Polymorphism in cytokine genes as prognostic markers in Hodgkin’s lymphoma


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Background: In Hodgkin’s lymphoma (HL), the production of cytokines by Reed–Sternberg cells and the surrounding tissue is thought to contribute to the biology of the disease. Cytokine expression can be altered by common single nucleotide polymorphisms (SNPs) in the 5'-promoter regions.

Patients and methods: We studied polymorphic allele variants of the cytokine genes interleukin (IL)-10 (T-3575A, G-2849A, C-2763A, A-1082G and C-592A), IL-6 (G-174C) and tumor necrosis factor-α (C-863A and G-308A) in 184 patients with HL, and analyzed for associations with treatment outcome.

Results: Carriers of the IL-10-592AA and the IL-6-174GG genotypes had a significantly lower probability of freedom from treatment failure (FFTF) with adjusted hazard ratios (HRs) for failure of 2.92 [95% CI (confidence interval) 1.58–5.41, P = 0.001] and of 1.75 (95% CI 1.04–2.92, P = 0.03), respectively. Reconstructing haplotypes from the five SNPs in the IL-10 promoter revealed that homozygous carriers of the IL-10.4 haplotype (T-G-C-A-A) had a worse FFTF (HR, 2.35; 95% CI 1.2–4.6, P = 0.01). In the Cox multivariate analysis, the IL-10-592AA, the IL-6-174GG genotypes and stage were independent prognostic factors.

Conclusions: Our study indicates that cytokine genotypes predict clinical outcome in patients with HL and points to the importance of the genetic background of the host for treatment response.

Key words: cytokine genes, Hodgkin’s lymphoma, IL-10, polymorphism, prognosis

introduction

Hodgkin’s lymphoma (HL) is characterized by rare neoplastic cells, the Hodgkin and Reed–Sternberg (HRS) cells, surrounded by a massive inflammatory infiltrate, mainly consisting of T cells, eosinophils and fibroblasts. The T-cell infiltrate in HL is characterized by a shift from a T helper 1 (Th1) immune response to T cells with T helper 2 (Th2) and regulatory T-cell characteristics, which play a pivotal role in immune suppression and induction of anergy [1]. These alterations are thought to create an immunologic favorable environment for the growth of the HRS cells. The interactions between the neoplastic and the reactive cells are mediated by cytokines and chemokines, which mediate proliferation and suppression of apoptosis of the HRS cells [2, 3]. Among cytokines implicated in the pathogenesis of HL, HRS cells have been shown to produce interleukin (IL)-10, IL-6 and tumor necrosis factor (TNF)α [2].

IL-10 is of particular interest in the immunopathology of HL as it is a critical anti-inflammatory cytokine known to suppress Th1-like immune responses and promote Th2 responses [2, 3]. IL-10 is produced by Th2 cells, B cells, monocytes and macrophages, and it antagonizes the production of proinflammatory cytokines, including TNF, IL-1 and IL-6, inactivating antigen presentation and cell-mediated immune response.

Conversely, IL-10 enhances humoral immunity by promoting the proliferation of B- and T cells. IL-10 has been shown to be expressed both by HL cell lines and HRS cells of 21%–36% of primary HL samples [2]. HRS cells are often latently infected by the Epstein–Barr Virus (EBV) and express several EBV-encoded viral proteins. IL-10 is more often expressed in EBV+ cases, which contain a higher percentage of IL-10+ HRS cells than the EBV− cases, suggesting that IL-10 may protect the EBV+ HRS cells from immune attack [4]. Moreover, IL-10 increases BCL-2 levels in B-, T cells and hematopoietic cells and protects them from apoptosis induced by glucocorticoids, doxorubicin and deprivation of growth factors. Elevated plasma levels of IL-10 have been reported in 30%–50% HL patients and have been associated with advanced stage of disease and poor clinical outcome [5–7].

Between 50% and 75% of the variability in IL-10, secretion is explained by genetic factors [8]. Nucleotide variations in the gene such as single nucleotide polymorphisms (SNPs) may affect transcription, translation or function of the gene product.
The IL-10 gene is known to contain several SNP, the most investigated in the promoter region are situated at positions -1082 and -592, numbered relative to the transcriptional start site in the proximal promoter and at positions -3575, -2849, -2763 in the distal promoter [9–14]. Several SNP are in linkage disequilibrium, and together they combine into the haplotypes commonly present in the population [10, 11]. So far, it has been difficult to determine the exact relationship between IL-10 genotypes and the corresponding cytokine production; it is thought that the combination of different polymorphisms into haplotypes is an important determinant [12–14].

