Jonasson, A; Eriksson, C; Jenkinson, HF; Kilestl, C; Johansson, I; Strmberg, N (2007) Innate immunity glycoprotein gp-340 variants may modulate human susceptibility to dental caries. BMC infectious diseases, 7. p. 57. ISSN 1471-2334 DOI: https://doi.org/10.1186/1471-2334-7-57

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Innate immunity glycoprotein gp-340 variants may modulate human susceptibility to dental caries

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Abstract

Background: Bacterial adhesion is an important determinant of colonization and infection, including dental caries. The salivary scavenger receptor cysteine-rich glycoprotein gp-340, which mediates adhesion of Streptococcus mutans (implicated in caries), harbours three major size variants, designated gp-340 I to III, each specific to an individual saliva. Here we have examined the association of the gp-340 I to III polymorphism with caries experience and adhesion of S. mutans.

Methods: A case-referent study was performed in 12-year-old Swedish children with high (n = 19) or low (n = 19) caries experiences. We measured the gp-340 I to III saliva phenotypes and correlated those with multiple outcome measures for caries experience and saliva adhesion of S. mutans using the partial least squares (PLS) multivariate projection technique. In addition, we used traditional statistics and 2-year caries increment to verify the established PLS associations, and bacterial adhesion to purified gp-340 I to II proteins to support possible mechanisms.

Results: All except one subject were typed as gp-340 I to III (10, 23 and 4, respectively). The gp-340 I phenotype correlated positively with caries experience (VIP = 1.37) and saliva adhesion of S. mutans Ingbritt (VIP = 1.47). The gp-340 II and III phenotypes tended to behave in the opposite way. Moreover, the gp-340 l phenotype tended to show an increased 2-year caries increment compared to phenotypes II/III. Purified gp-340 I protein mediated markedly higher adhesion of S. mutans strains Ingbritt and NG8 and Lactococcus lactis expressing Agl/II adhesins (SpaP or PAc) compared to gp-340 l and III proteins. In addition, the gp-340 I protein appeared over represented in subjects positive for Db, an allelic acidic PRP variant associated with caries, and subjects positive for both gp-340 I and Db tended to experience more caries than those negative for both proteins.

Conclusion: Gp-340 I behaves as a caries susceptibility protein.
Background

Dental caries is one of the most prevalent human infectious diseases with life style and genetic factors modifying disease activity [1-4]. The skewed distribution of caries in Western populations today and its weak association with traditional life style factors, e.g. sugar intake and oral hygiene [5], suggest genetic components in caries development. Early arguments for a genetic predisposition came from twin studies [6] and the Vipeholm study [1] showing large individual differences in caries development in spite of similar exposures to sugars.

Dental caries is a mixed species infection caused by an ecological shift from commensal toward cariogenic streptococci [2-4], including Streptococcus mutans [3]. Among potential caries susceptibility alleles or proteins are accordingly multiple salivary proteins [7], e.g. salivary agglutinin/gp-340 [8-10] and proline-rich proteins (PRPs) [10-12], involved in oral biofilm formation, tissue homeostasis and immunological surveillance [13-15]. While salivary agglutinin mediates aggregation (clearance) and adhesion (colonization) of S. mutans and other streptococci [16-18], PRPs primarily attach commensal streptococci and actinomycetes to teeth [13]. Accordingly, caries resistant subjects coincided with increased adhesion of commensal Actinomyces and the highly prevalent allelic PRP variants PRP-1 and PRP-2 [10]. By contrast, caries prone subjects coincided with increased salvia adhesion of S. mutans and Db, a low prevalence allelic acidic PRP variant [10]. Salivary agglutinin is the major adhesion and aggregation factor in saliva for S. mutans and is targeted by its major surface adhesin polypeptide, antigen I/II (AgI/II) [19]. Oral viridans streptococci generally express conserved, but species-specific, AgI/II polypeptides [19]. However, while the AgI/II adhesion SpaP (or PAc) expressed by S. mutans is the principal surface adhesin interacting with gp-340, the commensal organism Streptococcus gordonii expresses additional gp-340-interacting adhesins, including Hsa [20,21]. The AgI/II polypeptides interact with host cells and are potent activators of cell-mediated responses [19,22], and have been used for vaccine and anti-adhesion protection against S. mutans and dental caries [23,24]. We have shown salivary agglutinin to be identical to the scavenger receptor cysteine-rich glycoprotein gp-340 [9] and found three prevalent size variants of saliva gp-340, designated gp-340 I to III, each specific to individual donors [25]. However, the gp-340 I to III size polymorphisms have not been investigated as relates to susceptibility or resistance to dental caries or to differences in AgI/II-mediated adhesion of S. mutans.

