Case control study

Tumor necrosis factor and lymphotoxin-alpha genetic polymorphisms and risk of relapse in childhood B-cell precursor acute lymphoblastic leukemia: a case-control study of patients treated with BFM therapy

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Abstract

Background: Circulating levels of tumor necrosis factor (TNF) and lymphotoxin-α (LT-α) have been associated with outcome in solid and hematologic malignancies. Within the TNF gene and the LT-α gene, polymorphisms have been identified at nucleotide positions -308 and +252, respectively. The variant alleles for TNF are designated TNF1 and TNF2, the ones for LT-α LT-α (10.5 kb) and LT-α (5.5 kb). Of interest, TNF2 and LT-α (5.5 kb) were shown to be associated with higher TNF and LT-α plasma levels than their counterparts. In the present study, we investigated the associations of the above mentioned polymorphisms with risk of relapse in childhood acute lymphoblastic leukemia (ALL) treated according to Berlin-Frankfurt-Münster (BFM) protocols.

Methods: Matched case-control study of 64 relapsed and 64 successfully treated non-relapsed childhood B-cell precursor ALL patients of standard and intermediate risk for treatment failure.

Results: The odds ratio (OR) for the combined category of TNF1/TNF2 and TNF2/TNF2 genotypes in comparison to the TNF1/TNF1 genotype was 1.17 (95% confidence interval (CI) = 0.53 - 2.56, P = 0.697). The ORs for the LT-α (10.5 kb/5.5 kb) and the LT-α (5.5 kb/5.5 kb) genotypes with reference to the LT-α (10.5 kb/10.5 kb) genotype were 2.17 (95% CI = 0.84 - 5.58, P = 0.107) and 0.5 (95% CI = 0.09 - 2.66, P = 0.418), respectively.

Conclusions: Our results do not suggest a major role of the investigated genetic polymorphisms with regard to risk of relapse in standard- and intermediate-risk childhood B-cell precursor ALL treated according to BFM protocols.

Background

Tumor necrosis factor (TNF) and lymphotoxin-α (LT-α; formerly TNF-β) are cytokines with pleiotropic biological activities including, for example, the induction of programmed cell death and the regulation of immune cell proliferation and differentiation [1,2]. In a variety of studies, plasma levels of TNF or LT-α have been associated with outcome of certain autoimmune and infectious diseases as well as solid and hematologic malignancies [3–6]. Of interest, the secretion of TNF and LT-α is believed to be influenced by genetic polymorphisms within their genes located tandemly on the long arm of chromo-
some 6 within the MHC class III region. One of the best described of these polymorphisms is located at nucleotide position-308 within the TNF promoter region and affects a consensus sequence for a binding site of the transcription factor AP-2 [7,8]. Guanine at position-308 defines the common TNF1 allele and adenine the less common TNF2 allele. With regard to the LT-α gene, a polymorphism at nucleotide position 252 within the first intron was reported to influence LT-α plasma levels. This single nucleotide polymorphism (A252G) affects a phorbol ester-responsive element and distinguishes two alleles that have been designated LT-α (10.5 kb) and LT-α (5.5 kb) [9]. Both the TNF2 and the LT-α (5.5 kb) allele have been shown to correlate with elevated TNF or LT-α plasma levels. Besides a more severe outcome of autoimmune or infectious diseases and of particular interest to us, the TNF2 and the LT-α (5.5 kb) alleles have been associated with an adverse outcome in lymphoid malignancies [[10–15]].

In the present study, we genotyped a matched case-control study group of 64 relapsed and 64 non-relapsed patients with childhood acute lymphoblastic leukemia (ALL) for the above described genetic polymorphisms within the TNF and LT-α genes in order to assess their predictive potential with regard to relapse in childhood ALL.

