Association of FAS-670A/G and FASL–843C/T Gene Polymorphisms on Allograft Nephropathy in Pediatric Renal Transplant Patients

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Abstract

Objective: FAS and FASL polymorphisms are suggested to play an important role in tubulitis that is a major component of acute rejection. The aim of this study was to investigate the role of FAS-670A/G and FASL-843C/T gene polymorphisms on allograft nephropathy in pediatric renal transplant patients

Methods: Fifty three patients (22 males 31 females) aged 2 to 20 years (mean 12.3±0.6) who had renal transplantation and fifty healthy control subjects (25 males 25 females) were enrolled in the study. Pearson’s Chi Square test was used for the statistical analysis. Survival rates were estimated with the Kaplan Meier method. Age, sex, chronic renal failure etiology, treatment modality and duration and donor type were recorded. FAS-670A/G and FASL-843C/T gene polymorphisms were compared between renal transplant patients and normal healthy population as well as between renal transplant patients with and without acute rejection.

Findings: FAS-670A/G genotypes or alleles were not significantly different between control and transplant patients and among transplant patients with and without acute rejection (P>0.05 for all). FASL-843C/T genotypes and alleles were not different between transplantation and control groups (P>0.05 for all). However, FASL-843C/T alleles were significantly different between patients with and without AR (P=0.02). The percentages of C allele were higher in children with acute rejection (68.8% vs 44.6%).

Conclusion: FASL gene polymorphisms may play a major role in acute rejection while FAS polymorphisms have not been found to be different between patients with and without acute renal graft rejection.

Key Words: Renal transplantation; FAS ligand; Genetic polymorphism; Genotype; Kidney
**Introduction**

Allograft rejection that depends principally on human leukocyte antigen (HLA) polymorphism between donor and recipient is a complex multistage process involving T cells and immune components that are encoded by polymorphic genes\(^1\). Acute rejection is expected from the first week after transplantation on and is a cellular rejection involving T cell activation that involves a mononuclear cell predominant cellular inflammation morphologically\(^2-5\).

Recently, importance of protein molecules that participate in various stages of apoptosis are under investigation in the pathogenesis of renal graft rejection. FAS/FASL interaction seems to play a key role in acute allograft rejection and chronic allograft dysfunction\(^6\). Apo-1/FAS, also known as CD95, is a transmembrane protein and its major function appears to be the induction of apoptosis in cells expressing it after ligation by its ligand. Its natural ligand, FASL is a type II membrane protein belonging to the TNF family\(^7-9\). FAS is constitutively expressed on tubular cells while FASL is expressed by graft infiltrating T cells. Tubulitis is a major component of acute rejection and both FAS and FASL polymorphisms are thought to play role in tubulitis\(^1\). However, no previous study has evaluated the role of the polymorphisms in these genes together. Therefore, our study adds to the current literature the evaluation of the polymorphisms of FAS and FASL together in acute rejection in children with renal transplantation.

Recent identification of polymorphisms on the 5' flanking region on the human FAS gene has provided useful markers for investigation of graft rejection and survival\(^10\). The polymorphism is situated on a consensus sequence of the gamma-activated sequence (GAS) and thus may have a potential role in gene regulation\(^11\). Single nucleotide polymorphisms in the promoter region of the FAS at position –670 [FAS –670A/G] and FASL (T or C at position –843 [FASL –843T/C]) genes alter the transcriptional activity of these genes.

Therefore, the aims of this study was to investigate distribution of FAS gene promotor region -670 A/G and FASL gene promotor -843C/T polymorphisms in renal transplant as well as healthy control children and to investigate the role of these polymorphisms on allograft nephropathy in pediatric renal patients.

**Subjects and Methods**

**Subjects and Study Design:**

Fifty three consecutive children who had renal transplantation during 1991 to 2003 in Ege University Center of Pediatric Nephrology and Organ Transplantation were evaluated in this study as well as 50 random, age-matched, unrelated population of control children. All patients had received their first transplant. Age, sex, etiology of chronic renal failure, treatment modality and duration as well as the donor type were recorded.

Patients were followed at regular intervals in the surgical and nephrology clinics. Creatinine was measured at least monthly post-transplant. Renal biopsies were only performed if there were clinical indications with suspicion for allograft dysfunction. Acute rejection which is cellular rejection due to T cell activation encountered in first week after posttransplant was defined and graded according to the Banff criteria\(^3,12\). Chronic allograft nephropathy (CAN) was defined as a progressive decline in graft function accompanied by proteinuria and hypertension, which were not related to treatment interruption, recurrence of original disease, vascular, or urological complications\(^13\). Diagnosis of CAN was confirmed with a renal biopsy that showed histological changes consistent with Banff criteria\(^12,13\).

