Abstract: *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) are important etiologic agents in human dental caries. Using quantitative real-time polymerase chain reaction assays for the presence of those strains, we examined 145 outpatients with intellectual disability (ID), calculated the proportion of each of these strains to total bacteria, and compared dental caries incidence over 5 years. Plaque samples were collected from all erupted tooth sites, and dental examinations were performed annually to determine numbers of decayed, missing, and filled teeth (DMFT score; World Health Organization caries diagnostic criteria). Elevated DMFT scores were calculated as ∆DMFT, and sites of newly affected caries (∆SNAC) were identified. Sixty-six patients had both strains. The proportion of *S. mutans* to total bacteria was moderately correlated with DMFT in year 2, ∆DMFT in years 2 and 5, and ∆SNAC in years 2 and 5 (correlation coefficient = 0.470, *P* < 0.001), while the proportion of *S. sobrinus* to total bacteria was moderately correlated with DMFT in years 2 and 5, ∆DMFT in years 1, 2, and 5, and ∆SNAC in years 2 and 5 (correlation coefficient = 0.695, *P* < 0.001). Individuals with ID who harbored both bacterial strains had a higher risk of dental caries and a significantly higher proportion of *S. sobrinus* to total bacteria.

Keywords: caries risk, intellectual disability, real-time quantitative reverse transcriptase-PCR, *Streptococcus mutans, Streptococcus sobrinus*.

Introduction

Intellectual disability (ID) refers to sub-average general intellectual function originating during the development period and is associated with impaired adaptive behavior (1). Many reports have shown that individuals with ID have worse oral hygiene and higher rates of dental disease as compared with the general population (2-4). Until recently, studies of dental caries in individuals with ID primarily focused on children (5,6), although concerns regarding affected adults have recently been discussed (7). The oral health status of individuals with ID is related to their cognitive patterns, developmental anomalies (8), and a variety of other factors, including age, type of caregiver, and physical disability (3). Because of the many potential factors involved, it can be a challenge to improve the oral environment of a patient with ID. Thus, prevention and treatment protocols for improvement of oral health outcomes of adults with ID are clearly necessary (9).

Mutans streptococci (*Streptococcus mutans* [*S. mutans*] and *Streptococcus sobrinus* [*S. sobrinus*]) are important etiologic agents in human dental caries (10-13). These bacteria are the most common putative pathogens isolated from human dental plaque, and their prevalence
has been widely reported in epidemiologic studies (14-16). In addition, various selective media have been used for isolation, quantification, and characterization of mutans streptococi (17). Previous studies indicate that polymerase chain reaction (PCR) methods are more sensitive for detection than conventional culture techniques (18-20), even though they are qualitative analytic techniques and therefore unsuitable for accurate evaluation of caries susceptibility or activity (21). Quantitative analysis is essential for monitoring cell numbers and the proportions of cariogenic bacteria in oral specimens such as dental plaque and saliva (21).

Previous cross-sectional and longitudinal studies found that in preschool children with primary dentition the incidence of dental caries was significantly higher among those harboring both S. mutans and S. sobrinus than among those with S. mutans alone (20,22). In addition, a recent report noted that, in schoolchildren and adults with ID, dental caries experience was significantly greater in both permanent and primary teeth among those harboring both S. mutans and S. sobrinus than among those with S. mutans alone (13,23), which confirmed the findings of a similar study that used real-time quantitative reverse transcriptase-PCR (qRT-PCR) methods to investigate cultures (24,25).

The relative numbers of these commensal species are thought to be fairly constant in healthy individuals, and shifts in these proportions likely indicate disease activity (26). To further investigate the association between these pathogens and dental caries, quantification of bacterial proportions is essential. However, few longitudinal studies have examined the relation between these pathogens and caries incidence in general populations (25,27), and even fewer have investigated individuals with ID (28,29). Detection of these organisms is essential for dental caries prediction and subsequent treatment (22). Investigation of the association between these pathogens and caries risk with the distribution patterns of S. mutans and S. sobrinus in ID patients will assist in promoting caries prevention.

