Polymorphism analysis of CTLA-4 in childhood acute lymphoblastic leukemia

Liang Hui, Zhang Lei, Zeng Peng, Shan Ruobing* and Zhang Fenghua*
Qingdao Women & Children Hospital, Qingdao 266013, Shandong Province, China

Abstract: To investigate the correlation between cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphism and children with acute lymphoblastic leukemia (ALL). A total of 86 children of ALL (23 HR, 54SR) and 112 healthy controls was selected. The genptypes were determined by means of polymerase chain reaction (PCR) and the PCR product sequencing. Genotype and alleles frequency of SNP-318, SNP+49 and SNP-CT60 were compares among different groups. The frequency of TC,TT genotype and T allele in ALL children at SNP-318 position were statistically higher than controls. In HR group, the frequency of TC, TT genotype at SNP-318 position was statistically higher than SR group. There was no significantly difference in genotype and allele distribution of SNP+49 position among the HR patients, SR patients and control group. (2) The frequency of GG genotype and G allele in ALL children at SNP-CT60 position were significantly higher than controls. The genotype and allele distribution of SNP-CT60 position between different clinical risk groups were no significantly different. As a result of the increased frequency of TC, TT genotype and T allele at SNP-318, ALL children synthesized more CTLA-4 to deliver the inhibitive signal, and this lead to restraint of T cell activation. Such difference at SNP-318 position was obvious in HR children. The SNP+49 position is probably not the main regulating point in ALL. (2) In SNP-CT60 position, the G allele played the main part. The increase of G allele frequency result in the high expression of CTLA-4. such difference at SNP-318 position was obvious in HR children.

Keywords: Acute Lymphoblastic Leukemia, costimulatory molecules, CTLA-4.

INTRODUCTION

ALL is the most common childhood cancer, the incidence rate is about 3 ~ 4/10 million. The incidence of this disease in the rising, but its etiology and pathogenesis are not fully elucidated (Terracini, 2011). The current study suggests that the role of a variety of pathogenic factors in the bone marrow hematopoietic precursor cells to change the genetic background, followed by a variety of mechanisms leading to the occurrence and development of leukemia. Anti-tumor immunity, especially cellular immune abnormalities make tumor cells escape immune. T cells are the major effector cells, which depends on the dual signal activation, CTLA-4 is one of the important elements of the second signal system. Numerous studies have suggested that genetic polymorphism of the promoter and exon 1 in CTLA-4 gene may affect the expression of CTLA-4 molecule. This study examined polymorphisms of CTLA-4 gene promoter -318 sites +49 loci and CT60 sites in children with ALL to investigate genetic susceptibility and immunological pathogenesis.

MATERIALS AND METHODS

Clinical data

A total of 86 children with ALL admitted to Qingdao women and children's hospital from October 2010 to July 2011 were included in the ALL group. All patients were diagnosed according to "childhood acute lymphoblastic leukemia treatment recommendations"… also, in accordance with the standard clinical risk type… The patient group consisted of 54 males and 47 females aged 1 to 14 years old (mean =5.6± 3.02 years old) of which 54 cases of standard-risk, high-risk type 32 cases. All patients had not received corticosteroids and cytotoxic drugs and other treatment before enrollment.

The normal control group included 112 healthy children consisting of 50 males and 62 females aged 1.5 to 14 years old (mean =6.30 ± 2.76 years old). The age and sex ratios between the 2 groups were not significantly different. None of the enrolled subjects had other cancer, family history of autoimmune disease, history of infectious disease in the past 1 month, or received any glucocorticoid or other immunosuppressive treatment within the past 6 months. Other autoimmune diseases were also excluded. All patients were informed consent by the guardian. All subjects had no blood relationship.

The experiments were performed with informed consent from all patients and their families.

Ethical considerations

This study has complied with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/index.html). It was approved by the Faculty of Medicine

**Corresponding authors*: e-mails: yaojike1384@126.com, silvia_mpl@163.com

Ethics Committee of Qingdao Women and Children's hospital in July 2009. Before the study began, full details were given to all participants or guardian, who were assured of confidentiality and gave their informed written consent. All data were reported in a collective fashion, and participants could withdraw at any time without affecting their children's treatment.

Sample collection

For each of enrolled subjects, 2ml fasting venous blood was collected using EDTA anticoagulant and preserved in 20 degrees below zero.

The extraction of DNA

The peripheral blood DNA was extracted from each of the samples Using DNA Extraction Kit (non centrifugal column type DP319, Tiangen biotech, Beijing, China) according to the manufacturer's protocol. The concentration of DNA was measured using k5500 micro spectrophotometer. The one who's OD between 1.8~2.0 shows higher purity, which conforms to the standard. DNA concentration was adjusted to 10~100ng/ul, to -20 save.

All PCR primers were designed using Primer Premier 5.0 and produced by Shanghai Majorbio Company. Table 1 for a list of the primers used in this study.

