THE TUMOR NECROSIS FACTOR-A -308 G/A POLYMORPHISM
AND THE TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING
LIGAND POLYMORPHISMS, IN ASTHMATIC PATIENTS AND HEALTHY
SUBJECTS

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ABSTRACT
Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. Like other atopic diseases, asthma is a complex disorder caused by interactions between multiple genes of small to modest effect and equally important environmental factors.

The aim of this study was to determine the TNF-α -308 G/A polymorphism and the TRAIL polymorphisms, and their influence on asthma in asthmatic patients and healthy subjects.

The study population consists of 51 asthmatic patients (47 female and 4 male) and 72 healthy subjects (62 female and 10 male). The mean age of the asthmatic patients and healthy controls were 45.33±14.05, and 41.88±17.41 years, respectively. The asthmatic patients and healthy controls were similar with respect to their ages and sex characters. There was statistically a significant difference between the asthmatic patients and control groups in terms of TRAIL Arg141His, G422A (rs6557634) polymorphism (p=0.02). Statistically, there was not any significant difference between the asthmatic patients and control groups for TRAIL Thr209Arg, C626G (rs20575) TRAIL Glu228Ala, A683C (rs20576) and polymorphisms (p=0.57). Also, there was no significant difference between the asthmatic patients and control groups in terms of TNF-α-308 G/A polymorphism (p=0.90).
In our study, the TRAIL Arg141His G422A (rs6557634) polymorphism was detected for the first time in asthmatic patients, which may influence the susceptibility to the asthma.

Keywords: asthma, genetic polymorphism, TNF-α, TRAIL

Introduction
Asthma is a problem worldwide, with an estimated 300 million affected individuals. Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role (33).

The airway inflammation that occurs after repeated exposure to allergens or during the late-phase reaction results from a complex network of interactions between inflammatory cells (mast cells, eosinophils, macrophages, and activated T and B cells) and cells comprising the lung structure (endothelial cells, fibroblasts, bronchial epithelial cells, and smooth muscle cells) (11, 13).

Large numbers of activated eosinophils are present in the mucosa of asthmatic patients and they are thought to be the central effector cells in asthma (3).

The members of the tumor necrosis factor (TNF) family play critical roles as prominent mediators of immune regulation and the inflammatory response (28).

It is possible therefore that TNF-α is involved in mast cell/smooth muscle interaction, and that this is particularly important in the development of airway hyper-responsiveness (4).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was first cloned by Wiley et al. (35) and later by Pitti et al. (27). TRAIL may have an important role in the regulation of immune responses, inflammation and allergy.

It has been reported that TRAIL and its receptor systems are upregulated in the respiratory epithelium of asthmatic subjects and that this is correlated with eosinophilic airway inflammation (28).

Like other atopic diseases, asthma is a complex disorder caused by interactions between multiple genes of small to modest effect and equally important environmental factors. Asthma has an important genetic component but has not presented a clear pattern of inheritance, and heritability estimates of asthma vary between 36-79% (12, 18, 22).

Asthma is considered a good example of gene–environment interactions, although no single gene or environmental factor accounts for the disease (17).
The main focus of this study was to determine the TNF-α-308 G/A polymorphism and the TRAIL polymorphisms, and their influence on asthma in asthmatic patients and healthy subjects.

**Materials and Methods**

This study was carried out in accordance with the principles of Helsinki Declaration. Informed consent forms were signed by all subjects.

**Subjects**

The study population consists of 51 asthmatic patients (47 female and 4 male) and 72 healthy subjects (62 female and 10 male).

**Diagnosis of asthma**

Asthma diagnosis was based on international consensus reports on diagnosis and treatment of asthma and physicians recommendations (33). All asthmatic subjects were followed up at an outpatient clinic.

**Statistical analyses**

Allele frequencies between groups were compared using Chi-squared (χ²) statistics. Two-sided p values were considered statistically significant at p≤0.05. Statistical analyses were carried out with the statistical packages for SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

**Molecular analysis-DNA extraction**

Venous blood samples were collected in tubes containing ethylene diamine tetra acetic acid (EDTA). DNA was extracted from whole blood by salting out procedure (21).

