ORIGINAL ARTICLE

Association of interferon γ $T^{+874}A$ and interleukin 12 p40 promoter CTCTAA/GC polymorphism with the need for respiratory support and perinatal complications in low birthweight neonates

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Background: Data support the role of interferon (IFN)γ and interleukin (IL)12 in perinatal complications. IFNγ $T^{+874}A$ and IL12 p40 promoter CTCTAA/GC polymorphisms may have an effect on cytokine production.
Methods: DNA was extracted from dried blood samples of 153 low birthweight (LBW) infants and 172 healthy term infants. IFNγ and IL12 genetic polymorphisms were determined to investigate the association between polymorphisms and ventilation characteristics, bronchopulmonary dysplasia (BPD) and other perinatal disorders.
Results: The IFNγ^{+874}A allele was over-represented in LBW infants. Carriers of the IFNγ^{+874}T allele required mechanical ventilation and oxygen supplementation for time periods 41% and 35%, respectively, shorter than those required by those not carrying the IFNγ^{+874}T allele. Stepwise logistic regression analysis showed that carriers of the IFNγ^{+874}T allele were protected against BPD (odds ratio (OR) 0.35 (95% confidence interval (CI) (0.12 to 0.99))) and patent ductus arteriosus (OR 0.43 (95% CI 0.19 to 0.97)), whereas carriers of the IFNγ^{+874}A allele were at higher risk of severe hypotension (OR 3.40 (95% CI 1.01 to 11.52)) and respiratory distress syndrome (OR 4.03 (95% CI 1.30 to 12.50)). Carriers of the IL12 GC allele were protected against pneumonia (OR 0.32 (95% CI 0.14 to 0.75)). Carriers of the IL12 CTCTAA allele were at higher risk of developing necrotising enterocolitis (NEC; OR 2.37 (95% CI 1.01 to 5.53)).
Conclusions: Carrier state of the IFNγ^{+874}A allele presents an increased risk for premature birth and lung damage, as well as other perinatal complications. The risks of pneumonia and NEC are higher in heterozygotic carriers of the IL12 CTCTAA/GC polymorphism. Further studies are needed to determine whether these associations are the result of altered cytokine-producing capacity in infants carrying the tested alleles.

Respiratory dysfunction, clinically characterised by the need for oxygen supplementation, is a common complication in preterm infants and is usually caused by perinatal lung damage. Risk factors contributing to lung damage include inadequate immune response triggered by various stimuli, such as infection and medical intervention, causing postnatal oxygen toxicity, mechanical injury, barotrauma and overextension.[1–3] These factors also contribute to other perinatal complications that increase the need for mechanical ventilation in newborns.[4]

Both innate and adaptive immunity are immature in preterm infants. Innate responses are non-specific reactions to microbes and exogenous stimuli, whereas adaptive responses are specific to the type of microbe or stimulus. The immaturity of the immune system has been linked to the unbalanced activation of immune cells, which results in damage of susceptible organs such as the lungs.[3–8] The effector cells of immunity (lymphocytes, natural killer cells, macrophages and neutrophil granulocytes) have a central role in this process. These cells communicate with each other through several cytokines, including tumour necrosis factor (TNF)α, interleukin (IL)1, IL4, IL6, IL8, IL10, IL12 and interferon (IFN)γ.[4 9–11]

The production of cytokines is determined by inflammation, developmental factors and, to some extent, by genetic polymorphisms (single-nucleotide polymorphisms (SNPs)).[12–17] We and others have extensively investigated the association of some cytokine SNPs, such as TNFα $G^{-308}A$ variations, with preterm birth and perinatal disorders. Data have linked the carrier state of the TNFα^{-308}A allele to preterm birth, intraventricular haemorrhage (IVH) and bronchopulmonary dysplasia (BPD).[18–21] Recently, we found that carriers of the TNFα^{-308}A allele are at increased risk for longer ventilator support.[22]

Most of these studies focus on cytokines implicated in adaptive immune response. Data on the importance of SNPs of other genes encoding cytokines of innate immunity in perinatal disorders are much more limited. IFNγ and IL12 have a major role in the communication between macrophages and natural killer cells. IFNγ is the principal macrophage-activating cytokine, but it also stimulates neutrophils and natural killer cells.[10] IL12 is a disulphide-linked heterodimer consisting of two subunits (p35 and p40). Many cells seem to synthesise the p35 subunit, but only activated mononuclear phagocytes and dendritic cells produce the p40 component and, consequently, the biologically active cytokine. IL12 stimulates IFNγ production and the differentiation of Th1 cells, and improves the cytolytic functions of activated natural killer cells and CD8+ T lymphocytes.[11]