Here, we studied the genotypes of several polymorphisms in the IL-10, the IL-6 and TNFα genes in patients with HL, their association to patient characteristics and their role in patients’ clinical outcome.

patients and methods

patient characteristics

Our analysis included 184 patients (median age 32 years, range 14–77 years, 80 females and 104 males), diagnosed with HL from October 1984 to March 2005. The ethnic background was rather homogeneous: 183 patients were Caucasians, of whom 176 were Italians and one patient was African. Further patient characteristics are detailed in Table 1. Peripheral blood samples were obtained at the time of initial diagnosis in patients enrolled from 1999 on or during follow-up visits in remission or for relapse in patients treated before 1999. In 46 patients, DNA from archived lymph node biopsies was used. Therapy consisted of a combination with doxorubicin, bleomycin, vinblastine, dacarbazine (ABVD) in 108 patients, 17 patients were treated with combination therapy with mechloethamine, vincristine (Vincristina, Farmacia Italia SpA), procarbazine (Natulan, Sigmatea SpA), and prednisone-containing regimens and two patients received a combination therapy with etoposide, epirubicin, bleomycin, cyclophosphamide and prednisolone (VEBEP) [15, 16]. Patients with advanced stage disease (stage IIB with bulky disease IV) and C60 years of age were treated from 1999 to 2001 with a modified Stanford V regimen (substituting 6 mg/m² mechloethamine with 650 mg/m² cyclophosphamide (Endoxan, Baxter SpA)) (34 patients), and from October 2001 on with combination therapy with bleomycin (Belomicina, Aventis Pharma SpA), etoposide (Vepesid, Bristol-Myers Squibb Srl), doxorubicin (Adriblastina, Farmacia Italia SpA), cyclophosphamide, vincristine, procarbazine and prednisone (20 patients) [17, 18]. Three patients were treated with radiotherapy only, otherwise radiotherapy was included for consolidation in patients with limited stage disease and initial bulky disease.

Follow-up in failure-free patients was 5.8 years (median range 1.5–22.5 years). Patients were regularly evaluated every 3 months during the first 2 years, every 6 months up to 5 years and once a year up to 10 years. Follow-up visits included physical exam, routine laboratory tests and radiological exams as chest X-ray and abdominal ultrasound, while computed tomography scans were performed on clinician’s judgment. Patients with a follow-up of >10 years were contacted by telephone and visited on clinical suspect. Despite this program, 10 patients were lost to follow-up at a median of 5.5 years (range 3–11.3 years).

DNA extraction and amplification

DNA was extracted from peripheral blood leukocytes using DNAzol (Invitrogen, Carlsbad, CA), as previously described [19]. In 46 patients, DNA was extracted from paraffin-embedded diagnostic lymph node biopsies at diagnosis using QIAamp DNA mini kit (QIAGEN, Milan, Italy). All polymorphisms were evaluated by PCR amplification and restriction fragment length analysis (PCR-RFLP) according to previously published protocols with some minor modifications [20, 21]. In five patients, DNA was available both from mononuclear cells and from paraffin-embedded tissues and yielded the same genotyping results.

In brief, we used 50–100 ng of starting DNA, 300–400 nM of each primer and 1x HotMasterMix (Eppendorf, Hamburg, Germany) for each reaction. Thermal cycling consisted of an initial DNA denaturation at 95°C for 5 min and subsequent steps of 95°C for 1 min, 54–62°C (varying according to PCR, see below) for 1 min and 72°C for 1 min, repeated for 35–40 cycles, with a final extension step at 72°C for 10 min. IL-10 distal promoter polymorphisms (T-3575A, G-2849A, C-2763A) were genotyped according to the method described by Moraes et al. [19] using Tsp509I as restriction enzyme (New England Biolabs Ipswich, MA). A nested PCR for the simultaneous genotyping of IL-10 G-2849A and C-2763A was performed using the same conditions described above, with the exception of restriction digestions with AlwI (New England Biolabs) for G-2849A and Ddel (New England Biolabs) for C-2763A. This method did not always give reproducible results from paraffin-embedded tissues, and therefore data for IL-10-2849 and -2763 is missing for 36 patients.

