Urinary Excretion of MicroRNA-126 is a Biomarker for Hemangioma Proliferation

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Abstract

Background—Hemangiomas are unique endothelial cell tumors that spontaneously involute, which makes it difficult to interpret response to therapies. The objective of this work was to identify a potential biomarker in the urine of children with infantile hemangiomas that would facilitate testing new therapies.

Methods—A prospective longitudinal study in children with hemangiomas and age matched healthy controls was performed to determine whether microRNA (miR)-126, which is highly abundant in fetal endothelial cells, was more abundant in the urine of affected children. Prospective ultrasound measurements of hemangioma size and blood flow velocity were obtained as secondary endpoints to document longitudinal changes in untreated hemangiomas.

Results—Urinary miR-126 levels were significantly elevated in children with proliferating hemangiomas and relative levels of urinary miR abundance correlated with hemangioma size. Hemangiomas had elevated levels of miR abundance compared to healthy controls. Ultrasound data revealed that hemangioma proliferation typically stopped between 6 to 9 months of age. When hemangioma proliferation stopped urinary miR-126 levels in children with hemangiomas dropped to levels observed in healthy age matched controls.

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Conclusions—These are the first reported results to identify a potential miR biomarker in the urine of children with hemangiomas. Measurement of urinary levels of miR-126 could potentially be used to monitor hemangioma response to therapies.

Introduction

Hemangiomas are a unique tumor because 90% spontaneously involute, 10% threaten normal development, 1% threaten the life of the affected child and 50% of affected children have residual deformity(1–3). Judah Folkman described hemangiomas as pure angiogenesis (4–5) and the spontaneous involution of these tumors indicates that there is an "angiogenic switch" that stops tumor growth resulting in involution. The mechanisms underlying this "angiogenic switch" have not been identified in hemangiomas and the fact that most lesions spontaneously involute make it difficult to do rigorous clinical trials that can determine the extent to which observed effects are due to treatment or spontaneous involution. The FDA trial to evaluate oral propranolol, the most rigorous clinical trial ever performed on hemangioma patients (6), represents the current state of the art for therapeutic testing. That trial relied on photographic evidence and caliper measurements to document treatment effects (7). Identification of a biomarker would help predict hemangioma behavior and complications would advance our ability to test new treatment interventions by enabling investigators to determine whether observed involution was spontaneous or treatment induced.

The primary objective of this work was to identify a biomarker candidate expressed during hemangioma growth, but not during involution. Specifically, to observe expression of this biomarker candidate in untreated hemangioma patients over time to establish baseline patterns of expression. The ideal biomarker would be obtained non-invasively in urine. Based on our previous work, we had 2 potential candidates for a urine biomarker including monocyte chemoattractant protein-1 and 8-hydroxy-2′-deoxyguanosine (8-OHdG) (8–9). However, the concentration of these molecules in the urine could be affected by conditions other than hemangioma proliferation. We sought to identify a microRNA biomarker candidate. MicroRNA (miR) are small non-coding RNA molecules generally 21–25 nucleotides in length that regulate biologic responses by targeting multiple genes in a specific biologic pathway. Thus, miR are an upstream "hub" capable of targeting multiple downstream sites for amplified and potentially synergistic effects. In this case, we were looking for a miR that regulated angiogenesis and miR-126 is identified in the literature as a master 'angiomiR' (10–13). Furthermore, miR are excreted from cells in exosomes and have been identified in serum and urine as potential biomarkers for multiple pathologic conditions (14–16) making recovery from urine a viable option.

Doppler ultrasound is the screening test of choice to diagnose hemangioma on the basis of flow velocity patterns. Arteriovenous malformations and hemangioma have high flow velocities with hemangioma distinguished from arteriovenous malformations by the presence of tumor stroma (17). The secondary objectives of this work were to examine quantitative changes in hemangioma size and flow velocities as surrogate markers for hemangioma growth and involution and to establish the baseline patterns of changes of these values in children with untreated hemangiomas. These objectives were addressed through

the longitudinal collection of ultrasound measurements in children with hemangiomas. The biomarker data was compared to ultrasound measurements to determine how changes in biomarker abundancy correlate with changes in ultrasound measurements.