Polymerase chain reaction

The primers were designed and synthetised by the Shanghai Majorbio company refer to CTLA-4 gene nucleic acid sequence, on the basis of the downstream target sites on the nucleic acid sequence. -318 site and +49 site upstream primer F:5'-CAGAAAGTTAGCAGCCTAGT-3', downstream primer R:5'-ACCTTTGCAGAAGACAGGGA-3', product is 584bp; CT60 sites upstream primer F:5'-GCTTCATGAGTCAGCTTTGC-3', downstream primer R:5'-CTGTGTTAAACAGCATGCCA-3', product 161bp.

Preparation of PCR reaction system

Three sites with different DNA polymerase, -318 and +49 site by high fidelity PrimeSTAR HS, DNA Polymerase (TAKARA), Buffer (Mg2+plus) 10ul, dNTP Mixture (2.5mM) 4ul, Primer-F (5uM) 2ul.

Primer-R (5uM) 2ul, DNA4ul, PrimeSTAR HS DNA Polymerase (2.5U/ul) 0.5ul, reaction conditions: 95 denaturing 5min; denaturation 5sec at 98, 58 for 5sec, 72 for 45sec, a total of 30 cycles, 72 extension 10min. CT60 site using Premix Ex Taq (TAKARA), Premix Ex Taq (1.25U/25ul) 25yl, Primer-F (5uM) 2ul, Primer-R (5uM) 2ul, DNA7.5ul, ddH2O13.5ul, reaction conditions: 95 denaturing 5min; denaturation 5sec at 98, 62 for 30sec, 72 for 10sec, a total of 35 cycles, 72 extension 10min.

The identification and sequencing of PCR products

Amplification products were loaded onto 2% agarose gels containing loading buffer, stained with ethidium bromide and visualized under ultraviolet (UV) illumination.4ul products, plus the 1ul loading buffer, continuous 20min 2% agarose gel electrophoresis of 110V constant voltage, after the detection of the PCR product, observations in the gel imaging and photography. The electrophoretic bands of lighter, we commissioned the Shanghai Majorbio companies take the normal method of Sanger sequencing. Nucleic acid sequences by sequencing, as compared with the BLAST, and then use ChromasPro software to analyze the results of sequencing.

Sequence analysis

Genomic DNA obtained from PCR was sequenced by the Shanghai Majorbio Companies. The data were analyzed using SPSS17.0 software. Genotype and allele frequencies were calculated by direct counting method. Categorical variables were compared using χ^2 tests or Hardy-Weinberg balance test. The strength of correlation between gene and disease was evaluated using odds ratios and 95% confidence interval. The level of statistical significance was set at $P < 0.05$.

RESULTS

CTLA-4 gene -318, +49, CT60 polymorphism loci results -318 (T/C) [rs5742909] locus was shown in fig. 1. The product of PCR was 584bp, By sequencing the 3 genotypes: genotype CC, genotype CT, genotype TT, are shown in figs. 2-A, 2-B and 2-C.

The location on the genome SNP can be recognized this site, It can be the same as -318 sites with the same amplification system, Electrophoresis results are shown in fig. 1.

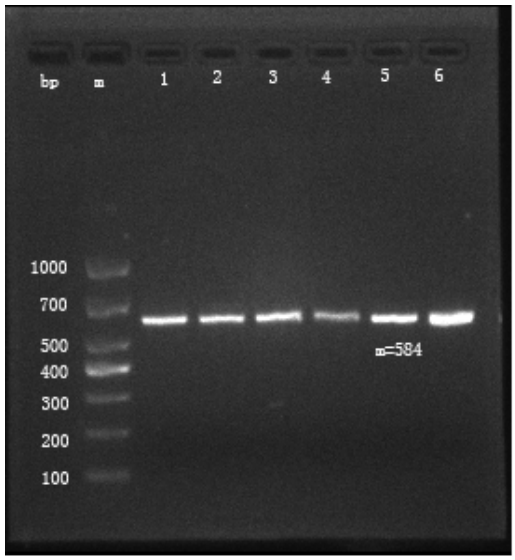

Fig. 1: PCR Production of SNP-318 (T/C) and SNP+49(A/G)
Maker 100/200/300/400/500/700/1000
Lane1-6 PCR (584bp)