Genotype distribution and allele frequencies of FAS/FASL genes were compared between renal transplant patient and normal healthy population. All patients who had had at least one episode of acute rejection within the first 6 months after transplantation were classified as having acute rejection\(^3\). Allele and genotype frequencies of renal transplant patients who had acute rejection were compared with those who did not have acute rejection. Informed consent was taken from patients or their families for inclusion into the study.
**Immunosuppressive Protocols:**

Treatment protocol in living related donors (LRD) until August 2001 included cyclosporine (CsA), prednisolone and azathioprine (AZA) (18 patients); basiliximab (Bsm) was added to treatment after August 2001 (5 patients). In cadaveric donors (CAD), immunosuppressive treatment protocol included AZA, prednisolone and anti-thymocyte globulin (ATG) until April 2002 and ATG was replaced with CsA if creatinine levels fell below 2.5 mg/dL (18 patients). After April 2002 FK506 was used instead of CsA (11 patients). To assess treatment efficacy in the patients, target serum levels of CsA at 0 and 2 hours were 150-250 ng/ml and 1000-1400 ng/ml respectively during the first three months while those after third month of treatment were 100-200 ng/ml or 700-1000 ng/ml. Doses of CsA were modified according to the serum levels of CsA at second hour. In patients who received FK506 treatment, target serum levels were 8-12 µg/ml during the first three months and 7-10 µg/ml after the third month. Prednisolone treatment was 20 mg/day during the first year but was tapered to 5 mg/day after the first year. Basiliximab was administered 2 hours before transplantation and fourth day after transplantation at a dose of 10 mg in children who weighed less than 35 kg and 20 mg in those who weighed more than 35 kg.

Acute Rejection treatment regimen included three days of methyl prednisolone (0.5 g/day for weight <30 kg and 1 g/day for weight >30 kg). ATG or OKT3 was used for steroid resistant cases.

**Genomic DNA preparation and quantitation:**

Genomic DNA was extracted from EDTA-anticoagulated whole blood samples using a commercially available genomic DNA purification kit (Nucleospin Blood L, Macherey-Nagel, Germany) following manufacturer’s instructions. DNA concentration was determined by the PicoGreen dsDNA quantitation kit (Molecular Probes Inc., Eugene, OR ) using the manufacturer’s instructions and diluted as 100ng/µl.

**PCR-restriction fragment length polymorphism (PCR-RFLP) genotyping for the –670A/G polymorphism of FAS:**

The FAS-670A/G polymorphism was typed as described previously, with some minor modifications. Briefly, amplification was carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA) in a 25 µl reaction mixture in 0.2 ml thin-wall PCR strip tubes (Axygen Scientific, Inc., CA) containing 1µl genomic DNA solution, GeneAmp Gold Buffer (15 mmol/l Tris-HCl, pH 8.0, 50 mmol/l KCl; PE Applied Biosystems), 1.5 mmol MgCl2, 50 µmol/l each of the dGTP, dATp, dTTP and dCTP (Promega, Madison, WI), 5 pmol each forward and reverse primers and 1.0 U AmpliTaq Gold polymerase (PE Applied Biosystems). The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 45 s, 62°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 7 min.

Primers used for PCR-RFLP. For FAS -670A/G polymorphism forward primer was 5’-CTACCT AAG AGC TAT CTA CCG TTC and reverse primer was 5’-GGG TGTCCA TGT TGT GGC TGC-3’ respectively.

Digest conditions. Amplified 331 bp PCR product (3µl) was digested in a 10µl final reaction volume using 1µl of Reaction Buffer 2 and 5 units of MvaI restriction enzyme (New England Biolabs, Beverly, MA), at 37°C overnight. Controls of known genotype were included for every set of digestions carried out. After digestion allele G yielded three fragments of 99, 188 and 44 bp, whereas allele A yielded two fragments of 99 and 232 bp. Digested fragments were separated on 3% agarose gels and visualized after ethidium bromide staining in the UVP BioDoc-It System (Upland, CA) Bioimaging systems.

