

Original Article

Increased Expression of the *ska* Gene in *emm49*-Genotyped *Streptococcus pyogenes* Strains Isolated from Patients with Severe Invasive Streptococcal Infections

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**SUMMARY:** Since 2000, *emm49*-genotyped *Streptococcus pyogenes* strains have been isolated from patients with severe invasive group A streptococcal infections in Japan, although they had not been isolated before 1999. We compare the characteristics of these strains with those of strains isolated from patients with non-invasive infections. *Sma*I-digested pulsed-field gel electrophoresis profiles of the isolates were almost indistinguishable between these groups, however, we found that *ska* (streptokinase gene) transcriptional levels in the strains isolated from patients with severe invasive infections were significantly higher than those in non-invasive infections.

INTRODUCTION

*Streptococcus pyogenes* (group A streptococcus; GAS) is one of the most common human pathogens. It causes a wide array of infections including acute pharyngitis (strep throat), impetigo, scarlet fever and cellulitis. Furthermore, GAS can also provoke the nonsuppurative sequelae of rheumatic fever and acute glomerulonephritis. Many streptococcal virulence factors involved in these diseases have been reported, including pyrogenic exotoxins, streptokinase and M protein. More than 90 M protein-derived serotypes have been identified, and a molecular approach to the identification of *emm* genes, which encode M proteins, has also been documented (<http://www.cdc.gov/ncidod/biotech/strep/emmdata.html>). An association between clinical symptoms and certain M types has also been seen. Since the late 1980s, streptococcal toxic shock syndrome (STSS) caused by *S. pyogenes* has become a serious problem in both developed and developing countries. The characteristic symptoms progress very rapidly and are fulminant from the onset. Patients develop renal impairment, necrosis of soft tissue, adult respiratory distress syndrome (ARDS), disseminated intravascular coagulopathy (DIC) and multiorgan failure (MOF) within scores of hours, leading to shock and death (1). The first defined case of STSS in Japan was reported in 1992 (2), and the strains of the *emm1* genotype have been found to be dominant in causing STSS in Japan (3). Since 2000, *emm49*-genotyped *S. pyogenes* strains have been isolated from patients with severe invasive GAS infections of STSS in Japan (4). In the present study, we compared some features of *emm49*-genotyped *S. pyogenes* strains isolated from patients with severe invasive and non-invasive infections and identified a difference in streptokinase gene (*ska*) expression.

MATERIALS AND METHODS

**Bacterial strains and growth conditions:** The *S. pyogenes* strains used in this study are described in Table 1 (4). *Escherichia coli* DH5 $\alpha$  was used as a host for plasmid construction. The strain was grown in liquid Luria-Bertani medium with shaking or on agar plates at 37°C. *S. pyogenes* was cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) without agitation or on tryptic soy agar supplemented with 5% sheep blood. Cultures were grown at 37°C in 5% CO<sub>2</sub>. When required, antibiotics were added to the medium at the following final concentrations: kanamycin, 25  $\mu$ g/ml for *E. coli* and 500  $\mu$ g/ml for *S. pyogenes*. The growth of *S. pyogenes* cells was turbidimetrically monitored at 660 nm using a mini photo 518R (Taitec, Tokyo, Japan).

**DNA manipulations:** DNA amplification by PCR, DNA restriction endonuclease digestion, ligation, plasmid preparation and agarose gel electrophoresis were performed follow-

Table 1. Strains of *emm49*-typed *S. pyogenes* used in this study

Strain number	Isolation year	Diagnosis or symptom <sup>1)</sup>
Isolates from patients of non-invasive infections		
Past strains		
B737/34/5	before 1963	unknown
DS-C274	1953	nephritis
Recent strains		
1566	2001	pus
KURUME51	2002	nephritis
K.H1651	2003	nephritis
Isolate from patients of severe invasive infections (5)		
NIH147	2000	NF, ARI
NIH200	2002	STSS
NIH226	2002	NF, ARI, LC
NIH230	2003	STSS
NIH269	2004	STSS

<sup>1)</sup> ARI, acute renal insufficiency; LC, liver cirrhosis; NF, necrotizing fasciitis; STSS, streptococcal toxic shock syndrome (16).