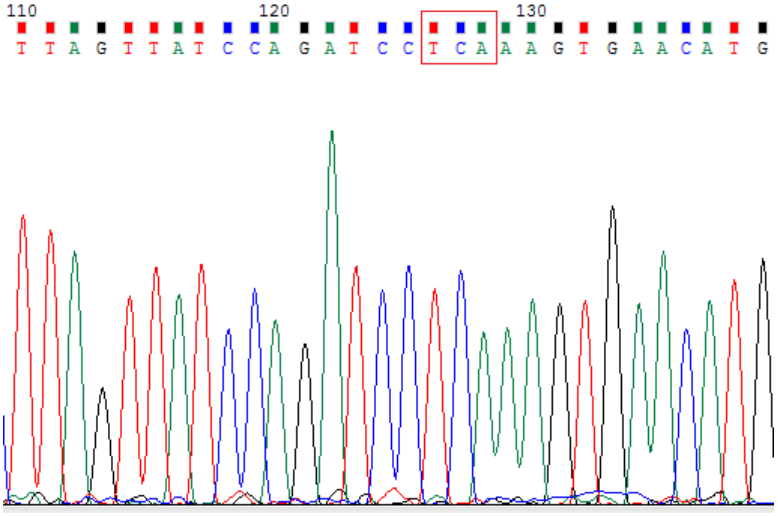

Fig. 2A: CC genotype of SNP-318(T/C)

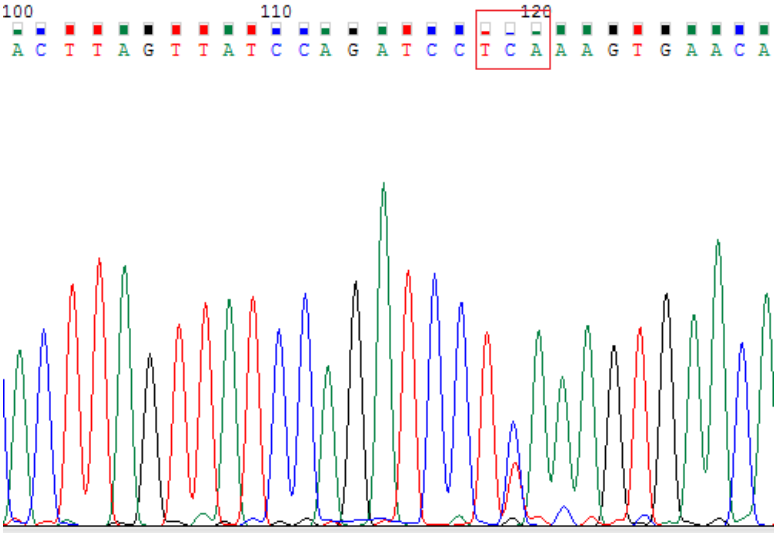

Fig. **2B**: TC genotype of SNP-318(T/C)

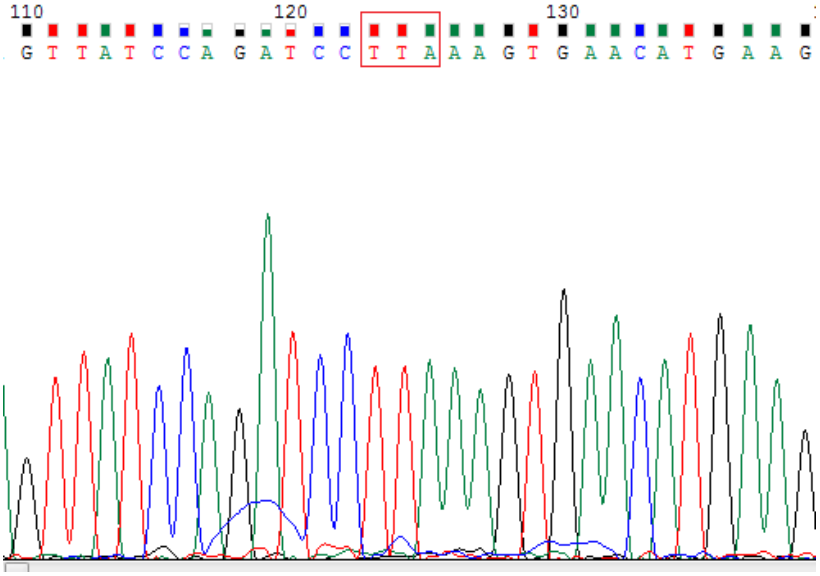

Fig. 2C: TT genotype of SNP-318(T/C)

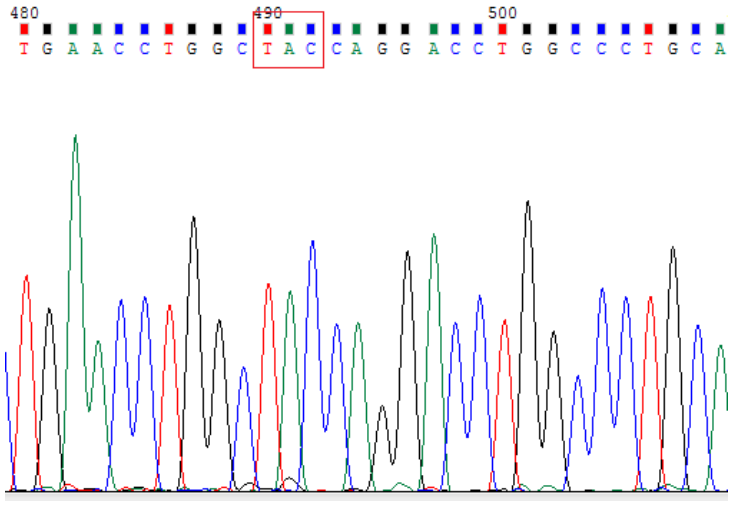

Fig. 3A: AA genotype of SNP+49(A/G)

