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GSTT1 gene abnormality in minimal change nephrotic syndrome with elevated serum immunoglobulin E

Kohei Miyazaki¹, Keisuke Sugimoto¹, Shoji Tsuji², Anna Iharada², Shinsuke Fujita¹, Hidehiko Yanagida¹, Naoki Sakata¹, Mitsuru Okada¹, Kazunari Kaneko² and Tsukasa Takemura¹

¹Department of Pediatrics, Kinki University School of Medicine, and

²Department of Pediatrics, Kansai Medical University, Osaka, Japan

Key words

allergy – children – glutathione S-transferase supergene family – polymerase chain reaction – reactive oxygen species – Th1/Th2 imbalance

Abstract. Introduction: Imbalance between T-helper 1 (Th1) and 2 (Th2) lymphocytes and effects of reactive oxygen species (ROS) upon glomerular capillary walls have been implicated in minimal change nephrotic syndrome (MCNS). Methods: By polymerase chain reaction and comparative genomic hybridization, we evaluated mutations of the GSTT1 gene (*GSTT1*), a member of the glutathione S-transferase (GST) supergene family associated with both protection of cells from ROS and control of allergic reactions and serum immunoglobulin (Ig) E. Results: Among 15 children with MCNS, IgE elevation (over 2,000 IU/l) and *GSTT1* deletion was found in 2 who showed severe allergic symptoms. Serum ROS concentrations in these 2 patients were significantly higher than in healthy controls or other MCNS patients. In addition, a Th2 shift caused by increased serum interleukin (IL)-4 was observed. Conclusion: These results suggest presence of a *GSTT1* abnormality in some children with MCNS having marked serum IgE elevations and various allergic complications. Defective ROS degradation and Th1/Th2 imbalance caused by *GSTT1* abnormality could initiate proteinuria leading to MCNS.

and allergic rhinitis, often occur in patients with MCNS [2]. Disordered Th1/Th2 balance arising from interferon (IFN)- γ synthesis, coupled with increases in Th2 cytokines, could underlie MCNS in some patients [3].

In recent years, oxidant/antioxidant imbalance has been reported to be involved in development of the nephrotic syndrome (NS) [4]. O₂- and other oxygen-derived intermediates that can modify organic molecules are referred to as reactive oxygen species (ROS), while nitric oxide (NO) and its derivatives are known collectively as reactive nitrogen species (RNS). An increase in ROS in the form of free radicals damages cellular molecules [5]. Under physiologic conditions in mammals, a balance is maintained between ROS formation and antioxidant defense [5]. Imbalance between ROS production and elimination is referred to as oxidative stress [5]. ROS are a key component in inflammation in many organs including the kidney [6]. Malondialdehyde (MDA), considered the main indicator of lipid peroxidation, promotes polymerization of components of the glomerular basement membrane to induce proteinuria [5].

The total antioxidant capacity (TAC) has been reported to be decreased at the time of proteinuria development in NS [7]. Both an enzyme system and non-enzymatic scavengers are involved in ROS degradation [5, 7]. The various antioxidants are present at a wide range of concentrations in body fluids and tissues, with some such as enzymes or glutathione existing mostly present within cells [8]. Enzymes of the glutathione S-transferase (GST) supergene family can act

Introduction

Though not fully elucidated, the cause of minimal change nephrotic syndrome (MCNS) is considered to involve abnormalities of cellular and humoral immunity such as increases in T-helper (Th) 2 cytokines reflecting a Th2-dominant state among T lymphocytes, as well as hypersensitivity of CD8⁺ cells to antigenic stimuli [1]. In addition, allergic disorders accompanied by an increase in serum immunoglobulin (Ig) E such as atopic dermatitis

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Correspondence to
Tsukasa Takemura, MD,
PhD
Department of Pediatrics,
Kinki University
School of Medicine,
377-2 Ohno-higashi,
Osaka-Sayama,
589-8511, Japan
tsukasa@
med.kindai.ac.jp

upon a wide variety of products of oxidative stress as substrates, and thus are critical in protection of cells and organs from ROS [9]. The GST supergene family includes *GSTM1*, *GSTT1* and *GSTP1* [10]. In addition, in null-function genotypes concerning the corresponding enzymes, allergic responses to various chemicals are enhanced, and serum IgE concentrations are much higher than in wild-type individuals [11].