In the present study, S. mutans and S. sobrinus were quantified in plaque samples using a qRT-PCR method. Then, the proportions of these bacteria to total mutans streptococci and the incidence of dental caries over a 5-year period were assessed in Japanese outpatients with ID. In addition, relevant clinical variables that might affect the incidence of dental caries, such as age, sex, ID level, and type of disability, were examined in relation to the incidence of dental caries.

### Materials and Methods

#### Subjects

This study enrolled 145 outpatients with ID aged 11-36 years (average: 22.5 ± 5.1 years). All had permanent dentition and had visited Hiroshima University Hospital in Hiroshima City. Those who had received antibiotics during the previous 3 months and patients with systemic diseases were excluded. Before the study, consent for participation was obtained from at least one of their guardians, in accordance with the ethical guidelines of the Declaration of Helsinki (1975). Ethical approval was obtained from the Ethical Committee of Hiroshima University (Epidemiology-No. 1143).

#### Dental examination

Dental examinations were performed in the Special Care Dental Clinic by a single well-trained dentist (M.O.) with the patient supine in a dental chair. World Health Organization caries diagnostic criteria were used to identify decayed, missing, and filled teeth (DMFT) index (30). DMFT scores were calculated on the day of plaque collection (baseline DMFT) and after 1 (DMFT1), 2 (DMFT2), and 5 (DMFT5) years. Change in DMFT score (ΔDMFT) was calculated at 1 (ΔDMFT1), 2 (ΔDMFT2), and 5 (ΔDMFT5) years after initial plaque collection. Sites of incident caries were determined as ΔSNAC, as indicated by medical records. The numbers of tooth sites (surfaces) with a newly affected dental cavity were counted as sites of newly affected caries (ΔSNAC). In patients with new dental caries requiring treatment of a previously treated tooth, DMFT and ΔDMFT scores would not change because ΔDMFT indicates number of teeth. However, ΔSNAC score would increase because it reveals new tooth sites (surfaces). By using ΔSNAC score, it is possible to quantify incident dental caries and more accurately evaluate caries activity. ΔSNAC scores at 1, 2, and 5 years after initial plaque collection are expressed as ΔSNAC1, ΔSNAC2, and ΔSNAC5, respectively. Dental treatment for a condition other than dental caries was excluded when determining ΔSNAC scores. For example, we excluded treatment for trauma, re-treatment for loss or damage during a restoration, treatment for chronic apical periodontitis, and treatment used as an abutment for a bridge. In the present study, DMFT, ΔDMFT, and ΔSNAC scores were used to evaluate the incidence of dental caries in patients with ID.

#### Classification and verification of patient information

Medical information on the present patients with ID that may have had an effect on the incidence of dental caries (age, sex, ID level, type of disability) was confirmed by
examining the relevant medical records for the period 2008-2013. ID level was classified as very severe, severe, moderate, and mild for IQ scores of less than 20, 20-35, 36-51, and 52-67, respectively. Type of disability was classified as mental retardation, autism, Down syndrome, and cerebral palsy.

Plaque sampling
Dental plaque was collected from all erupted teeth by brushing with a sterile toothbrush for 1 min, as previously described (13). The toothbrush was washed several times in a tube with 15 mL of sterile distilled water, to remove adherent plaque. The sample was then immediately transported to our research laboratory and stored at −20°C, after which genomic DNA was extracted.

DNA extraction from dental plaque samples
Plaque samples were first subjected to centrifugation at 1,600 × g for 20 min. Next, the supernatant was discarded and individual cell pellets were stored at −20°C until DNA isolation, for which the pellets were resuspended in 180 µL of enzymatic lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton X-100, 20 mg/mL lysozyme). A genomic DNA preparation from each plaque sample was obtained using a DNeasy Blood and Tissue Kit (Qiagen, Austin, TX, USA) for DNA extraction of gram-positive bacteria, to which RNase treatment was added (31). DNA concentrations in dental plaque samples were determined by measuring A260, and quality was estimated using the A260/A280 ratio.

