Association of gene polymorphism and serum levels of IL-12p40 with the susceptibility to non-Hodgkin’s lymphoma in Iraq

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(Submitted: 27 August 2017 – Revised version received: 02 September 2017 – Accepted: 11 October 2017 – Published online: 26 December 2017)

Introduction

Non-Hodgkin lymphomas are a diverse group of mature lymphoid neoplasms and it is the sixth most common cancer in the world, but it is well established that immune dysregulation especially by cytokines is one of major risk factors. This study investigates the association of genetic polymorphisms of IL-12P40 1188A/C and its circulating serum measurement in patients with advanced stage of NHLs in Iraq.

Methods

Fifty-five confirmed patients with advanced stages of NHLs and 40 apparently healthy individuals were used. Genetic polymorphism and circulating levels of IL-12p40 were examined by allele-specific polymerase chain reaction (AS-PCR) using specific primers and ELISA, respectively.

Results

The results of SNP IL-12P40 1188A>C had three genotypes, which were AA, AC and CC. These genotypes represented 32.73%, 38.18% and 29.09%, respectively, among NHL patients and 52.5%, 37.5% and 10%, respectively, among controls with a significant difference for the homozygous mutant genotype (OR = 4.667, 95% CI = 1.319–16.512, P = 0.017). Serum levels of IL-12p40 were significantly increased in an allele-dependent manner, which was linked to the CC homozygosity as being the highest.

Conclusions

This study has revealed a significant correlation between the gene polymorphisms of IL-12p40 and the induction of serum IL-12 with the risk to poor prognosis in patients with NHL. The correlation with the NHL grades and the prognostic value warrant further progressive investigation of developing prognostic biomarkers for NHL using patients at various disease stages.

Keywords

Non-Hodgkin's lymphoma, single nucleotide polymorphism, IL-12, allele-specific PCR

Materials and Methods

Study Subjects

This case control study included 55 patients with confirmed NHLs during the period from January 2015 to December 2015 from six teaching hospitals and medical centers in Baghdad Medical City (Teaching Hospital of Pediatric, Baghdad teaching Hospital and Hematology Center, Al-Mustansiriya University, Al-Kadhimiya Teaching Hospital). Family unrelated, apparently healthy 35 individuals were randomly selected to represent the control group. The mean ages of patients and control were 33.45 and 36.49 years, respectively. The stage of NHL disease of those recruited patients was mainly at the advanced stage of disease, so results will indicate this stage as such with no treatment history. This research was approved by the Ethics Committee of Scientific Research of the University. An informed consent to take part in the study was obtained from each participant after receiving approval of the experimental protocol by the Ethics Committee, which was essentially in line with the principles of the Declaration of Helsinki.
DNA Extraction and Genotyping

Three ml of venous blood was collected from each participant in EDTA tube. DNA was extracted from blood samples using ready kit (gSYNC™ DNA Mini Kit Whole Blood Protocol, Geneaid, Korea) according to the manufacturer’s instructions. Primers used for both genes are shown in Table 1. PCR conditions for IL-12p40 gene were an initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 1 min, and final extension at 72°C for 7 min. The primers of Toll-like receptor (TLR2) were used as an internal control in the amplification of IL-12p40 gene.

A ready 50 µl PCR master mix (Bioneer, Korea) was used for amplification for IL-12p40 gene. Template DNA (10 µl) from each sample and primers (5 µl from each) was added to each master mix tube. After mixing, the master mix tubes were transferred to the thermocycler (MyGenie 32 thermal block, Bioneer, Korea) which is previously programmed with the above protocol according to the gene to be amplified. The amplified products were determined by comparison with a commercial 1000 bp ladder (Kappa Biosystem, USA).

Statistical Analysis

The Statistical Package for the Social sciences (SPSS, version 14) was used for statistical analysis. The risk association between the genotype and NHLs susceptibility was estimated by the calculation of the adjusted odd ratio and 95% confidence intervals using bivariate logistic regression. For this analysis, subjects who were homozygous for the wild type allele were considered as a reference, and polymorphisms as dependent variables. Chi square test ($\chi^2$) was used to determine the significant difference between each two alleles. A $P$-value < 0.05 was considered statistically significant.

Results

Detection of IL-12p40 Polymorphism by Using Allele-Specific PCR

This study investigated the association of IL-12p40 1188A/C polymorphism with an incidence of NHL in patients and controls. A total of 55 NHL patients and 40 apparently healthy control individuals were recruited for this purpose.

Results of allele-specific PCR for the SNP IL-12p40 1188A/C in NHL patients and controls revealed specific amplification of 780 bp together with 254 bp of the internal control (Fig. 1).

IL-12p40 1188A/C had three genotypes which were AA, AC and CC. These genotypes represented 32.37%, 38.18% and 29.09%, respectively, in NHL patients and 52.5%, 37.5% and 10%, respectively, among controls. A significant difference for the homozygous mutant genotype CC (OR = 4.667, 95% CI = 1.319–16.512, $P$ = 0.017). At allelic level, the frequency of C allele in NHL patient was 48.18% compared with 28.75% in controls with significant difference (OR = 2.304, 95% CI = 1.250–4.249) (Table 2).