Genes encoding IL12 and IFNγ have several functional SNPs; of these, the most widely investigated SNPs are the IL12 p40

Abbreviations: BPD, bronchopulmonary dysplasia; IFN, interferon; IVH, intraventricular haemorrhage; IRDS, idiopathic respiratory distress syndrome; LBW, low birthweight; NEC, necrotising enterocolitis; PCR, polymerase chain reaction; PDA, patent ductus arteriosus; RFLP, restriction fragment length polymorphism; SNP, single-nucleotide polymorphism; TNF, tumour necrosis factor

promoter GC/CTCTAA and IFNγT^{+874}A.[13 15 23] We hypothesised that the carrier state of these SNPs influences the risk of some perinatal complications that may increase the need for ventilator support and also increase the risk for BPD. We tested this hypothesis in a heterogeneous population of very LBW infants.

PATIENTS AND METHODS

Patients

In our retrospective study, we analysed the medical records of consecutive LBW infants (birth weight ⩽1500 g) and healthy term neonates who had been born and treated at the Second Department of Gynecology and Obstetrics, Semmelweis University, Budapest, Hungary, between 2000 and 2003. At the beginning of treatment in the neonatal intensive care unit or nursery ward, informed consent of parents was obtained to collect dried blood samples from their infants for scientific purposes. Blood samples were taken on the fifth day of life or after the beginning of oral feeding. Dried blood samples were stored at the phenilketonuria (PKU) screening laboratory and were sent for genotyping. In our study, we did not enrol those infants (1) whose parents refused to participate in scientific projects (2% of LBW and 5% of term neonates), (2) who died before collection of dried blood samples (12% of LBW infants) or (3) who died before discharge to home or nursery ward (6% of LBW infants). The total drop-out rates of LBW and term infants were 19% and 5%, respectively. Finally, we enrolled 172 healthy term neonates (87 boys and 85 girls, median birth weight 3400 (range 2700–4200) g; median gestational age 40 (range 37–42) weeks; prevalence of intrauterine retardation (defined as gestational age-matched birth weight <10th centile) 0.03; and no reported perinatal complication) and 153 LBW neonates (76 boys and 77 girls, median birth weight 1180 (range 510–1500) g, median gestational age 29 (range 24–36) weeks; prevalence of intrauterine retardation 0.18). For LBW infants, we recorded the median duration of mechanical ventilation (4 (range 0–60) days) and length of total oxygen supplementation (12 (range 0–80) days), and the presence of BPD (n = 27). BPD was defined as oxygen dependency at 28 days' postnatal age or at 36 weeks' postmenstrual age.[24] Prenatally, 51 infants were treated with steroid; postnatally, 44 infants were treated with dobutamine and 23 with surfactant. Eleven underwent surgical intervention. Perinatal complications in the medical histories were recorded, such as pneumonia (n = 36), necrotising enterocolitis (NEC; n = 52), sepsis (n = 47), acute renal failure (n = 40), severe hypotension in infection (n = 49), idiopathic respiratory distress syndrome (IRDS; n = 80), patent ductus arteriosus (PDA; n = 50) and IVH (n = 47). We defined these complications according to internationally accepted criteria.[25 26]

Samples

DNA was extracted using an agent (Chelex, BioRad, Germany) according to the manufacturer's instructions. The institutional ethics committee approved the study (TUKEB 14/2003).

Genotyping

Genotyping was carried out with polymerase chain reaction (PCR) and, subsequently, with restriction fragment length polymorphism (RFLP) methods (table 1).

PCR amplifications were carried out in a buffer of final volume 50 µl, consisting of 25 pmol of each primer, 0.2 mmol/l of each deoxyribonucleotide triphosphate, 2.0 mmol/l magnesium chloride ($MgCl_2$) and 0.3 U *Taq* polymerase (Invitrogen, Carlsbad, California, USA). RFLP was carried out in a PCR product of volume 15 µl. The PCR and RFLP products were separated on 2.5% agarose gels (Invitrogen, Carlsbad, California, USA) and visualised under UV illumination, stained with 0.4 mg/l ethidium bromide (Amershan Pharmacia Biotech, Uppsala, Sweden).

