RESEARCH ARTICLE

Increased Risk of Thai Childhood Acute Lymphoblastic Leukemia with the *MiR196a2* T>C Polymorphism

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Abstract

Objectives: This study assessed associations of the *miR196a2* (rs11614913) T>C polymorphism withsusceptibility to childhood acute lymphoblastic leukemia (ALL) and clinical outcomes. **Materials and Methods:** Blood DNA samples from 104 childhood ALL patients and 180 healthy children were studied for the *miR-196a2* (rs11614913) polymorphism using a polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) approach. **Results:** The frequency of the *miR-196a2* (rs11614913) T allele in controls was 0.51 compared with 0.33 in ALL cases. In this study, CC, TC heterozygote and CC/TC genotypes were significantly associated with increase childhood ALL susceptibility compared with the TT wild type (OR =4.321, 95% CI = 2.091-8.930 p=0.000, OR = 2.248, 95% CI =1.103-4.579, p=0.024, OR = 2.921, 95% CI = 1.504-5.673 p=0.001, respectively). However, the *miR-196a2* (rs11614913) T>C polymorphism was not associated with demographic data or clinico-pathological data in ALL cases. **Conclusion:** CC, TC and CC+TC genotypes of *miR-196a2* (rs11614913) was significantly associated with increased susceptibility in Thai childhood ALL but not with clinical variables.

Keywords: Acute lymphoblastic leukemia- *miRNA-196a2*- polymorphism

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy. The long term survival rate of ALL is nearly 90% in developed countries (Tasian et al., 2015; Pui et al., 2016). However, in Thailand, the long term survival rate is lower (Wongmeerit et al., 2016). The pathogenesis of ALL includes environmental risk factors and genetic susceptibility (Deepa et al.,2015). MicroRNA (miRNA or miR), a noncoding RNA, is the novel epigenetic biomarkers. Previous studies have reported the role of miRNA in several biological process including cell proliferation, differentiation and apoptosis. (Bartel, 2004; Hwang and Mendell, 2006). Recently, it was found that the dysregulation of miRNA expression contributes to multistep processes of carcinogenesis (Jansson and Lund, 2012; Hayes et al., 2014). Single nucleotide polymorphisms (SNPs) in the miRNA gene region have effects on the function of miRNA and contribute to cancer susceptibility (Mishra et al., 2008).

Recently, several studies have investigated the association of *miR-196a2* (rs11614913) polymorphism with cancer susceptibility. (Srivastava K and Srivastava A, 2012; Ma et al., 2013). However, there are very few studies focus on the association of miR196-a2 (rs 11614913) polymorphism in childhood ALL. In Caucasian, there is no association between *miR-196a2* (rs11614913) polymorphism and risk of childhood ALL (Trevino et al., 2009). However, this significant association was found in Chinese population (Tong N. et al., 2014).

This research aims to investigate the association of *miR-196a2* (rs 11614913) polymorphism and childhood ALL susceptibility in Thailand. Moreover, the association of *miR-196a2* (rs 11614913) polymorphism and clinical outcomes was also studied.

Materials and Methods

Study Subjects

This research was 284 case-control study including 104 childhood ALL patients and 180 healthy cancer-free children at the Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital, Thailand. Patients were classified by risk-based assignment protocol (Smith et al., 1996), assessed by initial white blood cell counts, French- American-British morphology and lymphomatous disease. Moreover clinical and demographic data including age at diagnosis, sex, immunophenotype and chromosome abnormalities were retrospectively studied.

This research was approved by the Ethics Committee,

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Faculty of Medicine Ramathibodi Hospital, Mahidol University and inform consent was obtained from all the participants.

DNA Extraction

Genomic DNA extraction from EDTA blood of 194 healthy controls and 107 ALL patients and extracted by salting-out method (Miller SA et al., 1988). DNA was quantitated by a measurement of nano drop spectrophotometer and kept at -20oC prior to use.

Genotyping of the miR-196a2 (rs11614913) (T>C) polymorphism

The single nucleotide polymorphism of *miR-196a2* (rs11614913) (T>C) was performed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis. Genotyping of *miR-196a2* (rs11614913) (T>C) polymorphism was done using forward primer 5' CCC-CTT-CCC-TTC-TCC-TCC-AGA-TA 3' and reverse primer 5' CGA-AAA-CCG-ACT-GAT-GTA-ACT-CCG 3' as describe previously. (Shuma et al., 2015). DNA was amplified in 25 µL reaction mixture containing 100 ng of genomic DNA, 20 µM of each primer, 5 µL of 10 X PCR buffer with 1.5 mM MgCl2 and 1 unit of Taq (i-Taq, European Biotech network, Belgium). The PCR cycle was carried out in a Thermocycler (Bio-RAD, Hercules, CA, USA). The PCR conditions were the initial denaturation of 95^{o} C for 5 minute followed by 35 cycles of 94^{o} C for 35 second, 61^{o} C for 35 second, and 72^{o} C for 40 second, with a final elongation at 72^{o} C for 5 minute. The PCR products were found to be 149 bp in length by electrophoresis on 2% agarose gel, stained with 0.5 µg/ml ethidium bromide and then visualized using the gel documentation.