**PCR-RFLP genotyping for the –843C/T polymorphism of FASL gene:**

Genomic DNA was amplified using polymerase chain reaction (PCR) carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA) in a 25 µl reaction mixture as described above. The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 30 s, 45°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. A 114-bp fragment containing the promoter polymorphism C -843 T was amplified using the following primers:
Digest conditions. Amplified 114 bp PCR product (7µl) was digested at 37°C 3 hours in a 20µl final reaction volume using 2µl of Reaction Buffer 2 and 5 units of DraIII restriction enzyme (New England Biolabs, Beverly, MA). After digestion allele T yielded two fragments of 98 and 16 bp, whereas allele C was not digested and yielded as 114 bp. Digested PCR samples were subjected to electrophoresis on gels containing a mixture of 1.5% agarose (Sigma, St. Louis, MO) and 1.5 % NuSieve GTG (BMA, Rockland, ME).

Additionally, one sample for each of the three possible genotypes had formerly been confirmed by sequencing and served as standards in the restriction analysis. Sequencing was also performed for all individuals in which cosegregation of the mutant alleles did not occur. For sequencing PCR products for both genes were amplified using the same primers which are described above. Before sequencing the PCR products were purified using QIAquick PCR Purification Kit (Qiagen).

Dye terminator chemistry used in these reactions sequences was resolved using ABI 310 Genetic Analyser system. For sequence evaluation, the program SeqScape 2.0 was used.

Statistical Analysis:
Statistical analyses were performed by SPSS 11.0 (Chicago IL) computer program and Pearson’s Chi Square test. Survival rates were estimated with the Kaplan Meier method. P values less than 0.05 were regarded as statistically significant.

The Hardy-Weinberg equilibrium (HWE) assumption was assessed for case and control groups by comparing the observed numbers of different genotypes with those expected under HWE for the estimated allele frequency and comparing the Pearson goodness-of-fit statistic with a 2 distribution with 1 degree of freedom.

Findings

Subject Characteristics:
Mean age of the 53 patients (22 males, 31 females) included in this study was 12.3±0.6 years (range 2-20 years). The two most common etiologies of chronic renal failure were reflux nephropathy and chronic pyelonephritis (50% in total). Nineteen (35.8%) patients had received peritoneal dialysis while 33 (62.3%) had received hemodialysis and one (1.9%) patient had pre-emptive renal transplantation. Mean duration of dialysis was 21.7±2.3 months. The study population included 23 (43.4%) LRD and 30 (56.6%) CAD (Table 1).

Acute rejection was detected in 16 (29.6%) patients while chronic rejection was detected in 8 (14.8%) patients. Ten (18.5%) patients had more than one acute rejection episode.

Patient survival rates at 1, 3 and 5 years of transplantation were 93.7%, 89.2% and 77.9% respectively. Graft survival rates at 1, 3 and 5 years of transplantation were 90.6%, 84.5% and 81% respectively.

Distribution of FAS genotypes:
Comparison of FAS genotype in transplantation patients and the control group did not reveal a statistically significant difference. Frequencies of AA, AG and GG genotypes were 28.3, 62.3 and 9.4% of the transplant patients respectively (P=0.2) (Table 2).

| Parameter                  | No (%) |
|----------------------------|--------|
| **Gender**                 |        |
| Male                       | 23 (42.6) |
| Female                     | 31 (57.4) |
| **Treatment modality**     |        |
| Peritoneal                 | 20 (37) |
| Hemodialysis               | 33 (61.1) |
| Pre-emptive                | 1 (1.9) |
| **Donor type**             |        |
| LRD                        | 24 (44.4) |
| CAD                        | 30 (55.4) |

Table 1: Pretransplant characteristics of the patients (n=54)
The frequencies of FAS genotypes were also not significantly different between the patients who had acute rejection and those who did not. Frequencies of AA, AG and GG genotypes were 25, 62.5 and 12.5% in the rejection group while those in the group without rejection were 29.7, 62.2 and 8.1% ($P=0.8$) (Table 2). Comparison of the FAS alleles among transplanted children with and without acute rejection showed no significant difference ($P=0.7$) (Table 3).

**Frequencies of FAS alleles:**
Frequency of A allele in transplant patients was lower than that in the control group (59.4% vs 62%, $P=0.7$). Frequency of the G allele in the transplant and control patients was not significantly different (40.6% and 38% respectively) (Table 2). Comparison of the FAS alleles among transplanted children with and without acute rejection showed no significant difference ($P=0.7$) (Table 3).

**Distribution of FASL genotypes:**
FASL genotypes were not significantly different between transplantation patients and the control group. CC genotype was present in 35.8 and 32% of the transplantation and control patients respectively ($P=0.1$) (Table 3).

