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MiR-196a-2 Genotypes Determine the Susceptibility and Early Onset of Childhood Acute Lymphoblastic Leukemia

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Abstract. *Background/Aim: The roles of microRNAs (miRNAs) in tumorigenesis have attracted a lot of attention. The current study aimed at examining the association of the miR-196a-2 rs11614913 genotypes with susceptibility to childhood acute lymphoblastic leukemia (ALL) in Taiwan. Materials and Methods: This case-control investigation recruited 266 patients with childhood ALL and 266 healthy controls, and the miR-196a-2 rs11614913 genotypes of each participant were examined via the polymerase chain reaction-restriction fragment length polymorphism methodology. Results: The frequency of miR-196a-2 C allele in controls was 0.440 compared with 0.423 in ALL patients. In addition, there was no significant association between CT or CC genotypes with susceptibility to childhood ALL (OR=0.89 and 0.89, 95%CI=0.60-1.30 and 0.54-1.45, p=0.5427 and 0.6302). Furthermore, the frequencies of miR-196a-2 polymorphisms were not associated with age, gender and clinical outcomes in ALL cases. Conclusion: The miR-19a-2 genotypes are not associated with susceptibility to childhood ALL in Taiwan.*

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Acute lymphoblastic leukemia (ALL) occurs at all ages, and is the most common type of malignancy among children worldwide. The contribution of inherited factors to ALL are largely unknown. Epidemiological case-control studies have revealed several biomarkers that can be used for the prediction of childhood ALL (1-10). MicroRNAs (miRNAs) are endogenous non-coding RNAs that are capable of down-regulating protein-coding genes *via* targeting their mRNAs, degrading mRNAs or suppressing translation (11). In 2005, Lu and his colleagues analyzed the expression profiles of 217 miRNAs in a panel of human cancers (12). The down-regulation of these miRNAs was correlated with cancer developmental lineage and differentiation status (12). The differential expression of circulating miRNAs between cancer patients and healthy individuals is useful for cancer diagnosis, prognosis, and therapeutic outcomes, however, studies on the genomic levels of *miRNAs* are very few.

In recent years, mounting evidence has shown that rs11614913 (T>C), a common variant of *miR-196a-2*, may play a role in the development of several cancer types, including lung (13), breast (14), gastric (15), colorectal (16) and prostate cancer (17). As for childhood ALL, there are only two published studies, showing that variant C allele contributes to an increased risk of childhood ALL in Thailand (18), and China (19). Based on this information, we firstly aimed at genotyping the *miR-196a-2* rs11614913 profiles among Taiwanese children and analyzing the possibility the genotypes of *miR-196a-2* rs11614913 to serve as childhood ALL predictors.

Table I. *Distribution of miRNA-196a-2 rs11614913 genotypes between childhood ALL and control groups.*

	Genotype	ALL Cases	Controls	*p*-Value	Crude OR (95%CI)
rs11614913	TT	90 (33.8%)	83 (31.2%)		1.00 (Ref)
	CT	127 (47.8%)	132 (49.6%)	0.5427	0.89 (0.60-1.30)
	CC	49 (18.4%)	51 (19.2%)	0.6302	0.89 (0.54-1.45)
P_{trend}				0.8107	
Recessive	TT+CT	217 (81.6%)	215 (80.8%)		1.00 (Ref)
	CC	49 (18.4%)	51 (19.2%)	0.8244	0.95 (0.62-1.47)
Dominant	TT	90 (33.8%)	83 (31.2%)		1.00 (Ref)
	CT+CC	176 (66.2%)	183 (68.8%)	0.5171	0.89 (0.62-1.28)

ALL: Acute lymphoblastic leukemia; OR: odds ratio; CI: confidence interval. *p*-Values were calculated by Chi-square without Yates' correction.

Materials and Methods

Collection of childhood ALL cases and control participants. The research design and the detailed genotyping and analyzing procedures were approved by the committee of China Medical University Hospital Institutional Review Board. Briefly, cases with childhood ALL were recruited into the case group after diagnosis was confirmed by pediatric clinicians and pathologic examinations. Specifically, all children participated in this study with the consent of their parents or guardians, and provided 5 ml of their peripheral blood. Furthermore, a total of 457 volunteers aged under 18 years were diagnosed as cancer-free in accordance with the criteria set by the International Classification of Disease (9th revision, World Health Organization). Each childhood ALL case was matched according to age and gender with one of the 457 healthy volunteers. At last, 532 participants (266 cases and 266 controls) were recruited in the genotyping study. All the participants are Taiwanese, and were followed-up every 5 to 6 months.

