Evaluation of rs3102735 and rs2073617 Osteoprotegerin Gene Polymorphisms and the Risk of Childhood Acute lymphoblastic Leukemia in Zahedan Southeast Iran

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Received: 27, Apr, 2014
Accepted: 30, Jun, 2014

ABSTRACT

Introduction: Osteoprotegerin (OPG), a soluble decoy receptor secreted by osteoblasts, binds RANK-L, preventing stimulation of osteoclastogenesis. In the present study we aimed to investigate the impact of OPG variants and susceptibility to childhood acute lymphocytic leukemia (ALL) in a sample of Iranian population.

Methods: This case-control study was done on 98 ALL and 124 healthy children. We genotyped the polymorphisms using tetra-primer ARMS-PCR (T-ARMS-PCR).

Results: Our findings showed that neither rs3102735 nor rs2073617 variants were associated with ALL in a sample of Iranian population. Concerning rs3102735 polymorphism, the age of ALL predispositions was significantly higher in TC+CC genotype than TT genotype (P=0.032). Furthermore, the CSF involvement was significantly higher in ALL subjects carrying TC+CC genotype (p=0.044).

Conclusion: We found no association between OPG (rs3102735, rs2073617) gene polymorphisms and risk of childhood ALL. Further studies with larger sample sizes and various ethnicities are necessary to verify our findings.

KEYWORDS: Acute lymphocytic leukemia, Osteoprotegerin, OPG, Polymorphism

INTRODUCTION

Acute leukemia is a rapidly progressing disease that produces cells that are not fully developed. Although ALL can occur at any age, it is the most common type of leukemia in children and young adults younger than 20 years. ALL is a biologically, clinically, and etiologically heterogeneous disease the causes of ALL are not clear. The occurrence of pediatric leukemia has been linked to several environmental, maternal, and paternal characteristics and exposure to various environmental factors.¹

Receptor activator of NF-κB (RANK), its ligand RANKL and osteoprotegerin (OPG) are involved in bone metabolism. A functional interaction between RANKL and RANK is crucial for osteoclast differentiation, survival and activation.² RANKL (also called TNF ligand super family member 11; TNFSF11), a type II homotrimERIC transmembrane protein, is expressed by osteoblasts, osteocytes, bone marrow stromal cells, T cells and numerous
tumor cells.\textsuperscript{3–6} The type-I homotrimeric transmembrane protein RANK also called tumor necrosis factor receptor super family member 11A (TNFRSF11A) is not only expressed by osteoclast, T cells, dendritic cells, endothelial cells, and mammary glands but also by cancer cells such as prostate and breast.\textsuperscript{7–11} It has been shown that RANK-deficient mice develop osteopetrosis resulting from a lack of osteoclasts and absence of bone resorption.\textsuperscript{12, 13} OPG is a secreted homodimeric glycoprotein from the TNF receptor family, lacking a transmembrane domain and has homology to the CD40 protein.\textsuperscript{14} OPG binds RANKL and prevent RANK-RANKL interaction, thus inhibiting osteoclastogenesis.\textsuperscript{5,15} Transgenic mice over expressing OPG show osteopetrosis,\textsuperscript{14} while OPG-deficient mice are characterized by massive osteoclast activity and osteoporosis.\textsuperscript{16} It has been proposed that OPG is a positive regulator of microvessel formation and promotion of neovascularization,\textsuperscript{17} therefore, it may influence tumor progression.

OPG (also called as TNFRSF11B) is located on chromosome 8q23–24. OPG, which acts as decoy receptor for RANKL, is a potent inhibitor of osteoclastic bone resorption and has been investigated as a potential therapeutic modality for the treatment of both osteoporosis and tumour-induced bone disease.\textsuperscript{2, 18–20}

There is little data regarding the role of OPG polymorphisms and cancer risk.\textsuperscript{21, 22} To the best of our knowledge there is no data regarding the association between OPG polymorphisms and childhood ALL risk. Therefore, in this study was aimed to investigate the impact of OPG rs3102735 and rs2073617 polymorphisms with minor-allele frequencies (MAF) greater than 10\% on ALL development in a sample of Iranian population.

MATERIALS AND METHODS

Patients

This case-control study was performed on 98 children diagnosed with ALL and 124 age and sex matched healthy children in Zahedan, southeast Iran. The study design and the enrolment procedure have been described in previous publication.\textsuperscript{23} Demographic and clinical data including age, sex, hemoglobin (Hb), WBC and platelet count at diagnosis, and the status of organomegally, LAP (lymphoadenopathy) and CSF (cerebrospinal fluid) were summarized in table 1. Local ethics committee of Zahedan University of Medical Sciences approved the project, and informed consent was obtained from parents of cases and controls. DNA was extracted from peripheral whole blood using salting out method as described previously.\textsuperscript{24}