**Genotypic analysis of the TNF-α-308 G/A polymorphism**

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assays were used to determine TNF-α-308 G/A polymorphism. The oligonucleotide primers used to determine the ~308 G/A polymorphism within the TNF-α gene were as previously described (5). The primers, forward 5’-AGGCAATAGGTTTTGAGGGCCAT-3’ and reverse 5’-TCCTCCCTGCTCCGATTCCG-3’, were used to amplify the TNF-α gene. PCR was performed in a 25 μl volume with 100 ng DNA, 100 μm dNTPs, 20 pmol of each primer, 1.5 mM MgCl₂, 1x PCR buffer with (NH₄)₂SO₄ (Fermentas, Vilnius, Lithuania), 10% DMSO and 2U Taq DNA polymerase (Fermentas, Vilnius, Lithuania). Amplification was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK). PCR conditions were 2 min for initial denaturation at 95°C; 35 cycles at 95°C for 45 sec for denaturation, 1 min at 95°C for annealing and 90 s at 72°C for extension, followed by 7 min. at 72°C for final extension. The PCR products were digested with 10U BseGI (FokI, Fermentas, Vilnius, Lithuania) at 55°C for 4 h. Genotyping of the TRAIL gene was then determined by fragment separation at 120 V for 40-50 min on a 3% agarose gel that contained 0.5μg/ml ethidium bromide. A 100 bp marker (100 bp DNA Ladder, Fermentas) was used as a size standard for each gel lane. The gel was visualized under UV light using a gel electrophoresis visualizing system (Vilber Lourmat). The Nco I restricted products of TNF-α-308 G/A, GG, GA and AA genotypes, had band sizes of 87bp/20bp, 107bp/87bp/20bp and 107bp, respectively. Genotyping was based upon independent scoring of the results by two reviewers who were unaware of the case/control status.

**Genotypic analysis of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; Apo2L; TNFRSF10A; DR4) gene Exon 3, Arg141His, G422A (rs6557634) polymorphism**

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay was used to determine TRAIL Arg141His, G422A (rs6557634) polymorphism. The oligonucleotide primers that were used to determine the Arg141His, G422A (rs6557634) polymorphism in TRAIL gene were previously described (10, 15). The primers (GenBank Accession Number: NT_023666 and NT_18570975), forward 5’-ATCCTCTGGGAACTCTGTGG-3’ and reverse 5’-TACCACTCCCACTCTCTAGC–3’, were used to amplify a 230 bp PCR fragment. PCR was performed in a 25 μl volume with 100 ng DNA, 100 μm dNTPs, 20 pmol of each primer, 1.5 mM MgCl₂, 1x PCR buffer with (NH₄)₂SO₄ (Fermentas, Vilnius, Lithuania) and 2U Taq DNA polymerase (Fermentas, Vilnius, Lithuania). Amplification was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK). PCR conditions were 2 min for initial denaturation at 95°C; 35 cycles at 95°C for 45 sec for denaturation, 1 min at 59°C for annealing and 90 s at 72°C for extension, followed by 7 min. at 72°C for final extension. The PCR products were digested with 10U BseGI (FokI, Fermentas, Vilnius, Lithuania) at 55°C for 4 h. Genotyping of the TRAIL gene was then determined by fragment separation at 120 V for 40-50 min on a 3% agarose gel that contained 0.5μg/ml ethidium bromide. A 100 bp marker (100 bp DNA Ladder, Fermentas) was used as a size standard for each gel lane. The gel was visualized under UV light by using a gel electrophoresis visualizing system (Vilber Lourmat). The BseGI restricted products of TRAIL: Arg141 (allele G) and His141 (allele A) alleles had band sizes of 230 bp and 160bp+70bp, respectively.

**Genotypic analysis of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; Apo2L; TNFRSF10A; DR4) gene Exon 4, Thr209Arg, C626G (rs20575) polymorphism**

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay was used to determine TRAIL Thr209Arg, C626G (rs20575) polymorphism. The oligonucleotide primers used to determine the Thr209Arg, C626G (rs20575) polymorphism were previously described (10, 15). The primers (GenBank Accession Number: NT_023666 and NT_18570975), forward 5’-AAGGTCAAGGGACACGTCAGG-3’ and reverse 5’-GCTTCTGTGGTTTCTTTGAGG–3’, were used to amplify a 220 bp PCR fragment. PCR was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK). PCR conditions were 2 min for initial denaturation at 95°C; 35 cycles at 95°C for 45 sec for denaturation, 1 min at 95°C for annealing and 90 s at 72°C for extension, followed by 7 min. at 72°C for final extension. The PCR products were digested with 10U BseGI (FokI, Fermentas, Vilnius, Lithuania) at 55°C for 4 h. Genotyping of the TRAIL gene was then determined by fragment separation at 120 V for 40-50 min on a 3% agarose gel that contained 0.5μg/ml ethidium bromide. A 100 bp marker (100 bp DNA Ladder, Fermentas) was used as a size standard for each gel lane. The gel was visualized under UV light by using a gel electrophoresis visualizing system (Vilber Lourmat). The BseGI restricted products of TRAIL: Arg141 (allele G) and His141 (allele A) alleles had band sizes of 230 bp and 160bp+70bp, respectively.
in a 25 μl volume with 100 ng DNA, 100 μm dNTPs, 20 pmol of each primer, 1.5 mM MgCl₂, 1x PCR buffer with (NH₄)₂SO₄ (Fermentas, Vilnius, Lithuania) and 2U Taq DNA polymerase (Fermentas, Vilnius, Lithuania). Amplification was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK). PCR conditions were 2 min for initial denaturation at 95°C; 35 cycles at 95°C for 45 sec for denaturation, 1 min at 58°C for annealing and 90 sec at 72°C for extension, followed by 7 min at 72°C for final extension. The PCR products were digested with 10U Adel (DraIII, Fermentas, Vilnius, Lithuania) at 37°C for 14 h. Genotyping of the TRAIL gene was determined by fragment separation at 120 V for 40-50 min on a 3% agarose gel containing 0.5μg/ml ethidium bromide. A 100 bp marker (100 bp DNA Ladder, Fermentas) was used as a size standard for each gel lane. The gel was visualized under UV light using a gel electrophoresis visualizing system (Vilber Lourmat). The Adel restricted products of TRAIL: Thr209 (allele C) and Arg209 (allele G) alleles had band sizes of 164bp+56bp and 220bp, respectively.

