Association between the occurrence of matrix metalloproteinases 2 and 9 in parotid saliva with the degree of parotid gland damage in juvenile recurrent parotitis

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Objective: The study was aimed to investigate whether the occurrence of matrix metalloproteinases (MMP) 2 and 9 in parotid saliva of juvenile recurrent parotitis (JRP) patients is associated with the degree of glandular involvement.
Study design. Thirty-three JRP patients were included. Involvement of parotid gland was assessed by sialography. Parotid saliva was assayed for MMP-2 and MMP-9 by zymography. Medical charts were examined for number of recurrences, disease laterality, and time of follow-up. Logistic regression analysis between occurrence of either MMP, the clinical parameters, and sialographic staging was conducted.
Results. None of the clinical parameters under analysis were found to be associated with degree of sialographic involvement. Statistical associations were found between presence of MMP-9 and MMP-2 in parotid saliva and sialographic stage ($P = .017$; odds ratio [OR] 6.5, 95% confidence interval [CI] 1.4-30.4; and $P = .009$; OR 6.1; 95% CI 1.6-23.7; respectively).
Conclusions. Occurrence frequency of MMP-2 and MMP-9 in parotid saliva from affected glands of JRP patients was associated with degree of gland damage.

Juvenile recurrent parotitis (JRP) is an inflammatory disease affecting parotid glands of children whose etiopathogenesis remains an enigma.[1-8] The onset of the disease usually occurs around age 3. Frequently, the symptoms of JRP diminish and may fully disappear spontaneously after puberty.[3-7] The management of JRP is also a controversial issue, and different specialists have opted for different therapeutic strategies.[3,4,7,9-14]

The evaluation of parotid gland involvement in JRP, as in other clinical conditions affecting them, is also a matter of debate. Sialography is a fairly common standard test used in JRP assessment, although in the past few years ultrasonography has gained increasing popularity because of its lower invasiveness.[3,7,9-11,13-18] Sialographic and ultrasonographic imaging data produced by both methods correlate and are consistent with the degree of alteration of the glandular parenchyma.[10,12,17-18] However, neither of the methods provides insight on the activity of the degenerative processes that at any time may be affecting the parenchyma of the ill parotid gland.

Recently we reported that JRP patients at variance with healthy paired control subjects display matrix metalloproteinases (MMP) 2 and 9 in parotid saliva.[19] In that study, no reference to the degree of glandular involvement was made. Matrix metalloproteinases have been shown to occur in a number of degenerative diseases and have been associated with both tissue destruction and tissue remodeling.[20-24] The objective of the present study was to determine whether the occurrence of MMP-2 and MMP-9 in parotid saliva of JRP patients is associated with the degree of glandular involvement. To that aim, we performed a study of association between the occurrence of metalloproteinases MMP-2 and MMP-9 in parotid saliva, sialographic staging, and various clinical parameters probably related to parotid gland involvement, including: 1) number of recurrent episodes of JRP per gland; 2) laterality of the disease as assessed clinically; and 3) time since the diagnosis of the disease.

MATERIALS AND METHODS

Patients

A group of patients was made up of children and adolescents with diagnosed JRP who are under current surveillance at the Pediatric Maxillofacial Surgery Division of the San Juan de Dios Hospital in Santiago, Chile. Patients had been admitted at the medical center