Restriction fragment length polymorphism (RFLP)

PCR products were digested by *MspI* (NEB, UK). The product sizes were verified on 3% agarose gel electrophoresis stained with 0.5 µg/ml ethidium bromide and then visualized using the gel documentation. The product presented three different patterns: a single 149 bp fragment for the TT genotype; two fragments of 125 and 24 bp for the CC genotype; and three fragments of 149, 125 and 24 bp for the CT genotype, respectively.

Statistical analysis

Comparisons of genotypes between cases and controls group were analyzed by binary logistic regression analysis. The associations between the clinical data and the distribution of the *miR-196a2* genotypes in ALL patients were calculated by Chi-square test. Crude odds ratios (OR) and 95% confidence intervals (CI) were also calculated. P values less that 0.05 were considered statistically significant. All of the statistical analysis using SPSS version 17.0 program (SPSS Inc., Chicago, USA).

Results

Characteristic of the study population

The demographic data of ALL cases and control subjects as well as clinical data of ALL patients are summarized in Table 1. There were 104 ALL cases and 180 cancer-free controls. The demographic data shown that there was no significant difference in the frequency of sex distribution between ALL cases and controls (p=0.193). There was significant difference in the frequency of age distribution between ALL cases and control (p=0.009). Among ALL patients, there were 101 cases with known immunophenotype including 12 cases with T- ALL (11.88%), 78 cases with Early pre-B ALL (77.23%), 10 cases with pre-B ALL (9.90%) and 1 case of Mixed cell type ALL. The data of treatment protocol collected in 91 cases including 41 cases (45.05%) of low risk, 41 cases (45.05%) of standard risk and 9 cases (9.90%) of high risk ALL.

Association between miR-196a2 (rs11614913)T>C polymorphism and the susceptibility of childhood ALL

The allele frequency of *miR-196a2* T allele in control was 0.51 compared with 0.33 in ALL cases (Table 2). The frequencies of the *miR-196a2* (rs 11614913) genotypes was 29.45% for TT, 43.33 % for CT and 27.22 % for CC in controls, whereas the data was 12.50%, for TT, 41.35% for CT and 46.15% for CC in ALL cases. There were significant differences between the distribution of genotype frequencies of *miR-196a2* (rs11614913) between ALL cases and controls. It was found that *miR-196a2* (rs11614913) variant CC, TC heterozygote and CC/TC genotypes were significantly associated with increase childhood ALL susceptibility compared with TT wild type.(OR =4.321, 95% CI = 2.091-8.930 p=0.000, OR = 2.248, 95% CI =1.103-4.579, p=0.024, OR = 2.921, 95% CI =, 1.504-5.673 p=0.001, respectively).

Association between genotypes of the miR-196a2 (rs11614913)T>C polymorphism and clinicopathological data in childhood ALL patients

Table 1. Frequency Distribution of Selected Variables in Cases and Controls

Demographic characteristics	ALL (%) n=104	Controls (%) n=180
Sex		
Males	65 (62.50)	98 (54.44)
Females	39 (37.50)	82 (45.56)
Age at diagnosis		
< 6 years	64 (61.54)	137 (76.11)
>6 years	40 (38.46)	43 (23.89)
Immunophenotype (n=101)		
T cell	12 (11.88)	-
Non T cell	89 (88.12)	-
Early pre-B	78 (87.64)	-
Pre-B	10 (11.24)	-
Mixed	1 (1.12)	-
Risk classification (n=91)		
low risk	41 (45.05)	-
Standard risk	41 (45.05)	-
High Risk	9 (9.90)	-

Table 2. *MiR-196a2* T>C Genotypes Distribution and Allele Frequency in ALL And Controls

Genotypes	ALL (%) n=104	Controls (%) n=180	OR (95%CI)*	p-value**
TT	13 (12.50)	53 (29.45)	1.000 (Reference)	
TC	43 (41.35)	78 (43.33)	2.248	0.024
CC	48 (46.15)	49 (27.22)	4.321	0
TC+CC	91 (87.50)	127 (70.55)	2.921	0.001
Allele frequency				
MiR196a2 T allele	0.33	0.51		
MiR196a2 C allele	0.67	0.49		