In this study of children with MCNS showing allergic symptoms, we evaluated mutations of *GSTT1*, whose product carries out ROS degradation and allergic response modulation involving regulation of serum IgE.

Subjects and methods

Subjects

Among 63 pediatric patients with MCNS treated at our institution, we identified 29 children with MCNS accompanied by allergic disease such as bronchial asthma, allergic rhinitis, or atopic dermatitis. Among the 29 patients, 15 who experienced remission without immunosuppressant treatment including corticosteroids, were enrolled in the present study. In the 15 patients, serum IgE concentrations exceeded 2,000 IU/l in 2, ranged from 500 to 1,000 IU/ml in 6, and ranged from 295 to 500 IU/l in 7. The remaining 14 patients receiving immunosuppressants were excluded from this study on account of such treatment.

Polymerase chain reaction (PCR)

Genomic DNA was recovered in the aqueous phase and precipitated with ethanol/sodium acetate. The polymerase chain reaction (PCR) was carried out as described previously [12], testing for *GSTT1* deletion using primers able to serially detect fragments ~ 4,000 bases in length, proceeding from the 5'- to the 3'-end of *GSTT1*. Specific primers were constructed based on previously published sequence data for human *GSTT1* coding regions [13]. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation

at 94 °C for 30 sec, annealing at 63 °C for 30 sec, and extension at 72 °C for 4 min. *STAT3* and *Tyrosine kinase 2 (TyK2)* mutations also were examined by using a direct sequencing method described previously [14]. Analysis of *GSTT1* was performed after we received approval from the Ethics Committee at Kinki University and obtained written informed consent from the patient's family members.

Array comparative genomic hybridization

Array comparative genomic hybridization (CGH) was used to screen for genes showing upregulation or downregulation in each subject. We obtained genomic DNA from a reference sample (46,XY) (Promega p/n G1471) and the present patients. CGH was performed using prefabricated oligo-CGH arrays (244 kb arrays; Agilent Technologies, Palo Alto, CA, USA) consisting of ~ 244,000 in-situ-synthesized 60-mer oligonucleotides spanning the entire genome, resulting in an average genomic distance of ~ 12 kb. These probes included both coding and noncoding sequences on every human chromosome. Hybridization was carried out according to the manufacturer's instructions, and results were visualized by means of CGHAnalytics 3.4 software (Agilent Technologies).

Measurement of ROS and reactive oxygen metabolites (d-ROMs)

Total serum ROS was evaluated using H₂O₂ production by polymorphonuclear leukocytes according to a previously described method [15]. Serum concentrations of degradation-related reactive oxygen metabolites (d-ROMs); degradation products of ROS were measured spectrophotometrically in Carratelli units (UCARR, 1 CARR U = 0.08 mg hydrogen peroxide/dl), using commercial kits (d-ROMs test; Diacron International, Grosseto, Italy) and a FRAS4 analyzer [16]. Then dROMs/ROS ratio then was calculated as a median value.

Table 1. Patient profiles.

Variable	Patient 1	Patient 2
Age (y)/gender	13/M	5/M
Age at onset of NS, y	3	2
Type of NS	FRNS/SRNS	FRSN
Histology	MCNS	MCNS
Serum IgE, IU/l (normal, below 250)	7,800 – 9,600	6,200 – 10,300
Serum IL-4, pg/ml (normal, below 6.0)	83.9	54.6
Serum INF- γ , IU/ml (normal, below 1 : 2)	1.24	0.82
neutrophil phagocytosis/sterilizing function (normal, 70%/90%)	70/95	78/93
CAP RAST <i>staphylococcal</i> endotoxin/fungi	positive/positive	positive/positive
Allergic symptoms	dermatitis/rhinitis	asthma/dermatitis
dROM/ROS ratio	2.07	2.48
gene abnormalities of <i>STAT3/Tyk2</i>	none/none	none/none

FRNS = frequently relapsing nephrotic syndrome; SRNS = steroid-resistant nephrotic syndrome; MCNS = minimal change nephrotic syndrome.