Methods

RNA Extraction and Quantitative Real-Time PCR

Detection of miRNA was performed as previously described (18–19). Briefly, total RNA from cells was extracted using miRVana miRNA isolation kit according to the manufacturer's protocol (Ambion/Life Technologies). Levels of miR expression in each sample were determined by miR TaqMan assays and TaqMan microRNA reverse transcription kit, followed by quantitative real time PCR (qPCR) using Universal PCR Master Mix (Applied Biosystems/Life Technologies). The probe sequences used for miR qPCR were: (See Table 1).

Results of qPCR were reported as fold change in the target gene relative to the endogenous control miR and calculated using the $2^{-(\Delta Ct)}$ method as previously described (18, 20–22):

Human Subjects Protocol

A prospective longitudinal study was done in children with hemangioma (n=34) and age matched healthy volunteers (n=34) (ClinicalTrials.gov identifier: NCT01598116). Subjects were enrolled through the Hemangioma and Vascular Malformation Clinic and the Primary Care Clinic at Nationwide Children's Hospital. Inclusion criteria were the presence of a hemangioma (HE) on the skin 1 cm diameter with diagnosis confirmed by Doppler ultrasound, and age 5 months to ensure that data was captured while hemangioma were still in the proliferation stage. Exclusion criteria were treatment of hemangioma prior to or during the study, taking steroids or propranolol for other co-morbidities, hemoglobinopathies, PHACES syndrome, rapidly involuting or non-involuting congenital hemangiomas, and parents/guardian unable to provide informed consent. Doppler ultrasound was used to confirm the diagnosis of hemangioma. Data collection was done at 2, 4, 6, 9, 12, 18 and 24 months of age +/− 2 weeks. Bag urine samples and Doppler ultrasound were obtained on the same visit for hemangioma subjects and urine alone for healthy controls. Subjects that missed study visits were allowed to continue in the study.

Urine RNA Isolation

Detection of microRNA in the urine was performed using the mercury™ RNA Isolation Kit-Biofluids (Exiqon) kit according to the manufacturer's protocol. A minimum sample volume of 3ml urine was needed for testing and samples were centrifuged to remove pelleted debris. The quality and quantity of total RNA was measured using NanoDrop 2000 Spectrophotometer as described earlier (23). RNA quality was detected by uv absorbance at 260nm/280nm. Samples with $OD_{260nm/280nm}$ ratios 1.6 and 2.0 were used for qPCR measurements. 100ng of total RNA was used to make cDNA for each miRNA.

Ultrasound Evaluation

Gray scale evaluation was used to assess the size and borders of the hemangioma. Color Doppler images were obtained to evaluate degree of internal vascularity using the same sensitivity setting to monitor changes over time. Spectral Doppler recordings were performed to assess the relative amount of diastolic flow within the lesion, to reflect the degree of arteriovenous shunting. When possible, angle corrected velocities were recorded to allow monitoring of proliferation or involution of the hemangioma. Systolic wave form velocity measurements and resistive indices were calculated in triplicate (if possible) to evaluate changes in the vascular flow over time during hemangioma growth and involution phases. Since precise orientation of feeding or internal vessels was not always possible to determine due to vessel tortuosity, the Doppler frequency shift was also recorded. Studies were performed using a Philips iU22 ultrasound system with high frequency linear array transducers operating between 8 and 17 MHz. Calculation of tumor volume was done as described by Feldman et al (24).