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Table 2. Plasmids used in this study

Plasmid name	Relevant characteristics	Reference
pABG5	promoter probe vector carrying <i>rofA</i> promoter	9
pABG0	Deleted <i>rofA</i> promoter from pABG5	This study
pABG1	pABG0 containing <i>ska</i> promoter derived from B737/34/5	This study
pABG2	pABG0 containing <i>ska</i> promoter derived from DS-C274	This study
pABG3	pABG0 containing <i>ska</i> promoter derived from 1566	This study
pABG4	pABG0 containing <i>ska</i> promoter derived from KURUME51	This study
pABG5	pABG0 containing <i>ska</i> promoter derived from K.H1651	This study
pABG6	pABG0 containing <i>ska</i> promoter derived from NIH147	This study
pABG7	pABG0 containing <i>ska</i> promoter derived from NIH200	This study
pABG8	pABG0 containing <i>ska</i> promoter derived from NIH226	This study
pABG9	pABG0 containing <i>ska</i> promoter derived from NIH230	This study
pABG10	pABG0 containing <i>ska</i> promoter derived from NIH269	This study

ing standard techniques (5). PCR reactions were performed using TaKaRa Ex Taq (Takara Bio, Tokyo, Japan). Nucleotide sequences were determined by automated sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, Calif., USA).

**Transformation of bacteria:** CaCl<sub>2</sub>-competent *E. coli* cells were prepared and transformed following a standard protocol (5). Electrocompetent *S. pyogenes* cells were prepared as described in a previous study (6). Briefly, *emm49*-genotyped strains were inoculated in 10 ml THY containing 20 mM glycine. Cells were washed twice with 0.5 M sucrose. Two hundred microliters of the suspension was mixed with 1  $\mu$ g of DNA at 0°C, and mixture was then transferred into a chilled Bio-Rad electroporation cuvette (inter-electrode distance = 0.2 cm) and subjected to a single voltage shock (25 kV/cm, 400  $\Omega$ , 25  $\mu$ F), using a Gene Pulser™ apparatus (Bio-Rad, Hercules, Calif., USA). Immediately after the pulse, 10 ml of THY medium was added. The sample was incubated at 37°C for 2 h, and then plated onto THY agar containing the antibiotic of interest.

**Pulsed-field gel electrophoresis (PFGE):** PFGE was performed as described previously (7).

**Determination of *spe* (superantigen) and other virulence genes:** PCR amplification was performed under optimal conditions to detect the *spe* genes with specific primers (8).

**Construction of the *ska-phoZF* transcriptional fusion plasmid:** To construct a plasmid that could measure the *ska* transcriptional level, we used the plasmid, pABG5 (9), that has the *phoZF* gene as a reporter gene. We deleted only the promoter region of the *phoZF* gene but not its SD region from pABG5, resulting in pABG0. We amplified a putative *ska* promoter region of each strain by PCR with a pair of primers, *ska*-proF (GGG GAT CCT ACT TGC ATC TCT GGA AAA TAG) and *ska*-proR (GGG AAT TCG TCC CCT CAA CAG TAC CAG CAA). We then cut the PCR product with *Bam*HI and *Eco*RI, and inserted it into the *Bam*HI - *Eco*RI site upstream of the *phoZF* SD region of pABG0, resulting in pABGska1 to pABGska10 (Table 2).

**Assay of PhoZF alkaline phosphatase activity:** A reporter gene (*phoZF*) for the analysis of transcription encodes a chimeric protein consisting of both the amino-terminal domain of protein F and the carboxy-terminal domain of the alkaline phosphatase (PhoZ) of *Enterococcus faecalis*. PhoZF is freely secreted from the cell and can readily be detected in the cell-free supernatant, since it retains alkaline phosphatase activity. Alkaline phosphatase activity was measured following the method described by Alexander et al. (9). Each strain was

