Differential Expression of *PTEN*-Targeting MicroRNAs *miR-19a* and *miR-21* in Cowden Syndrome

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Germline mutations in the gene encoding phosphatase and tensin homolog deleted on chromosome ten (*PTEN* [MIM 601728]) are associated with a number of clinically distinct heritable cancer syndromes, including both Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRRS). Seemingly identical pathogenic *PTEN* mutations have been observed in patients with CS and BRRS, as well as in patients with incomplete features of CS, referred to as CS-like (CSL) patients. These observations indicate that additional, unidentified, genetic and epigenetic factors act as phenotypic modifiers in these disorders. These genetic factors could also contribute to disease in patients with CS, CSL, or BRRS without identifiable *PTEN* mutations. Two potential modifiers are *miR-19a* and *miR-21*, which are previously identified *PTEN*-targeting miRNAs. We investigated the role of these miRNAs by characterizing their relative expression levels in *PTEN*-mutation-positive and *PTEN*-mutation-negative patients with CS, CSL, or BRRS. Interestingly, we observed differential expression of *miR-19a* and *miR-21* in our *PTEN*-mutation-positive patients. Both were found to be significantly overexpressed within this group ($p < 0.01$) and were inversely correlated with germline PTEN protein levels. Similarly, the relative expression of *miR-19a* and *miR-21* was differentially expressed in a series of *PTEN*-mutation-negative patients with CS or CSL with variable clinical phenotypes and decreased full-length PTEN protein expression. Among *PTEN*-mutation-positive patients with CS, both miRNAs were significantly overexpressed ($p = 0.006$–0.013). Taken together, our study results suggest that differential expression of *PTEN*-targeting *miR-19a* and *miR-21* modulates the PTEN protein levels and the CS and CSL phenotypes, irrespective of the patient's mutation status, and support their roles as genetic modifiers in CS and CSL.

Introduction

Cowden syndrome (CS [MIM 158350]) is an underdiagnosed autosomal-dominant disorder characterized by multiple hamartomatous lesions and a vast phenotypic spectrum that includes predisposition to malignancies of the breast, thyroid, and endometrium.[1] The majority of patients with CS (85%) have been found to harbor pathogenic germline mutations in the gene encoding phosphatase and tensin homolog deleted on chromosome ten (*PTEN* [MIM 601728]), a tumor-suppressor gene located on 10q23.[2] The inactivation of PTEN protein, an antagonist of the phosphatidylinositol-triphosphate kinase (PI3K)-signaling pathway, in CS results in the constitutive activation of Akt.[3–8] As a consequence, hyperactive Akt-mediated signaling of several cascades results in increased and uncontrolled cellular survival and proliferation.

Germline mutations in *PTEN* also cause a subset of Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM 153480]), a related hamartomatous tumor syndrome that shares, in addition to its genetic etiology, a few of the clinical manifestations seen in CS.[3] Interestingly, identical pathogenic *PTEN* mutations have been observed among patients with these seemingly disparate disorders.[9] Furthermore, these same identical mutations have also been observed in two additional hamartomatous tumor syndromes, Proteus syndrome (PS [MIM 176920]) and Proteus-like syndrome (PLS), as well as in a subset of patients referred to as CS-like (CSL) patients who do not meet the full diagnostic criteria for CS.[1,3] Whereas germline *PTEN* mutations have been identified in the majority of patients diagnosed with CS and BRRS, for approximately 15% and 35%, respectively, as well as for 90% of patients with CSL, the pathogenic mutations have yet to be identified.[2,9] Interestingly, decreased PTEN protein expression has been observed in a large number of these *PTEN*-mutation-negative patients (Waite and Eng, unpublished observation). The lack of detectable germline *PTEN* mutations in subsets of patients with CS, CSL, or BRRS with altered PTEN protein expression, as well as the imprecise genotype-phenotype correlation associated with these syndromes, suggests the presence of alternate mechanisms that contribute to PTEN dysfunction and to the development and progression of the disease.

MicroRNAs (miRNAs), a novel class of negative gene regulators, have recently been shown to regulate the expression of several tumor suppressors and oncogenes and contribute to carcinogenesis.[10–14] To date, two miRNAs, *miR-19a* and *miR-21*, have been reported to specifically target and to downregulate *PTEN*.[15,16] Furthermore, the overexpression of each of these miRNAs has been correlated with decreased PTEN levels in human cancer.[17,18]

Because PTEN dysfunction is common in patients with CS, CSL, and BRRS, irrespective of their mutation status, we hypothesized that aberrant *miR-19a* and/or *miR-21* expression could modulate PTEN protein levels and, thereby, modulate phenotypic expression in patients with these syndromes. To investigate this hypothesis, we chose to characterize the relative expression levels of both *miR-19a* and *miR-21* in *PTEN*-mutation-positive and

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Table 1. Summary of Control and Patient Clinical Features