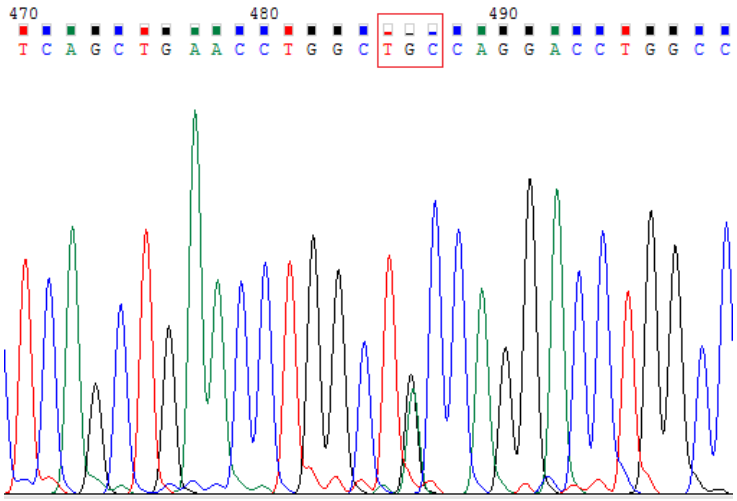

Fig. 3B: AG genotype of SNP+49 (A/G)

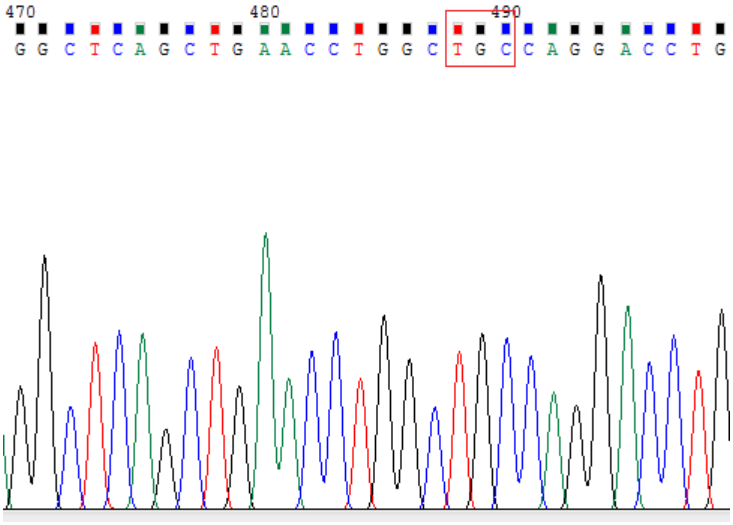

Fig. 3C: GG genotype of SNP+49(A/G)

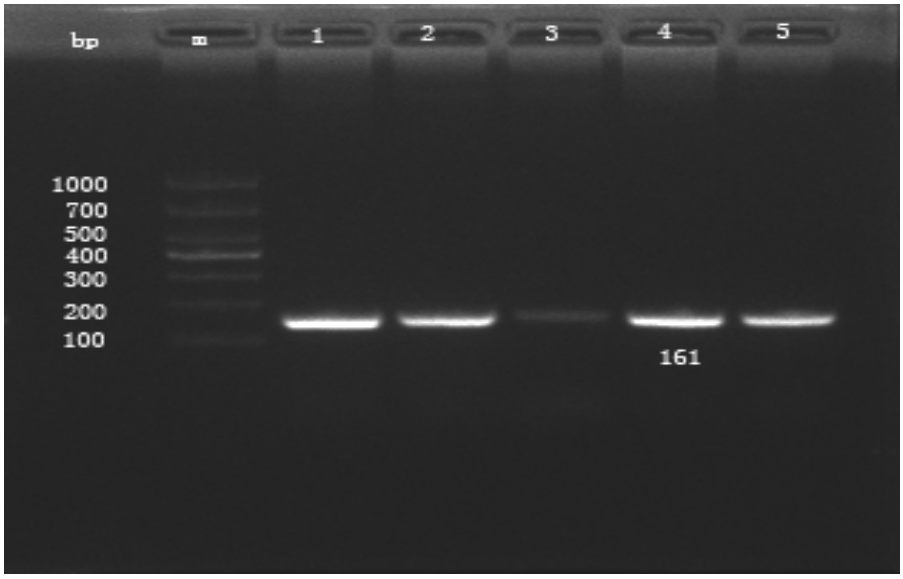

Fig. 4: PCR Production of SNP-CT60(A/G)
Maker 100/200/300/400/500/700/1000 - Lane1-5 PCR products (161bp)

The product of PCR was 584bp, by sequencing can be found in 3 genotypes: genotype AA, genotype AG, genotype GG, 3-A, 3-B were found in the diagram, fig. 3C.