Patient presentation and findings

We identified 2 patients possessing *GSTT1* abnormality by CGH. These 2 patients showed remarkably high serum IgE concentrations together with intractable, severe atopic dermatitis and/or severe asthma according to Japanese guidelines for childhood asthma or atopic dermatitis [17, 18]. Profiles of the 2 children with high serum IgE are summarized in Table 1. While in the remaining 13 children with serum IgE concentrations below 1,000 IU/l showed no *GSTT1* abnormality (Figure 1, 2). These 13 patients also had at least one allergic disease; the severity of illness varied between individuals.

Patient 1

A 13-year-old boy developed NS at the age of 3 years, and was diagnosed with MCNS by renal biopsy specimen findings at the age of 4 years. He had relapsed 12 times by the time of the present study, showing repeated steroid-sensitive or -resistant NS, sometimes requiring immunosuppressants such as cyclophosphamide, cyclosporin A (CyA) and mizoribin. At present, remission has been maintained by administration of CyA. The serum IgE concentration was 7,800 – 9,600 IU/l (normal, < 295 IU/l). The serum concentration of interleukin (IL)-4, which stimulates IgE production, was very high (83.9 pg/ml; normal < 6), while the

concentration of interferon (INF)- γ , which inhibits IgE production, remained normal (1.24 pg/ml; normal range < 1 – 2). Intractable skin eruptions accompanied by *Staphylococcus aureus* skin abscesses occurred over the trunk and all limbs, and severe allergic rhinitis was evident. Neutrophil function was normal, but a CAP radioallergosorbent test (RAST) gave positive results for *staphylococcal* endotoxin and for fungi. *STAT3* and *Tyk2*, genes found responsible for the hyper-IgE syndrome, were normal. No susceptibility to infection was noted. The serum ROS concentration was 151 mean fluorescence intensity unit (MIF), a significantly higher value than those in MCNS patients without *GSTT1* abnormality (median value 94.7) or in healthy control subjects with no nephrosis or allergic disorder (median value 92.5) [16, 19]. The serum dROM/ROS ratio was significantly low in this patient with *GSTT1* abnormality (2.07) compared with that in MCNS patients with no *GSTT1* abnormality (median 3.54) or that in healthy control subjects (3.47) [19], indicating deficient ROS degradation.

Patient 2

A 5-year-old boy developed NS at the age of 2 years, relapsing 4 times during the following year. Renal biopsy was performed because of steroid dependence, leading to the diagnosis of MCNS. Since he continued to experience relapses and remissions, he has received cyclophosphamide to prevent relapse. He also