Genotypic Analysis of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; Apo2L; TNFRSF10A; DR4) gene Exon 5, Glu228Ala, A683C (rs20576) Polymorphism:

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay was used to determine TRAIL Glu228Ala, A683C (rs20576) polymorphism. The oligonucleotide primers used to determine the Glu228Ala, A683C (rs20576) polymorphism in TRAIL gene was described previously (10,15). The primers (GenBank Accession Number: NT_023666 and NT_18570975), forward 5’-ATCCCACCTGGCCAGCTTTCCA–3’; reverse 5’-AGACAGGAGTCTCGGGCTGCT–3’ were used to amplify the 201bp PCR fragment. PCR was performed in a 25 μl volume with 100 ng DNA, 100 μm dNTPs, 20 pmol of each primer, 1.5 mM MgCl₂, 1x PCR buffer with (NH₄)₂SO₄ (Fermentas, Vilnius, Lithuania) and 2U Taq DNA polymerase (Fermentas, Vilnius, Lithuania). Amplification was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK). PCR conditions were 2 min for initial denaturation at 95°C; 35 cycles at 95°C for 45 sec for denaturation, 1 min at 61°C for annealing and 90 sec at 72°C for extension, followed by 7 min at 72°C for final extension. The PCR products were digested with 10 U TaqI (Fermentas, Vilnius, Lithuania ) at 65°C for 4 hr., the genotyping of the TRAIL gene was determined by fragment separation at 120 V for 40-50 min. on a 3 % Agarose gel containing 0.5μg/ml ethidium bromide. A 100 bp marker (100 bp DNA Ladder, Fermentas) was used as a size standard for each gel lane. The gel was visualized under UV light using a gel electrophoresis visualizing system (Vilber Lourmat). The TaqI restricted products of TRAIL; Glu228 (allele A) and Ala228 (allele C) alleles had band sizes of 110bp+91bp and 201 bp, respectively.

All procedures were conducted in a manner blind to the case status and other characteristics of the participants. Scoring of gels and data entry was conducted independently by two persons. We performed the PCRs and evaluated the results without information concerning the groups of the subjects. At least 10% of the samples were retested, and the results were 100% concordant.

Results and Discussion

There were 51 asthmatic patients (47 female, 4 male) with a mean age of 45.33±14.05 years, and 72 healthy controls (62 female, 10 male) with a mean age of 41.88±17.41 years. The asthmatic patients and healthy controls were similar in terms of their age and sex characteristics.

Statistically, there was not any significant difference between the asthmatic patients and those in the control group in respect of TNF-α-308 G/A polymorphism (p=0.90) (Table 1).

There was a significant statistical difference between the asthmatic patients and the control group in terms of TRAIL Arg141His, G422A (rs6557634) polymorphism (p=0.02) (Table 2).

There was not any statistically significant difference between the asthmatic patients and the control group for TRAIL Thr209Arg, C626G (rs20575) polymorphism (p=0.57) (Table 3).

Also, there was not any statistically significant difference between the asthmatic patients and the control group in concern to TRAIL Glu228Ala, A683C (rs20576) polymorphism (p=0.36) (Table 4).