	Controls (n = 20)[a]	*PTEN*-Mutation-Positive Patients (n = 28)[a]	*PTEN*-Mutation-Negative Patients (n = 130)[a]
Gender			
Male	7 (35.0)	12 (42.9)	10 (7.7)
Female	13 (65.0)	16 (57.1)	120 (92.3)
Phenotype			
CS	–	17 (60.7)[b]	63 (48.5)
CSL	–	5 (17.9)	62 (47.7)
BRRS	–	5 (17.9)	5 (3.8)
Mutation			
R130X	–	13 (46.4)	–
R233X	–	5 (17.9)	–
R335X	–	10 (35.7)	–
Clinical Features			
Macrocephaly	–	25 (89.3)	53 (40.8)
Pathognomonic	–	16 (57.1)	28 (21.5)
Breast CA	–	7 (43.8)[c]	80 (66.7)[c]
Thyroid CA	–	2 (7.1)	32 (24.6)
Endometrial CA	–	2 (12.5)[c]	14 (11.7)[c]

Pathognomonic features include the mucocutaneous lesions associated with CS (trichilemmomas, acral keratoses, and papillomatous papules).
[a] Values are expressed as n (%).
[b] One *PTEN*-mutation-positive sample had features consistent with both CS and BRRS.
[c] Frequency among female patients.

PTEN-mutation-negative patients with CS, CSL, and BRRS relative to healthy control subjects.

Subjects and Methods

Study Subjects

A total of 178 unrelated subjects were included in the present study, including 20 control subjects and 158 patients with CS, CSL, or BRRS (Table 1). Mutation analysis of the entire *PTEN* coding sequence and its exon-intron boundaries and promoter region was performed for all patients with CS, CSL, or BRRS who were included in the present study.[2] Additionally, all patient samples were also screened for PTEN protein expression by Western blot analysis. Briefly, patient protein lysates were isolated from immortalized lymphoblastoid cell lines (LBCLs) by the Genomic Medicine Biorepository and protein concentrations were determined by use of the BCA assay, with BSA as a standard. Thirty micrograms of protein was applied to nitrocellulose with a dotblot apparatus (Bio-Rad, Hercules, CA, USA). Equal protein loading was confirmed by staining of the nitrocellulose blots with Ponceau stain (Bio-Rad). Nitrocellulose blots were then subjected to Western blot analysis with α-PTEN (monoclonal antibody clone 6H2.1, Cascade Biosciences, Portland, OR, USA) at 1:1000 dilution and then incubated with appropriate secondary antibody as previously described.[7] Proteins were visualized via enhanced chemiluminescence. Full-length PTEN protein levels were compared to controls and scored by two individuals as either normal, decreased (<50% loss, compared to controls), half (~50% of controls), or faint (>50% loss, compared to controls). *PTEN*-mutation-negative patients with decreased, half, or faint full-length PTEN protein levels were also scanned for large deletions and rearrangements, and those with the latter were excluded. Among the patients with CS, CSL, or BRRS, 28/158 of the individuals selected for inclusion were previously found to harbor pathogenic germline mutations (i.e., mutation-positive patients). Mutation analysis in the remaining 130/158 patients revealed no detectable germline *PTEN* mutations (i.e., mutation-negative patients). For interrogation of *miR-19a* and *miR-21* expression in patients with CS, CSL, or BRRS without germline *PTEN* mutations but with deregulated PTEN protein levels, all 130 mutation-negative patients selected for inclusion in the current study were previously found to have decreased full-length PTEN protein expression relative to normal controls (data not shown). All subjects were enrolled by referral from centers throughout the United States, Canada, and Europe after granting informed consent in accordance with procedures approved by the human-subjects-protection committees of each respective institution.

Among the cohort of *PTEN*-mutation-positive patients included in our analysis were 13/28 patients with R130X mutations (c.388 C/T), 5/28 patients with R233X mutations (c.697 C/T), and 10/28 patients with R335X mutations (c.1003 C/T) (Figure 1 and Table 1). Of those patients with R130X mutations, 9/13 had classic CS, 1/13 exhibited a CSL phenotype, and 2/13 had classic BRRS. Additionally, 1/13 patients with an R130X mutation had phenotypic features consistent with both CS and BRRS, and this patient thus was considered as a CS/BRRS overlap patient. Among the patients with an R233X mutation, 1/5 met classic CS criteria, 3/5 were considered as having CSL, and 1/5 had classic BRRS. Lastly, of patients with R335X mutations, 7/10 met classic CS criteria, 1/10 had CSL, and 2/10 had BRRS.

Among the cohort of *PTEN*-mutation-negative patients with decreased full-length PTEN expression, 63/130 had classic CS, 62/130 had CSL, and 5/130 were diagnosed with BRRS. All mutation-positive and mutation-negative patients with CS included in the present study were classified in accordance with criteria established by the International Cowden Consortium and incorporated into the practice guidelines of the National Comprehensive Cancer Network.[1]

Cell Lines and Culture

LBCLs from all healthy control samples and those from patients with CS, CSL, or BRRS were generated by the Genomic Medicine Biorepository. LBCLs were cultured in RPMI-1640 media supplemented with 20% FBS and 100 units/ml each of Penicillin and Streptomycin and maintained at 37°C with 5% CO_2.