Genotyping methodology for the miR-196a-2 rs11614913. Genomic DNA was extracted from the blood of each subject, aliquoted, and stored at –20˚C as a working stock. The genotyping of *miR-196a-2* (rs11614913) (T>C) single nucleotide polymorphism was performed *via* the typical polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) using the following primer pairs: forward 5'-CTT-CCC-TTC-TCC-TCC-AGA-TA-3' and reverse 5'-CGA-AAA-CCG-ACT-GAT-GTA-ACT-C-3', modified from those published by Sushma and his colleagues (20). DNA was amplified in a 25-μl mixture containing 100 ng of DNA, 20 μM of forward primer, 20 μM of reverse primer, 5 μl of 10X reaction buffer, and 1 unit of hot-start DNA polymerase. The PCR was performed in a PCR Thermocycler (Bio-RAD, Hercules, CA, USA). The PCR protocol was set as first step: initial denaturation at 94˚C for 5 min; second step: 35 cycles of denaturation at 94˚C for 30 s, annealing at 64^{o}C for 40 s, and extension at 72˚C for 45 s; third step: a final extension step at 72˚C for 10 min. The success of PCR was 100%, as checked by 3% agarose gel electrophoresis. Then, the produced PCR amplicons were subjected to *Msp* I digestion, and the products were subjected to 3% agarose gel electrophoresis. The different genotypes were identified as follows: i) a single 146 bp fragment indicated the wild-type TT genotype; ii) two products of 122 and 24 bps indicated the homologous variant CC genotype; iii) three products of 146, 122 and 24 bps indicated the heterozygous variant CT genotype.

Table II. *Allelic frequencies for miRNA-196a-2 rs11614913 polymorphism among childhood ALL and control groups.*

Allele	Cases, n (%) (n=532)	Controls, n (%) (n=532)	Adjusted OR (95%CI)[a]	*p*-Value[b]
T	307 (57.7)	298 (56.0)	1.00 (Reference)	
C	225 (42.3)	234 (44.0)	0.93 (0.73-1.19)	0.5775

OR: Odds ratio; CI: confidence interval. [a]Data adjusted for age and gender status. [b]Based on chi-square test without Yates' correction.

Statistical analysis. The Student's *t*-test was applied in order to examine the differences in age between the control and childhood ALL groups. The Pearson's chi-square test without Yates' correction was applied to examine the differential percentages of *miR-196a-2* rs11614913 genotypes between case and control groups. Crude odds ratios (OR) and 95% confidence intervals (CI) are shown in Table I. In Table II, the ORs are adjusted for age and gender. *p*-Values less than 0.05 were considered statistically significant.

Results

This study included 266 Taiwanese childhood ALL cases (148 males and 118 females) and 266 age- and gender-matched healthy children. The age and gender of each participant is summarized and compared between childhood ALL patients and control individuals in Table III. Since we matched for age and gender during the selection of the controls, there is no difference in these aspects between the case and control groups (Table III).

The genotypic frequencies for *miR196a-2* rs11614913 genotypes were determined among a Taiwanese childhood ALL population and are shown in Table I. The *miR196a-2* rs11614913 genotypic distribution in the control group fitted well with the Hardy-Weinberg equation ($p>0.05$). The TT, CT and CC proportions were 33.8, 47.8 and 18.4%, in the childhood ALL patient group, and 31.2, 49.6 and 19.2% in the control group. Based on statistical analysis, the

Table III. *Distribution of onset age and sex of the 532 subjects, 266 childhood ALL patients and an equal number of healthy controls.*

Index	ALL cases, n=266			Healthy controls, n=266			*p*-Value
	n	%	Mean±SD	n	%	Mean±SD	
Onset age, year			7.0±4.4			8.3±4.8	0.6483[a]
Gender							1.0000[b]
Boy	148	55.6%		148	55.6%		
Girl	118	44.4%		118	44.4%		

ALL: Acute lymphoblastic leukemia; SD: standard deviation. [a]Based on Student's *t*-test; [b]based on chi-square test without Yates' correction.

Table IV. *Summary of genotypic findings for miRNA-196a-2 rs11614913 polymorphism in childhood ALL risk.*

Author	Year	Case/control, n	Population	Methodology	Highlight findings
Tong	2014	570/673	China	PCR-RFLP	TC and CC/TC genotypes are associated with increased childhood ALL risk
Rakmanee	2017	104/180	Thailand	PCR-RFLP	CC, TC and CC/TC genotypes are associated with increased childhood ALL risk
Chen	2020	266/266	Taiwan	PCR-RFLP	No positive association

PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism.

distributions of *miR196a-2* rs11614913 genotypes were not differentially distributed between the childhood ALL case and control groups (*p* for trend=0.8107). The homozygous and heterozygous variant *miR196a-2* rs11614913 genotypes were similar in the control group and the case group (*p*=0.6302 and 0.5427, OR=0.89 and 0.89, 95%CI=0.54-1.45 and 0.60-1.30, respectively) (Table I). In the recessive and dominant models, no positive association was found (Table I).