Table 1. Clinical characteristics of patients with childhood acute lymphoblastic leukemia

| Characteristics          | Values |
|--------------------------|--------|
| Age at diagnosis         | 6.23±3.82 |
| Sex (Male/Female)        | 58/40  |
| WBC ($\times 10^3$)      | 34.11 ± 50.8 |
| HB (mg/dL)               | 7.54±2.42 |
| PLT ($\times 10^6$)      | 59.74±48.58 |
| Organomegally Positive   | 80 (81.6\%) |
| Negative                 | 17 (17.3\%) |
| Lymphoadenopathy Positive| 55 (56.1\%) |
| Negative                 | 42 (42.9\%) |
| CSF involvement Positive | 6 (6.1\%)  |
| Negative                 | 92 (93.9\%) |

Genotyping

The OPG genomic sequence (NT-008046) was obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The polymorphisms were searched and primers were designed for T-ARMS-PCR, which is a simple and rapid technique for recognition of single nucleotide polymorphism (SNPs).\textsuperscript{25–27} Two external primers (control band) and two allele-specific primers (inner primers) are used for detection of each SNPs (table 2). The location of SNPs and T-ARMS-PCR assay are shown schematically in Figure 1.

PCR reactions consisted of a total volume of 20 µL containing 250 µM dNTPs, 0.5 µM of each primer, 1.5 mM MgCl$_2$, 1 U Taq DNA polymerase, and 50 ng genomic DNA. The PCR cycling conditions were initial denaturation at 95°C for 5 min followed by 30 cycles for rs3102753, 35 cycles for rs2073617, 35 cycles for rs3102735 at 95°C for 30s and, annealing temperature 30s at 59°C
for rs3102735, 66°C for rs2073617 and 30 s at 72°C, with a final extension of 72°C for 10 min. The PCR products were verified onto 2% agarose gels containing 0.5µg/ml ethidium bromide, and observed under UV light. For rs3102735 T/C variant, the product sizes were 202-bp for T allele, 299-bp for C allele and 435-bp for control bond (Figure 2). The product sizes were 263-bp for G allele, 345-bp for A allele and 562-bp for control band for rs2073617 polymorphism (Figure 3). To ensure genotyping quality, approximately 20% of random samples were regenotyped and found no genotyping error.

Table 2. Primer sequences for detection of OPG gene polymorphisms rs3102735 and rs2073617 polymorphisms

| Primers             | Sequence (5’→3’) | Size (bp) |
|---------------------|------------------|-----------|
| rs3102735 T/C       |                  |           |
| FO                  | TAAAGCGGCTGTATTCTGCATTTC | 453bp     |
| RO                  | AAGCGCATTTGCGCTCTCCTGG |           |
| FI (T allele)       | GGGTCGGCTCTGCCCCCCTT | 202 bp    |
| RI (C allele)       | TCAAGCTAACTTCTAGACCCGGGAAGTG | 299bp    |
| rs2073617 G/A       |                  |           |
| FO                  | GAGGTTCGGAGACACGCTGGAGCC | 562bp     |
| RO                  | CACACAGTGGGCACCTCCGGTG |           |
| FI (G allele)       | GGGGGTGTGCAGAAAGCTCCATGG | 263bp   |
| RI (A allele)       | GCCCCAGCCTGAAAGCGTTATG | 345bp     |

Statistical analysis

Statistical analysis was done by statistical package SPSS 18 software. Data were analyzed by independent sample t-test and χ2 test. Odds ratio (OR) and 95% confidence intervals (95% CI) were calculated from logistic regression analyses to find out the possible association between the variants and ALL. A p-value less than 0.05 were considered statistically significant.

RESULTS

The study group involved 98 ALL patients (58 male, 40 female; age: 6.2±3.7 yrs) and 124 healthy subjects (58 male, 66 female; age: 5.8±2.2 yrs).
significant difference was found between the groups concerning age (p=0.272) and sex (p=0.074). The frequencies of allelic/genotypic of OPG rs3102735 and rs2073617 polymorphisms are shown in Table 3. The results showed that no significant association was found between OPG polymorphisms and ALL risk in our population. While in rs3102735 polymorphism, the age of predisposition to ALL was significantly higher in TC+CC genotype than TT genotype (P=0.032). Furthermore, the CSF involvement was significantly higher in ALL subjects carrying TC+CC genotype (p=0.044). No relation was denoted between the OPG rs2073617 variant and clinical characteristics of ALL children (table 4).

The genotype frequency of the OPG polymorphisms was examined for Hardy-Weinberg equilibrium (HWE). The rs3102735 and rs2073617 polymorphisms in controls were in HWE ($\chi^2=0.82$, p=0.364 and $\chi^2=0.23$, p=0.629, respectively).

**DISCUSSION**

OPG that encoded by the TNFRSF11B gene is a key negative regulator of osteoclastogenesis. It binds to RANKL preventing it from activating RANK. Genetic polymorphisms within the TNFRSF11B, RANK and RANKL genes have been extensively studied not only for association with osteoporosis but also with other disorders such as rheumatoid arthritis, cardiovascular disease and cancer metastasis.

In this study we examined the impact of OPG rs3102735 and rs2073617 polymorphisms on the risk of childhood ALL in a sample of Iranian population. The results showed no association between the polymorphisms and ALL in our population. Regarding the rs3102735 polymorphism, the age of predisposition to ALL was significantly higher in TC+CC genotype than TT genotype (P=0.032). Additionally, ALL subjects carrying TC+CC genotype had significantly CSF involvement (p=0.044).

