Interleukin-10 -1082 G/A gene polymorphisms in Egyptian children with CAP
A case–control study

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Abstract
Community-acquired pneumonia (CAP) is one of the leading causes of death worldwide. Cytokines are involved in the pathogenesis of CAP. To date, only a few studies concerned the association of interleukin-10 (IL-10) gene polymorphisms with CAP.

In this study, we aimed to investigate whether the -1082(G/A) polymorphism in the promoter region of the IL-10 gene is involved in susceptibility to and the outcome of CAP, and we also measured the serum level of IL-10 to assess its relation to such polymorphism.

This was a case–control study included 100 patients with CAP, and matched with age, gender, and ethnicity of 100 healthy control children. IL-10 -1082(G/A) gene polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism, while the serum IL-10 levels were measured by ELISA method.

Compared to the controls subjects, the frequencies of the IL-10 -1082 AA genotype and A allele were observed to be overrepresented in patients with CAP (51%; odds ratio [OR] = 2.8; 95% confidence interval [CI]: 1.5–5.3 for the AA genotype; P < 0.01) and (70%; OR: 1.95; 95% CI: 1.27–3.00 for the A allele; P < 0.01, respectively). We found that patients with the GG genotype had significantly higher serum IL-10 levels (46.7 ± 9.5 pg/mL) compared to those with AG genotype (21.8 ± 4.5 pg/mL) and AA genotype (11.5 ± 3.3 pg/mL). P < 0.01, respectively. Our data revealed a significant positive association between the -1082 GG genotype and susceptibility to severe sepsis, acute respiratory failure, and hospital mortality (OR: 3.8; 95% CI: 1.3–11.2; P < 0.01).

We demonstrate for the first time, to the best of our knowledge, that IL-10 -1082 (G/A) gene polymorphism may contribute to susceptibility to CAP in Egyptian children. Moreover, we observed that the presence of a G allele or GG genotype at the -1082 position of the promoter region of the IL-10 gene constitute risk factors for developing severe sepsis, acute respiratory failure, and hospital mortality among patients with CAP.

Abbreviations: ARF = acute respiratory failure, CAP = community-acquired pneumonia, CI = confidence interval, IL-10 = interleukin-10, OR = odds ratio, SIRS = systemic inflammatory response syndrome, SNP = single nucleotide polymorphism.

Keywords: children, community-acquired pneumonia, gene polymorphisms, interleukin-10

1. Introduction

Community-acquired pneumonia (CAP) is one of the most common and serious infections in children, with a prevalence of 34 to 40 cases per 1000 in industrialized countries. In the developing world, CAP is even more common and more severe and is the largest killer of children. Lung injury resulting in acute respiratory failure (ARF) is the primary complication of CAP. The mechanism underlying lung injury is complex and involves a variety of molecular and cellular processes that may be
2. Methods

This was a prospective case–control study performed in Zaqazig University Children Hospital, and outpatient clinics in the same hospital from August 2013 to October 2015. One hundred children, who had CAP as diagnosed in the Department of Pediatrics in the same hospital, were enrolled in this study. The age of the patients ranged from 2 months to 13 years (median, 3.4 years). Diagnosis of CAP was defined by previously published guidelines;[11] an acute illness of less than 14 days of symptoms, the presence of a new chest radiographic infiltrate or consolidation confirmed by a radiologist trained in reading and interpreting radiographs according to the WHO guidelines[12] (blinded to the patient genotype), and clinical features compatible with pneumonia. The clinical features required were 1 of the following: fever more than 37.8°C, hypothermia less than 36°C, peripheral blood count >10,000/µL or <4500/µL or >15% immature neutrophils; and 2 of the following: tachypnea (respiratory rate >2 SD from the mean for age), dyspnea, or hypoxemia (pulse oximetry <94% on room air on initial evaluation without a known mixing heart lesion). Pneumonia was considered as community-acquired if the patient had no history of hospitalization during the 2 weeks prior to admission.[13]

2.1. Exclusion criteria

1. Patients hospitalized within the past 30 days
2. Clinical diagnosis of bronchiolitis
3. Children with a preexisting lung disease, particularly asthma
4. Postoperative children
5. Patients receiving treatment with corticosteroids equivalent to prednisolone 20 mg/day for more than 14 days
6. Children with a congenital heart disease, or a chronic liver or kidney disease
7. Patients with immuno deficiency
8. Patients who have undertaken chemotherapy or immunosuppressive drugs in the past 60 days

The following data were collected at admission: sociodemographic data, comorbidities, and prehospital admission treatment. Severity of pneumonia was assessed for each child on admission.