Bacterial strains and PCR primers
*S. mutans* ATCC25175 and *S. sobrinus* ATCC33478 were utilized as the bacterial strains. The primers and probes used in this study targeted the *gtfB* and *gtfT* genes of *S. mutans* and *S. sobrinus*, respectively, while another universal primer that targeted the eubacterial 16S rRNA gene was used to quantify the total bacterial load in the samples. The primers and probes (Table 1) were described previously (21,32).

qRT-PCR
For each qRT-PCR assay, 20 µL of a mixture containing 1 µL of lysed cells, 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 200 nM of sense and antisense primers, and a 250-nM TaqMan probe were placed in each well of a 96-well plate. Amplification and detection were performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems) with the following cycle profile: 50°C for 2 min, 95°C for 10 min, and 60 cycles of 95°C for 15 s and 58°C for 1 min. The critical threshold cycle (*C*ₜ) was defined as the cycle at which fluorescence became detectable above the background and was inversely proportional to the logarithm of the initial number of template molecules. The copy number for each target bacterial gene was normalized to the copy number of the 16S rRNA gene, using a simplified comparative *C*ₜ method (21,33).

Statistical analysis
Descriptive statistical analyses were performed using a software statistical package (SPSS 14.0, Inc., Chicago, IL, USA). The means and standard deviations for incremental increases in dental caries were determined using ∆DMFT and ∆SNAC. Pearson correlation coefficients were used to evaluate associations of caries incidence with microbiological findings, age, sex, ID level, and type of disability. The *t*-test was used to compare the means for incremental increases in dental caries (∆DMFT and ∆SNAC).

Results
Among a total 145 subjects, *S. mutans* and *S. sobrinus* were detected in 134 (92.4%) and 69 (47.6%), respectively (Table 2). Sixty-eight (46.9%) subjects were positive for *S. mutans* alone, 3 (2.1%) for *S. sobrinus*

### Table 1 Oligonucleotide primers and probes used in this study (Yoshida et al., 2003)

| Designation | Sequence | Amplicon size | Target |
|-------------|----------|---------------|--------|
| **Primers** |          |               |        |
| Smut3368-F  | 5'-GCCTACAGCTCAGAGATGCTATTCT-3' | 114 | gtfB |
| Smut3481-R  | 5'-GCCATACACCTCTGCATGTAATGTA-3' | 88 | gtfT |
| Ssob287-F  | 5'-TTCAAGCCAAGACACGCTAGT-3' | 69 | 16S rRNA |
| Ssob374-R  | 5'-CCACGCTGTGAGATCGCTG-3' | 250 | 16S rRNA |
| Uni52-F     | 5'-CGCTCAGTATCATCGGGATGATCAAATG-3' | 72 | 16S rRNA |
| Uni220-R    | 5'-TGCTGACGGGCGGTGTGTT-3' | 16S rRNA |
| **Fluorescent probes** | | | |
| Smut3423T  | 5'-FAM-TGGAAAYGACGGTCGTAATGGA-TAMRA-3' | 250 | 16S rRNA |
| Ssob298T  | 5'-FAM-CCTGCTCCACCGCAAAAGGCA-3' | 16S rRNA |
| Uni117T   | 5'-FAM-CACCGTGAATACGTTCCCGGGC-3' | 16S rRNA |
alone, and 66 (45.5%) for both; 8 (5.5%) were negative for both *S. mutans* and *S. sobrinus*.

Among the 66 subjects who were positive for *S. mutans* and *S. sobrinus*, 49 (74.2%) were men and 17 (25.8%) were women (Table 3). Regarding the severity of ID, 12 (18.2%), 45 (68.2%), 6 (9.1%), and 3 (4.5%) patients were classified as very severe, severe, moderate, and mild, respectively. With regard to type of disability, 31 (47.0%), 23 (34.8%), 2 (3.0%), and 10 (15.2%) were classified as having mental retardation, autism, Down syndrome, and cerebral palsy, respectively.

