAs are also demonstrated in Fig. 2. MiR-203a and miR-184 were found to be downregulated in all samples, a result consistent with the initial experiment. While the sample size is too small to judge possible implications about chemo-responsiveness, it is interesting to note that Patient 7, who did not receive pre-operative treatment, showed miRNA deregulations of higher amplitude in comparison to cases that were pre-treated and showed a certain level of regression. Similarly high fold changes were observed in only one case (Patient ID: 8), which showed no response to chemotherapy at all (no regression by the time of surgical removal).

Comparing found miR-184 expression levels to the pilot experiment, six samples (Patient IDs: 2-4, 6-8) showed a similar or higher fold change, while one sample displayed an underexpression of lower magnitude, close to the -4.00 fold change reported by Ludwig et al. (2016) for blastemal Wilms' tumor. As the authors did not publish variance between their samples, we applied a one sample t-test to compare fold changes in all of our samples to -4.00 (following a logarithmic transformation). The difference was significant (p=0,0002). MiR-184 has been proposed as a tumor suppressor in nasopharyngeal carcinoma (Zhen et al., 2013) as well as small-cell lung cancer (Zhou et al., 2015), and based on our results, such a role seems possible in a subset of blastemal Wilms' tumors, even if we consider the small sample size.

Downregulation of miR-203a is perhaps even more interesting. An *in silico* network analysis (He et al., 2016) suggested that E2F transcription factor 3 (E2F3) may be a target for miR-203a – this was recently confirmed in gastric cancer (Yang et al., 2017). E2F3 overexpression in Wilms' tumor was noted by some authors (Kort et al., 2008; An et al., 2013], but we are the first to suggest the loss of miR-203a as a possible cause. On a similar note, the let-7 family of miRNA precursors, which plays a major role in the development of Wilms' tumors (Wegert et al., 2015; Gadd et al., 2017) may also be under the control of E2F3 (Bueno et al., 2010). Putting these observations into perspective with our own contribution and a single paper available in the literature on miR-203a expression (Liu et al. 2013), we can conclude that the downregulation of miR-203a may or may not be blastema-specific, but this particular miRNA should be included in future efforts of Wilms' tumor profiling.

5. Conclusions

Utilizing FFPE archives as miRNA sources may have clinical value in Wilms' tumor, as more knowledge on miRNA deregulations in different subtypes may be needed to assess expression signatures as indicators of chemo-responsiveness. Despite the small sample size of our study, results are suggestive for the downregulation of miR-203a and miR-184 having a possible role in the pathogenesis of blastemal Wilms' tumors.

Author contributions

Chief investigator: GB
Chief clinician: ZM
Providing clinical samples and patient care: ZM, ÉRG, RB, MC, PV
Pathological diagnosis and figures: TM, ZS
Performing laboratory experiments: GB, ZB
Writing manuscript: GB
Revising manuscript: ZM, ÉRG, RB, MC, TM, BZ, PV, ZS, BN
Supervising researcher: BN
Supervising clinician: ÉRG

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Conflict of interest

The authors declare no conflicts of interest.

Compliance with ethical standards

All procedures involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from the parents of all individual participants in the study.