Table 1: Characteristics of 64 relapsed case subjects and 64 successfully treated matched control subjects with acute lymphoblastic leukemia selected from trials ALL-BFM 86 and ALL-BFM 90

|                      | Cases (%) | Controls (%) |
|----------------------|-----------|--------------|
| Trial                |           |              |
| ALL-BFM 86           | 35 (54.7) | 35 (54.7)    |
| ALL-BFM 90           | 29 (45.3) | 29 (45.3)    |
| Sex                  |           |              |
| Male                 | 42 (65.6) | 42 (65.6)    |
| Female               | 22 (34.4) | 22 (34.4)    |
| Age (y)              |           |              |
| <1                   | 1 (1.6)   | 1 (1.6)      |
| 1-9                  | 56 (87.5) | 56 (87.5)    |
| 10-14                | 6 (9.4)   | 7 (10.9)     |
| 15-18                | 1 (1.6)   | -            |
| WBCa (10^3/µl)       |           |              |
| <10                  | 42 (65.6) | 41 (64.1)    |
| 10-<50               | 21 (32.8) | 22 (34.4)    |
| ≥ 50                 | 1 (1.6)   | 1 (1.6)      |
| Immunophenotype      |           |              |
| c-ALLb               | 54 (84.4) | 54 (84.4)    |
| pre-B-ALLc           | 10 (15.6) | 10 (15.6)    |
| Risk groupd          |           |              |
| standard             | 23 (35.9) | 23 (35.9)    |
| intermediate         | 41 (64.1) | 41 (64.1)    |
| high                 | -         | -            |
| DNA indexe           |           |              |
| <1.16                | 30 (46.9) | 30 (46.9)    |
| ≥ 1.16               | 12 (18.8) | 7 (10.9)     |
| not examined         | 22 (34.4) | 27 (42.2)    |
| Genotype             |           |              |
| normal               | 4 (6.3)   | 10 (15.6)    |
| 11q23 aberrations    | -         | 1 (1.6)      |
| t(1;19)              | 1 (1.6)   | -            |
| t(9;22)              | -         | -            |
| other                | 15 (23.3) | 13 (20.3)    |
| not examined         | 44 (68.8) | 40 (62.5)    |

a white blood cell count b common acute lymphoblastic leukemia c precursor B-cell acute lymphoblastic leukemia d therapy stratification in risk groups was mainly based on initial leukemic cell mass estimate and initial treatment response [16–18] e ratio of DNA content of leukemic G0/G1 cells to normal diploid lymphocytes
Methods

Patients and study design
The present study utilizes patients and data from the ALL-BFM 86 and ALL-BFM 90 multicenter trials of childhood ALL, conducted by the BFM study group. Design, conduct, analysis, and results of the ALL-BFM 86 and ALL-BFM 90 trials are described in detail elsewhere [16,17]. In both trials treatment was stratified into three branches (standard, intermediate, and high risk), mainly according to the leukemic cell mass estimate and treatment response. Treatment (in most cases induction, consolidation, reinduction, maintenance) consisted of intensive multiagent chemotherapy regimens employing standard drugs (e.g. prednisone, vincristin, daunorubicin, L-asparaginase, cyclophosphamide, cytarabine, 6-mercaptopurine, 6-thioguanin, methotrexate). Parts of the study group received cranial radiotherapy.

The establishment of the present case-control study group has been described previously [18]. Briefly, relapsed patients from ALL-BFM 86 and ALL-BFM 90 with an available remission peripheral blood or bone marrow smear were included as cases into the study group if they could be matched to a successfully treated patient with an available remission peripheral blood or bone marrow smear (control individual) according to the following criteria: sex, age at diagnosis (± 6 months), white blood cell count (WBC) at diagnosis (± 10,000/µl), immunophenotype, trial, risk group, and treatment arm within the risk group of the respective trial. The latter criterion assured similarity of treatment between cases and controls. Controls had to have a minimum follow-up of 5 years. In case of relapses occurring later than 5 years of diagnosis, the follow-up for the control subject had to be at least as long as the time from date of initial diagnosis to date of relapse diagnosis in the case subject. If more than one control subject was available, the subject with the closest initial WBC at diagnosis with reference to the case subject was chosen. All spare remission peripheral blood or bone marrow smears were derived from official routine remission control examinations at time points during the first 6 month of treatment according to the study protocols of ALL-BFM 86 and 90.