Statistics

The objective of this longitudinal observational study is to collect preliminary data of the changes of selected biomarkers from ultrasound measures (volume and blood flow velocity) and urine samples (MCP-1 and miR) in children with hemangioma, and compare the difference with age matched control at different time point. A sample size of 26 patients and 26 normal age matched control subjects was proposed to detect an effect size of 0.8 standard deviation difference based on a 2 sided 2-sample t-test with 80% power at $\alpha=0.05$. All data analysis was conducted using SAS 9.4 (SAS Inst. Inc.). Linear mixed models for repeated measures with autoregressive (AR) variance-covariance structure to estimate the changes of tumor volume, miR-126 and blood flow velocity over time. For each measure, the means and the standard errors at each time point were estimated using the LSMEANS statement in the GLIMMIX procedure. The covariance structure was chosen based on AIC/BICC criteria. Sensitivity analyses were conducted use different models and covariance structure to ensure that the results are consistent with the selection of the models. The correlations between the miR-126 levels and the tumor volumes at each time point (4, 6 and 9 month) were summarized using the Spearman correlation due to the non-normal distribution of the variables. The miR-126 differences between the HE patients and the healthy controls were compared using the 2 sample-t-test. Sensitivity analysis was also conducted using 2-sample rank test. A p value of <0.05 was considered statistically significant.

Results

Identification of a housekeeping miR for qPCR standardization was done by measuring commonly reported urinary miR in samples from healthy age matched controls (n=6) and children with hemangiomas (n=6). Results are summarized as mean ± SD and differences between control and HE groups analyzed using Student's t-test. Normality test of the residual from the mixed models and the sensitivity analysis using 2-sample rank test method were also conducted to ensure the robustness of our conclusions from the t-test. Data were normalized to values observed for the healthy age matched controls and reported as fold-change differences for children with hemangiomas. miR-30a is known to be highly abundant

in urine from children and had equivalent abundance in both subject groups(Table 2), so this was selected as the housekeeping miR(25).

All urine samples that had enough volume to yield sufficient amounts of adequate quality miR of were analyzed by qPCR. miR-126 abundance was significantly elevated in the HE group at age 4 months and 6 months of age, but was not significantly different from healthy controls at age 9 months (Fig. 1). The slight increase in miR-126 abundance at 9 months is attributed to 2 HE children with elevated miR-126 levels at that time point as the other 7 subjects had levels of miR-126 abundance similar to healthy controls. A temporal pattern for miR-126 levels was identified with elevation occurring at 4 and 6 months of age in the HE group that normalized in most affected children by age 9 months.

Ultrasound measurements of hemangioma size and blood flow velocity were obtained to understand how these values change as hemangiomas progress from the proliferative to the involuting phase and to serve as an objective comparator for the miR-126 biomarker data. The numbers of subjects with both valid miR-126 and ultrasound measures were 14, 13, and 9 at time point of 4, 6 and 9 month respectively. The mean and standard errors of the blood flow velocities and volumes were estimated using the LSMEANS statement based on the linear mixed model for repeated measures. Mean blood flow velocities peaked at 4 months of age and had progressive decline to age 24 months with flow velocities that were roughly half the peak flow velocities observed at age 4 months (Fig. 2a). Blood flow velocity may peak at 4 months because this is when the rate of tumor growth is greatest based on the change in tumor volume from 2–4 months. Hemangioma size did not increase past 6 months of age and began to decrease in size between 9–12 months of age (Fig. 2b). Representative ultrasound images for measuring flow velocity in a single subject are shown in Fig. 2c–e. Temporal changes in volume are consistent with clinical observations where hemangiomas reach a plateau in size during and gradually involute. In this cohort there was no additional growth after 6 months of age and that plateau in hemangioma growth occurred between 6–9 months of age.