T60 (A/G) [A6230G, electrophoresis of rs3087243] sites results in fig. 4.

The product of PCR was 161bp, by sequencing identified 3 genotypes: genotype AA, genotype AG, genotype GG, respectively is shown in figs. 5-A, 5-B and 5-C.

Genetic balance

Through chi square test, the control group was in the Hardy-Weinberg equilibrium, has a group of representative.

Single factor analysis was adopted. corellation between the polymorphism in (SNP-318, +49, CT60 of the CTLA-4 gene and ALL

The distribution and diversity of locus in the case group and control group in the genotype and allele frequencies are shown in table 1. The frequency of TC, TT genotype and T allele in ALL children at SNP-318 position were statistically higher than controls (χ^2=8.799, P=0.012; χ^2 =9.527, P=0.002). In HR group, the frequency of TC, TT genotype at SNP-318 position was statistically higher than SR group (χ^2=6.24, P=0.044; χ^2 =6.599, P=0.01). There was no significantly difference in genotype and allele distribution of SNP+49 position among the HR patients, SR patients and control group. (χ^2=2.101, P=0.35; χ^2=0.205, P=0.651). The frequency of GG genotype and G allele in ALL children at SNP-CT60 position were significantly higher than controls (χ^2=6.365, P=0.041); χ^{22}=6.761, P=0.009). The genotype and allele distribution of SNP-CT60 position between different clinical risk groups were no significantly different (χ^2=2.625, P=0.269; χ^2=2.923, P=0.087).

Single factor analysis of the polymorphism of TC, TT genotype and T allele in ALL children at SNP-318, +49 and ct60 position and acute lymphoblastic leukemia in different clinical risk.

The distribution and difference of CTLA-4 gene SNP-318, +49, CT60 polymorphism in children with different clinical risk genotype frequency and allele frequency display in table 2, CTLA-4 gene SNP+49 (A/G) in high risk group of AA genotype and A allele frequency was higher than that of low risk group, there was statistically significant difference (χ^2=10.482, P=0.005; χ^2=6.696, P=0.01). CTLA-4 gene SNP-CT60 (A/G) there was no significant difference of distribution in the high risk group in the frequencies of alleles and genotypes (χ^2=2.626, P=0.269; 2=2.923, P=0.087).

DISCUSSION

Acute leukemia is a heterogeneous hematopoietic malignancies, the cause may be associated with virus infection, physical and chemical factors and their genetic susceptibility. In the process of tumor development, immune disorder of the organism plays an important role.

Research on immune cells and molecules on tumor has the potential to reveal the mechanism of the occurrence and development of tumor, guide the future direction of tumor immunotherapy.

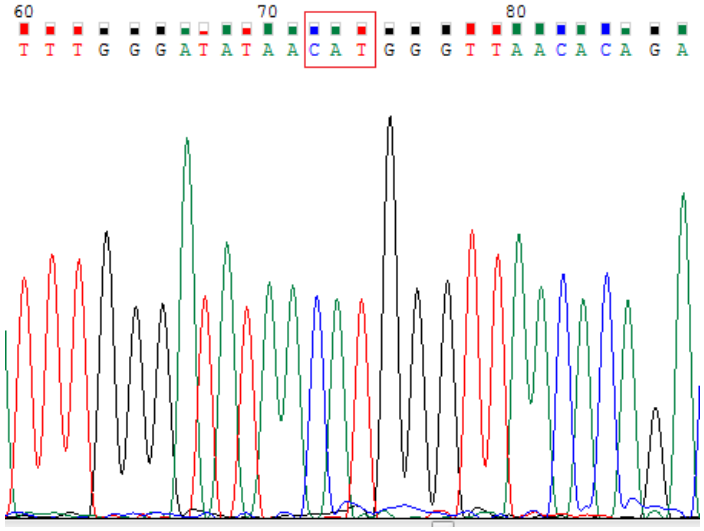

Fig. 5A: AA genotype of SNP-CT60(A/G)

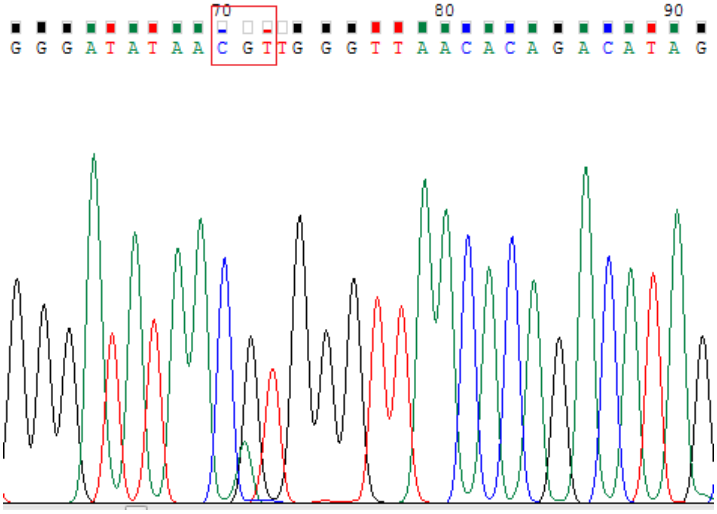

Fig. 5B: AG genotype of SNP-CT60(A/G)