Gp-340 [8,9,26] or DMBT1 (deleted in malignant brain tumour, [27]) are protein homologs, encoded by the same dmbt1 gene. They are mucin-like multidomain proteins, composed of 14 repeating scavenger receptor cysteine-rich SRCR domains intercalated by SID domains and followed by CUB and ZP domains. In saliva, gp-340 exists as an oligomer complexed with secretory immunoglobulin A (S-IgA) [16,28]. Salivary gp-340/agglutinin aggregates a wide array of bacteria and viruses via O-glycosylated Ser/Thr-rich SID repeats and N-glycans [9,20,29,30]. It behaves differently in fluid versus surface adsorbed form [20,21]. While fluid phase gp-340 aggregates only certain streptococcal phenotypes, surface adsorbed gp-340 selectively adhere other phenotypes (even of the same bacterial species) [20,21]. Moreover, the SRCR, CUB and ZP domains interact with multiple protein ligands [14]: SpD and SpA collectins [31], lactoferrin [32], complement factor C1q [33], S-IgA [28], and with MUC5B [34]. Gp-340/DMBT1 are present on macrophages, in lung and brain tissues and in gastric and intestinal mucosa [26], and activate macrophages [31] and PMN cells as well as affect the differentiation mode of epithelial cells [14]. Accordingly, the gp-340/DMBT1 proteins are considered pattern recognition molecules in various host innate defences [14].

The partial least squares (PLS) multivariate projection technique derives its usefulness from its ability to deal with multiple and noisy variables and multicollinearity in data structures [35,36]. The PLS technique is designed to handle multiple variables measured on relatively few subjects (so-called “short and fat” data structures) compared to traditional statistics that measures a few variables on many subjects (so-called “long and lean” data structures). It has been applied in genomics, proteomics and metabolomics [36], in biochemistry to delineate the chemical features of the RGRPQ peptide derived from the caries resistance PRP-1 polypeptide [37,38], and to delineate biomarkers or other clinical traits in human diseases [36,10,39].

The aim of the present study was to utilize the PLS method to correlate the gp-340 I to III size variants with caries experience and saliva adhesion of S. mutans in children with high (n = 19) or low (n = 19) caries experience, as well as to substantiate possible mechanisms behind identified associations. The results reveal a positive association of gp-340 I with both caries experience and saliva adhesion of S. mutans, and that purified gp-340 I protein mediates increased AgI/II-mediated adhesion of S. mutans.

Methods

Study groups, clinical recordings and saliva measurements

Twelve-year-old high caries cases (n = 19) and low caries referents (n = 19) from three Public Dental Health Clinics in Sweden were used in the present study. The 12-year-old children were nested within the northern portion of a Swedish nationwide cohort study of 3,400 children [5,10]. The cases were randomly selected from the children with 4 or more new enamel/dentin lesions during
the latest year (mean baseline DMFS = 5.0), and the refer-
ents were matched for gender and living area from caries
free individuals (baseline DMFS = 0). Caries was recorded
at base-line and after 2 years (2-year increment of dentin
and enamel lesions). The study was approved by the Eth-
ics Committee at Umeå University, Umeå, Sweden.

Data collection and saliva analyses were largely performed
as described [5,10]. Briefly, a questionnaire was used for
analyses of life style factors, e.g. sugar intake, oral hygiene,
fluoride exposure etc, and fresh whole saliva for analyses
of saliva factors, e.g. flow rate, pH, buffer capacity etc.
Parotid saliva, collected on ice using Lashley cups and 3 %
citric acid stimulation, was stored frozen (-80°C) in ali-
quots for subsequent Db and gp-340 phenotyping and the
ability to mediate adhesion of S. mutans to saliva-coated
hydroxyapatite [10]. Typing of Db+ subjects (hetero-
or homozygous) versus Db- subjects (completely lacking Db
but harbouring two or more of the allelic PRP-1, PRP-2,
Pf or Pa variants), used native alkaline electrophoresis as
described [10]. Missing saliva data for some measure-
ments resulted in final analyses using either 36, 37 or 38
subjects.