2.2. Severity criteria

Patients were further subdivided according to the severity criteria sourced from the management guidelines of the British Thoracic Society.[13] Any of the following led to a classification of “Severe disease”: tachypnea (RR > 70 for infants < 1 year old, RR > 50 for children > 1 year old), dyspnea, oxygen saturation <92%, oxygen given, nasogastric feeds, intravenous fluid infusion, septicemia, empyema, high dependency, or intensive care. “Mild” included immediate home discharge or hospital stay of < 3 days and no oxygen, no intravenous or nasogastric feeds, or ‘Moderate’ with neither category (e.g., body temperature = 38.5°C, RR between 50 and 70 breaths/min, moderate recession or breathlessness, taking infrequent feeds, and vomiting but no signs of dehydration). Patients were subjected to clinical, laboratory, and radiological follow-ups to detect any improvements. The occurrence of ARF or severe sepsis was identified to quantify illness severity. The endpoints of the clinical outcome were defined as intensive care unit admission and mortality.

In the presence of a documented infection, severe sepsis was defined as SIRS in combination with organ failure.[14] ARF was defined as an oxygen saturation <90% on room air, or a PaO2 <60 mmHg.

One hundred healthy children, of comparable age and gender, who attended Pediatric Department for preoperative evaluation for elective surgery, were enrolled as a control group. Patients and controls belonged to the same ethnic group: African Caucasian. All patients and controls included were subjected to proper history taking, thorough clinical examination. Laboratory investigations were done for all studied patients and included: complete blood count (CBC) including blood indices, ESR and C-reactive protein (CRP), sputum and blood culture and sensitivity tests, and liver function and kidney function tests.

2.3. Blood sampling

Blood samples were drawn from all subjects at admission and divided into 2 portions: 2 mL of whole blood was collected into tubes containing EDTA, for genomic DNA extraction. Serum was separated immediately from remaining part of the sample and stored at −20°C till the time of analysis.
2.4. Genomic DNA extraction
Genomic DNA from venous blood samples of CAP patients and healthy controls were extracted using a genomic DNA extraction kit (Puregene Blood Kit, Gentra, Valencia, CA) according to the manufacturer’s protocol. DNA quantification was done using an Eppendorf BioPhotometer (NY). DNA was stored at −20°C.

2.5. IL-10 Genotyping
All subjects were genotyped for IL-10 polymorphism by polymerase chain reaction–restriction fragment length polymorphism. For the polymorphism at position -1082 of IL-10, a 238 base-pair region was amplified by using the sense primers 5’TTCCCGAGGTAGAGCAGCCT-3’ and the antisense primer 5’GATGGGGTGGAAGTTGAA-3’ as described before.[13] A 2.5-μl PCR reaction mixture contained 10 ng of genomic DNA, 10 pmol of each of 5’- or 3’- primer, 100 μmol/L dNTP, 50 mmol/L KCL, 10 mmol/L Tris-HCL (pH 8.3) 1.5 mmol/L MgCl2 and 1 U of AmpliTaq polymerase. Amplifications were performed using a Perkin-Elmer 2400 thermal cycler according the following parameters: 95°C for 3 minutes followed by 29 cycles of 95°C for 30 seconds, 64°C for 20 seconds, and 72°C for 30 seconds. A final extension at 72°C was performed for 10 minutes. PCR products were digested overnight at 37°C using 2.5 U of restriction enzyme. The digested products were analyzed on a 2% agarose gel stained with ethidium bromide.