Statistical analysis

Hardy–Weinberg equilibrium was calculated to evaluate the relationship between measured and expected genotype frequencies. Categorical data were analysed using the χ^2 test. The number of days on oxygen supplementation and mechanical ventilation were log transformed. We applied multiple linear regression analysis to test the effect of genotypes on ventilation characteristics. We used stepwise binary logistic regression to determine the independent association between BPD and polymorphisms. These associations were adjusted for gestational age and clinical characteristics. We also tested the association of the genotypes with perinatal complications other than BPD; for this purpose, we also used a stepwise binary logistic regression approach. The level of significance was set at $p<0.05$. All calculations were carried out with the statistical software package SPSS V.10.0.

RESULTS

The distribution of the investigated genotypes fulfilled Hardy–Weinberg criteria in each population studied. The prevalence of IFNγ T^{+874}A genotypes (TT/TA/AA) in term and LBW infants was 0.33/0.46/0.21 and 0.17/0.56/0.25, respectively, with a significantly higher prevalence of the IFNγ^{+874}A allele among LBW infants (0.44 *v* 0.54, odds ratio (OR) 1.50, 95% confidence interval (95% CI) 1.10 to 2.05). The prevalence of IL12 p40 promoter CTCTAA/GC (I/D) genotypes (II/ID/DD) in term and LBW infants was 0.33/0.47/0.20 and 0.28/0.50/0.20, respectively, with similar prevalence in both groups (0.44 *v* 0.46).

In our stepwise linear regression model, the need for ventilator support was associated with gestational age at birth, and the presence of IVH and IFNγ^{+874}T genotype; carriers of the IFNγ^{+874}T allele required mechanical ventilation and oxygen supplementation for time periods 41% and 35% , respectively, shorter than that required by infants with IFNγ^{+874}AA genotype (table 2). The IL12 polymorphism showed no association with ventilation characteristics.

We also analysed the association between genotypes and perinatal complications. The χ^2 test showed that the distribution of IL12 genotypes (II/ID/DD) is different only in LBW infants with and without pneumonia (0.23/0.57/0.20 *v* 0.47/0.36/0.17, OR 1.76, 95% CI 1.13 to 2.76, p = 0.014, respectively). The stepwise binary logistic regression analysis, however, showed that the carrier state of the IFNγ^{+874}T allele is an independent determinant of BPD (OR 0.35, 95% CI 0.12 to 0.99). The IL12 polymorphism by itself was not associated with risk for BPD; however, we found an increased risk for pneumonia in carriers of the IL12 CTCTAA allele (OR 1.76, 95% CI 1.13 to 2.76). Binary logistic regression analysis also showed an association between the IFNγ A^{+874}T genotype and several perinatal complications that increase the need for ventilator support. We found that carriers of the IFNγ^{+874}T allele were protected against PDA (table 2 shows corresponding ORs), and carriers of the IFNγ^{+874}A allele were at higher risk for severe hypotension and IRDS. These associations were adjusted for gestational age and other risk factors.

For the IL12 p40 promoter CTCTAA/GC polymorphism, we found that carriers of the IL12 GC allele were at lower risk for pneumonia, and carriers of the IL12 CTCTAA allele were at higher risk for NEC. Heterozygosity was associated with a decreased risk for pneumonia and an increased risk for NEC compared with homozygotes for the tested IL12 polymorphism.

We also tested for an association between the common carrier state of the IFNγ^{+874}A×IL12 GC alleles and perinatal

Table 1 Conditions for polymerase chain reaction and restriction fragment length polymorphism

	IFNγ $T^{+874}A$ polymorphism	IL12 GC/CTCTAA polymorphism
Forward primer	5′-TTC TTA CAA CAC AAA ATC AAG TC-3′	5′-TGT TCT AAT GTG GGG GCC ACG-3′
Reverse primer	5′-AGT ATT CCC AAA AGG CTT ATG T-3′	5′-CTG TTT GTC AGC AGA CCT TCC T-3′
Denaturation temperature (20 s)	94°C	94°C
Annealing temperature (20 s)	50°C	55°C
Extension temperature (30 s)	72°C	72°C
Restriction enzyme	8 U of *Alw26**	10 U of *Taiİ**
Overnight restriction, temperature	37°C	65°C
Product size	340/26 bp A allele 366 bp T allele	205/22 bp CTCTAA allele 223 bp GC allele

IFN, interferon; IL, interleukin
* Provided by New England Biolabs, Beverly, Madison, USA

complications. We found that infants simultaneously carrying the IFNγ^{+874}A and IL12 GC alleles were at increased risk for severe hypotension with infection.