Isolation of Total RNA and cDNA Synthesis

Total RNA was isolated from control and patient LBCLs with the *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol and, subsequently, subjected to DNase treatment with DNA-free (Ambion). For analysis of *PTEN* expression levels, 1 μg of DNase-treated RNA was converted to cDNA with Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) as specified by the manufacturer.

In order to assay mature *miR-19a* and *miR-21* expression in our control and patient samples, stem-loop reverse transcription (RT) was performed with TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA). For each miRNA, individual RT reactions were carried out with 10 ng of DNase-treated RNA and either *miR-19a*-, *miR-21*-, or U6 small nuclear RNA (RNU6)-specific

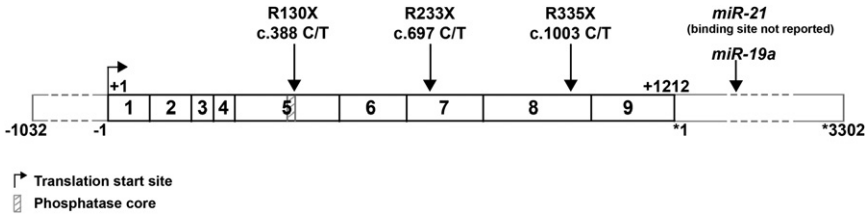

Figure 1. Schematic Diagram of *PTEN*'s mRNA Sequence

PTEN's full-length mRNA sequence (NM_000314.4), including its 1032 nucleotide (nt) 5′ UTR, 1212 nt coding sequence, and 3302 nt 3′ UTR, is shown along with the *miR-19a* binding site (position *1208 to *1228). *miR-21*'s binding site has not been reported. Additionally, the positions for the three truncating mutations R130X, R233X, and R335X are shown. *PTEN*'s translation start site and the region encoding PTEN's phosphatase core are also annotated.

TaqMan MicroRNA RT primers (Applied Biosystems). Twenty-microliter RT reactions were performed with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol.

Real-Time Quantitative PCR

Relative expression of the full-length *PTEN* transcript was assayed for all samples with 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems), 10 mM forward primer (5′-TCCACAAACAGAACAAGATG-3′), 10 mM reverse primer (5′-CTGGTCCTGGTATGAAGAAT-3′), and 20 ng of template cDNA. Thermal cycling conditions comprised 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s followed by 60°C for 1 min with an ABI 7500 Sequence Detection System (Applied Biosystems). Real-time PCR reactions were also performed for *GAPDH*, a normalization control (F: 5′-GGGCTGCTTTTAACTCTGGTAA-3′ and R: 5′-ATGGGTGGAATCATATTGGAAC-3′).

Mature *miR-19a* and *miR-21* expression was assayed with a 20 μl reaction containing 10.0 μl TaqMan 2X Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), 1.0 μl 20X TaqMan MicroRNA assay (Applied Biosystems), and 1.3 μl of RT product. Real-time PCR conditions were performed as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s followed by 60°C for 1 min with an ABI 7500 Sequence Detection System (Applied Biosystems). Additionally, RNU6 expression was assayed for normalization.

All reactions were performed in triplicate, and relative gene-expression determinations were made with the comparative delta-delta C_T method ($2^{-\Delta\Delta CT}$) as described by Livak et al.[19]

Statistical Analysis

After expression analysis was performed, statistical comparisons of relative levels of *PTEN*, *miR-19a*, and *miR-21* expression between all patient groups and control samples were performed with Welch's t test. All results were considered significant at the $p < 0.05$ level. In addition, we performed classification and regression tree (CART) analysis as implemented in the R software package.

Results

Differential Expression of *miR-19a* and *miR-21* in Three Groups of Patients with CS, CSL, or BRRS with Germline *PTEN* Mutation R130X, R233X, or R335X

In the present study, we sought to investigate whether miRNAs previously shown to target and regulate PTEN protein expression are differentially regulated in patients with CS, CSL, and BRRS relative to healthy controls and, more specifically, whether these miRNAs are modifiers of these phenotypes. To address this question, we initially chose to assess the relative expression of *miR-19a* and *miR-21* in a series of 28 *PTEN*-mutation-positive patients sharing identical truncating mutations located in each of *PTEN*'s three most frequently mutated exons (Figure 1) and in 20 normal controls.[3] Included among these patients were 13 carriers of R130X mutations, five carriers of R233X mutations, and ten carriers of R335X mutations. Previous studies have reported a broad clinical spectrum in patients even within each of these three mutation groups.[9,20] Similar observations were apparent in this collection, given that nine patients with CS, one with CSL, one with CS and BRRS, and two with BRRS were among those with R130X mutations; one patient with CS, three with CSL, and one with BRRS were among those with R233X mutations; and seven patients with CS, one with CSL, and two with BRRS were among those with R335X mutations (Table S1, available online).