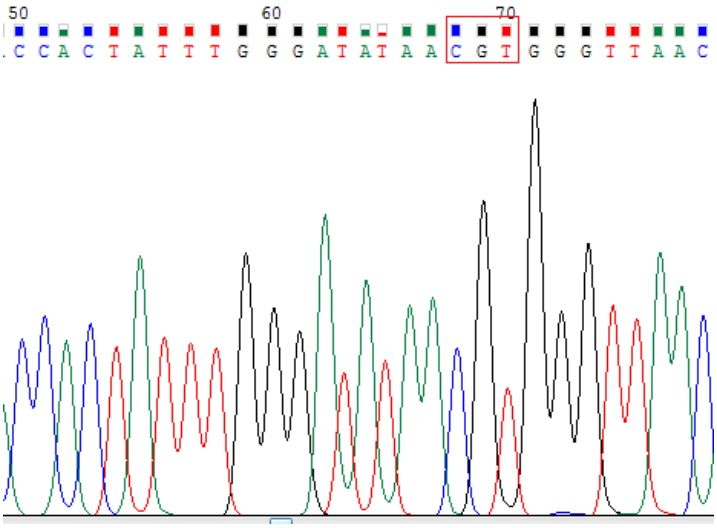

Fig. 5C: GG genotype of SNP-CT60 (A/G)

Table 1: Association of CTLA-4 gene polymorphism with the risk of ALL

SNPs		Cases [n(%)]	Controls [n(%)]	X~ 2	P	OR
-318	Genotype					
	CC	48(55.81%)	84(75%)	8.799	0.012	1
	CT	31(36.05%)	25(22.32%)			2.170(0.273~5.063)
	TT	7(8.14%)	3(2.68%)			4.083(0.659~10.176)
	Alle					
	C	127(73.84%)	193(86.16%)	9.527	0.002	1
	T	45(26.16%)	31(13.84%)			2.206(1.187~3.597)
+49	Genotype					
	AA	9(10.47%)	15(13.39%)	2.101	0.35	0.917(0.363~2.317)
	AG	41(47.67%)	42(37.50%)			1.491(0.817~2.721)
	GG	36(41.86%)	55(49.11%)			1
	Alle					
	A	59(34.3%)	72(32.14%)	0.205	0.651	1.102(0.723~1.68)
	G	113(65.7%)	152(67.86%)			1
CT60	Genotype					
	AA	3(3.49%)	8(7.14%)	6.365	0.041	1
	AG	15(17.44%)	34(30.36%)			1.176(0.273~5.063)
	GG	68(79.07%)	70(62.5%)			2.590(0.659~10.176)
	Alle					
	A	21(12.21%)	50(22.32%)	6.761	0.009	1
	G	151(87.79%)	174(77.68%)			2.066(1.187~3.597)

Studies suggest that tumor associated macrophages (TAM) through the activation of cell signaling pathways, secrete various growth factors, expression of various chemokine mechanisms involved in tumor invasion, metastasis, angiogenesis and inhibit the process of peripheral immune function. Study of tumor associated immune, immune regulation genes also is one of the focus. Polymorphism of immunity related genes may affect the immune response between individuals of different and various tumor susceptibility.

For example, TNF, IL gene polymorphism and gastrointestinal cancer, stomach cancer risk associated with (He et al., 2000; Saito et al., 2001), CTLA-4 gene may be one of the immune related genes, the existing research results, related CTLA-4 gene polymorphism and pancreatic cancer, renal cancer, cervical cancer, lymphoma and leukemia disease (Piras et al., 2005; Lang et al., 2011). At present, there is no relative report gene polymorphism in children with acute leukemia.

CTLA-4 belongs to the immunoglobulin superfamily, is expressed on activated CD4+ and CD8+T lymphocytes and activation of B lymphocytes. In cells, up regulate the expression of CTLA-4 and CD28, and the competitive and the antigen presenting cell ligands on the B7 binding. Unlike CD28, the combination of CTLA-4 and B7 after transfer negative feedback signals play a role of immune regulation, response by mediating T cell apoptosis, inhibit the secretion of IL-2, cell cycle control and remote signal model of T cells down regulate pathway or termination, inhibition of T cell proliferation, activation.