2.6. Measurement of serum interleukin-10 (IL-10) levels
IL-10 plasma levels were measured using an enzyme-linked immunosorbent assay (ELISA; CLB, Pelikine Compact human IL-10 ELISA kit, Amsterdam, The Netherlands). The sensitivity of the assay was 1 pg/mL and the assay was performed according to the manufacturer’s instructions. The manufacturer reports an intraassay coefficient of variation of less than 10% and an interassay coefficient of variation of less than 10%.

2.7. Statistical analysis
IL-10 -1082G/A genotype and allele frequencies in patients and controls were tested for Hardy–Weinberg equilibrium. Chi-square test was used to determine differences in the frequencies of the different IL-10 -1082G/A genotypes between patients and controls and between clinical outcomes within CAP patients. In case of statistically significant results, logistic regression analysis was performed with the significant variable in combination with clinical characteristics of the disease. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated for disease susceptibility and CAP outcome in relation to the IL-10 -1082G/A polymorphism. The Student t test and analysis of variance were used to compare numeric variables within groups, depending on the distribution of the data. P value < 0.05 was considered to be statistically significant. All data were analyzed using the Epi Info statistical software (version 6.2, World Health Organization, Geneva, Switzerland).

2.8. Ethics
Informed parental consent was obtained to be eligible for enrollment into the study. The study was done according to the rules of the Local Ethics Committee of Faculty of Medicine, Zagazig University, Egypt. Our institutional review committee of ethical research approved the study.

3. Results
Our study included 100 patients with CAP, their age ranged from 2 months to 13 years (median 3.4 years). Of these patients, 53 were males (53%) and 47 were females (47%). The control group included 100 healthy controls. Our study included 100 patients with CAP, their age ranged from 2 months to 13 years (median 3.4 years). Of these patients, 53 were males (53%) and 47 were females (47%). The control group included 100 healthy controls. The IL-10 -1082 genotype distribution differed between patients and controls. The AA homozygous genotype was overrepresented (51%) among CAP patients, while the AG genotype was underrepresented (5%) compared to the control group (9%).

The distribution of IL-10 -1082G/A genotypes, alleles, and serum IL-10 levels in patients with CAP and controls are summarized in Table 1. Both groups were in Hardy–Weinberg equilibrium, with no significant Chi-squared values for the observed and expected genotype frequencies.

The IL-10 -1082 G/A genotype distribution differed between patients with CAP and healthy controls. The AA homozygous genotype was overrepresented (51%) among CAP patients, compared with controls (27%). Homozygous subjects had a 2.8-fold increased risk of developing CAP (OR = 2.8; 95% CI: 1.5–5.3; P < 0.01), while heterozygous AG/GG were not representative for CAP patients (OR = 0.36; 95% CI: 0.19–0.67; P < 0.01).

Table 1: Demographic and clinical characteristics of patients with CAP.

| Characteristics                          | n (%)      |
|-----------------------------------------|------------|
| Age, median (range)                     | 3.4 (2 months–13 years) |
| Gender                                  |            |
| Male                                    | 53 (53)    |
| Female                                  | 47 (47)    |
| Pneumonia severity                      |            |
| Mild                                    | 39 (39)    |
| Moderate                                | 35 (35)    |
| Severe                                  | 26 (26)    |
| ICU admission                           |            |
| No                                      | 75 (75)    |
| Yes                                     | 25 (25)    |
| Severe sepsis                           |            |
| No                                      | 79 (79)    |
| Yes                                     | 21 (21)    |
| Acute respiratory failure               |            |
| No                                      | 88 (88)    |
| Yes                                     | 12 (12)    |
| Hospital mortality                      |            |
| No                                      | 92 (92)    |
| Yes                                     | 8 (8)      |

CAP = community-acquired pneumonia, ICU = intensive care unit.

Our data revealed that patients with CAP had significantly higher serum IL-10 levels compared to the control group (21.5 ± 4.7 vs 6.7 ± 1.5; P < 0.01), Table 2.