For the analysis of IL-10 G-1082A, C-592A and TNFα G-308A SNPs, we used the multiplex mismatched PCR-RFLP assay, described by Tseng et al. [20]. PCR products were digested simultaneously using EcoNI (New England Biolabs) and fragments obtained were identified on 4% agarose gel.

Table 1. Characteristics of 184 patients with Hodgkin’s lymphoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number</th>
<th>%</th>
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<tr>
<td>Age, years (n = 184)</td>
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<td></td>
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<tr>
<td>&lt;45</td>
<td>133</td>
<td>72</td>
</tr>
<tr>
<td>&gt;45</td>
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<td>Gender (n = 184)</td>
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<tr>
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<td>Histotype (n = 178)</td>
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<td></td>
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<tr>
<td>Other histotype</td>
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<tr>
<td>Stage (n = 184)</td>
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<td></td>
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<tr>
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<td>IIB–IV</td>
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<tr>
<td>B symptoms (n = 184)</td>
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<td></td>
</tr>
<tr>
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<tr>
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<td>Therapy (n = 184)</td>
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<td></td>
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<td>Stanford V</td>
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<td>BEACOPP</td>
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<td>1</td>
</tr>
<tr>
<td>RT only</td>
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<td>2</td>
</tr>
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<td>1994–1998</td>
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<td>20</td>
</tr>
<tr>
<td>1999–2005</td>
<td>119</td>
<td>65</td>
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</table>
in situ hybridization for EBV

Samples from 71 HL patients were analyzed for EBV infection. In situ hybridization of EBV-encoded small RNAs (EBERs) on formalin-fixed, paraffin-embedded tissue sections was performed using a cocktail of fluorescein isothiocyanate-labeled oligonucleotides complementary to the two nuclear EBER (1/2) RNAs, following the manufacturer’s instructions (Dako; Dakopatts, Golstrup, Denmark), and as previously described [21]. A case of infectious mononucleosis served as positive control for EBV; an intestinal biopsy of an individual with a negative serology for EBV served as negative control.

statistical analysis

Haplotypes for the IL-10 promoter region were inferred using PHASE, version 2.1 [22], including all five loci in sequence. Haplotype numbering is according to Eskdale et al. [11] and Kurreman et al. [12]. Linkage disequilibrium was estimated by calculating Lewontin’s D’ coefficients for each pair of loci using the Arlequin program. Fisher’s exact test was used to examine for associations of IL-10 genotypes with patient characteristics. The primary survival end point was freedom from treatment failure (FFTF), with progression during treatment, lack of complete remission at the end of first-line treatment, relapse and death from any cause counted as adverse events. Survival curves were estimated using the Kaplan–Meier product limit method. Kaplan–Meier plots presented are unadjusted survival curves. Log-rank tests were used for analysis for differences in FFTF and Cox proportional hazards model for relative risk of failure. As patients have been treated with different regimens during different time periods (Table 1), all analyses were adjusted for kind of chemotherapy and era of enrolment. Cox proportional hazards model was also used for multivariable analysis including SNPs and patient characteristics that had prognostic significance in the univariate analysis (IL-10-592, IL-6-174 and stage). Computations were performed using the Stata 6.0 software (Stata Corp., College Station, TX).

results

allele and genotype frequencies of cytokine gene polymorphisms and clinical outcome in HL

We studied polymorphisms at five positions in the 5’-region of the IL-10 gene (-3575, -2849, -2763, -1082, -592), at two positions in the TNFα gene (-863 and -308) and at one position in the IL-6 gene (-174), in 184 patients with HL. Genotype distribution and allele frequency of the polymorphisms in the patient cohort are given in Table 2. Using the Fisher’s exact test, we found no associations between polymorphisms and patients characteristics as age, gender, histotype, stage and B symptoms. We studied the EBV status in 71 samples from HL patients. EBV was detected in Hodgkin and Reed–Sternberg cells in 20 of 71 (28%) cases tested. No associations between EBV and cytokine polymorphisms were detected.