The mean numbers of total bacteria, *S. mutans*, and *S. sobrinus* in subjects who were positive for both bacteria were $1.44 \times 10^9 \pm 1.59 \times 10^9$, $7.01 \times 10^5 \pm 2.79 \times 10^6$, and $4.18 \times 10^5 \pm 1.94 \times 10^6$ cells/mL, respectively (Table 4). Furthermore, the proportions of *S. mutans* and *S. sobrinus* to total bacteria were $4.33 \times 10^{-2} \pm 9.51 \times 10^{-2}$ and $3.38 \times 10^{-2} \pm 7.79 \times 10^{-2}$, respectively.

Among patients who harbored both bacteria, the values for DMFT, ∆DMFT, and ∆SNAC increased from 9.23 to 13.42, from 1.15 to 4.20, and from 1.70 to 7.21 between baseline and year 5, respectively (Table 5). ∆SNAC5 was significantly higher than ∆DMFT5 ($P < 0.01$).

The proportion of *S. mutans* to total bacteria was moderately positively correlated with DMFT2, ∆DMFT2, ∆DMFT5, ∆SNAC2, and ∆SNAC5, and the proportion

| Table 2 | Distribution of mutans streptococci in individuals with ID |
|---------|------------------------------------------------------------|
| Organisms present | Number (%) of subjects |
| *S. mutans* | *S. sobrinus* |
| + – | 68 (46.9) |
| + + | 66 (45.5) |
| – + | 3 (2.1) |
| – – | 8 (5.5) |
| Total | 145 |

| Table 3 | Numbers with breakdown according to classification for subjects |
|---------|---------------------------------------------------------------|
| Classification | Number (%) of patients |
| Sex | |
| men | 49 (74.2) |
| women | 17 (25.8) |
| Intellectual disability | |
| Very severe | 12 (18.2) |
| Severe | 45 (68.2) |
| Moderate | 6 (9.1) |
| Mild | 3 (4.5) |
| Type of disability | |
| Mental retardation | 31 (47.0) |
| Autism | 23 (34.8) |
| Down syndrome | 2 (3.0) |
| Cerebral palsy | 10 (15.2) |

| Table 4 | Numbers of total bacteria, *S. mutans*, and *S. sobrinus* and the proportions of these strains to total bacteria in subjects harboring both bacterial strains |
|---------|---------------------------------------------------------------------|
| Mean ± standard deviation | |
| Total bacteria (cells/mL) | $1.44 \times 10^9 \pm 1.59 \times 10^9$ |
| *S. mutans* (cells/mL) | $7.01 \times 10^5 \pm 2.79 \times 10^6$ |
| *S. sobrinus* (cells/mL) | $4.18 \times 10^5 \pm 1.94 \times 10^6$ |
| *S. mutans*/total bacteria (%) | $4.33 \times 10^{-2} \pm 9.51 \times 10^{-2}$ |
| *S. sobrinus*/total bacteria (%) | $3.38 \times 10^{-2} \pm 7.79 \times 10^{-2}$ |

| Table 5 | Caries incidence in subjects harboring both bacterial strains (2008-2013) |
|---------|--------------------------------------------------------------------------|
| Period | Mean ± standard deviation |
| DMFT | 2008 (baseline) | 9.23 ± 6.54 |
| DMFT1 | 2009 (after 1 year) | 10.38 ± 7.04 |
| DMFT2 | 2010 (after 2 years) | 11.36 ± 7.60 |
| DMFT5 | 2013 (after 5 years) | 13.42 ± 7.54 |
| ∆DMFT | 2008-2009 (for 1 year) | 1.15 ± 1.60 |
| ∆DMFT2 | 2008-2010 (for 2 years) | 2.64 ± 2.97 |
| ∆DMFT5 | 2008-2013 (for 5 years) | 4.20 ± 3.49 |
| ∆SNAC1 | 2008-2009 (for 1 year) | 1.70 ± 1.64 |
| ∆SNAC2 | 2008-2010 (for 2 years) | 2.97 ± 3.31 |
| ∆SNAC5 | 2008-2013 (for 5 years) | 7.21 ± 7.24* |

Statistically significant difference between ∆DMFT5 and ∆SNAC5; *$P < 0.01$, t-test.
of \textit{S. sobrinus} to total bacteria was moderately positively correlated with DMFT2, DMFT5, all ADMFT values, ΔSNAC2, and ΔSNAC5 (Table 6). Furthermore, age was moderately positively correlated with DMFT at all time points.