In patients with the GG genotype, the frequency of severe sepsis (54.5%) was significantly higher than in patients with the AG (28.9%) and AA genotypes (7.8%). AA genotype was protective.
Distribution of IL-10 (-1082) A/G genotypes, alleles and serum IL-10 levels in patients with CAP and controls.

| Genotype        | Patient group n (100) % | Control group n (100) % | OR (95% CI) | P      |
|-----------------|-------------------------|-------------------------|-------------|--------|
| IL-10 (-1082)   |                         |                         |             |        |
| AA              | 51 (51)                 | 27 (27)                 | 2.8 (1.5–5.3) | <0.01  |
| AG              | 38 (38)                 | 55 (65)                 |             |        |
| GG              | 11 (11)                 | 18 (18)                 |             |        |
| Alleles         |                         |                         |             |        |
| A               | 140 (70)                | 109 (54.5)              | 1.95 (1.27–3.0) | <0.01  |
| G               | 60 (30)                 | 91 (45.5)               |             |        |
| Serum IL-10 (pg/mL) | 21.5 ± 4.7          | 6.7 ± 1.5               |             | <0.01  |

Values in parentheses are percentages or data are presented as mean ± SD. *P value < 0.05 indicates a significant difference. Chi-square test. CAP = community-acquired pneumonia, CI = confidence interval, IL-10 = interleukin-10. OR = odds ratio, SD = standard deviation.

AA genotype was associated with serum IL-10 levels (21.5 ± 4.7 pg/mL) compared to AG genotype (11.5 ± 3.3 pg/mL) and GG genotype (11.2 ± 2.3 pg/mL), P < 0.01, respectively. The frequency of ARF in patients with the AA genotype was significantly higher (11.5 ± 4.5 pg/mL) compared to those with AG genotype (5.3 ± 2.3 pg/mL) and GG genotype (5.0 ± 2.2 pg/mL), P = 0.0015.

**4. Discussion**

CAP is one of the leading causes of death worldwide despite advances in diagnostic methods, antimicrobial, and intensive care treatment. A variety of pro- and anti-inflammatory cytokines are involved in the regulation of the inflammatory response to pulmonary infections. Among these cytokines, IL-10 is the most potent anti-inflammatory cytokine as it downregulates the release of proinflammatory cytokines and chemokines, prevents antigen-specific T-cell activation, inhibits T-cell expansion, and potentiates the release of inflammatory modulator IL-1 receptor antagonist. It also has immunosuppressive properties. IL-10 plays a key role in the pathogenesis, severity and the outcome of SIRS, sepsis, and septic shock. Studies concerning CAP-associated lung injury in children are limited, conflicting results are often inferred from adult studies.
Because of potential immune-modulatory effects of IL-10 and its importance as a major antiinflammatory cytokine, IL-10 gene polymorphisms might affect individual susceptibility to CAP, severity of illness, and the outcome of disease.

In the present study, we found a significant difference in the genotype and allele frequency of IL-10 -1082G/A polymorphism between children with CAP and the control group. The AA genotype and A allele were overrepresented in patients with CAP compared to the control group. In addition, we observed that individuals with the AA genotypes had a 2.8-fold higher risk for developing CAP, thus revealing that patients were more susceptible to CAP. This finding has never been reported in children with CAP.

In an attempt to explain our results, we studied the serum levels of IL-10 in our patients which were significantly elevated in comparison to the control group. Our finding was in agreement with Glynn et al.[18] who studied circulating IL-10 in 38 adult patients with CAP compared to 25 healthy subjects. They reported that plasma IL-10 levels were higher in patients with CAP suggesting a potential immune-modulatory role for IL-10 in controlling the inflammatory cytokine response in CAP. Furthermore, we observed that our patients with the GG genotype had significantly higher serum IL-10 levels compared to those with the AG and AA genotypes.

These results were concordant with those of Stanilova et al.[20] who reported that the A to G switch at position -1082 of the IL-10 gene was associated with increased IL-10 production, and the AA, AG, and GG genotypes being associated with low, intermediate, and high IL-10 production, respectively. We suppose that the low production of IL-10 observed in our patients with the AA genotype at the onset of infection may be a reason for the production of a high quantity of proinflammatory cytokines and the development of CAP.