Multiple linear regression analysis

The duration of mechanical ventilation and oxygen supplementation in days was log transformed to achieve normal distribution. The parameters were adjusted for gestational age and perinatal complication possibly affecting ventilation characteristics. Gestational age was also a significant ($p<0.001$) predictor. B is the raw regression coefficient; e^B is the ratio of the durations of ventilation to oxygen supplementation between infants with and without IFN T allele.

Stepwise binary logistic regression

The association between genotype and perinatal complications was adjusted for gestational age and known risk factors. Gestational age was a significant ($p<0.05$) predictor of each dependent variable; IVH was a significant ($p<0.01$) predictor of ventilation durations and BPD; severe hypotension was a significant ($p<0.05$) predictor of pneumonia; and IRDS was a significant ($p<0.05$) predictor of severe hypotension in infection. β is the standardised regression coefficient.

DISCUSSION

The lungs are some of the most susceptible organs during the perinatal period. Clinical experience supports that prematurity itself along with several perinatal complications may trigger lung damage that is clinically characterised by increased need for ventilator support. Studies have shown that an exaggerated production of inflammatory cytokines increases the risk for chronic mechanical ventilation and BPD.[1 4 21 27]

In this study, we tested the association of functional SNPs of two inflammatory cytokines IFNγ and IL12 with the length of postnatal oxygen requirement and risk for BPD and other perinatal disorders. Although the observed distributions of the IFNγ and IL12 genetic polymorphisms in healthy newborns are similar to reference values reported in other populations, the IFNγ^{+874}A allele occurred more frequently in LBW infants.[14 28] This finding implies that IFNγ is associated with the pathogenesis of preterm birth. Indeed, recent data collected in women also suggest that the maternal carrier state of this SNP may contribute to recurrent pregnancy loss and, possibly, to premature birth.[29 30] However, we tested only infants' genotypes and had no data about the mothers. As half of the alleles in the offspring originate from the mother, we can only speculate whether this association is attributable to the infant's genotype or is the result of the mother's genotype. Therefore, further studies on the simultaneous genotyping of parents and offspring should be conducted to determine the role of the IFNγT^{+874}A SNP in preterm birth.

On testing the association between genotype and ventilation characteristics of infants, we found that carriers of the IFNγ^{+874}T allele required mechanical ventilation and oxygen supplementation for a time period about 40% shorter than that required by infants without this allele. As the presence of the IFNγ^{+874}T allele is linked to increased IFNγ levels, it is tempting to speculate that our results are due to altered IFNγ levels.[13] Newborn infants with low in vitro production of IFNγ were at higher risk for longer mechanical ventilation in the presence of respiratory syncytial virus infection.[31]

Table 2 Association of the investigated genotypes with ventilation characteristics and perinatal complications

Results of multiple linear regression analysis

Dependent variable	Independent variables	p	B	e^B
Log (duration of mechanical ventilation)	Carrier state of the IFN T allele	0.002	−0.533	0.59
Log (duration of oxygen supplementation)	Carrier state of the IFN T allele	0.003	−0.438	0.65

Results of stepwise binary logistic regression analysis

Dependent variable	Independent variables	p	β	$e^β$ = OR, 95% CI
Bronchopulmonary dysplasia	IFN T allele	0.049	−1.054	0.35, 0.12 to 0.99
	IFN AA×IL12 ID genotype	0.042	1.488	4.43, 1.06 to 18.6
Patent ductus arteriosus	IFN T allele	0.043	−0.837	0.43, 0.19 to 0.97
Idiopathic respiratory distress syndrome	IFN A allele	0.016	1.395	4.03, 1.30 to 12.5
Severe hypotension in infection	IFN A allele	0.049	1.225	3.40, 1.01 to 11.5
Pneumonia	IL12 D allele	0.009	−1.135	0.322, 0.138 to 0.750
	IL12 DI genotype	0.016	−1.076	0.341, 0.142 to 0.819
Necrotising enterocolitis	IL12 I allele	0.046	0.862	2.369, 1.013 to 5.533
	IL12 DI genotype	0.004	1.069	2.914, 1.410 to 6.015

β, standardised regression coefficient; B, raw regression coefficient; e^B, ratio of the durations of ventilation to oxygen supplementation between infants with and without IFN T allele; IFN, interferon; IL, interleukin.