IL-12p40 Levels in NHL patients and controls

In regard to IL-12 production in this study, Figure 2 shows the mean serum levels of IL-12p40 in NHL patients and controls. NHL patients produced higher levels of IL-12p40

Table 1. Nucleotide sequences of primer sets and their corresponding genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers $5'\rightarrow3'$</th>
<th>Amplicon</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12p40</td>
<td>Consensus F: ATCTTGGAGCGAATGGGCAT</td>
<td>780 bp</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>R1: TTGTTTCAATGAGCATTTAGCATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R2: GTTTCAATGAGCATTTAGCATCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>F: CCTGGCAAGTGGACCATTCATCG</td>
<td>254 bp</td>
<td>[16]</td>
</tr>
<tr>
<td>(internal</td>
<td>R: GGCCACTCCAGTAGGTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F, forward primers set; and R, reverse primer pairs; TLR, toll-like receptor.

Fig. 1 Genotyping of IL-12p40 1188A/C allele’s distribution in patients with NHL by using allele-specific (AS)-PCR. Gel electrophoresis and detection method were as described for Figure 3-1. M: 100 bp DNA marker. The 780 bp represents the amplification of IL-12p40 1188A/C, while the 254 bp represents the amplification of TLR2 gene as an internal control. The IL-12p40 A/C run side-by-side on the gel and the lanes order for IL-12p40 A are 1, 3, 5, 7, and 9 whereas IL-12p40 C alleles are run in lanes 2, 4, 6, 8, and 10.
(156.44 ± 54.188 pg/ml) than the control group (107.34 ± 56.957 pg/ml) with a significant difference ($t = 4.136, P = 0.001$) (Fig. 2).

Analysis of the correlation of serum levels of IL-12p40 among the three genotypes (AA, AC and CC) in NHL patients revealed that CC genotype carriers had higher levels of IL-12p40 (119.51 ± 55.678 pg/ml) than either AC genotype carriers (138.68 ± 63.102 pg/ml) or AA genotype carriers (155.35 ± 61.877 pg/ml) with significant differences between CC and AA genotype ($P = 0.04$) (Fig. 3).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases $N = 55$</th>
<th>Control $N = 40$</th>
<th>$P$-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12p40 1188A/C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>18(32.73%)</td>
<td>21(52.5%)</td>
<td>0.056</td>
<td>1.0</td>
</tr>
<tr>
<td>AC</td>
<td>21(38.18%)</td>
<td>15(37.5%)</td>
<td>0.293</td>
<td>1.633(0.655–4.074)</td>
</tr>
<tr>
<td>CC</td>
<td>16(29.09%)</td>
<td>4(10%)</td>
<td>0.017</td>
<td>4.667(1.319–16.512)</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>57(51.82%)</td>
<td>57(71.25%)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>53(48.18%)</td>
<td>23(28.75%)</td>
<td>2.304(1.250–4.249)</td>
<td></td>
</tr>
</tbody>
</table>

N: total number; OR, odds ratio; CI, confidence interval; p, probability.
Discussion

The current cross-sectional study revealed that the SNP of IL-12p40 1188A/C (rs3212227) had three genotypes: AA, AC and CC. Both AC and CC genotypes were associated with a significant increased risk to NHL in studied patients at advanced stage of disease compared to healthy controls. The mean circulating serum levels of IL-12p40 in NHL patients had significantly higher levels than the control group. This elevation was linked with the CC homozygosity as being the highest inducer, and AA was the lowest.

IL-12 has a pleotropic activities where it plays an important role to induce Th1 immune response, and cancer immunotherapy. It also can stimulate IFN-gamma production, activation of cytotoxic T and natural killer cells, and impairs angiogenesis in tumours. It can suppress tumour development due to induction of apoptosis in cancerous cells. IL-12 therapy increased the median number of circulating CD8 T lymphocytes in patients with relapsed NHL.

In the present study, The SNP IL-12p40 1188AC/CC genotypes were associated significantly with an increased risk of NHL patients in Iraq population. There is a general consensus that AC/CC genotypes are associated with an increased risk to overall cancer development. This was exemplified by similar association between the C allele of IL-12p40 1188A/C polymorphism and bladder cancer, cervical cancer, nasopharyngeal carcinoma, and esophageal cancer. Notably, the 1188A/C polymorphism is also implicated in an increased risk for psoriasis and soriatic arthritis as well as type 2 diabetes susceptibility. It has been found that IL-12p40 of the circulating serum cytokines were significantly associated with FL and/or DLBCL. On the contrary, the serum IL-12p40 levels were significantly higher in controls than those in osteosarcoma patients with genotype CC and AC/CC were associated with the risk of osteosarcoma. Furthermore, 1188A variant was shown to be correlated with reduced levels of IL-12p40 subunit of IL-12, while 1188C variant associated with increase this subunit of the cytokine, which is similar to the finding of the present study. However, several analyses indicated that the C allele could lead to a decrease in the expression of IL-12p40, which consequently results in a lack of IL-12 protein. On the other hand, the results of this study are inconsistent with a previous study, where the A allele was found but not C to be associated with different cancers including the NHL and in TB infected patients, that resulted in lower plasma levels of IL-12 in normal control rather than in TB infected patients. Thus, it is expected there will be an increase of IL-12 associated CC genotype resulting in robust cell-mediated immune response, as with the current result that revealed a strong relationship C allele with high levels of IL-12 which was more prevalent in NHLs patients than in healthy controls.