The contribution of IL-10 to the immune response during respiratory infections has been evaluated on different models with varying results. Recent experimental work[21] confirmed that the absence of IL-10 in the early stages of pneumococcal pneumonia renders the host more susceptible to death, due to excessive neutrophil recruitment into the lung and production of proinflammatory cytokines, which lead to excessive inflammatory response, which can be associated with a poor prognosis. In other words, a genetically predetermined antiinflammatory cytokine profile may lead to a compensatory immunosuppression response and contribute to progression to sepsis and its complications.[15] However, future more extended studies are needed to refine this assumption.

In accordance with our results, Glynn et al.[18] found that plasma IL-10 levels were higher in patients with CAP who met the criteria for SIRS compared to patients who did not. The authors concluded that IL-10 concentrations correlated with severity of illness and may be of prognostic importance. Wu et al.[17] reported that cytokine expression was markedly increased in rapidly fatal cases of CAP, but only BAL IL-8 and BAL IL-10 were significantly higher in the rapidly fatal group than in the late mortality group. This research revealed that both local and systemic IL-10 appear to be good markers for predicting mortality in severe CAP with ARF without response to initial treatment. Gogos et al.[24] added that in severe sepsis, sustained
overproduction of IL-10 was the main predictor of severity and fatal outcome. On the contrary, Lowe et al reported that the A allele of the -592 polymorphism, but not other SNPs in the IL-10 gene promoter, was associated with lower stimulated IL-10 release and increased mortality in critically ill patients. Our results were also different from those of Montón et al who studied both lung and systemic inflammatory responses in severe pneumonia, but found no evident association between lung and systemic cytokine levels and clinical outcome, a finding which may be due to their small sample size.

Discrepancies between previously published studies and ours might be explained by the differences in age, study design or geographic/ethnicity, or by gene–gene or gene–environmental interactions. The genetic predisposition to CAP could be polygenic, with many variants in multiple gene loci, playing an important role. However, it is possible that IL-10 -1082G/A SNP may not be functional by itself, but may be in linkage disequilibrium with other functional mutations as several mutations occurring in this promoter region have been associated with various diseases. In other words, this polymorphism may either have a direct effect on transcription or represent linkage disequilibrium with another yet-to-be-identified marker. Further measurements of circulating IL-10 levels and genotyping of other IL-10 promoter polymorphisms (such as microsatellites, -819 [C/T] and -592 [C/G]) should be examined in CAP to confirm our findings in different populations.

A few studies in the literature concerned the association of human IL-10 gene polymorphisms with the susceptibility to CAP, clinical course, and the outcome of disease. To the best of our knowledge, ours is the first such study performed in an Egyptian population. However, the small sample size was one of our limitations in this study; we suggest that multicenter approaches may be necessary to attain larger sample size. Another limitation in our study was that cytokine profile was not measured serially during the course of illness. Therefore, the patterns of cytokine expression could not be determined to know whether sustained overproduction of IL-10 associated with the IL-10 -1082 GG genotype is a major predictor of fatal outcome. Further studies and more genetic information on ethnicities from different parts of the world will provide an additional understanding of the possible role of cytokine gene polymorphisms in CAP aiming to improve diagnosis, assess severity, and thus seek for new treatment modalities of such disease.

5. Conclusion

We demonstrate for the first time, to the best of our knowledge, that IL-10 -1082G/A gene polymorphism may contribute to susceptibility to CAP in Egyptian children. Moreover, we observed that the presence of a G allele or GG genotype at the -1082 position of the promoter region of the IL-10 gene constitute risk factors for developing severe sepsis, ARF, and hospital mortality among patients with CAP.

Finally, it is supposed that genetic information will be used in the future by clinicians to define different subtypes of the disease and to stratify CAP patients according to their risk for poor outcome. Genotyping will also be used to determine optimal drugs and dosage for treating patients while minimizing adverse effects.

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