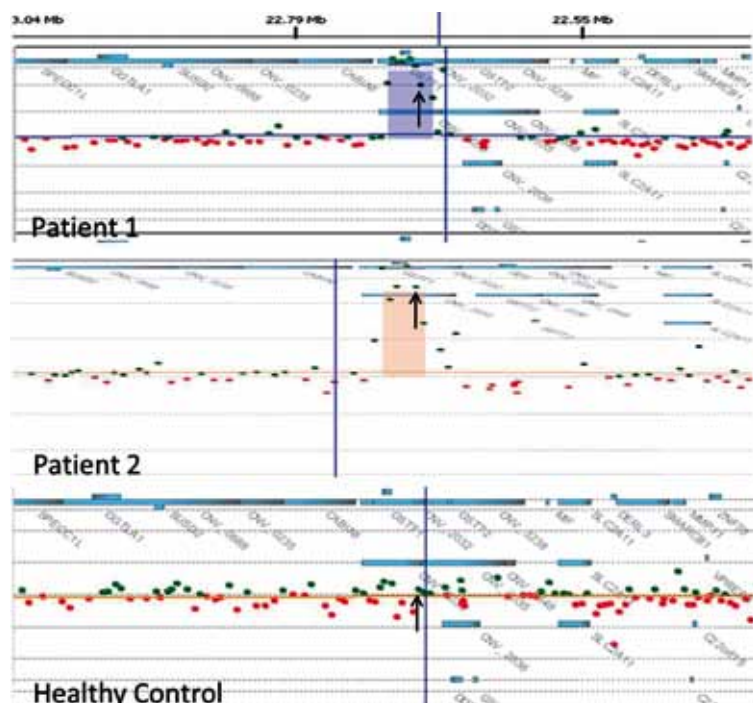


Figure 1. The CGH findings in the 2 patients (Patient 1 and Patient 2) compared with those in a healthy control. Array CGH disclosed nearly complete deletion of *GSTT1*, indicated by arrows.

has been treated by steroid inhalation and oral pranlukast hydrate for moderately severe bronchial asthma. The serum IgE concentration was very high (6,200 – 10,300 IU/l), and serum IL-4 was increased (54.6 pg/ml); the serum INF- γ concentration remained normal (0.82 pg/ml). Neutrophil function was normal. A CAP RAST gave positive results for *staphylococcal* endotoxin and for fungi. No *STAT3* or *Tyk2* abnormalities were found. He achieved complete remission with administration of CyA. The serum ROS concentration was 141 despite CyA administration, significantly higher than those in MCNS patients without a *GSTT1* abnormality or in healthy controls. The serum dROM/ROS ratio (2.48) was lower than in MCNS patients with no *GSTT1* abnormality (3.54) or healthy controls (3.47), indicating insufficient ROS degradation.

To confirm deletions, PCR was carried out. Since *GSTT1* is 8.2 kb in length, we employed a pair of primers able to serially detect fragments 4,000 bases in length from the 5'- to the 3'-end of *GSTT1* coding region to perform PCR. In Patients 1 and 2, no amplification band was detected (Figure 2), confirming the CGH results.

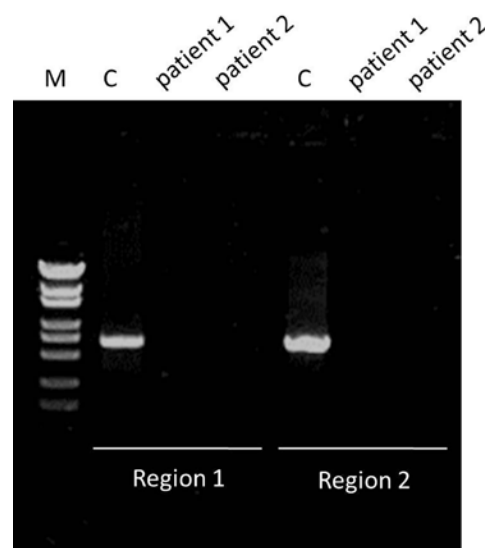


Figure 2. Results of *GSTT1* amplification by PCR using specific primer pairs. Deletion testing using a pair of primers able to serially detect fragments ~4,000 bases in length from the 5'- to the 3'-end of the *GSTT1* coding region (Region 1 and Region 2) demonstrated a nearly complete defect of *GSTT1* in these 2 patients.

Discussion

MCNS sometimes accompanies allergic disorders with increased serum IgE, suggesting a shared pathogenetic mechanism. Causes and mechanisms underlying MCNS have not been fully clarified, but corticosteroids and other immunosuppressants can reduce proteinuria; further, allergic diseases such as bronchial asthma and atopic dermatitis may coexist. Some reported patients developed MCNS after vaccination or an insect bite [1]. All of these clinical features suggest an immunologic abnormality involving hypersensitivity to antigenic stimulation. In MCNS patients, the CD8⁺ T-cell antigen receptor structure exhibits a diversity not observed in healthy individuals, in which the receptor apparently is sensitive to multiple antigenic stimuli.