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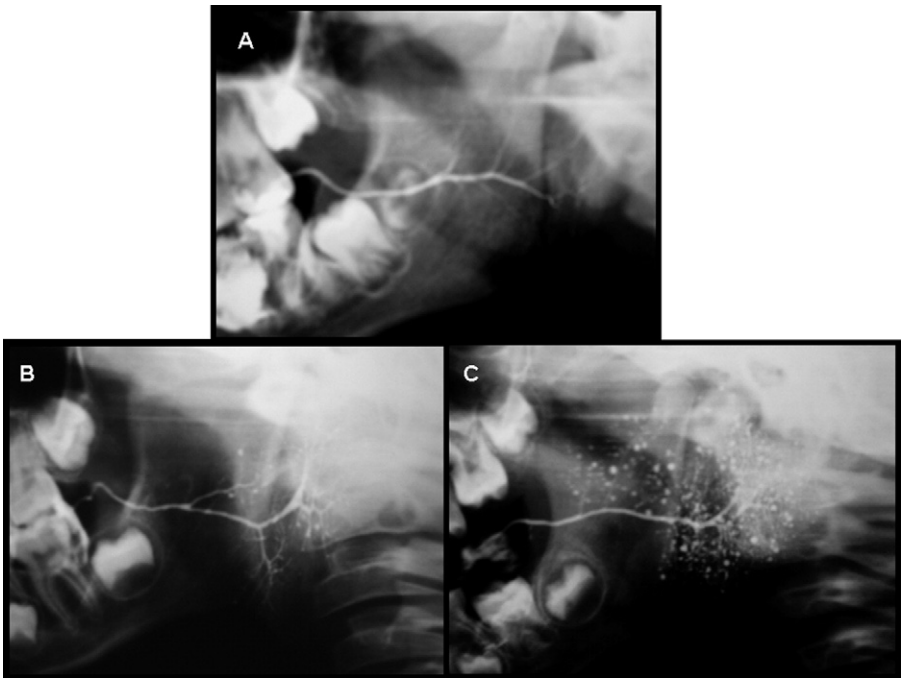

Fig. 1. Sialographic staging. **A,** Normal (stage 0). **B,** Moderate (stage 1). **C,** Severe (stage2).

between 1993 and 2005. Since their admission, patients are checked clinically once a month and the affected parotid glands are lavaged with an iodide/iodine solution administered via the Stensen duct. Sialographic assessment is performed yearly. This routine is repeated until a normal sialographic image of the gland is recovered. A total of 33 patients who attended the medical center for routine medical review at any time during the first 2 weeks of June 2006 were included in the present study. For each patient, samples of saliva were collected from both parotid glands before lavage. Medical charts of each patient were reviewed to draw the following data: date of admission at the medical center, birth date, gender, disease laterality, and number of recurrences per affected gland. Parents of the study participants signed an informed consent. The Ethics Committee of the Faculty of Dentistry, University of Chile, approved the study protocol, which was in accordance with the Declaration of Helsinki.

Degree of involvement of parotid gland as assessed by sialographic staging

Two independent and precalibrated operators classified the degree of involvement of the ductal tree of parotid glands by analyzing the sialogram obtained during the most recent yearly sialographic assessment. To this end, 3 stages were defined, namely: stage 0 or normal sialography, stage 1 (moderate changes; both main, secondary and tertiary ducts of the glands are unaltered and a few radiopaque dots are seen), and stage 2 (more severe changes; the main duct can be altered, both secondary and tertiary ducts have disappeared, and a high density of radiopaque dots are seen; Fig. 1). Stages 1 and 2 correspond to a gradation of the punctate pattern of sialectasis defined by Blatt.[25]

Collection of saliva

One-milliliter samples of right and left parotid saliva were collected by a single trained operator using a collection device previously described by Morales et al.[26] Saliva was collected after the application of a single drop of 2% citric acid to the tongue of the donor. Samples were maintained in ice-cooled containers during the collection and processed immediately.

Zymography

Aliquots of parotid saliva corresponding to 5 μg protein were subjected to electrophoresis in 10% sodium dodecyl sulfate–polyacrylamide gels containing 1 mg/mL gelatin and processed for MMP analysis. The presence or absence of clear bands having identical mobilities as pure MMP-2 and MMP-9 was determined. Unspecific proteases (nonmetalloproteinases) were controlled by incubating salivary samples with a mixture of 50 mmol/L N-ethylmaleimide and 50 mmol/L phenyl-methyl-sulphonyl-fluoride. Metalloproteinase activity was confirmed by incubating the gels in activation buffer supplemented with 5 mmol/L EDTA.[19]

Statistics

For age, months of follow-up, and number of episodes, average and standard deviation or median and range were calculated. Statistical analyses were performed by Student *t* test or the Wilcoxon Mann-Whitney test as appropriate. For laterality, level of gland damage as assessed by sialography, and occurrence of MMP-9 and MMP-2, frequencies were determined. In these cases, statistical analyses were performed by the chi-square test or the Fisher exact test, as appropriate. Group comparisons for quantitative variables were performed by using the Kruskal-Wallis nonparametric test. Association between variables was assessed by logistic regression analysis. Stata version 8.0 statistical software was used. Statistical significances were set at a value of $P < .05$.