Cytokines play a key role in the coordination and persistence of the chronic inflammatory process in asthma. Eosinophils secrete cytokines and chemokines, including TNF-α and –β, transforming growth factor (TGF)-α and –β, macrophage inflammatory protein 1α, Interleukin (IL)-1, IL-3, IL-5, IL-6, IL-8, and Granulocyte-macrophage colony-stimulating factor (GM-CSF). The secretion of these factors may continue eosinophil participation in the inflammatory response, in part, by promoting their survival (6, 7, 23, 25, 26, 30, 31).

We detected statistically significant difference between the asthmatic patients and the control group in respect to TRAIL Arg141His, G422A (rs6557634) polymorphism. This is the first study about the TRAIL Arg141His, G422A (rs6557634) polymorphism in asthmatic patients and its influence on asthma. Whether TRAIL gene polymorphism increased or decreased production of some cytokines that might cause the regulation of the inflammation in the airways is not known.

There was not any statistically significant difference between the asthmatic patients and the control group in terms of TRAIL Thr209Arg, C626G (rs20575) polymorphism (p=0.57)

Weckmann et al. reported that recombinant TRAIL induced pathogenic features of asthma and stimulated the production of CCL20 in primary human bronchial epithelium cells. The increase of the TRAIL has also been detected in sputum of asthmatics. The function of TRAIL in the airway epithelium...
might identify this molecule as a target for the treatment of asthma (34).

Alveolar macrophages and eosinophils from asthmatics express more TRAIL on their cell surface and TRAIL prolongs the lifespan of eosinophils in vitro (9, 28).

TRAIL may promote eosinophilia and T helper 2 (Th2) cell activation. Thus, TRAIL is a critical regulator of eosinophilic inflammation and allergen-specific T cell activation in the lung. TRAIL may have an important role in the regulation of immune responses, inflammation and allergy (8).

It has been reported that TRAIL and its receptor systems are upregulated in the respiratory epithelium of asthmatic subjects and that this is correlated with eosinophilic airway inflammation (16).

TNF-related ligand, TRAIL is a member of the TNF superfamily of cytokines and is capable of inducing apoptosis in a variety of transformed cells in vitro (35).

Solarewicz-Madejek et al. reported that demonstration of bronchial smooth muscle cells (SMC) death, both by apoptosis and necrosis indicated the essential role of T cells and eosinophils in the bronchial tissue injury, particularly in the severe asthma. In this study, bronchial SMCs were detected as an important target of the inflammatory attack by T cells and eosinophils. The consequent turnover of the SMCs might finally account for morphological changes and functional contraction/relaxation disorder in bronchial wall, particularly in severe forms of asthma (32).

Holgate ST reported that the airway epithelium in asthma was fundamentally abnormal with increased susceptibility to environmental injury and impaired repair associated with activation of the epithelial-mesenchymal trophic unit (17).

Robertson et al. first described that TRAIL concentrations were significantly increased in allergic asthmatic subjects after antigen challenge, and resulted in the local production of TRAIL by airway cells. These data suggest that TRAIL might act either as an integral membrane protein via direct cell-to-cell contact or as a soluble effector, to prolong survival of BAL eosinophils in the asthmatic airway (28).

We could not detect any difference for TNF-α-308 G/A polymorphism in patients with asthma and healthy controls. Our study results indicated that the frequency of TNF-α-308G/A polymorphisms might not seem to influence susceptibility to
asthma. Our results were compatible with the study reported by Aytekin et al. (2).

In a reported meta-analysis by Gao et al., the TNF-α-308G/A polymorphisms was concluded to be a significant risk factor for development of asthma (14).

Many studies have demonstrated that TNF polymorphisms, particularly TNF-α promoter polymorphisms, were in association with asthma and related phenotypes like: raised immunoglobulin E (IgE) levels, bronchial hyperreactivity and wheezing (1, 20, 24, 29).

Li et al. found association between the polymorphism TNF-α -308 G/A (corresponding to 1800629) with childhood asthma and wheezing (19).

The main limitation of our study was the lack of quantitative and/or immunohystological assessment of the TRAIL in the serum and airway tissue, and the study population was not large enough.

It is yet to be determined how the TRAIL gene polymorphisms affect the pathogenesis of the asthma.

Conclusions

The pathogenesis of asthma, a complex disease, involves gene-gene interactions as well as gene-environment interactions. Multiple modest risk factors work synergistically to influence asthma disease susceptibility. Our results suggest that the TRAIL Arg141His, G422A (rs6557634) polymorphism may contribute to the susceptibility of asthma development in some cases. Further studies are needed to identify the role of the TRAIL Arg141His, G422A (rs6557634) polymorphism in the pathogenesis of asthma.

REFERENCES


19. Li Y.F., Gauderman W.J., Avol E. et al. (2005) Haplotype analysis of a 100 kb region spanning TNF-LTA identifies a polymorphism in the LTA promoter region that is