In addition to the variable clinical spectrum observed among patients with identical mutations, levels of *PTEN* mRNA expression were also found to be variable. Among all 28 *PTEN*-mutation-positive patients, expression of *PTEN* transcript levels were significantly reduced relative to controls ($p = 0.015$) (Table 2). However, when we divided this group on the basis of the three different germline *PTEN* mutations found in these patients, significantly decreased *PTEN* transcript expression was only observed in patients with the R130X and R233X mutations ($p < 0.001$) (Table 2) and not in carriers of the R335X mutation ($p = 0.287$). Similarly, we observed differential expression of *miR-19a* and *miR-21* among our mutation-positive samples. Overall, the average relative expression of both *miR-19a* and *miR-21* was higher in mutation-positive patients compared to controls ($2^{-\Delta\Delta CT} = 1.54$ versus 1.08, $p = 0.003$; and $2^{-\Delta\Delta CT} = 1.49$ versus 1.15, $p = 0.006$, respectively) (Figure 2A and Table 2). For mutation-positive patients with R130X and R233X mutations, *miR-19a* was found to be overexpressed as compared to that of controls ($p = 0.037$ and $p = 0.015$, respectively), whereas *miR-21* was overexpressed only in carriers of the R130X mutation ($p = 0.044$). *miR-19a* and *miR-21* were not differentially expressed in carriers of the R335X mutation relative to our control population. Interestingly, 90% of patients with this mutation had decreased full-length PTEN protein levels, compared to only 54% of those with R130X mutations. Moreover, although *miR-19a* and *miR-21* were not differentially expressed in carriers of the R335X mutation, the majority of these patients (70%) exhibit classic CS

Table 2. Summary of Comparisons of Relative Levels of *PTEN* Transcript, *miR-19a*, and *miR-21* Expression among Control Samples and *PTEN*-Mutation-Positive and *PTEN*-Mutation-Negative Patient and Patient Subgroup Samples

	PTEN	*miR-19a*	*miR-21*
PTEN+	0.015	0.003	0.006
R130X	< 0.001	0.037	0.044
R233X	< 0.001	0.015	0.184
R335X	0.287	0.137	0.147
PTEN+ dec. versus *PTEN*+ norm.	0.336	0.009	0.003
R130X/R233X dec. versus R130X/R233X norm.	0.234	0.020	0.008
PTEN+ CS	0.069	0.006	0.013
PTEN+ CSL	0.055	0.034	0.085
PTEN+ BRRS	0.209	0.932	0.772
PTEN−	0.969	< 0.001	0.141
PTEN− CS	0.900	< 0.001	0.835
PTEN− CSL	0.544	< 0.001	0.018
PTEN− BRRS	0.148	0.492	0.215
PTEN+ versus *PTEN*−	0.001	0.977	< 0.001
PTEN+ CS versus *PTEN*− CS	0.020	0.878	0.015
PTEN+ CSL versus *PTEN*− CSL	0.079	0.368	0.029
PTEN+ BRRS versus *PTEN*− BRRS	0.067	0.496	0.326

p values were determined from t tests comparing the relative expression of *PTEN*, *miR-19a*, and *miR-21* between controls and patients or patient subgroups, except where indicated. Significant results are indicated as underlined.
"*PTEN*+" denotes *PTEN*-mutation-positive patients.
"*PTEN*−" denotes *PTEN*-mutation-negative patients.
"*PTEN*+ dec." denotes *PTEN*+ patients with decreased PTEN protein expression. PTEN protein status "calls" are illustrated in Figure S1.
"*PTEN*+ norm." denotes *PTEN*+ patients with normal PTEN protein expression. PTEN protein status "calls" are illustrated in Figure S1.
"R130X/R233X dec." denotes patients with R130X and/or R233X mutations with decreased PTEN protein expression.
"R130X/R233X norm." denotes patients with R130X and/or R233X mutations with normal PTEN protein expression.

features, compared to only 56% of patients with R130X and/or R233X mutations.

miR-19a and *miR-21* Overexpression are Associated with Decreased Full-Length PTEN Protein Levels and Clinical Phenotype in *PTEN*-Mutation-Positive Patients with CS, CSL, or BRRS

Our data show that *PTEN* transcript and PTEN protein levels, as determined by Western blot analysis, do not correspond well among many of the mutation-positive patients included in this analysis, suggesting that other biological processes are likely involved in the regulation of these products (Table S1). On the basis of this observation, we then chose to examine the relationship between the relative expression of each miRNA and PTEN protein levels in these patients. More specifically, we examined levels of *miR-19a* and *miR-21* expression in mutation-positive patients with normal full-length PTEN protein expression compared to those with decreased full-length PTEN protein levels. Interestingly, both miRNAs were significantly overexpressed in *PTEN*-mutation-positive patients with decreased full-length PTEN protein levels as compared to those with normal full-length PTEN protein levels (*miR-19a*: $2^{-\Delta\Delta CT} = 1.69$ versus 1.13, $p = 0.009$; *miR-21*: $2^{-\Delta\Delta CT} = 1.65$ versus 1.07, $p = 0.003$, respectively) (Figure 2B and Table 2). Moreover, patients with R130X and/or R233X mutations with decreased full-length PTEN protein expressed approximately 54% higher levels of *miR-19a* ($p = 0.020$) and 60% higher levels of *miR-21* ($p = 0.008$) relative to patients with R130X and/or R223X mutations with normal PTEN protein levels (Table 2).