We next analyzed whether the cytokine gene polymorphisms were associated with prognosis. Homozygous carriers of the IL10-592A allele had a decreased FFTF (Figure 1A). The adjusted probability of failure-free survival at a median time of observation of 5 years for homozygous IL-10-592AA patients was 20%, while it was 62% for heterozygous and for 77% for homozygous patients for the -592C allele (P = 0.002, log-rank test; Table 2). The adjusted hazard ratio (HR) for failure of IL-10-592AA was 2.92 [95% CI (confidence interval), 1.58–5.41, P = 0.001]. The genotypes at position -1082, -2763, -2849 and -3575 in the IL-10 did not influence failure-free survival (Table 2).

The IL-6-174GG genotype was also associated with poor failure-free survival (P = 0.03) (Figure 1B). The adjusted HR for failure of IL6-174GG was 1.75 (95% CI 1.04–2.92, P = 0.03). No associations between genotype distributions of TNFα at position -308 and -863 and prognosis was found. Among clinical parameters, stage only had a strong prognostic impact (P = 0.02). The Cox multivariate analysis showed that the IL-10-592AA, the IL-6-174GG genotype and stage were independent prognostic factors (P = 0.001, 0.01 and 0.02, respectively) (Table 3).

linkage disequilibrium and haplotype structure in the IL-10 gene and prognosis in HL

As there is strong linkage disequilibrium between the polymorphic sites in the promoter region of the IL-10 gene, we reconstructed haplotypes from the single SNPs using the PHASE program, to analyze whether haplotypes would be
more informative than single SNPs with respect to prognosis. Haplotype diversity was limited: five frequent (>5%) haplotypes described 86% of the observed haplotypic variation over the five SNP loci. The A allele at position -592 is in strong linkage disequilibrium with other polymorphic allele sites in the IL-10 promoter, in particular the A allele at position -1082 (Lewontin’s D’ coefficient: 0.94). These alleles in combination with the -3575T, the -2849G and the -2763C alleles form the IL-10.4 haplotype (T-G-C-A-A). Homozygous carriers of the IL-10.4 haplotype had a worse FFTF (P = 0.01). (Figure 2). The adjusted 5-year FFTF probability was 35% for homozygous carriers of the IL-10.4 haplotype and 68% for patients with no or one IL-10.4 allele. The other major haplotypes (IL10.2, IL10.3, IL10.5 and IL10.1) had no impact on FFTF.

Including the IL10.4 and IL-10-592 AA genotype into a multivariate Cox analysis, only the IL-10-592 AA maintained independent prognostic significance (P = 0.3 and P = 0.005, respectively), indicating that this polymorphism is the single most powerful predictor of failure-free survival among IL-10 polymorphisms and that the haplotype analysis did not give additional information.

discussion

Cytokines play a pivotal role in the pathogenesis of HL by acting in a highly complex, coordinated network [2]. IL-10 is a regulatory cytokine, whose main role in vivo is to limit inflammatory response. IL-10 and its polymorphisms have been reported to play a role for both the susceptibility and prognosis of various diseases, including infectious and autoimmune diseases [10, 23]. Recently, IL-10 polymorphisms have been associated with acute graft-versus-host disease, transplant-related mortality and survival after allogenic stem-cell transplantation [24]. IL-10 polymorphisms can influence the risk of developing non-HL [25, 26]. For HL, there are only few data on limited patient numbers [27, 28].

When looking at prognosis, Lech-Maranda et al. [25] showed that patients with diffuse large B-cell lymphoma carrying the homozygous IL10-1082AA genotype had a poorer freedom from progression and overall survival in comparison to carriers of the G allele. We show that homozygous carriers of the A allele at position -592 in the IL-10 promoter region is a negative prognostic factor for failure-free survival.