Scatter plots were used to visually evaluate whether urinary miR-126 abundance correlated with hemangioma size at ages 4, 6, and 9 months. All subjects that had miR-126 qPCR values and ultrasound volume measurements were included in the analysis. At 4 months of age there was a moderately strong correlation between HE size and urinary miR abundance (Fig. 3a). Increased urinary miR-126 abundance was detectable for all HE. At 6 months there was a moderately strong correlation between HE size and miR-126 abundance. All HE had increased miR-126 abundance compared to controls. This was most pronounced at age 6 months (Fig. 3b). In the 6 month group there was 1 subject that was treated as an outlier and not included in the calculation of the Spearman correlation co-efficient. In this subject the relative abundance of miR-126 was higher at 4 months (11.18 fold change) compared to 6 months (9.93 fold change) with a corresponding decrease in tumor volume from 18.6 cm^3 to 13.2 cm^3 indicating that this HE was beginning to involute prior to age 6 months. The clinical course for this subject indicates that this was likely a rapidly involuting congenital hemangioma, which should have been excluded. By 9 months of age miR-126 abundance for all HE subjects decreased, so that there was no statistically significant difference in values between HE and age matched controls. For 2 subjects relative miR-126 abundance was high,

but it was decreasing. The decrease from 6 months to 9 months in 1 subject was 62.58 to 31.21 fold and 15.17 to 13.6 fold for the other subject. All the other subjects had miR-126 abundance levels that essentially dropped to levels observed with healthy controls even though the HE were still present (Fig. 3c). This indicates that the expression of miR-126 is not needed to maintain HE tumor volume and that the biology of the HE has changed.

Discussion

In 1998 the National Institutes of Health Biomarkers Definition Working Group defined biomarkers as, "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." (26). There have been previous attempts to identify urine biomarkers for hemangioma. In a study reported by Marler et al, elevation of urinary MMP-2, MMP-9, bFGF or VEGF was not observed in children with infantile hemangioma, but they did identify 2 high molecular weight matrix metalloproteases in 60% of children with hemangiomas (27). Kleber et al monitored urinary MMP-2 and MMP-9 levels in response to propranolol treatment of hemangiomas and found increased MMP-2 levels, but no changes in MMP-9 levels (28). However, MMP-2 and MMP-9 are found in the urine of healthy children in response to normal tissue remodeling, so the levels of these proteins are not necessarily specific for hemangioma growth (29). At this time there is no urine biomarker that has been adopted on a widespread clinical basis to monitor growth of infantile hemangioma.

There is evidence that points to miR-126 as a potential biomarker for hemangiomas as it is the most abundant miR in fetal endothelial cells (30–31). Endothelial cell specific expression of miR-126 regulates endothelial cell proliferation, development of fetal vasculature and vascular integrity (31–32). Expression of miR-126 is substantially elevated during fetal development and drops significantly post-partum (31, 33–35), which is consistent with the theory of hemangiomas as fetal rests (10, 12). These results provide first evidence that elevated urinary miR-126 levels can be measured and used as an indicator of a hemangioma growth in affected children. The elevated levels and correlation between HE size and miR-126 abundance at 4 and 6 months, the drop in miR-126 expression once proliferation stops, along with essentially undetectable levels of miR 126 in controls indicates that HE are the source of the miR-126. Thus, miR-126 meets the biomarker criteria as, "a characteristic that is objectively measured and evaluated as an indicator of … pathogenic processes."

This is the first reported longitudinal ultrasound data for changes in blood flow velocity and tumor volume in children with untreated hemangiomas. As with the miR biomarker, it is important to establish the changes in ultrasound measurements over time to determine whether this method of monitoring HE has merit for future studies. In addition, the ultrasound data was collected to provide quantitative data that documents changes in hemangioma size and to correlate these findings with miR126 abundance levels. Ultrasound data is challenging to use because HE size and blood flow will change if the child is crying and we could not control for this variable. Nonetheless, the changes in blood flow velocity and tumor volume observed in this study are consistent with a report by Chang, et al. that performed serial measurements (using tape measures) of hemangioma growth on 367

children. They found that 80% of HE growth was complete by age 5 months and 97% had completed proliferation by age 9 months (36). Both ultrasound and miR-126 data reported in this study are consistent with this clinical picture. Thus, ultrasound monitoring of flow velocity and size may be a reasonable surrogate for monitoring changes in hemangioma growth and proliferation, but collection of additional data is need to validate this method of monitoring.