The significance of IL-12p40 subunit in CC genotype carriers compared with either AC genotype carriers or AA genotype carriers indicates that the variant IL-12p40 1188C causes an increase in the production of this subunit in an allele-dependent manner, as clearly demonstrated by this study where the NHL patients with homozygous mutant genotype (CC) had the highest level of IL-12p40 subunit and have graded down with the heterozygous genotype (AC), to the lowest with the homozygous wild genotype (AA).

Whether this elevation can be attributed to the effective role of IL-12 alone as anti-tumour therapeutic, is still not well understood considering the complex nature of other activities of the immune system in cancers in general and NHLs in particular. With respect to the elevation of IL-12 and its association with C allele, this issue is still controversial. It gets more complicated when the elevation of total IL-12 levels was seen in lymph nodes tissue micro-environment, whether this overproduction includes IL12-p40 is not yet clear.

The discrepancy is that the increased production of IL-12 does not mean that IL-12p70 is overproduced. The induction rather involves only the IL-p40 subunit. The homodimers of this subunit antagonizes IL-12p70 activity by binding to the β1 subunit of the IL-12 receptor. Therefore, the increased production of this subunit, in fact, causes reduction in the activity of IL-12 and hence reduces the efficiency of cell-mediated response and increases the susceptibility to the malignancy. The SNP IL-12p40 1188A/C is located in the 3’UTR of the gene. This region although does not encode for a protein, it can influence the amount of translated protein through other mechanisms including effects on mRNA stability or on transcriptional activity. Thus the SNP affects the gene silences and could regulate the level of IL-12p40 mRNA expression. Moreover, there was no association between the IL-12p40 promoter genotypes and the severity of psoriasis was found. This SNP is linked to the susceptibility to psoriasis and specifically accompanied by the overproduction of IL-12p40, which suggests that IL-12p40 may act as a proinflammatory mediator. However, such effect is more likely not to be attributed only to mutant IL-12p40 rather than to be multifactorial and is warranted further analysis.

There are other heterodimeric cytokines in addition to IL-12p40, such as IL-23, IL-27, and IL-35, whose subunits consist of either or both the IL-12 p35 and p40 chains. Its high affinity to IL-23 receptor and hence abolish IL-23 role in immune response. The main role of IL-23 involves the stimulation of Th17 cells to produce IL-17, which has an important role in the immunity against NHL. Therefore, specific therapeutic antibodies for either IL-12 subunits may lead to the dysfunction of several cytokines during therapy. Similarly, inherited variations of IL-12 genes (particularly, functional SNPs) may dramatically alter the expression and/or protein structure of more than one cytokine. However, IL-12 therapy in NHL patients warrants further careful investigation of the drug candidates. It is important to note that the high levels of IL-12p40 were commonly seen in patients with a good prognosis, therefore it is suggested to serve as a tumour progression and/or prognostic marker. The latter aligned well the current study where the level of circulating IL-12p40 combined with its genetic polymorphisms can efficiently serve as a good prognostic marker. This conclusion needs further analysis after addressing the limitations of this study. There are two caveats in this study. First, patients recruited were from an advanced stage of NHL, so results won’t reflect disease stages/progression. Second, there were no results available on the pre-diagnosis cytokine levels of those patients. Therefore, these limitations will be warranted by designing a larger scale additional investigations to precisely define the correlation between genetic polymorphism, cytokine levels with NHL outcomes and subtyping. On the other hand, the genetic variants of IL-12p40 may or may not
have relationship with the fact that the tumour develops metastasis which may suggest its role, if any, in initiating the cause rather than a consequence of metastasis.\(^\text{50}\)

**Conclusion**

The SNP of IL-12p40 1188A/C had three genotypes: AA, AC and CC. Both AC and CC genotypes of the SNP IL-12p40 1188A/C were associated with a significantly increased risk to NHLs among Iraqi patients. The allele frequency distribution was also correlated with an overproduction of circulating IL-12p40 reaching its maximum levels with the CC mutant homozygosity in patients with advanced stage of NHL. Taken together, the results strongly suggest that the allele C of the SNP IL-12p40 1188A/C may serve as a contributing risk factor for poor prognosis in patients with NHL.

The correlation with the NHL grades and the prognostic value warrant further progressive investigation of developing prognostic biomarkers for NHL using patients at various disease stages.

**Acknowledgements**

The author would like to thank Ms. Mayasah Ali for her technical assistance and Dr. Qasim Sherhan for manuscript comments and data analyses.

**References**


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