Next, we subdivided our *PTEN*-mutation-positive group on the basis of their clinical diagnoses (CS, CSL, or BRRS) and compared their relative levels of *miR-19a*, *miR-21*, and *PTEN* expression to those of the control group. Mutation-positive patients with CS displayed relative overexpression of both *miR-19a* and *miR-21* compared to controls ($p = 0.006$ and $p = 0.013$, respectively) (Figure 2C and Table 2). A modest association was observed between the mutation-positive group of patients with CSL and *miR-19a* expression status ($p = 0.034$), whereas *miR-21* overexpression in this same group did not reach statistical significance when compared to controls ($p = 0.085$), which was due in part to the small sample size in this latter subgroup. No differences in *PTEN* transcript expression were observed in either phenotypic subgroup. Lastly, *PTEN* transcript, *miR-19a*, and *miR-21* expression did not differ between the mutation-positive patients with BRRS and the controls ($p > 0.20$).

Additionally, *PTEN* transcript, *miR-19a*, and *miR-21* expression did not differ between *PTEN*-mutation-positive patients with CS and *PTEN*-mutation-positive patients with CSL ($p > 0.200$). Both *miR-19a* and *miR-21* levels were lower in patients with BRRS as compared to levels in patients with CS (*miR-19a*: $2^{-\Delta\Delta CT} = 1.07$ versus 1.63 and *miR-21*: $2^{-\Delta\Delta CT} = 1.25$ versus 1.53) and in patients with CSL (*miR-19a*: $2^{-\Delta\Delta CT} = 1.07$ versus 1.67 and *miR-21*: $2^{-\Delta\Delta CT} = 1.25$ versus 1.56), although statistical significance was only reached in comparisons of *miR-19a* expression ($p < 0.05$).

Differential Expression of *miR-19a* and *miR-21* in *PTEN*-Mutation-Negative Patients with CS, CSL, or BRRS

Differential expression of *miR-19a* and *miR-21* in *PTEN*-mutation-positive patients with CS, CSL, or BRRS with variable PTEN protein expression suggests that these miRNAs play a role in modulation of both the PTEN protein expression and the disease phenotype in patients with these syndromes. On the basis of these results, we further hypothesized that these miRNAs could play a similar role in patients with CS, CSL, or BRRS who lack detectable *PTEN* mutations. To investigate this, we chose, therefore, to assess the relative expression of *miR-19a* and *miR-21* in 130 selected patients with CS, CSL, or BRRS with both undetectable *PTEN* mutations and decreased full-length PTEN protein expression.

In contrast to the *PTEN*-mutation-positive patients, decreased expression of *PTEN* transcript levels was not

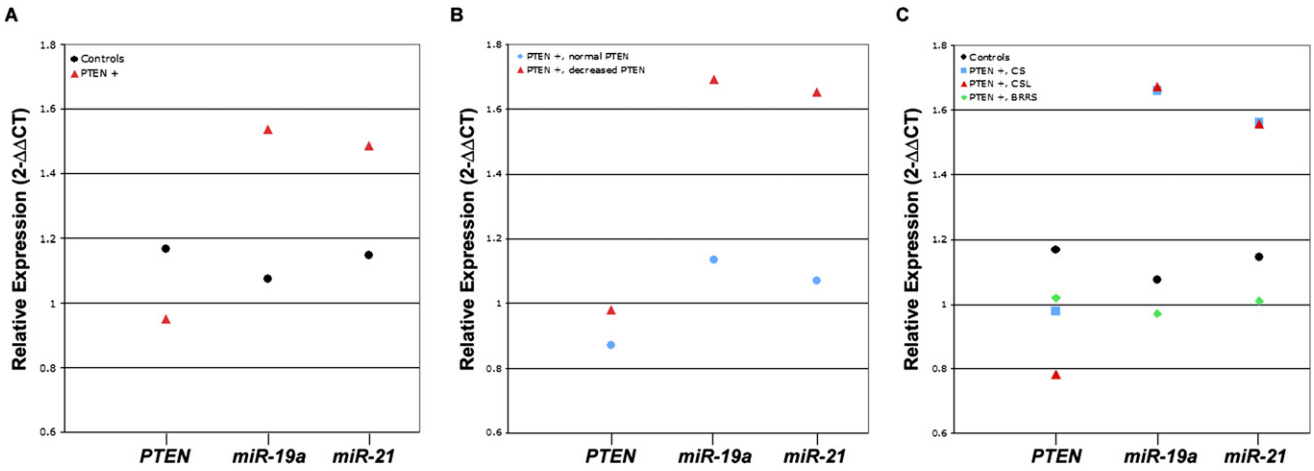

Figure 2. Relative Expression Values for *PTEN* Transcript, *miR-19a*, and *miR-21* in *PTEN*-Mutation-Positive Patients