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**Table 2:** Distribution of FAS genotypes, FAS -670A/G alleles and FASL genotypes in control and transplant groups

|                      | Transplant | Healthy |
|----------------------|------------|---------|
| **FAS genotypes**    |            |         |
| AA                   | 15 (28.3)  | 20 (40) |
| AG                   | 33 (62.3)  | 22 (44) |
| GG                   | 5 (9.4)    | 8 (16)  |
| **P value**          |            | 0.2     |
| **FAS -670A/G alleles** |          |         |
| A                    | 63 (59.4)  | 62 (62) |
| G                    | 43 (40.6)  | 38 (38) |
| **P value**          |            | 0.7     |
| **FASL genotypes**   |            |         |
| CC                   | 19 (35.8)  | 16 (32) |
| CT                   | 17 (32.1)  | 24 (48) |
| TT                   | 17 (32.1)  | 10 (20) |
| **P value**          |            | 0.2     |
| **FASL-840 C/T alleles** |         |         |
| C                    | 55 (51.9)  | 56 (56) |
| T                    | 51 (48.1)  | 44 (44) |
| **P value**          |            | 0.5     |

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**Table 3:** Distribution of FAS genotypes, FAS -670A/G alleles and FASL genotypes in transplantation cases with and without rejection

|                      | Rejection (+) | Rejection (-) |
|----------------------|---------------|---------------|
| **FAS genotypes**    |               |               |
| AA                   | 4 (25)        | 11 (29.7)     |
| AG                   | 10 (62.5)     | 23 (62.2)     |
| GG                   | 2 (12.5)      | 3 (8.1)       |
| **P value**          |               | 0.8           |
| **FAS -670A/G alleles** |           |               |
| A                    | 18 (56.3)     | 45 (60.8)     |
| G                    | 14 (43.7)     | 29 (39.2)     |
| **P value**          |               | 0.7           |
| **FASL genotypes**   |               |               |
| CC                   | 8 (50)        | 11 (29.7)     |
| CT                   | 6 (37.5)      | 11 (29.7)     |
| TT                   | 2 (12.5)      | 15 (40.5)     |
| **P value**          |               | 0.1           |
| **FASL-840 C/T alleles** |           |               |
| C                    | 22 (33)       | 33 (44.6)     |
| T                    | 10 (31.2)     | 41 (55.4)     |
| **P value**          |               | 0.02          |
groups respectively. Frequencies of CT and TT genotypes were 32.1% each in the transplantation group while 48 and 20% respectively in the control group ($P=0.2$) (Table 2). Difference in the frequencies of FASL genotypes was not statistically significant between patients with and without acute rejection but CC genotype was more common in patients with acute rejection (50% vs 29.7%) while TT genotype was more common in patients without acute rejection (40.5% vs 12.5%) ($P=0.12$) (Table 3).

**Frequencies of FASL alleles:**

Frequencies of FASL alleles were not significantly different between control patients and transplanted patients ($P=0.5$) (Table 2).

On the other hand, FASL allele frequencies were significantly different between the patients with acute rejection and the ones without acute rejection ($P=0.02$). The frequency of C allele in children with acute rejection was 68.8% while that in the ones without acute rejection was 44.6%. Frequencies of the T allele in children with and without acute rejection were 31.2% and 55.4% respectively (Table 3).

**Discussion**

FAS/FASL cell death pathway is one of the major pathways involved in cytotoxic T lymphocyte mediated apoptosis\[14-16\]. It is the major pathway for activation induced cell death leading to elimination of activated T cells essential for lymphocyte homeostasis\[17\]. Interaction of FAS and FASL induces a cytolytic pathway leading to caspase mediated apoptosis\[18\]. In this aspect it is a marker of renal damage initiation\[18,19\]. On the other hand, FASL in renal tubular cells induces elimination of antigen activated CD4+ T lymphocytes\[18\]. Therefore, the development of acute rejection depends on a balance between these mechanisms besides many other factors.

Apoptosis via FAS/FASL system has been demonstrated to play role in pathogenesis of many diseases that include cholestasis induced hepatocyte injury, indirect acute lung injury after shock, autoimmune lymphoproliferative syndrome and Hashimoto thyroiditis as well as Grave's disease\[20-23\]. Moreover, it is thought to modulate tumorigenesis as demonstrated in cholangiocarcinoma, hepatocellular carcinoma, breast cancer and colon cancer\[24-27\].