The allele frequencies of *miR196a-2* rs11614913 were analyzed to validate the findings in Table I, and the results are presented in Table II. The results showed that the variant C allele in childhood ALL cases was 42.3% compared with 44.0% in the controls. There was no association between *miR196a-2* rs11614913 C allele and susceptibility to childhood ALL (OR=0.93, 95%CI=0.73-1.19, *p*=0.5775) (Table II).

Discussion

microRNAs are non-coding 20-22 nucleotide single strand RNAs, which are involved in cancer cell processes such as cell proliferation, differentiation, apoptosis, metastasis, and tumorigenesis (21). MicroRNAs can be classified as oncogenic or tumor suppressive, whose expression levels are increased and decreased in cancer patients. Recently, the investigations of miRNAs in ALL in children is becoming one of the hotspots of research.

Most miRNAs are down-regulated in tumor cells and this repression can cause cellular transformation which leads to tumor development. Among them, *miR196a-2* rs11614913 C to T single nucleotide substitution can modify its expression and function, leading to altered interactions with its target genes (such as *annexin-A1*) and increased susceptibility to cancer (22, 23). In literature, there is accumulating evidence showing that *miR196a-2* rs11614913 genotypes are associated with several types of solid cancer including breast (13), prostate (14), gastric (15), colorectal (16) and lung cancer (17). However, there is still no conclusive evidence regarding the non-solid tumor leukemia. Thus, the contribution of *miR196a-2* rs11614913 genotype to childhood ALL, the most common type of childhood cancer, is not clear.

As for childhood ALL, there are only two published studies. First, Tong and her colleagues in China had genotyped 570 childhood ALL patients and 673 non-cancer controls for *miR196a-2* rs11614913, and found that the TC heterozygote and CC/TC genotypes were associated with increased childhood ALL risk (19). Then, in 2017, Rakmanee and his colleagues collected 104 cases and 180 controls to conduct a similar genotyping study with the same PCR-RFLP methodology, and showed that the variant C allele contributed to an increased risk of childhood ALL in Thailand (18). In the current study, we provided evidence showing that *miR196a-2* rs11614913 polymorphism was not significantly associated with susceptibility to childhood ALL

in a relative moderate population (case/control=266/266) in Taiwan (Tables I and II). The different results between the previous studies and current one on *miR196a-2* rs11614913 genotypes, may be mainly due to different ethnic groups. We concisely summarized the information about the investigated population, genotyping methodology, and highlighted the findings of these three valuable studies in Table IV.

We were also interested in identifying the contribution of *miR196a-2* rs11614913 genotype in basic and clinical indexes such as gender, age, white blood cell count, risk classification and immune-phenotype. The results of stratification analysis showed that there was no statistically significant difference between *miR196a-2* rs11614913 polymorphism regarding gender, age at diagnosis, initial white blood cell count, risk classification or immune-phenotype (data not shown). This may also be because of the small sample size. A study with a larger sample size should be performed in the future to determine the clinical implications of the genotypes of *miR196a-2* rs11614913.

We cannot arbitrarily exclude the possibility that *miR196a-2* rs11614913 polymorphism may indeed play a role in the etiology of childhood ALL. MiR196a-2 may interact with its target genes, and be involved in complex tumor processes. For instance, in the near future we are going to investigate the single or synergistic effects of the genotypes of *miR196a-2* and target genes in childhood ALL, such as that of *annexin-A1*. Annexin-A1 is a calcium-dependent phospholipid binding protein that is critical in carcinogenesis and metastasis. It has been reported that miR196a-2 may act as a tumor suppressor or oncogene by directly bind with *Annexin-A1* gene and affecting its expression in breast tumor tissues (24).

In conclusion, *miR196a-2* rs11614913 polymorphism was not significantly associated with susceptibility in Taiwan childhood ALL. Our findings are not in agreement with previous findings in Thailand and China. Validation of the contribution of *miR196a-2* rs11614913 for early detection and prediction of childhood ALL risk is warranted in other populations.

Conflicts of Interest

All Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Research Design: Chen CC, Hsu PC and Shih LC; Questionnaire Collection and Analysis: Hsu PC, Pei JS, and Chen CC; Genotyping: Wang YC, Kuo CC; Chang WS, and Tsai CW; Statistical Analysis: Hsu YN, and Chao CY; Manuscript Writing: Pei JS and Bau DT; First Revision: Chang WS, and Pei JS; Second Revision: Tsai CW, and Bau DT.

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