Regression lines for the association of ΔSNAC5 with quantitative levels of \textit{S. mutans} and \textit{S. sobrinus} in plaque samples from subjects harboring both bacteria are shown in Fig. 1. Those levels are expressed as proportions of \textit{S. mutans} and \textit{S. sobrinus} to total bacteria.

**Discussion**

This 5-year longitudinal study evaluated caries risk in outpatients with ID harboring both \textit{S. mutans} and \textit{S. sobrinus}. We used real-time qRT-PCR to investigate the clinical factors of age, sex, ID level, and type of disability.

The male:female ratio was 49:17, which is consistent with data from previous surveys of patients with autism.
and mental retardation (34,35). The vast majority of our subjects had severe or very severe ID, most likely because individuals with moderate or mild ID can be cared for by general practitioners in Japan. The rate of Down syndrome in the present study (3.0%) was lower than that (6.3%) in a previous report (36). Individuals with Down syndrome are highly susceptible to periodontal disease and thus some were excluded from the present analysis because of tooth loss caused by periodontal destruction (37,38).

*S. mutans* and *S. sobrinus* are the most commonly isolated bacterial species associated with dental caries and are regarded as the main pathogenic microorganisms of the disease (10,11,14,15). The present findings show that the prevalence of mutans streptococci was 94.5% in subjects aged 11-36 years, which is consistent with the findings of a previous study of Down syndrome patients aged 1-48 years (29). However, other studies have reported lower prevalence rates for mutans streptococci in children (13,20,27,39). Because *S. sobrinus* becomes established later than *S. mutans* in the oral cavity of children older than 3 years (40), it is likely that the retention rate for *S. sobrinus* in the permanent dentition of children with ID would be greater than in normal children. The high detection rate of *S. sobrinus* in the present study is not a finding particular to patients with ID but is instead the result of the older age of the subjects. In addition, the percentages of subjects with positive test results for *S. mutans* and *S. sobrinus* were 92.4% and 47.6%, respectively, and 45.5% had both *S. mutans* and *S. sobrinus*. These findings are similar to those reported in previous PCR studies of children aged 3-5 years (25) and 12 years (41) and adults aged 25-55 years (42). The inconsistencies between past and present findings are at least partially attributable to the detection methods used and ethnic backgrounds and ages of the study subjects.

In the present study, the proportions of *S. mutans* and *S. sobrinus* to total bacteria were 92.4% and 47.6%, respectively, and 45.5% had both *S. mutans* and *S. sobrinus*. These findings are similar to those reported in previous PCR studies of children aged 3-5 years (25) and 12 years (41) and adults aged 25-55 years (42). The inconsistencies between past and present findings are at least partially attributable to the detection methods used and ethnic backgrounds and ages of the study subjects.

In the present study, the proportions of *S. mutans* and *S. sobrinus* to total bacteria are consistent with the findings of previous studies of preschool children (25,26). However, there were large variations in the proportions of *S. mutans* and *S. sobrinus* to total bacteria, perhaps because of differences in oral conditions and the wide age range of the study subjects.