Gp-340 phenotyping

Gp-340 I to III typing of parotid saliva was done by West-
ern blot using mAb143 directed to the gp-340 protein
core [9,25]. Saliva samples were boiled in sample buffer
(62.5 mM Tris, 10.1 % glycerol, 2 % SDS, 0.01 % pyronin)
for 5 minutes. Proteins were separated by SDS-PAGE
using precasted 5 % polyacrylamide gels (Bio-Rad, Her-
cules, CA) and running buffer (25 mM Tris, 192 mM gly-
cin, 0.1 % SDS), pH 8.3. Separated proteins were trans-
ferred to an Immobilon-P transfer membrane (0.45
µm, Millipore, Billerica, MA) using 65 mA/membrane for
60 minutes. Subsequently, the membranes were incu-
bated with 5% non-fat dried milk in TBS-T (50 mM Tris,
150 mM NaCl, and 0.05 % Tween 20), pH 7.4, overnight
at 4°C. The blocked membranes were overlaid with
mAb143, diluted 1:100,000 in TBS-T with 5 % non-fat
dried milk, for one hour at room temperature. After
repeated washes with TBS-T, the membranes were incu-
bated for one hour with horseradish peroxidase-conju-
gated goat anti-mouse IgG (Nordic Biosite, Stockholm,
Sweden) in TBS-T containing 5 % non-fat dried milk.
After repeated washes, bands were detected using chemilumi-
nescence (SuperSignal Substrate, Pierce, Rockford, IL).

Gp-340 I to III phenotyping of saliva from the 38 children
was performed using three gp-340 I to III saliva pheno-
types as typing references. The majority of gp-340 I to III
phenotypes, including all I and III phenotypes, were dis-
tinguished in a single electrophoretic analysis. Some gp-
340 II phenotypes required one or two additional electro-
phoretic runs to safely be distinguished from III. All sali-
vas, except one with a double band character, adhered to
the single band and size typing criteria.

Purification of gp-340 I to III proteins

The gp-340 I to III protein variants were purified from
parotid saliva from three donors as described [9,25].
Briefly, fresh parotid saliva diluted 1:1 in 10 mM phos-
phate buffered saline (PBS, K2HPO4, 150 mM NaCl), pH
6.8, was mixed with a suspension S. mutans Ingbritt (5 × 109
cells/ml) and allowed to aggregate for 60 minutes at
37°C. After addition of 50 mM EDTA to the pelleted
aggregates, released gp-340 was purified by gel filtration
(Superdex 200 26/60; Pharmacia, Uppsala, Sweden). Pro-
tein concentration and purity of isolated gp-340 I to III
proteins were determined by the DC protein assay (Bio-
Rad) with bovine serum albumin (BSA) as a standard,
densitometric analyses of Coomassie Blue-stained gels
and by Western blotting with mAb143.

Bacterial strains and culturing

S. mutans strains Ingbritt, NG8 and mutant 834 were
grown in Brain Heart Infusion broth (BHI; Difco labora-
tories, Detroit, MI) or Jordan broth [20] at 37°C for 14–
16 hours. The isogenic S. mutans 834 Δpac mutant was
generated from wild type strain NG8 by allelic replace-
ment [40] and cultured as described above except for
addition of erythromycin (5 µg/ml) to the media [20].
The pac and spaP genes were cloned into the vector
pTREX1-usp45LS and expressed on the surface of wild
type Lactococcus lactis MG1363 as described previously
[21]. Lactococci strains were grown in M17 broth (Merck,
Darmstadt, Germany) at 30°C for 14–16 hours with or
without (wild type MG1363) addition of erythromycin (5
µg/ml) to the media. The bacterial cells were [35S]-labelled
by adding [35S]methionine to the growth medium prior to
culturing as described [20].

Adhesion of bacteria to gp-340 I to III proteins

Bacterial adhesion to hydroxyapatite beads coated with
purified gp-340 protein was analysed [20]. Briefly, after
hydration of the hydroxyapatite beads (5 mg/well, Macro-
Prep ceramic hydroxyapatite Type II, 80 µm, Bio-Rad) in
buffered KCl (1 mM KH2PO4, K2HPO4 buffer, pH 6.5, con-
taining 50 mM KCl, 1 mM CaCl2, and 0.1 mM MgCl2)
overtight at 4°C, the beads were washed with gp-340 pro-
tein (1–6 or 2 µg/ml in buffered KCl) for 60 minutes at
room temperature. The beads were blocked with 5% BSA
for 60 minutes, washed, and incubated with [35S]methio-
nine-labelled bacteria (5 × 108 cells/ml in buffered KCl
supplemented with 0.5% BSA) for 60 minutes at room
temperature. After washings, the numbers of bound bac-
teria were measured by scintillation counting.
**PLS modelling**