Relative expression values for *PTEN* transcript and each miRNA are shown for (A) controls and all mutation-positive patients, (B) mutation-positive patients who express normal PTEN protein and those who express decreased PTEN protein, and (C) controls and mutation-positive patients subdivided on the basis of their clinical phenotypes.

All relative expression values are expressed as n-fold change ($2^{-\Delta\Delta CT}$).

PTEN+ = *PTEN*-mutation-positive patients.

observed in mutation-negative patients ($p = 0.969$). Interestingly, similar to our mutation-positive patients, mutation-negative patients displayed relative overexpression of *miR-19a* as compared to our control population ($p < 0.001$) (Figure 3 and Table 2). However, contrary to what was observed in mutation-positive patients, no difference in *miR-21* expression was observed in this group ($p = 0.141$).

Following this analysis, *PTEN*-mutation-negative patients were subsequently subdivided on the basis of their clinical diagnoses (CS, CSL, or BBRS). We then carried out analyses of genotype-phenotype association based on these groupings. Similar to the mutation-positive cohort, *miR-19a* overexpression was observed both in mutation-negative patients with CS and in mutation-negative patients with CSL ($p < 0.001$ in both groups) (Table 2). In contrast, *miR-21* was underexpressed in mutation-negative patients with CSL compared to controls ($p = 0.018$), whereas no difference was observed in patients with CS from this same group ($p = 0.835$). We did not detect any difference in *miR-19a* and *miR-21* expression among our mutation-negative patients with BRRS ($p > 0.215$).

miR-21 is Differentially Expressed between *PTEN*-Mutation-Positive and *PTEN*-Mutation-Negative Patients

Because *miR-19a* is overexpressed in patients with CS or CSL irrespective of *PTEN* mutation status, whereas overexpressed *miR-21* occurs only in *PTEN*-mutation-positive patients, we then chose to compare the relative expression of each miRNA between patients with and without *PTEN* mutations. *miR-21* was significantly overexpressed in *PTEN*-mutation-positive patients relative to patients without *PTEN* mutations ($2^{-\Delta\Delta CT} = 1.48$ versus 1.05, $p < 0.001$) (Figure 3 and Table 2), whereas *miR-19a* expression did not differ between the two groups ($p = 0.977$). *PTEN* transcript levels were significantly lower in the mutation-positive patient group ($p = 0.001$). When subdivided on the basis of their clinical diagnoses, *miR-21* overexpression was observed both in groups of mutation-positive patients with CS and in groups of mutation-positive patients with CSL relative to mutation-negative patients with these same phenotypes ($p = 0.015$ and $p = 0.029$, respectively), whereas *miR-19a* expression did not differ among these subgroups ($p > 0.50$).

miR-21 Expression Could Contribute to Phenotypic Features Associated with CS

To investigate whether differential expression of *miR-19a* and *miR-21* was associated with the cancers commonly seen in CS, as well as with other clinical features of the syndrome, we compared their relative expression between patient groups with and without each of the key phenotypic features of CS. These comparisons failed to yield any significant associations with breast cancer, thyroid cancer, or macrocephaly between miRNA or *PTEN* expression among all patient samples and patient subgroups ($p > 0.05$), probably due to small sample sizes in subgroup analyses. We did observe a trend of overexpression of *miR-21* in patients diagnosed with endometrial cancer as compared to those not diagnosed with this cancer, irrespective of mutation status ($2^{-\Delta\Delta CT} = 1.39$ versus 1.06 for *PTEN*-mutation-positive patients and $2^{-\Delta\Delta CT} = 1.37$ versus 1.01 for *PTEN*-mutation-negative patients; $p = 0.12$-0.15). Despite our small sample size, we did find that *miR-21* was significantly overexpressed among patients with CS and one or more pathognomonic feature (adult Lhermitte-Duclos disease, trichilemmomas, acral keratoses, and papillomatous papules) relative to those without any of these features ($p = 0.02$).