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Figures and tables

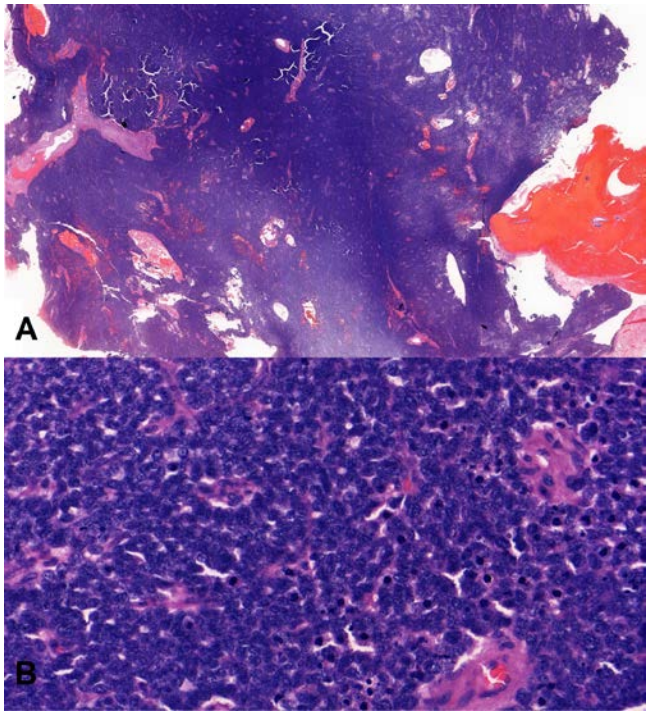

Fig. 1 Blastemal Wilms' tumor from one of our samples (Patient ID: 2). At 10x magnification (A), diffuse tumor tissue can be seen with necrotic elements. At 400x magnification (B), undifferentiated cells are visible, many of them mitotic or necrotic. Nuclei are hyperchromatic and cytoplasm is almost completely absent

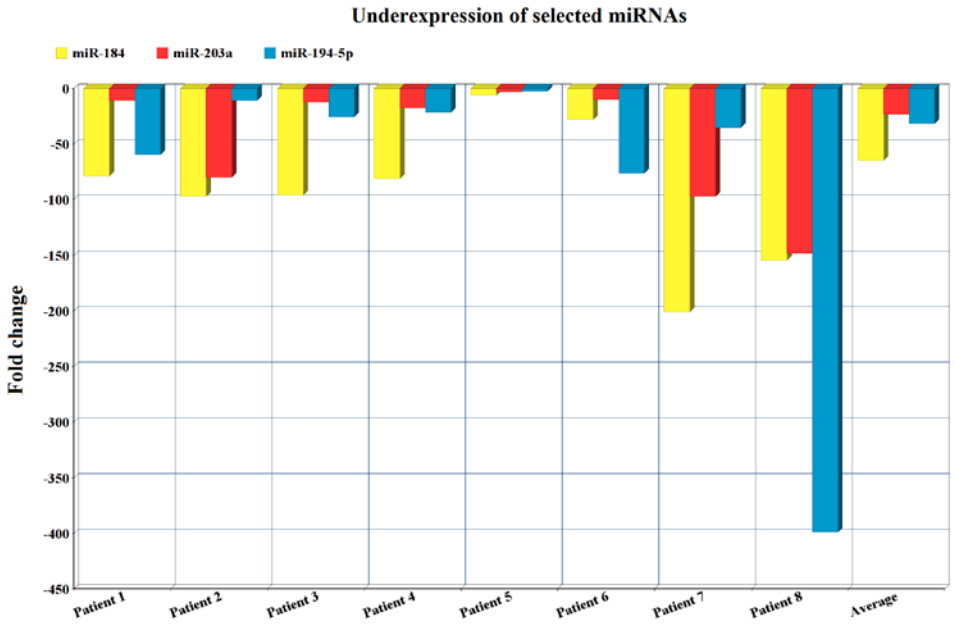

Fig. 2 Fold change in miR-184, miR-203 and miR-194-5p expression in all studied patients. Patients 1–3 and 5–8 remained in remission, while Patient 4 produced a relapse. Patient 5 had an unusual, cortisol-producing tumor. Patients 1–6 showed varying degrees of regression in response to preoperative chemotherapy, while Patient 7 did not receive the therapy due to an initial misdiagnosis, and Patient 8 showed no discernible response at all

Table 1 Enrolled patients with miRNA concentrations obtained from their surgical samples

Patient ID	Age	Sex	Date of operation	miRNA concentration in tumor	miRNA concentration in control
1.	3 years	Female	2012. 01.	>75 ng/µl	>75 ng/µl
2.	2 years	Male	2012. 02.	>75 ng/µl	>75 ng/µl
3.	1 year	Male	2013. 05.	>75 ng/µl	>75 ng/µl
4.	3 years	Female	2014. 06.	>75 ng/µl	>75 ng/µl
5.	5 years	Female	2014. 07.	>75 ng/µl	>75 ng/µl
6.	2 years	Male	2014. 10.	48.4 ng/µl	70.1 ng/µl
7.	8 years	Male	2013. 12.	>75 ng/µl	28.8 ng/µl
8.	1 year	Female	2015. 12.	>75 ng/µl	>75 ng/µl