Since combination of genotypes into haplotypes may be even more important for the production of cytokines, we extended our analysis to other polymorphisms in the IL-10 promoter region. The IL-10.4 haplotype which includes the A allele at

<table>
<thead>
<tr>
<th>Variable</th>
<th>P</th>
<th>Adjusted HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10-592 AA versus AC/CC</td>
<td>0.001</td>
<td>2.88</td>
<td>1.53–5.41</td>
</tr>
<tr>
<td>IL-6-174 GG versus GC/CC</td>
<td>0.01</td>
<td>1.94</td>
<td>1.15–3.28</td>
</tr>
<tr>
<td>Stage IIb-IV versus I-IIIA</td>
<td>0.02</td>
<td>2.28</td>
<td>1.15–4.53</td>
</tr>
</tbody>
</table>

*Explains the adjustment of the hazard ratio. Hazard ratios are adjusted for kind of chemotherapeutic regimen and era of enrolment. One hundred and eighty-three patients were included. FFTF, freedom from treatment failure; HR, hazard ratio; CI, confidence interval.
position -592 was associated with poor outcome. However, after adjusting for stage, the IL-10-592 genotype remained the strongest predictor for treatment failure.

Some limitations of our study have to be considered. It is a retrospective study, and for this a potential bias in patient selection cannot be ruled out. In the attempt to avoid a bias for selecting surviving patients only, we also genotyped DNA from 46 archived lymph node biopsies, including 12 deceased patients. Analysis of failure-free survival according to the time of enrolment suggested that there was no significant selection of patients with favorable prognosis (data not shown). Given the heterogeneity in type and period of treatment, we adjusted all analyses for these two factors. Moreover, limiting the analysis to 108 patients treated with ABVD and stratifying for the period of treatment, the IL-10-592 AA genotype conferred a risk for failure with a HR of 3.71 (95% CI 1.88–7.33, \( P < 0.001 \)), the IL-6-174 GG genotype conferred a HR of 1.94 (95% CI 1.01–3.74, \( P = 0.047 \)) and the HR for the homozygous IL10.4 haplotype was 2.91 (95% CI 1.4–6.0). The IL10-592 and the IL-6-174 genotypes maintained their prognostic significance also in the multivariate analysis including stage as the most important clinical prognostic factor; however, independence from the international prognostic score (IPS) could not be assessed as many factors necessary for IPS calculation were not available for analysis.

So far, it has been difficult to determine the exact relationship between IL-10 genotype and the corresponding cytokine production, and conflicting results have been reported. A recent study [10, 12] on the genetic control of IL-10 production. Some authors have reported increased IL-10 production in vitro associated with the presence of the -1082G allele or G-C-C haplotype (defined by three SNPs at positions -1082, -819 and -592 of the IL-10 gene) [10, 12]. Other investigators, however, reported that the -1082G allele and A-A-G-C-C haplotype (defined by five SNPs at positions -3575, -2763, -1082, -819 and -592) is associated with decreased IL-10 production [9, 13]. These differences may be due to differences in the cell type and kind of stimulation, or differences in the cell source from normal individuals or patients, whose immune functions may be altered by the disease. Studies including a higher number of patients and prospectively collecting serum samples will be necessary to assess the relationship between genetic polymorphism and cytokine levels in HL.

In addition to the IL-10 genotype, the IL-6 polymorphic site at position -174 had a prognostic role. Patients carrying the high-producing homozygous IL-6-174G allele had a poorer treatment outcome. Higher expression of IL-6 may confer a growth advantage for HRS cells. Recent publications have identified the high-producer IL-6-174G allele as a risk factor for HL. In a study on twins, Cozen et al. [29] observed that an increasing number of C alleles at position -174 was associated with a significantly decreased risk of HL. Cordano et al. [30] observed a decreased risk for HL in young adults with increasing C alleles without reaching statistical significance. In contrast to our findings, the latter authors reported that overall and disease-specific survival of these patients from the Scotland and Newcastle Lymphoma Group Registry did not differ according to the IL-6 genotype [30].

It has been suggested that EBV-positive HL is associated with delayed exposure to EBV in young adults, and to the loss of the normal balance between latent EBV infection and immunity in older adults. Since cytokines, and in particular IL-10, are believed to play an important role in EBV infections [23], we looked at IL-10 genotypes in 22 EBV-positive and 50 -negative cases. We found no differences in the genotype frequencies.

In conclusion, our study indicates that cytokine genotypes, in particular the IL10 -592 genotype, predict clinical outcome in patients with HL and points to the importance of the genetic background in determining patients’ prognosis. Larger, prospective HL studies are needed to confirm our findings.

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references