There is little data regarding the role of OPG polymorphisms and cancer risk. Ney et al have found that OPG rs3102735 variant increased the risk of breast cancer in Caucasian. No significant association was found between 149 T/C and 950 T/C polymorphisms in the putative promoter region of OPG and prostate cancer. However, those patients with TC and TT genotypes in the 950 T/C polymorphism had a significantly increased risk of extraprostatic and metastatic disease.

Sonmez et al have investigated the OPG gene variants C950T (promoter), C1181G (exon 1), and myeloma bone disease. They found that 1181 G/950 T alleles and 950 TT/1181 GG genotypes might play a role in the development of bone disease.

Ney et al have investigated OPG rs3102735 and rs2073618 polymorphisms in breast cancer. They found that rs3102735 variant increased the risk of breast cancer. It has been shown that OPG rs10505346 polymorphism is associated with prostate specific antigen (PSA) level and could be a prognostic factor for the recurrence of PSA in prostate cancer patients receiving radical prostatectomy. Narita et al have found no association between 149 T/C (rs3134071) and 950 T/C polymorphisms and prostate cancer. While, those patients with TC and TT genotypes in the 950 T/C polymorphism had a significantly increased risk of extraprostatic and metastatic disease.

In conclusion, our finding showed that OPG1 polymorphisms were not associated with the risk of ALL in a sample of Iranian population. Subjects carrying rs3102735 TC+CC genotype had significantly higher age of ALL predispositions as well as CSF involvement. Larger sample sizes with different ethnicities are required to validate our findings.

**Table 3. Genotypic and allelic frequencies of OPG1 polymorphisms in ALL patients and control subjects**

| OPG1 polymorphisms | ALL n (%) | Control n (%) | OR (95%CI) | P-value |
|---------------------|-----------|---------------|------------|---------|
| rs3102735           |           |               |            |         |
| TT                  | 83 (84.7) | 105 (84.7)    | 1.00       | -       |
| TC                  | 10 (10.2) | 19 (15.3)     | 0.66 (0.29-1.59) | 0.421   |
| CC                  | 5 (5.1)   | 0 (0.0)       | -          | -       |
| TC+CC               | 15 (15.3) | 19 (15.3)     | 1.0 (0.48-2.08) | 0.981   |
| T                   | 176 (89.8)| 229 (92.3)    | 1.00       | -       |
| C                   | 83 (41.8) | 115 (46.4)    | 1.37 (0.71-2.64) | 0.339   |
| rs2073617           |           |               |            |         |
| AA                  | 32 (32.7) | 37 (29.8)     | 1.00       | -       |
| AG                  | 50 (51.0) | 59 (47.6)     | 0.98 (0.53-1.79) | 0.947   |
| GG                  | 16 (16.3) | 28 (22.6)     | 0.66 (0.30-1.43) | 0.333   |
| AG+GG               | 66 (67.3) | 87 (70.2)     | 0.88 (0.49-1.55) | 0.664   |
| A                   | 114 (58.2)| 133 (53.6)    | 1.00       | -       |
| G                   | 82 (41.8) | 115 (46.4)    | 0.83 (0.57-1.21) | 0.386   |
Table 4. Association between OPG polymorphisms with clinical demographic and characteristics of ALL patients

| Genotype | Age | Sex | WBC | Hb | PLT | LAP | Organomegally | CSF involvement |
|----------|-----|-----|-----|----|-----|-----|---------------|----------------|
|          |     |     | M   | F  |     | Yes | No | Yes | No | Yes | No |
| rs3102735 |     |     |     |    |     |     |    |     |    |     |    |
| TT       | 5.9±3.6 | 48 | 35 | 32.0±45.3 | 7.7±2.4 | 62.0±50.1 | 48 | 35 | 69 | 14 | 3 | 80 |
| TC+CC    | 8.2±4.3 | 10 | 5  | 45.3±66.3 | 6.7±2.7 | 45.9±36.9 | 7  | 7  | 11 | 3  | 3 | 12 |
| P-value  | 0.032 | 0.581 | 0.356 | 0.192 | 0.172 | 0.772 | 0.707 | 0.044 |
| rs2073617 |     |     |     |    |     |     |    |     |    |     |    |
| AA       | 6.4±4.4 | 20 | 12 | 40.3±64.0 | 7.7±1.9 | 64.7±60.9 | 17 | 15 | 29 | 3  | 3 | 29 |
| AG+GG    | 6.1±3.5 | 38 | 28 | 31.3±43.7 | 7.5±2.7 | 57.5±42.4 | 38 | 27 | 51 | 14 | 3 | 63 |
| P-value  | 0.724 | 0.668 | 0.427 | 0.725 | 0.521 | 0.667 | 0.166 | 0.389 |

ACKNOWLEDGEMENTS
This work was funded by a dissertation grant (MSc thesis of ME) from Zahedan University of Medical sciences. The authors thank to the patients and healthy subjects who willingly participated in the study.

CONFLICT OF INTEREST
All authors declare no conflict of interest.

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