Genotype analysis
Genomic DNA was isolated from remission bone marrow or peripheral blood smears as described before [18]. Genotypes for TNF and LT-α were determined by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis. The -308 TNF promoter polymorphism was analyzed by incorporating it into an Ncol restriction site by introducing a single base change within the forward primer [7]. Primer sequences were: forward 5'-AGCAATAGTTTGTAGCCAT-3'; reverse 5'-TCCTGCCTGATCCG-3'. The LT-α polymorphism at nucleotide position +252 was analyzed by PCR amplification of a 368 bp fragment using the following primer pair: forward 5'-CTCCTGCACCTGCTGCCTGGATC-3'; reverse 5'-GAAGAGACGTTCAGGTGGTGTCAT-3' [10]. The amplified PCR products were digested overnight with Ncol and analyzed on 3.0 % Nusieve (TNF) or 3.0 % conventional agarose gels (LT-α). In case of presence of the TNF1 allele, the amplified 107 bp fragment from the TNF promoter is cut into two fragments of 87 and 20 bp, a fragment amplified from TNF2 remains uncut [7]. The amplified PCR products were digested overnight with Ncol and analyzed on 3.0 % Nusieve (TNF) or 3.0 % conventional agarose gels (LT-α). In case of presence of the TNF1 allele, the amplified 107 bp fragment from the TNF promoter is cut into two fragments of 87 and 20 bp, a fragment amplified from TNF2 remains uncut [7]. The 368 bp fragment from LT-α is unaffected by Ncol digestion in case of presence of a LT-α (10.5 kb) allele while a PCR product amplified from a LT-α (5.5 kb) allele is cut into two fragments of 133 and 235 bp [10].

Table 2: Distribution of tumor necrosis factor (TNF) and lymphotoxin-α (LT-α) genotypes and their association with the occurrence of relapse in 64 case subjects and 64 successfully treated matched control subjects with acute lymphoblastic leukemia from ALL-BFM trials 86 and 90

|                      | Cases (%) | Controls (%) | ORa (95 % CIb) | P value |
|----------------------|-----------|--------------|----------------|---------|
| TNF                  |           |              |                |         |
| TNF1/TNF1            | 41 (64.1) | 43 (67.2)    | 1.00           |         |
| TNF1/TNF2            | 21 (32.8) | 20 (31.3)    | 1.17c (0.53-2.56) | 0.697  |
| TNF2/TNF2            | 2 (3.1)   | 1 (1.6)      |                |         |
| LT-α                 |           |              |                |         |
| 10.5 kb/10.5 kb      | 25 (39.1) | 30 (46.9)    | 1.00           |         |
| 10.5 kb/5.5 kb       | 32 (50.0) | 23 (35.9)    | 2.17d (0.84-5.58) | 0.107  |
| 5.5 kb/5.5 kb        | 7 (10.9)  | 11 (17.2)    | 0.50d (0.09-2.66) | 0.418  |

a odds ratio b confidence interval c odds ratio for the combined category of TNF1/TNF2 and TNF2/TNF2 genotypes d odds ratio for the combined category of LT-α (10.5 kb/5.5 kb) and LT-α (5.5 kb/5.5 kb) with reference to LT-α (10.5 kb/10.5 kb) was 1.5 (95% CI = 0.68-3.32; P = 0.317)
**Statistical analysis**

After frequencies were calculated for descriptive purposes, correlation analyses (contingency coefficients for nominal data, Spearman correlation coefficients for ordinal data, Pearson correlation coefficients for continuous data) were computed to investigate the interrelationships between TNF genotype, LT-α genotype and important clinical prognostic variables such as sex, age at diagnosis, WBC at diagnosis, and immunophenotype. Differences in the distribution of categorical variables were analyzed by X² or Fisher’s exact test. The association between TNF and LT-α genotypes and relapse of leukemia was examined by use of conditional logistic regression analysis to calculate odds ratios and their 95% confidence intervals. Genotypes and genotype combinations were used as categorical variables in the analyses. The association of genotypes with time to relapse was analyzed by log rank tests. Computations were performed using SAS software (SAS-PC Version 6.04, SAS Institute Inc., Cary, NC).