T lymphocytes with the FASL -843CC genotype had heightened FASL expression that is associated with increased activation-induced cell death of the T cells stimulated by MCF-7 cells or phytohemagglutinin compared with the FASL -843TT genotype. Therefore, breast cancer patients with the FASL -843CC genotype had higher apoptotic tumor-infiltrating lymphocytes in their cancer tissues than those with the FASL -843TT genotype\[26\]. Aguilar-Reina et al have reported that the grade of necroinflammatory activity and the stage of fibrosis in patients with chronic hepatitis C infection was correlated with -670A > G variant of FAS gene\[28\].

Beside these, FAS/FASL system has an important role in progressive renal disease and organ rejection in renal, liver and cardiac transplantation\[9,29,30\]. For example, liver transplant recipients bearing the FAS -670AA genotype showed significantly lower graft survival rate than those bearing the AG genotype\[31\]. Cappelessou et al have detected that FAS -670GG genotype of the donor was associated with lower level of rejection episodes in renal transplant cases\[31\]. Therefore, the aim of this study was to evaluate FAS/FASL polymorphisms in children who had undergone renal transplantation and compare these polymorphisms in children with and without acute rejection as well as healthy controls.

Although immunosuppressive protocols continue to improve, acute rejection is still a cause of early graft loss after renal transplantation\[32\]. Humoral immune and host-mediated cellular responses play role in acute rejection\[18\]. Cytotoxic T lymphocyte mediated apoptosis is thought to play a major role in response to major histocompatibility alloantigens during acute rejection\[14,16\]. Antigens trigger T lymphocyte activation which is followed by CD4+ and CD8+ T lymphocyte, macrophage and natural killer cell infiltration into the tissues\[14\]. This T lymphocyte dominant leukocyte infiltrate in the cortical parenchyma displays the distinctive histological finding in
Acute rejection as well as tolerance is under genetic control involving MHC polymorphisms and various other genes. Such single nucleotide polymorphisms may modify the susceptibility of recipient T cells to FASL mediated apoptosis. However, previous studies have failed to detect a relationship between FAS gene polymorphisms and acute rejection. Similarly, FAS gene polymorphisms were not found to be different between transplant and control cases. Not only in transplantation but also in animal models of obstruction induced renal tubular cell apoptosis, mRNA expression of FASL and associated caspases was found to be increased. These findings are similar to our findings in children that displayed similar allele frequencies of FAS gene in control and transplant cases as well as cases with and without acute rejection. This finding has led to the second part of our hypothesis that polymorphisms of the FASL gene that are the other arm of this pathway might play a role in acute rejection.

FASL expression has been found to be increased in peripheral blood and urine samples as well as in biopsy specimens from patients with acute rejection. Similarly, FASL expression has been found to be significantly correlated with subacute graft rejection. These findings support our finding that this specific FASL polymorphism plays a significant role in acute rejection in children who underwent renal transplantation.

Apoptosis is crucial for normal maintenance of immunologic tolerance and for function of many nonlymphoid tissues. One of the many signals that induce apoptosis include ligation of the appropriate death signaling receptors one of which is FAS. FAS/FASL system that has a role in acute and subacute rejection through the induction of apoptosis, is also involved in ischemia reperfusion injury that is also an important mechanism for renal damage after transplantation. Therefore these findings are important in interpretation of our findings that demonstrated FASL gene polymorphisms are associated with acute rejection after transplantation.

In an animal study, it has been demonstrated that apoptosis in cholestasis induced hepatocyte injury was decreased in FAS deficient mice. This is different from our study in that we have not detected a difference in FAS alleles or genotypes in our patients with and without acute rejection. A similar observation of decreased apoptosis was detected in FAS/FASL deficient mice that showed marked decrease in apoptosis in acute lung injury. This finding is consistent with our finding of a significant difference detected in FASL alleles in patients with acute rejection.

The major limitation of this study is the relatively low number of patients included. We found non-significant relations between acute rejection and genotype except for the FASL alleles. This might be attributed to type 1 inflation due to the great number of comparisons that were performed. However, genotypes of transplant patients with and without acute rejection and those of healthy controls were not very similar. Therefore further studies with a larger sample may provide more information about the issue.

**Conclusion**

Apoptosis, which is crucial for immunologic tolerance, has a major role in development of acute rejection. One of the signaling pathways of apoptosis that includes FAS/FASL has been found to be related to acute graft rejection. The results of the present study indicate that FASL gene polymorphisms play a major role in acute rejection while FAS polymorphisms have not been found to be different between patients with and without acute renal graft rejection. Determination of this relationship may be
helpful as the first clue for a future therapeutic target.

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Conflict of Interest: None

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