It can be inferred, blocking CTLA-4 may enhance the immune response, enhance the ability of anti tumor. In vitro studies showed in mouse melanoma cell lines and prostate cancer cell lines (Youlin et al., 2012), blocking CTLA-4 can promote T cell proliferation and INF-secretion and enhance anti-tumor immunity, which has a synergistic effect with activation of 4-1BB/4-1BBL pathway. CTLA-4 has been regarded as an important regulatory factor for the inhibition of antitumor immunity.

Darivach cloned the CTLA-4 gene from the total DNA of human, at the same time that it is located in 2q31-2q33, was a single copy gene, the size of about 4kb. CTLA-4 gene contains 4 exons and 3 introns. Exon 1 108BP long, encoding a hydrophobic amino acid residues of the leading strand; exon 2 348bp long, encoding CTLA-4 V region of 116 amino acid residues, including encoding outer membrane fragments, are the ligands of B7; exon 3 111bp in length, encoding 37 amino acid residues, several residues including the extracellular part of residues, began to transmembrane hydrophobic residues and cytosolic functional area, as the transmembrane region; exon 4 102bp in length, encoding cytosolic CTLA-4 most amino acid residues, cytoplasmic area; followed by the 3 'untranslated region 1150bp, containing about 60 AT repeats, stability control mRNA.

In the crowd, DNA sequence variation among individuals called gene polymorphism.

The CTLA-4 gene polymorphism of are recognized as follows: located in the first exon of +49 A/G allele; in -

Table 2: Association of CTLA-4 gene polymorphism with the different clinical risk ALL

SNP		HR[n(%)]	SR[n(%)]	x~2	P	OR
-318	Genotype					
	CC	10(37.04%)	38(64.41%)	6.24	0.044	1
	CT	13(48.15%)	18(30.51%)			2.744(1.013~7.438)
	TT	4(14.81%)	3(5.08%)			5.067(0.972~26.411)
	Alle					
	C	33(61.11%)	94(79.66%)	6.599	0.01	1
	T	21(38.89%)	24(20.34%)			2.492(1.229~5.056)
+49	Genotype					
	AA	7(25.93%)	2(3.39%)	10.482	0.005	12.25(2.114~70.986)
	AG	12(44.44%)	29(49.15%)			1.448(0.515~4.075)
	GG	8(29.63%)	28(47.46%)			1
	Alle					
	A	26(48.15%)	33(27.97%)	6.696	0.01	2.392(1.226~4.666)
	G	28(51.85%)	85(72.03%)			1

318 promoter alleles of T/C; and is located in the fourth exon 3 'untranslated region (AT)) n repeats; located in the first intron of +1822 C/T allele. CT60 A/ is located in the 3 'UTR of the G-spot. There are other -1722T/C allele, -1661A/G allele. Promoter is an important part of gene, plays an important role in the regulation of gene expression, if the promoter is part of change (mutation), to regulate gene expression disorder. Studies have shown that, CTLA-4 gene regulator -318 site C/T polymorphisms affecting transcription, CTLA-4 gene promoter -318 sites for the T crowd in the stimulated CTLA-4 expression level is C people, even if does not stimulate the expression level of CTLA-4 mRNA under the state of high (Ligers et al., 2001). Wang research that the promoter activity of higher -318 site is T, unregulated the expression of CTLA-4 molecule (Wang et al., 2002). Our research shows that children with acute lymphoblastic leukemia -318 loci TT, CT genotype increased, the frequency of T allele increased, the difference was statistically significant (χ^2=8.799, P=0.012). Accordingly, expression and regulation of carrying the genotype with the T lymphocyte surface CTLA-4 molecules increases, transfer activation inhibitory signal enhancement, the inhibition of T cell activation, anti tumor immunity, may be more susceptible to cancer. Previous research also shows that children with acute lymphoblastic leukemia T cell surface CTLA-4 expression was significantly higher than that of control group. Research shows that 318 locus involved in the pathogenesis of SSc, AS, GD and other autoimmune diseases. Unlike the neoplastic diseases, excessive activation of the immune system is in autoimmune diseases, therefore speculated that the distribution of CTLA-4 gene polymorphism in these two kinds of diseases in the opposite. Impact of genetic polymorphism in CTLA-4 expression in T lymphocytes in the pathogenesis of it. In addition, the distribution of the sites in different clinical risk in children is different, in high-risk groups TT, TC genotype frequency and allele T frequency was significantly increased, the difference was statistically significant (χ^2=2.625, P=0.03; χ^2=2.923, P=0.021).

It can be speculated that, compared to children with low risk, the site of children at risk for more regulation CTLA-4 expression on the surface of T lymphocyte activation, inhibition of signal to deliver a stronger, more obvious inhibition of T lymphocytes activation, anti-tumor weaker. On the other hand, presumably under different genetic background, the locus of control of CTLA-4 expression in children with higher risk, so after the onset showed clinical risk higher. Our previous study showed higher expression of CTLA-4, its clinical risk is high, confirmed this point.