Performing a longitudinal study on infants and small children with minimal compensation or incentive for participation, i.e. no treatment, was a significant challenge. Many subjects either dropped out, missed study visits or the urine samples were not adequate for testing. As a result the number of valid samples at each time point was much smaller than expected. The team is greatly indebted to the parents who did bring their children for 2 hour study visits as their only real incentive was to help future children born with hemangiomas. Nonetheless, the results provide critical first evidence that justifies performing additional studies to validate miR-126 as a biomarker. These are the first reported results of a miR biomarker for hemangiomas and the only reported urine biomarker for hemangiomas with elevated expression only during the proliferative phase. This work provides a new paradigm for identifying the transition from hemangioma growth to involution. These results provide an important baseline for future studies to test whether urinary miR-126 levels decrease in response to therapeutic intervention. The use of quantitative values derived from biologic events that can be used as surrogate endpoints to monitor hemangioma proliferation and involution will enable investigators to test new treatments for hemangioma, so that all children with hemangiomas can be safely treated.

Acknowledgments

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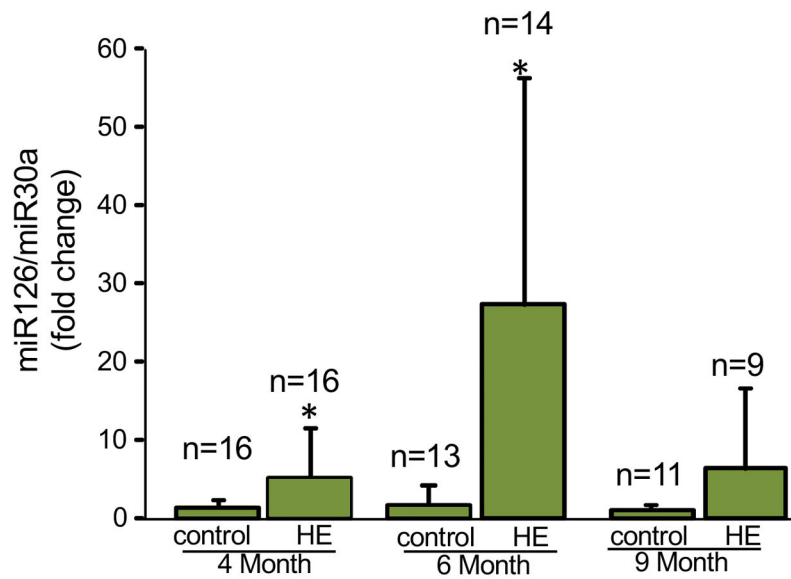
Figure 1. miR 126 is more abundant in urine of children with hemangiomas during proliferative phase only

Urine specimens collected from children with HE and healthy age matched controls at 4, 6 and 9 months of age show significantly increased abundance of miR126 during HE proliferation (4 & 6 months) which subsequently reverts to baseline level when proliferation stops (9 months). Results are expressed as mean ± SD; *p <0.05 Students' t-test, n= number of subjects analyzed.
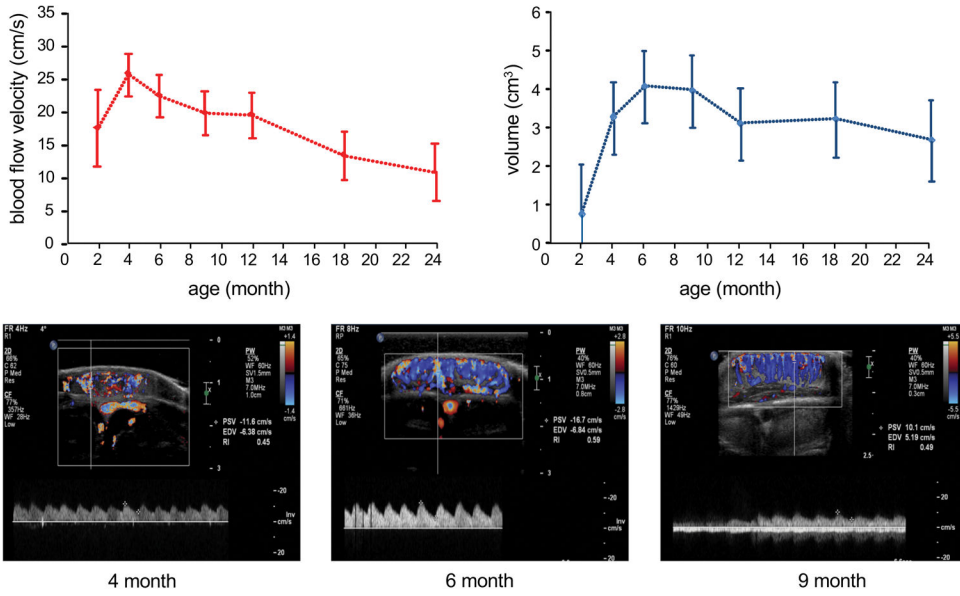
Figure 2. Ultrasound measurements track longitudinal changes in hemangioma blood flow and size