Since many characteristics associated with ID may increase the risk of oral disease (43), persons with ID may have worse oral hygiene and more dental disease than the general population (2-4). Various classification systems are available to assess prevalence, risk, and prevention of dental caries and promote oral health (44). DMFT scores in patients with ID are thought to be higher than those of the general population. In the present longitudinal study, we used ΔSNAC and patient medical records (2008-2013) to assess the risk of dental caries. To evaluate the incidence of dental caries, ΔSNAC scores were used as a new straightforward variable for accurately determining the number of new caries. ΔSNAC is useful for all patients, regardless of the presence of ID, who have permanent dentition and a high DMFT score. In cases of new dental caries in a previously treated tooth, the DMFT score does not change. To determine ΔSNAC, we excluded restorations for a cause other than dental caries, including treatment for trauma, re-treatment for loss or damage during restoration, treatment for chronic apical periodontitis, and treatment used as an abutment for a bridge. Use of the ΔSNAC score enabled quantification of incident dental caries and more accurate evaluation of caries activity. In the present study, the increase in ΔSNAC score was significantly greater than that in ADMFT score (Table 6), which suggests that ΔSNAC better reflects current rather than past dental caries activity.

In the present study, we chose real-time qRT-PCR to monitor bacterial cell number and the proportions of cariogenic bacteria, as it is the most accurate and reliable tool to investigate caries risk in clinical plaque samples. Specific primers used to amplify the species-specific target sequence can provide detection specificity. It has also been suggested that a TaqMan assay is accurate and useful for absolute and relative quantification of cariogenic bacteria in oral specimens (21). The proportion of *S. mutans* to total bacteria in this study was moderately positively correlated with DMFT2, ∆DMFT2, ∆DMFT5, ΔSNAC2, and ΔSNAC5. Furthermore, the correlation coefficients of DMFT, ∆DMFT, and ΔSNAC with the proportion of *S. mutans* increased in a longitudinal manner from 0.158 to 0.312, from 0.218 to 0.450, and from 0.114 to 0.470, respectively. In addition, the proportion of *S. sobrinus* to total bacteria was moderately positively correlated with DMFT2, DMFT5, ∆DMFT at all time points, ΔSNAC2, and ΔSNAC5, the last of which had the strongest correlation (r = 0.695, P < 0.001). The correlation coefficients of DMFT, ∆DMFT, and ΔSNAC with the proportion of *S. sobrinus* increased in a longitudinal manner from 0.210 to 0.451, from 0.352 to 0.581, and from 0.230 to 0.695, respectively. In addition, quantitative levels of *S. mutans* and *S. sobrinus* in plaque samples were significantly associated with caries prevalence. We noted a significantly higher proportion of *S. sobrinus* to total bacteria, which is in agreement with previous studies of preschool children (25,45,46). Children with a higher proportion of *S. sobrinus* to *S. mutans* in dental plaque have a higher incidence of early childhood caries (25). In contrast, another study found a
significant correlation between DMFT score and quantitative level of salivary S. mutans but not S. sobrinus (27). Because of the relatively wide age range and small number of subjects in our study, additional studies are needed in order to confirm the present clinical findings.

Development and progression of dental caries result from interactions between oral microorganisms, dietary carbohydrates, and tooth enamel (47). In addition, it is thought to be necessary to examine the background characteristics of patients with ID, as such characteristics might affect development and progression of dental caries (4). In the present study, we assessed the effects of age, sex, ID level, and type of disability on dental caries incidence and proportions of caries-causing bacteria. Age was moderately positively correlated with DMFT1, DMFT2, and DMFT5. The correlation coefficients of DMFT with age increased in a longitudinal manner from 0.522 to 0.436. However, sex, ID level, and type of disability were not correlated with DMFT, ∆DMFT, or ASNAC. However, the present sample size was small, and further investigations are required. In addition, basic research is needed in order to determine why the risk of dental caries increases in patients with a high proportion of S. sobrinus and to identify the mechanisms involved.

In conclusion, our results show that dental caries risk is higher among individuals with ID harboring both S. mutans and S. sobrinus and among those with a significantly higher proportion of S. sobrinus to total bacteria. Furthermore, individuals with ID harboring both S. mutans and S. sobrinus have a significantly higher incidence of dental caries than do those with S. mutans alone.

Conflict of interest
The authors have no conflict of interest to declare.

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