Regulation of the CTLA-4 gene of another recognized polymorphism site is located in the first exon of the +49 locus. Exon 1 encodes the leading strand, leading strand can guide the synthesis of CTLA-4 molecular transport into the endoplasmic reticulum, and in the +49 locus (A/G) mutation leads to the leading strand seventeenth amino acid threonine to alanine, affect the leading chain conformation, thereby affecting the CTLA-4 molecular transport, so that the expression of CTLA-4 molecule on the surface and the reduced cell membrane. Polymorphism in exon 1 of CTLA-4 affecting CTLA-4 function: CTLA-4 exon 1 of G gene frequency was increased, the inhibitory function of the CTLA-4 of the corresponding reduction. Our study shows that differences in children with acute lymphoblastic leukemia +49 genotype and allele frequencies were not statistically significant (χ^2=6.365, P=0.24; χ^2=0.205, P=0.651), suggesting that the polymorphism in children with acute lymphoblastic leukemia risk not related.

The CT60 site is the recent discovery of a polymorphic loci, we investigate the relevance of this locus and B lymphocytic leukemia children, cases of GG genotype and G allele frequency was significantly increased, with significant difference (χ^2=6.365, P=0.041; χ ~ 2=6.761, P= 0.009). In according to the different clinical risk stratification of patients showing the differences between high and low risk group distribution were not statistically significant (χ^2=2.626, P=0.269; χ 2=2.923, P=0.087). This study is consistent with most domestic, suggesting that CTLA-4 gene may be a variety of human tumor susceptibility gene in common. Different from the research in Chinese population, the Cozar study showed that the frequency of AA genotype was higher in patients with renal cell carcinoma in a Spanish population of CT60 sites, but there was no obvious correlation in colon cancer. This polymorphism may play different roles in different tumors of different races, the specific mechanism is not clear. Ueda found that CT60 G allele and soluble CTLA-4 expression decreased, CT60 locus determines the efficiency and sCTLA splicing of mRNA production, so as to determine the soluble CTLA4 (sCTLA4) relative to the amount of full-length CTLA4 (flCTLA4), especially GG based homozygous for individuals with AA genotype, reduce sCTLA4 yield reduction. The CT60 G allele frequency of in case group than in the control group, which indicated that the level of sCTLA-4 patients decreased. Generally, the soluble form of surface membrane proteins and their competitive ligand binding and inhibition. Reduced levels of sCTLA-4, strengthen the interaction of B7-mCTLA4 in a certain extent, and induced the loss of T cell function, which directly inhibits the activation of T cells, and weakened the anti-tumor immunity of the body, eventually promoted tumor.

CONCLUSION

This study analyzes the relationship between CTLA-4 gene -318 site, +49 site, CT60 gene polymorphism and Chinese children with acute B lymphoblastic leukemia. The results showed that there were different -318 T allele, CT60 G allele distribution in the case group and the control group, suggesting that the two loci may be the susceptibility gene locus leukemia. But the sample size is relatively small, some genotypes and alleles were lower, more research sample size would be more convincing.

In addition, Ipilimumab, a monoclonal antibody blocking CTLA-4, has been approved by the FDA for the treatment of advanced melanoma. Can still needs to be further explored through polymorphism detection of CTLA-4 gene to evaluate the effect of the drug for the treatment of refractory leukemia for.

REFERENCES

He X, Jiang L, Fu B and Zhang X (2002). Relationship between interleukin-1b and interleukin-1 receptor antagonist gene polymorphisms and susceptibility to gastric cancer. *Chinese Med J-Peking,* **82**(10): 685-688.

Lang C, Chen L and Senlin L (2012). Cytotoxic T-Lymphocyte Antigen-4 + 49G/A Polymorphism and Susceptibility to Pancreatic Cancer. *Dna Cell Biol,* **31**(5): 683-687.

Ligers A, Teleshova N and Masterman T *et al*. (2001). CTLA-4 gene expression is influenced by promoter and exon 1 polymorphisms. *Genes Immun,* **2**(3): 145-152.

Piras G, Monne M and Uras A *et al* (2005). Genetic analysis of the 2q33 region containing CD28-CTLA-4-ICOS genes: association with non-Hodgkin's lymphoma. *Br. J. Haematol.,* **129**(6): 784-790.

Saito S, Kasai Y, Nomoto S *et al* (2001). Polymorphism of tumor necrosis factor in esophageal, gastric or colorectal carcinoma. *Hepato-Gastroenterol,* **48**(38): 468-470.

Wang XB, Zhao X and Giscombe R *et a1* (2002). A CTLA-4 gene polymorphism at position-318 in the promoter region affects the expression of protein. *Genes lmmurt.,* **3**(4): 233-234.

Youlin K, Li Z, Xiaodong W, Xiuheng L and Hengchen Z (2012). Combination immunotherapy with 4-1BBL and CTLA-4 blockade for the treatment of prostate cancer. *Clin Dev Immunol,* Epub 439235.