Binding of the transcription factor NF- κ B to intranuclear DNA in response to certain antigenic stimuli also has been reported to be enhanced at onset or recurrence of MCNS [16]. In cultures of peripheral blood lymphocytes, T-helper lymphocytic activity tended to show a Th2-dominant state in MCNS patients, with consequent increases in such

serum cytokines as IL-4, -5, -9, -10 and -13 [2]. Increases in these vascular permeability factors have been proposed to cause structural or functional impairment of the glomerular basement membrane to result in leakage evident as heavy proteinuria [20]. However, serum concentrations of the above cytokines vary considerably between patients, and MCNS has not been induced in experimental animals by administration of these cytokines alone. One might understand then that implicating a particular cytokine as the cause of MCNS therefore would be difficult.

Animal experiments have shown that antioxidant administration decreases excessive glomerular capillary wall permeability caused by serum ROS increases [21]. The kidney is highly vulnerable to deleterious effects of ROS; a single intravenous injection of puromycin-aminonucleoside causes marked proteinuria as a result of attack by ROS [22]. In addition, a reduction of glomerular nephrin expression was demonstrated to be closely related to oxidative stress reaction in an animal model of nephritis [23]. Recently, studies in patients with idiopathic NS have shown an imbalance between oxidant and antioxidant activity [4]. Previous studies have shown increased oxidative stress in leukocytes of patients with NS [24]. ROS, synthesized mainly by peripheral neutrophils, presumably have essential roles in many physiologic and pathophysiologic processes [25]. The action of ROS upon development of MCNS remains unclear, but one might consider that ROS production is increased not only in idiopathic NS but also in puromycin-aminonucleoside nephropathy, where the lipid membrane is directly damaged [26, 27]. Impaired antioxidant status enhances lipid peroxidation [28]. Evidence suggests that during the proteinuric stage of primary NS, circulating lipids bind to and become trapped by the extracellular matrix, where they undergo oxidation that increases ROS production [29]. Accordingly, permeability of the glomerular capillary wall could be increased by ROS elevation in patients with NS, inducing proteinuria.

Individual capacity to tolerate oxidative stress varies, possibly reflecting genetic variability involving *GSTT1*. Our patients showed significantly increased serum ROS concentrations compared with NS patients having no *GSTT1* abnormality or with healthy controls

with no nephrosis or allergic disorder. Inhibition of ROS degradation by a *GSTT1* abnormality likely may favor development of proteinuria by increasing permeability of the glomerular capillary wall or directly damaging that structure. Abnormalities in the GST supergene family also have been found to increase allergic responses to stimuli such as diisocyanates [11]. Similarly, GST supergene family variant was found to be associated with an increase in IgE after challenge with multiple allergens [10]. Impaired ROS degradation together with increased serum IgE caused by the *GSTT1* abnormality could promote development of NS.

The present study suggested that some children with MCNS who have very high serum IgE concentrations may have a *GSTT1* abnormality, resulting in inhibition of ROS degradation and increased IL-4 causing Th1/Th2 imbalance inducing proteinuria. Characteristics of NS in our 2 patients reported here include numerous relapse (10 times or more), representing repeated steroid-sensitive or -resistant NS. They required multiple immunosuppressive agents, indicating intractable NS. Most of our 13 patients without *GSTT1* abnormality responded well to corticosteroid therapy, with a mean relapse numbers of 2.74. Although these 2 patients with *GSTT1* abnormality showed transient remission at the time of this writing, more intense immunosuppressive therapy than that used in NS patients with no *GSTT1* abnormality was required. Since NS may persist for life in children carrying *GSTT1* abnormality, ongoing treatment throughout life with consideration of risk-benefit tradeoffs may be necessary. Additionally, since both patients showed moderately mixed severe allergic symptoms, appropriate control of allergic reactions is necessary, and diets including foods with antioxidant benefits would appear to be indicated.

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