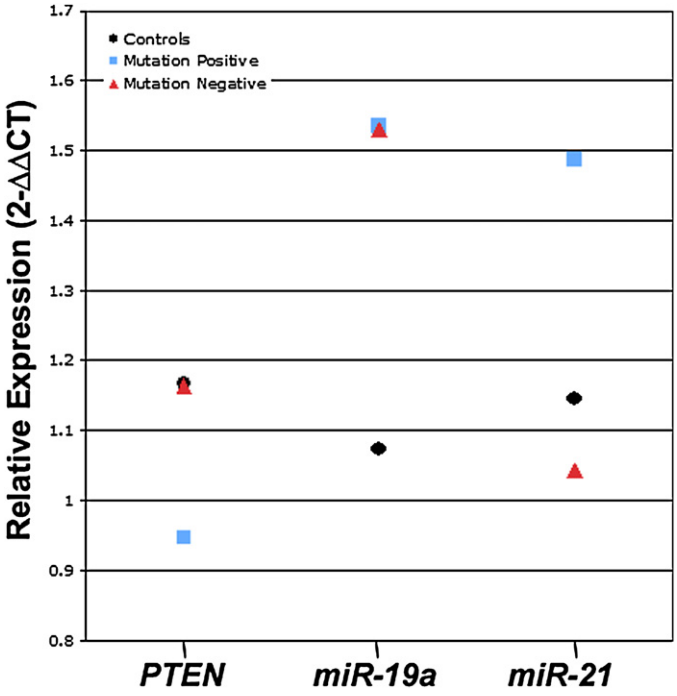

Figure 3. Relative Expression Values for *PTEN* Transcript, *miR-19a*, and *miR-21* among *PTEN*-Mutation-Positive Patients, *PTEN*-Mutation-Negative Patients, and Controls
All relative expression values are expressed as n-fold change ($2^{-\Delta\Delta CT}$).

To examine the predictive value each miRNA might contribute to the CS phenotypes observed in *PTEN*-mutation-negative patients, we performed CART analysis in an attempt to identify subgroups of patients at higher risk of developing four major clinical features associated with this syndrome (breast cancer, endometrial cancer, thyroid cancer, and macrocephaly) by using relative expression values for both *miR-19a* and *miR-21*. Additional CART analysis was performed for the CS and CSL phenotypes. Approximately 76% of mutation-negative patient samples with relative *miR-19a* expression > 2.26 had developed breast cancer. Similarly, 70% of those with high *miR-21* expression ($2^{-\Delta\Delta CT} > 1.81$) also developed breast cancer. Although the total number of mutation-negative patients included in our study who developed endometrial cancer was small ($N = 16$), none of these patients were among the subgroups identified with relatively low *miR-19a* and *miR-21* expression ($2^{-\Delta\Delta CT} < 0.87$ and < 0.64, respectively).

Macrocephaly was observed in 64% of *PTEN*-mutation-negative patients with *miR-19a* expression > 2.60, and this feature was observed in 71% of patients with *miR-21* expression > 1.94. Analysis of the CS and CSL phenotypes revealed that 72% of mutation-negative patients with high *miR-19a* expression ($2^{-\Delta\Delta CT} > 2.32$) had classic CS, whereas 67% of those with low *miR-19a* expression ($2^{-\Delta\Delta CT} < 0.75$) had CSL. Similarly, 79% of patients with high *miR-21* expression ($2^{-\Delta\Delta CT} > 1.56$) had CS. CART analysis was not able to identify any unique clusters of patients with thyroid cancer among the mutation-negative patients with the use of either miRNA as a predictor.

Each phenotype was also assessed with the use of the relative expression of both miRNAs jointly as phenotypic predictors. Although expression of neither miRNA was predictive of thyroid cancer, as was the case when each was considered separately, high *miR-21* expression proved to be the single strongest predictor for endometrial cancer, macrocephaly, and the CS or CSL phenotype given that the joint analysis with both *miR-19a* and *miR-21* together identified the same clusters as did *miR-21* considered independently.

Discussion

Alternate mechanisms of *PTEN* dysfunction are becoming increasingly germane in the *PTEN* hamartoma tumor syndromes, particularly in CS and BRRS.[21–24] Our investigation of two *PTEN*-targeting miRNAs, *miR-19a* and *miR-21*, suggests that their differential expression modulates PTEN protein levels and the CS or CSL phenotype in patients with these syndromes, irrespective of their *PTEN* mutation status.

Variable PTEN protein levels were inversely correlated with *miR-19a* and *miR-21* expression levels in *PTEN*-mutation-positive patients and, more specifically, only in patients with R130X and/or R233X mutations. In patients with the R335X mutation, in which the relationship among miRNA expression, PTEN protein levels, and clinical diagnosis was absent, seven of ten had classic CS phenotypic features. This observation suggests that the R335X *PTEN* genotype strongly influences phenotype, and indeed, this is corroborated by the *miR-19a*- and/or *miR-21*-independent overall decreased full-length PTEN protein levels in this group of patients with the R335X mutation. In contrast, we believe that differential expression of *miR-19a* and *miR-21*, directly correlated with PTEN protein levels in R130X and/or R233X mutation carriers, could help modulate phenotype beyond the germline mutations.