The partial least squares (PLS) projection method was performed using the Simca-P software (version 10.5, Umetrics AB, Umeå, Sweden) as described [35,36]. PLS establishes the information in x variables that relates to the variation in Y in a multivariate model. An X matrix, containing life-style (e.g. sugar intake, oral hygiene, use of fluorides) and saliva (e.g. allelic PRP variants, pH, buffer capacity, gp-340 I to III) variables were modelled against two different Y matrices. One Y matrix was composed of eight individual caries measures (i.e. fillings, and dentine or enamel caries at various tooth surfaces as described) [10], and another Y matrix consisted of saliva adhesion of *S. mutans* (i.e. adhesion of *S. mutans* to hydroxyapatite beads coated with parotid saliva diluted 1:1). The X and Y matrices are described in detail elsewhere [10], except for the present inclusion of gp-340 I to III as qualitative variables in the X matrices. The associations between each x-variable and the Y matrix are expressed as PLS regression coefficients and VIP-values (Variable Importance in Projection), where a VIP>1 indicates that the x-variable is influential for explaining Y.

**Statistics**

Differences between group means (bacterial adhesion) were tested with Student’s unpaired t test (2 groups) or ANOVA followed by Tukey’s test (>2 groups). Differences in distribution were tested with the Chi²-test. Group differences in caries scores (DMFS or caries increment) and gp-340 antibody staining/amounts were tested with the Mann Whitney U test. All tests were 2-sided except for caries increment, and the significance level was set at p < 0.05.

**Results**

**The gp-340 I phenotype correlated with susceptibility to caries**

The children with high (n = 19) or low caries experience (n = 19) were phenotyped for gp-340 I to III protein variants, based on their saliva gp-340 protein banding pattern by anti-gp-340 mAb143 in Western blot (Figure 1). All subjects except one displayed the gp-340 I, II or III phenotypes (10, 23 and 4, respectively). A single subject with a gp-340 double band character [25] was not considered in the subsequent analyses.

In multivariate PLS modelling (including the multiple life style and saliva variables), the gp-340 I to III phenotypes were correlated with caries experience (Figure 2A). The variable set rendered a two component PLS model explaining (R²) and predicting (Q²) the variance in caries experience at an acceptable level (R² = 0.56, Q² = 0.20). In this model, the gp-340 I phenotype correlated (VIP = 1.37) with a high caries experience, while the gp-340 II and III phenotypes tended to behave in the opposite way (VIPs<1) (Figure 2A). The correlation between gp-340 I phenotype and caries occurred at a level similar to traditional factors (e.g. sugar intake and oral hygiene) and to novel host factors (e.g. saliva adhesion of *S. mutans* and the susceptibility protein Db) (Figure 2A). The PLS model was stable, i.e. the correlations for the gp-340 I to III phenotypes remained the same when modelling was done with 1/3 of the subjects randomly and consecutively excluded (data not shown).

**The gp-340 I saliva phenotype mediated increased adhesion of *S. mutans* and Ag/II/II polypeptides**

PLS was used to correlate the gp-340 I to III phenotypes with the ability of saliva from the children to mediate adhesion of *S. mutans* to saliva-coated hydroxyapatite (Figure 2B). A one component PLS model with R² = 0.51 and Q² = 0.27 was generated. The gp-340 I phenotype coincided with a high adhesion of *S. mutans* Ingbritt (VIP = 1.47), while the opposite tended to be true for gp-340 phenotypes II (VIP<1.0) and III (VIP = 1.06) (Figure 2B).

We next investigated if purified gp-340 proteins I to III mediated different adhesion levels of *S. mutans* through recognition by Ag/II proteins (Figures 3A to 3C). The gp-340 I protein promoted markedly higher adhesion of *S. mutans* Ingbritt and NG8 compared to gp-340 II and III (Figure 3A, 46%, 25% and 16% adhering cells, respectively, p < 0.001 for I vs II or III). *S. mutans* mutant 834, derived from *S. mutans* NG8 and abrogated in expression...
of PAc (Agl/II) protein, showed no adhesion to gp-340 I (Figure 3B). Moreover, the gp-340 I protein mediated several fold higher adhesion than gp-340 II and III of Lactococcus lactis expressing AgI/II polypeptides from strains Ingbritt and NG8 (SpaP and PAc, respectively) (Figure 3C). Wild-type vector control L. lactis MG1363 cells not expressing any Agl/II protein did not adhere to gp-340 proteins I, II or III (Figure 3C). Taken together, these results suggest that gp-340 I is preferentially recognized by the S. mutans Agl/II polypeptide and promotes high affinity adhesion and oral colonization by pathogenic S. mutans bacteria.