Table 2 Fold change in miRNA expressions (in tumor tissue compared to a tumor-free region of the same sample) in patient 1, obtained by PCR array

miRNA	**Fold change**	**miRNA**	**Fold change**	**miRNA**	**Fold change**
let-7a-5p	-1.62	*miR-182-5p*	11.96	*miR-29b-3p*	-5.78
let-7b-5p	-2.85	*miR-183-5p*	7.36	*miR-30c-5p*	-3.97
let-7c	-2.68	*miR-184*	-78.79	*miR-31-5p*	-1.47
let-7f-5p	1.16	*miR-194-5p*	-59.30	*miR-3163*	-1.07
miR-100-5p	1.40	*miR-195-5p*	-1.42	*miR-32-5p*	-1.04
miR-101-3p	-1.60	*miR-196a-5p*	4.53	*miR-330-3p*	1.09
miR-106b-5p	3.05	*miR-19b-3p*	2.13	*miR-331-3p*	1.24
miR-125a-5p	-1.27	*miR-200b-3p*	-12.55	*miR-34a-5p*	5.21
miR-125b-5p	-2.04	*miR-200c-3p*	-24.59	*miR-34b-3p*	-5.90
miR-126-3p	-31.34	*miR-203a*	-10.85	*miR-34c-5p*	2957.17
miR-126-5p	-2.46	*miR-205-5p*	-1.48	*miR-361-5p*	1.53
miR-128	3.66	*miR-20a-5p*	2.99	*miR-365a-3p*	-2.31
miR-133a	-2.06	*miR-20b-5p*	1.96	*miR-3662*	1.71
miR-135a-5p	2.45	*miR-21-5p*	1.71	*miR-3666*	-1.49
miR-135b-5p	15.03	*miR-218-5p*	1.42	*miR-374b-5p*	1.11
miR-141-3p	-25.99	*miR-22-3p*	-3.73	*miR-375*	-2.30
miR-143-3p	-2.57	*miR-221-3p*	-1.91	*miR-425-5p*	1.39
miR-145-5p	-3.41	*miR-222-3p*	-2.23	*miR-449a*	2.71
miR-146a-5p	-1.01	*miR-223-3p*	1.21	*miR-455-5p*	-3.68
miR-146b-5p	3.76	*miR-224-5p*	1.51	*miR-494*	-1.39
miR-148a-3p	-1.45	*miR-23b-3p*	-1.46	*miR-616-3p*	1.27
miR-15a-5p	-1.27	*miR-24-3p*	-1.17	*miR-7-5p*	1.65
miR-15b-5p	1.68	*miR-25-3p*	3.76	*miR-9-3p*	-2.95
miR-16-5p	-1.06	*miR-26a-5p*	316095.29	*miR-92a-3p*	1.21
miR-17-5p	3.29	*miR-26b-5p*	-1.06	*miR-93-5p*	-
miR-17-3p	4.29	*miR-27a-3p*	-1.99	*miR-96-5p*	-
miR-181a-5p	4.86	*miR-27b-3p*	-1.77	*miR-99a-5p*	-2.85
miR-181b-5p	4.17	*miR-296-5p*	4.38	*miR-99b-5p*	-1.07

Table 3 Fold change in miRNA expressions (in tumor tissues compared to tumor-free regions of the same samples) in all patients. Average fold changes were calculated from the mean of ΔΔCt values for all cases, and for cases that involved pre-operative chemotherapy (with the omission of Patient 7)

	miR-184	**miR-203a**	**miR-34c-5p**	**miR-194-5p**
Patient 1	-78.79	-10.85	2957.17	-59.30
Patient 2	-97.01	-79.89	-1.24	-10.93
Patient 3	-95.67	-12.47	-1.64	-25.63
Patient 4	-81.01	-17.39	14.12	-21.71
Patient 5	-5.90	-2.87	1.09	-2.53
Patient 6	-28.05	-9.99	2.43	-76.11
Patient 7	-200.85	-97.01	-2.13	-35.26
Patient 8	-154.34	-148.06	-23.92	-398.93
Average of all cases	-64.61	-22.96	2.39	-31.53
Average of cases treated pre-operatively	-54.95	-18.69	3.02	-31.03