Similarly, both miRNAs were differentially expressed in a series of *PTEN*-mutation-negative patients with CS and CSL with variable clinical phenotypes and decreased expression of full-length PTEN protein. Importantly, decreased expression of *PTEN* transcript levels was not observed in these mutation-negative patients, suggesting that their decreased PTEN protein expression is probably due to dysregulation at the protein level. Taken together, our data in both mutation-positive and mutation-negative patients demonstrate that *miR-19a* and *miR-21* can modulate PTEN protein levels. Because PTEN's sufficient activity is dependent on its protein levels, perturbation of its expression can enhance disease progression and facilitate tumorigenesis.[25] Therefore, our data suggest essential roles for *miR-19a* and *miR-21* in the modulation of the diverse clinical and molecular phenotypes observed in CS.

The identification of clear genotype-phenotype correlations has, for the most part, proven elusive in CS and prompted speculation that other loci contribute to the variable clinical spectrum observed in this syndrome.[9,20] Although there is a lack of genetic heterogeneity in CS, recent studies have suggested that other genetic factors, such as modifier loci, contribute to disease susceptibility and the variable phenotypes observed in patients with this disorder.[1,9,26,27] Interestingly, a study by Freeman et al. demonstrated that phenotypic differences, including the onset and incidence of tumor formation, differed among mice with identical *PTEN* mutations yet with different genetic backgrounds.[26] These differences correspond well with the vast clinical spectrum common in patients with CS and BRRS, suggesting that genetic modifiers, which probably include other genes, miRNAs, and proteins (for example PICT-1), account for the observed differences in human patients.[28] Our data strongly suggest that *miR-19a* and *miR-21*, at least in part, contribute to this phenotypic variability.

Interestingly, in accordance with this, neither *miR-19a* nor *miR-21* were differentially expressed in patients with the BRRS phenotype. Although we interpret this with caution because of the small number of patients with BRRS included in our study, this observation suggests that although these disorders are allelic, their underlying modifiers differ. If this can be replicated in a larger series of patients with BRRS, then it is tempting to speculate that *miR-19a* and/or *miR-21* modulation of *PTEN* leads to CS or CSL, whereas the absence of this modulatory influence, probably together with other mechanisms, results in the BRRS phenotype.

Although the results of our investigation of *miR-19a* and *miR-21* in patients with CS and CSL are highly suggestive of their role in these diseases, the precise mechanism by which these miRNAs are deregulated in these patients is currently unknown. Both miRNAs localize to chromosomal regions in which loss of heterozygosity in sporadic cancer has been described.[27,29–31] However, these loci have not been genetically linked to CS, CSL, or BRRS. Furthermore, although genetic polymorphisms at miRNA-binding sites have recently been shown to alter miRNA:target gene interactions, no genetic alterations have been identified in *PTEN*'s 3′ untranslated region (UTR) near *miR-19a*'s reported binding site (*miR-21*'s precise binding site is unknown).[1,3,32] Although these alterations are likely to result in the loss of miRNA function, it is interesting to speculate whether genetic variations in *PTEN*'s 3′UTR further contribute to the variable phenotypic spectrum of CS and CSL by altering the binding of other potential miRNAs. To begin to address this question, we recently screened *PTEN*'s 3′UTR for genetic alterations in a subset of *PTEN*-mutation-negative patients with decreased full-length PTEN protein expression. However, we failed to identify any sequence differences in these individuals (Zbuk and Eng, unpublished data). Given these data, we hypothesize that the differential expression of *miR-19a* and *miR-21* that we observed in our patients with CS or CSL probably results from the dysregulation of their expression, perhaps through the oncogenic activation of *miR-19a*'s and *miR-21*'s primary miRNA or through the aberrant processing of their mature miRNA. Although the mechanisms that contribute to the oncogenic activation of these miRNAs are currently unknown, our data suggest that, both in the presence and absence of specific *PTEN* mutations, this dysregulation modulates disease phenotype. On the basis of our findings, additional investigation for better understanding of these potential mechanisms in patients with CS and CSL is warranted.

Our study is the first patient-oriented study to examine *miR-19a* and *miR-21* expression in CS, CSL, and BRRS. Along with other, yet to be identified, genetic modifiers, these miRNAs might contribute to disease susceptibility and phenotypic modulation and could serve as potential biomarkers of phenotypic variation associated with each syndrome. It is our hope that these findings will improve our understanding of the pathogenesis of CS, CSL, and BRRS in patients, both in those with defined germline *PTEN* mutations and in those for which traditional mutational-scanning methodologies have been unable to uncover a genetic etiology. We believe that an improved understanding of the role of these, and other, modifier loci in CS, CSL, and BRRS will enable more accurate clinical and molecular phenotyping and lead to advances in both the diagnostic and the preventive care afforded to patients with these and related syndromes as well as with sporadic neoplasias in which the PTEN pathway is germane.

Supplemental Data

One supplemental figure and one supplemental table are available with this article online at http://www.ajhg.org/.

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.gov/Omim/

National Comprehensive Cancer Network, www.nccn.org
Genomic Medicine Biorepository, www.lerner.ccf.org/gmi/gmb/methods.php
R software package, http://cran.r-project.org

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