* Odds ratio and p-value were calculated by comparison of control and ALL for TC, CC and CC+CT versus TT; ** significant at the 0.05 level of significance

Table 3. The Association between *MiR196a2* T>C Genotypes and Clinico-Pathological Data of ALL Patients

Demographic data	MiR-196a2 T/T	CT or CC	p-value
Sex (n=104)			
Male	9	56	0.592
Female	4	35	
Age at diagnosis (n=104)			
< 6 years	7	57	0.542
>6 years	6	34	
Initial WBC count (n=97)			
<50,000 cell /µl	6	73	0.625
>50,000 cell /µl	2	16	
Immunophenotype (n=101)			
T cell	2	10	0.676
Non T cell	11	78	
Early –pre-B	10	68	
Pre-B	1	9	
Mixed	0	1	
Risk classification (n=91)			
High Risk	2	7	0.399*
Low risk or Standard risk	10	72	
Low risk	5	36	
Standard risk	5	36	

*, p-value were calculated by comparison of genotypes between high risk and low risk or standard risk

In this study, there was no association between *miR-196a2* (rs11614913) T>C polymorphism and clinical data including gender, age at diagnosis, initial WBC count, immunophenotype and risk classification of ALL patients (Table 3).

Discussion

MiRNAs are non coding RNA that inhibit the expression of protein coding genes by either mRNA degradation or translational repression. MiRNAs regulate cell proliferation, differentiation and apoptosis. Polymorphism of miRNA precursor may affect mature microRNA function (Ryan BM et al., 2010). Recently, several investigators study the association of *miR-196a2* (rs11614913) polymorphism and cancer susceptibility. It was found that *miR-196a2* (rs11614913) T allele was significantly associated with decreased susceptibility to breast cancer in Caucasian (Hoffman, et al., 2009; Dai et al., 2016). In contrast, TT genotype of *miR-196a2* (rs 11614913) was associated with an increased risk of gastric cancer in Chinese (Gu and Tu, 2016). Moreover, it was shown that CC genotype of *miR-196a2* (rs 11614913) was significantly associated with oral squamous cell carcinoma in Indian (Sushma et al., 2015) and lung cancer in Chinese. (Tian et al., 2009 and Yuan et al., 2013).

To evaluate the association between *miR-196a2* (rs11614913) polymorphism and susceptibility to Thai childhood ALL, case-control study was investigated in Thai children whose frequency of gender match between cases and controls. However, there is significant difference of age distribution between cases and controls. The previous study shown that the major allele of *miR-196a2* (rs11614913) is C allele in Caucasian population whereas it is T in Chinese population (Chen et al., 2011). In this study, we found that the major allele in our control group was T allele which was 29.45% compared with 35.2% in Chinese population (Tong N et al., 2014). There were significant differences in the distribution of *miR-196a2* (rs11614913) genotypes between ALL cases and controls. The *miR-196a2* (rs11614913) variant CC, TC heterozygote and CC/TC genotypes were significantly associated with increase childhood ALL susceptibility compared with TT wild type. The association of *miR-196a2* (rs11614913) genotypes with susceptibility to Thai childhood ALL in this study is similar to Chinese study (Tong N et al., 2014). They revealed that TC heterozygote and CC/TC were associated with an increased risk of childhood ALL in Chinese. However, in this study, the frequencies of *miR-196a2* (rs11614913) T>C polymorphism were not associated with demographic data and clinical outcomes in ALL cases.

There are several target genes of miR-196a2. *MiR-196a2* may regulate different genes in different cell type or under different condition in a specific cell type. Genotype-phenotype correlation analysis revealed that CC homozygote in *miR-196a2* was associated with significantly increased *miR-196a2* expression (Hu et al.,2008). Recently, it was found that the target genes of *miR-196a2* are homeobox family of genes which is called Hox genes. They involve in hematopoietic cell growth and differentiation. There was some evidences revealed that

HOXC8, which suppressed cell migration and metastasis, is the target gene of *miR-196a2* (Tong N et al., 2014). Another possible target genes of *miR-196a2* are LSP1 (lymphocyte specific protein 1) and GDF3 (Growth/ differentiation factor 3 precursor) (Hu et al.,2008). However, there is still no study about the target genes of *miR-196a2* in ALL patients. Further study should be done to investigate the pathogenesis about the interaction pathway between *miR-196a2* and its target genes in acute lymphoblastic leukemia.

In conclusion, *miR-196a2* (rs11614913) T>C polymorphism was significantly associated with susceptibility to Thai childhood ALL and was not significantly associated with clinico-pathological variables.

Conflict of interest

The study presented no conflict of interest.

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