incubated statically at 37°C in a 5% CO<sub>2</sub> atmosphere until the optical density reached approximately 0.7 at 660 nm. To inhibit the degradation of alkaline phosphatase by cysteine protease, SpeB, 28  $\mu$ M trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E-64; Sigma-Aldrich, St. Louis, Mo., USA), which is an inhibitor of cysteine proteinase, was added to the THY. Cells were removed by centrifugation (13,000  $\times$  g, 1 min, 25°C), and triplicate samples of 200  $\mu$ l aliquots of cell-free supernatant from each strain were added to the wells of a 96-well microtiter plate in each experiment. A 1-mg/ml solution of *p*-nitrophenyl phosphate (Sigma-Aldrich) in 1.0 M Tris-HCl (pH 8.0) was then added to each well. After incubation at room temperature for 4 h, the absorbance was measured at 405 nm.

**Statistical analysis:** Statistical difference was determined by the Mann-Whitney U test.

## RESULTS

**PFGE analysis of *S. pyogenes emm49* strains:** Using PFGE to separate *Sma*I-digested DNA fragments, we compared the genomic profiles of a total of 10 *emm49* strains isolated from patients of both non-invasive infections (5 strains) and severe invasive infections (5 strains) (Figure 1). The PFGE profiles of 3 strains derived from severe invasive infections (NIH200, NIH230, NIH269) and 2 strains derived from non-invasive infections (1566 and KURUME51) were indistinguishable, and the profiles of the other strains were also somewhat similar to each other. There were no distinctive discriminations in the PFGE profiles between the non-invasive and severe invasive strains.

**Distribution of virulence genes:** In our previous study, we found that recent *emm3*-genotyped strains acquired *speL* genes (7); we therefore examined in the present study whether recent *emm49*-genotyped strains acquired *spe* or other virulence genes. All isolates showed the *speB*, *speF*, *speG*, *scpA*, *ska*, *sagA* and *slo* genes, however, none of the isolates had the *speC*, *speJ*, *speL* (M3), *speL* (M18) or *speM* genes. We found no differences in the possession of known virulence genes.

**Expression of the streptokinase gene (*ska*):** In all cases of severe invasive infections, renal impairment is accomplished in the early stage (7, our unpublished data). The M49 (*emm49*) strain is known to be the most frequently related to pyoderma-associated poststreptococcal glomerulonephritis. Streptokinase production was found to be required for the induction of poststreptococcal glomerulonephritis in an

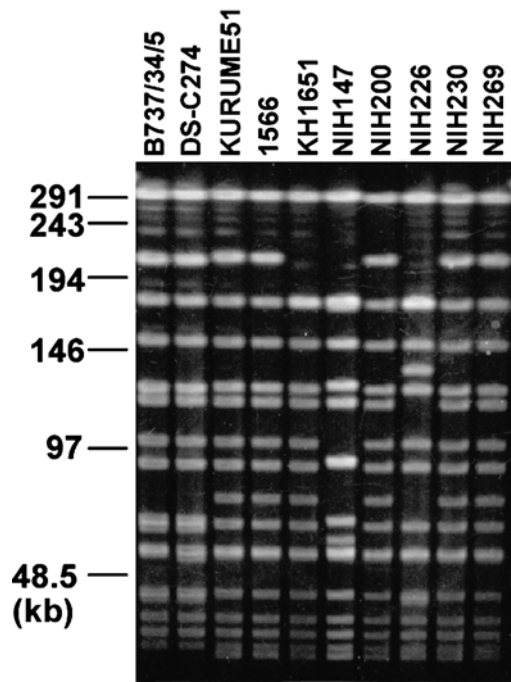


Fig. 1. Ethidium bromide staining of *Sma*I-digested genomic DNAs of *emm49*-genotyped strains after separation by PFGE. Strain number is indicated on each lane. Sizes of lambda concatemers are shown on the left.