What is already known on this topic

- Inflammatory cytokines (tumour necrosis factor α, interleukin (IL)1β, IL8, etc) play a key part in the pathomechanism of perinatal complications including bronchopulmonary dysplasia.
- Interferon γ and IL12 are the most important inflammatory cytokines of innate immunity.
- Cytokine levels could be affected by certain genetic polymorphisms.

What this study adds

- This study suggests an association between perinatal complications, including bronchopulmonary dysplasia, and the carrier state of interferon γ $T^{+874}A$ and interleukin (IL)12 p40 promoter CTCTAA/GC polymorphisms.

Although no data are available on the association between BPD and altered IL12 and IFNγ levels, our finding that the IFNγ^{+874}T allele is protective, whereas the simultaneous presence of IFNγ^{+874}AA and IL12 GC/CTCTAA genotype is a risk factor for BPD, is in line with the current theories about the implication of disturbed cytokine network in BPD.[3 4] Increased levels of other inflammatory cytokines, such as IL1β, TNFα and IL8, have a key role in the pathomechanism of lung damage and BPD.[4 21 22 24 27] These cytokines also play a part in the regulation of IL12 and IFNγ production; therefore, it is reasonable to postulate that IL12 and IFNγ also contribute to BPD.[32] The association between IFNγ and IL12 genotypes with BPD would support this hypothesis.

The need for mechanical ventilation and oxygen supplementation is influenced by several perinatal complications.[1–4 25 33 34] We therefore also examined the independent association of the IL12 promoter GC/CTCTAA and IFNγT^{+874}A genetic polymorphisms with NEC, pneumonia, infection and sepsis, because inflammation plays a key part in the pathomechanism of all four perinatal complications.[25] Although we found no association between sepsis and the tested genotypes, we observed that alleles with low IFNγ levels increase the risk for hypotension in patients with sepsis. We also found that patients with inherited susceptibility for low IL12 levels (ie, those with the IL12 promoter CTCTAA allele) are at increased risk for NEC and pneumonia. The causative role of these genetic variants in these perinatal complications should be verified with serial determinations of IL12 and IFNγ levels.

We also showed that patients who are predisposed to low IFNγ levels are at increased risk for PDA. No data on animals and humans are available about the possible implication of IFNγ in PDA; further studies should be carried out to elucidate this issue. We also found that infants with low IFNγ levels are at increased risk for IRDS. Although the main risk factor for IRDS is immaturity, inflammatory cytokines such as IL1β and TNFα do affect lung development and protect against IRDS.[35 36] Our results raise the possibility that IFNγ genotype may influence the infant's risk for IRDS through its effect on inflammatory cytokine levels. Again, this speculation needs experimental verification.

Our study has limitations. The estimated drop-out rate of LBW infants in our study was approximately 20%, which may be a selection bias. However, genotype distributions fulfilled the Hardy–Weinberg criteria in each population tested, which suggests that the investigated genotypes did not have a major effect on perinatal mortality. The lack of cytokine levels is another limitation; serial testing along with genotyping may disclose a causative role among the investigated SNPs, cytokine levels and perinatal morbidity. Furthermore, it should also be considered that cytokines do not act in an isolated manner; rather, they exert their effect in a complex network. The SNPs of other cytokines have been tested in several studies on newborns. Most of the data concern the association of perinatal morbidity with TNFα SNPs.[18–22] Probably, it would be of interest to test the interaction among TNFα, IL12 and IFNγ genotypes in a larger cohort of preterm infants.

In summary, our results suggest several associations between perinatal morbidity and the IFNγ and IL12 SNPs. Although the change in cytokine-producing capacity is an attractive hypothesis for our results, the causative role of these SNPs should be investigated in further studies.

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