The gp-340 I phenotype coincides with an increased caries increment and the caries susceptibility protein Db

To validate our findings from the PLS model (using eight dependent Y caries measures), we also analysed the gp-340 I versus II/III phenotypes for differences in 2-year caries increment by means of traditional statistics (Figure 4A). The caries increment was higher for gp-340 I compared to gp-340 II/III phenotypes (p = 0.027, Figure 4A). In addition, the gp-340 I versus II/III phenotypes did not differ significantly in gp-340 amounts as inferred from mAb143 staining of gp-340 in individual salivas upon Western blotting (data not shown).

We have previously reported that the allelic acid PRP protein variant Db correlates with a high caries experience [10]. The present findings showed the gp-340 I protein to be more common among Db+ subjects (5/11 = 45 %), than among Db- subjects (4/26 = 15%, p = 0.051) harbouring other allelic acidic PRP variants (i.e. PRP-1, PRP-2, PIF, Pa) (Figure 4B). The phenotypes positive for both gp-340 I and Db experienced more caries than those negative for both proteins (4.8 versus 1.5 DMFS, respectively, p = 0.023, Figure 4C).

Discussion

This study suggests for the first time a potential role for gp-340/DMBT1 polymorphisms in human diseases beyond cancer, as it implies the gp-340 I protein as a caries susceptibility protein. Accordingly, the gp-340 I phenotype correlated positively with caries experience when analysed among other variables by PLS modelling, as well as coincided with an increased 2-year caries increment. Moreover, the gp-340 I phenotype correlated positively with saliva adhesion of S. mutans, an intermediate caries measure, and purified gp-340 I protein mediated increased adhesion of the same organism and its major Agl/II surface adhesin. Finally, the gp-340 I protein was overrepresented in subjects positive for Db, another caries susceptibility factor.

It is possible that gp-340 I acts as a caries susceptibility protein by increasing the adhesion and colonisation of S. mutans. Gp-340 I positive subjects displayed increased saliva adhesion of S. mutans, a function previously associ-
A role for gp-340 I in susceptibility to caries is consistent with its potential link to Db, a caries susceptibility PRP protein variant or allele. Gp-340 I was over represented in Db+ as compared to Db- subjects, and gp-340 I+/Db+ phenotypes experienced more caries than those negative for both proteins. The gp-340 and PRP scavenger protein families are located on separate chromosomes, 10 and 12, respectively, but may cooperate in adhesion or molecular networking to neutralize non-self ligands in saliva. Notably, both gp-340 I and Db correlate positively with saliva adhesion of S. mutans [10] and gp-340 interacts with multiple salivary proteins, e.g. S-IgA, lactoferrin, SpD and MUC5B, and co-operate with SpD to neutralize influenza virus in saliva [30].

The present work further emphasizes the usefulness of the PLS method to identify potential target molecules for host susceptibility or resistance in small clinical samples. Notably, the potential PRP-1 resistance polypeptide targeted by this approach releases via bacterial proteolysis an RGRPQ peptide affecting key properties of biofilm formation (i.e. adhesion, proliferation and local pH) in vitro and in vivo [37,38]. Whether the gp-340 size variants are subject to similar proteolytic events or will provide similar drug candidates remains to be determined. Moreover, the potential gp-340 I and Db susceptibility markers are present at about a 15–20% prevalence level. However, it is reasonable to assume that the predictive value of single susceptibility factors – similar to many other potentially polygenetic diseases – will be low for the multifactorial and chronic caries disease [45]. Finally, further studies on
host polymorphisms and their evaluation in disease profiling and risk assessment using larger clinical samples may reveal the usefulness of the gp-340 and other host polymorphisms in risk assessment of caries in a clinical setting.

Conclusion
This report shows that the scavenger protein gp-340 size variant I coincides with host susceptibility to dental caries, and that increased Agl/I- mediated adhesion of the cariogenic bacterium S. mutans may be an underlying mechanism.

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
AJ: planning, adhesion experiments, PLS modeling, data analyses. CE: gp-340 purification and adhesion experiments. CK, HFJ, IJ and NS: overall design and planning, co-ordination and writing of the final manuscript. All authors contributed to writing of the final manuscript.

All authors read and approved the final manuscript.

Acknowledgements
This work was supported by grants from the Swedish Medical Research Council (9106), the Wellcome Trust (064832), and the County Council of Västerbotten. The assistance of Ulla Ohman in experimental parts and guidance in PLS modelling by Conny Wikström (Umetrics AB, Umeå) are acknowledged.

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Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2334/7/57/prepub