**Results and Discussion**

Table 1 shows the distribution of matching variables and genetic analyses (DNA index and karyotype) in the 64 re-lapsed case subjects and the 64 successfully treated control subjects as previously described [18]. Within our entire study population of 128 patients, 84 (65.6%) patients were homozygous for the TNF1 allele, 41 (32.0%) patients were heterozygous (TNF1/TNF2) and 3 (2.3%) patients were homozygous for the TNF2 allele. With regard to LT-α, we observed 55 (43.0%) patients being homozygous for the LT-α (10.5 kb) allele and 55 (43.0%) patients being heterozygous (LT-α (5.5 kb)/ LT-α (5.5 kb)). Eighteen (14.1%) patients were homozygous for the LT-α (5.5 kb) allele. The prevalences observed in our study are similar to the ones reported by Demeter et al. in a healthy German control sample [15]. Thus, it seems unlikely that TNF or LT-α genotypes are associated with the pathogenesis of childhood B-cell precursor ALL in the German population. With regard to clinically important variables, no particular associations between the TNF and LT-α genotypes and sex, age at diagnosis, WBC at diagnosis, immunophenotype, and risk group were observed (data not shown). Table 2 shows the prevalences of the investigated TNF and LT-α genotypes by case-control status of our study subjects. In addition, Table 2 contains information on the association of the -308 TNF promoter polymorphism and the intronic LT-α polymorphism at nucleotide position 252 with risk of ALL relapse in our study subjects. For both genotypes, no particular associations with risk of ALL relapse were observed (Table 2). As reported in previous studies, the TNF1 allele was significantly linked to the LT-α (10.5 kb) allele as was the TNF2 allele to the LT-α (5.5 kb) allele (X² test; \( P < 0.01 \)) [10,13]. The strong association between polymorphic TNF and LT-α alleles made Warzocha and colleagues to use haplotypes for their outcome analysis in a study on lymphoma outcome related to TNF and LT-α alleles [10]. In their study, the authors found that the presence of at least two TNF or LT-α high-producer alleles (TNF2 or LT-α (5.5 kb)) was an independent risk factor for progression-free survival in adult diffuse large-cell lymphoma patients [10]. When we analyzed the risk of relapse conferred by so-called high-producer TNF/LT-α haplotypes (at least two high-producer alleles) in comparison to low-producer TNF/LT-α haplotypes (less than two high-producer alleles) we also failed to observe any meaningful association in our study population (odds ratio (OR) = 1.08; 95% confidence interval (CI) = 0.51 - 2.30; \( P = 0.841 \)). However, based on the discordant pairs observed in our study, we only had a power of 80% to detect risks greater than 3.0 conferred by the TNF1/TNF2 and the LT-α (10.5 kb)/LT-α (5.5 kb) genotypes or by the high-producer TNF/LT-α haplotypes. Therefore, we neither can exclude smaller effects on treatment outcome mediated by the above mentioned exposures nor reasonably exclude clinically important effects of the rare TNF2/TNF2 or the LT-α (5.5 kb)/LT-α (5.5 kb) genotype. Furthermore, we investigated possible influences of the analyzed TNF and LT-α genotypes on time to relapse or on site of ALL relapse (isolated bone marrow relapse; combined and isolated CNS relapse; combined and isolated testis relapse) and, as above, did not detect any associations.

From the data presented in this study, we are not able to generalize our findings to childhood ALL patients of all immunophenotypic subgroups since we only investigated common and pre-B-cell ALLs. Similarly, we were not able to assess an association of the investigated TNF and LT-α genotypes on risk of relapse in high-risk childhood ALL patients since also these patients were not part of our study group. The latter point may be interesting to pursue as Demeter and colleagues, in a study on TNF and LT-α polymorphisms in chronic lymphocytic leukemia (CLL), detected an increase of the LT-α (10.5 kb) allele at more advanced disease stages [15]. Thus, additional investigations including childhood ALL patients of all clinically relevant subgroups are needed to lead to more conclusive results. However, for the subgroup of childhood B-cell precursor ALL of standard and intermediate risk treated according to BFM regimens that was analyzed in the present study, the investigated genetic TNF and LT-α polymorphisms do not seem to play a major role with regard to risk of relapse.

**Conclusions**

In a matched case-control group of 64 relapsed and 64 successfully treated childhood B-cell precursor ALL patients (all at standard or intermediate risk), the TNF gene polymorphism at nucleotide position -308 and the
LT-α gene polymorphism at nucleotide position +252 were not significantly related with risk of ALL relapse. Our results do not suggest a major role of the investigated genetic polymorphisms with regard to risk of relapse in childhood B-cell precursor ALL of standard and intermediate risk treated according to BFM protocols.

Competing interests
None declared.

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