a, Changes in mean blood flow velocity (+/− SD) for all subjects at each time point in the study indicate increasing flow velocities that peak at 4 months of age then progressively decline. **b,** HE size (volume) peaks at 6 months marking the end of HE proliferation, remains stable in size between 6–9 months and then begins to decrease in size. Representative color Doppler ultrasound images from single subject at **(c)** 4 months, **(d)** 6 months and **(e)** 9 months of age. The calculated flow velocity measurements (cm/s) are displayed on the right hand side of each image where PSV= peak systolic velocity and EDV= end diastolic velocity.
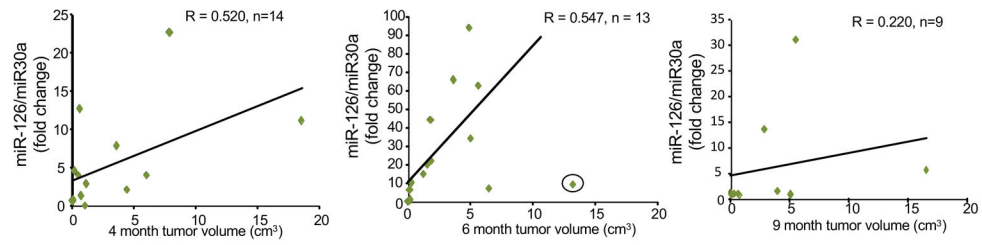
Figure 3. Urinary miR126 abundance correlates with tumor size

The Spearman correlation coefficient (R) indicates a moderately strong positive correlation between urinary miR126 abundance and tumor volume that is observed during HE proliferation at **(a)** 4 months and **(b)** 6 months of age, but not at 9 months of age **(c)**. The circled value in panel B was excluded as a rapidly involuting congenital hemangioma, n= number of subjects analyzed.

Table 1

The probe sequences used for miR qPCR.

miR ID	Sequence
RNU44	CCTGGATGATGATAGCAAATGCTGACTGAACATGAAGGTCTTAATTAGCTCTAACTGACT
U6snRNA	TGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT
snoRNA202	GCTGTACTGACTTGATGAAAGTACTTTTGAACCCTTTTCCATCTGATG
miR30a	UGUAAACAUCCUCGACUGGAAG
miR-126	UCGUACCGUGAGUAAUAAUGCG
miR126*	CAUUAUUACUUUUGGUACGCG

Table 2

Identification of normalization miR in urinary samples from 6 month old subjects (n= 6/group)

miR	control	HE
snoRNA202	1.0 ± 0.44	0.56 ± 0.44*
U6 snRNA	1.0 ± 0.32	2.1 ± 0.84
RNU44	1.0 ± 0.41	0.35 ± 0.21*
miR-30a	1.0 ± 0.22	1.1 ± 0.18