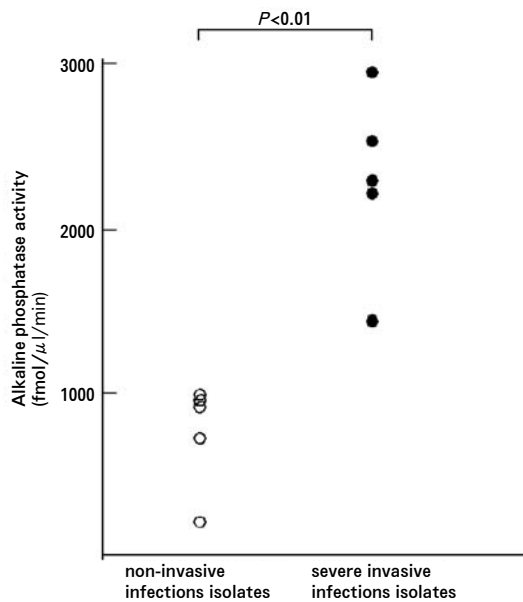


Fig. 2. Transcriptional level of the *ska* gene in strains from severe invasive infections and non-invasive infections. To measure the transcriptional levels of *ska* genes, we used a constructed *ska-phoZF* transcriptional fusion plasmid and measured alkaline phosphatase activity. Mean activity of each strain was shown after the 5 times independent experiments.  $P < 0.01$  by the Mann-Whitney U test.

experimental mouse model (10). Thus, we hypothesized that renal impairment in the early stage of severe invasive infections by *emm49* strains might be due to increased expression of the streptokinase gene. We introduced the *ska-phoZF* transcriptional fusion plasmids, pABGska1 to pABGska10, into each *emm49* strain corresponding to the origin of the promoter, and measured alkaline phosphatase activity, which was found to be significantly higher in isolates from patients

with severe invasive infections than in those from the recent or old non-invasive infections ( $P < 0.01$ ) (Figure 2). As a control, we noted that the activities of all *emm49* strains with only vector pABG0 were almost identical at less than 5 fmol/μl/min. These data suggest that the transcriptional level of the *ska* gene in isolates from severe invasive infections is higher than that in isolates from non-invasive infections. To examine whether this increase in *ska* transcription in isolates from severe invasive infections is due to a change in the sequence in the *ska* promoter region, we compared each of the sequences of the inserted promoter region. The promoter sequences were the same among the 10 strains (data not shown), suggesting that the increase in *ska* transcription in the strains of severe invasive infections is not due to a change in *ska* promoter sequences.

## DISCUSSION

In the present study, we demonstrated that the expression of the *ska* gene in the recent *emm49* isolates of severe invasive infections was significantly higher than that in recent and old *emm49* isolates of non-invasive infections. To the best of our knowledge, this is the first observation showing that isolates from severe invasive infections as clearly different from those from non-invasive infections.

What causes the enhancement of the transcriptional level of the *ska* gene? This enhancement was not caused by a change in the *ska* promoter region. CsrR (CovR) is a transcriptional repressor of the *ska* gene (11). The *csrR* mutant is known to form a mucoid colony (12), however recent isolates of severe invasive infections in the present study did not show mucoid colonies, indicating that the increased expression of the *ska* gene is not due to the *csrR* mutation. The *ska* gene is up-regulated by some activators such as *pell/sagA* RNA and FasBCAX (13-15). Such factors or other unknown factors may be involved in the enhancement of *ska* gene expression.

In a previous study, we reported that recent *emm3*-type strains of *S. pyogenes* have acquired a phage DNA fragment and show an extra DNA fragment in PFGE analysis (7). However, in the *emm49* strains in the present study, we could not discriminate the isolates of severe invasive infections (for example, NIH200, NIH230, NIH269) from those of recent non-invasive infections (1566, KURUME51) by PFGE (Figure 1), although we did find a significant difference between them in the expression of the *ska* gene. We speculate that minor changes in certain genes, such as point mutations and small fragment insertions, might occur and induce this increased expression of the *ska* gene. As streptokinase activity is known to be involved in the induction of poststreptococcal glomerulonephritis (10), enhancement of streptokinase production in *emm49* isolates may be a causative factor for STSS induction. Although this may be characteristic of strains from severe invasive infections, the clarification of *ska* gene enhancement will lead to the identification of the mechanism of severe invasive infections.

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