RESULTS

Patients

The average age of the 33 patients (12 girls and 21 boys) at the time of the study was 11.2 ± 4.7 years. By then, average ages were 10.8 in the female group (range 5-16) and 11.4 in the male group (range 6-22). The average age of the patients at the time of diagnosis was 6.2 ± 3.6 years (range 1-13). By that time, the average age of the girls was 6.6 ± 3.6 years (range 1-13) and the average age of the boys was 5.9 ± 3.6 (range 1-13). No statistical difference between the ages of both gender groups was observed.

Sialographic staging

All of the altered sialograms displayed the punctate pattern of sialectasis described by Blatt.[25] According to sialographic staging criteria defined under Materials and Methods, out of 46 clinically affected glands 3 were at stage 0 (normal sialographies), 29 were at stage 1, and 14 were at stage 2 (more severely affected). Among the 20 clinically unaffected glands, 16 displayed normal sialographies but 4 of them exhibited sialographic alterations corresponding to stage 1 (Fig. 1 and Table I).

Clinical variables

Laterality of the disease, number of episodes per affected gland, and time of follow-up (the time elapsed between the date of admission at the medical center and the date of saliva collection) were tested as clinical variables that might be related to the level of gland damage (Tables I and II). Of 33 patients in the study, 20 (8 girls and 12 boys) had a single affected gland and 13 (4 girls and 9 boys) were bilaterally affected. In the entire population, time of follow-up was widely distributed in a range from 4 to 148 months (median 56 months); the average number of episodes per affected gland was 2.7 ± 1.2 (range 1-7).

Salivary MMP-2 and MMP-9

The presence of metalloproteinases MMP-2 and MMP-9 in saliva collected separately from both parotid glands of each JRP patient was determined by zymographic analysis (Fig. 2). Of 66 salivas, 34 were found to be MMP-9 positive. Twenty-nine of the salivas testing positive for MMP-9 were obtained from clinically affected glands, whereas 5 were produced by clinically unaffected glands. Of these clinically unaffected glands, 4 were in sialographic stage 1 and 1 was in stage 2. Twenty-one of the 66 tested salivas were MMP-2 positive. Twenty of these MMP-2–positive salivas were obtained from clinically affected glands and 1 from a clinically unaffected gland in sialographic stage 1 (Table III).

Association studies

To determine whether the variables taken into consideration in the study were effectively associated with the degree of gland damage, we performed a bivariate analysis between each of the variables and the sialographic stage of the glands. In this analysis, the degree of parotid damage as evidenced by sialography showed a statistically significant difference between unilateral and bilateral patients. This difference was observed only in the case of stage 0 glands (chi-squared test: $P = .008$; Table II). This analysis also showed that glands in different sialographic stages had experienced a similar number of episodes (Kruskal-Wallis test: $P = .476$; Table II). In addition, the time of follow-up was found to be similar for glands displaying different sialographic stages (Kruskal-Wallis test: $P = .912$; Table II). When bivariate analysis was applied to the occurrence of metalloproteinases in saliva in relation to the sialographic stage of the glands, frequencies of both MMP-2 and MMP-9 showed statistically significant increases in salivas produced by glands displaying higher degrees of damage (chi-squared test: $P < .001$; Table III).

Logistic regression analysis was used to determine associations between clinical parameters of the disease and the sialographic stage of the glands. Clinical parameters included in the study were disease laterality, number of episodes per gland, time of follow-up, and sialographic stage of the glands. No statistical associations were found between laterality and either sialographic stage ($P = .905$; odds ratio [OR] 0.936, 95% confidence interval [CI] 0.319-2.745), number of episodes ($P = .703$; OR 1.099, 95% CI 0.676-1.786), or time of follow-up ($P = .113$; OR 1.015, 95% CI 0.997-1.033).

Table I. Clinical, sialographic, and sialochemical parameters of juvenile recurrent parotitis patients

		Number of episodes		*Sialographic stage*		*MMP-9*		*MMP-2*	
Patient	*Laterality*	*R**	*L**	*R*	*L*	*R*	*L*	*R*	*L*
1	Left	0	2	0	0	−	−	−	−
2	Left	0	3	0	1	−	−	−	−
3	Left	0	2	0	1	−	−	−	−
4	Left	0	2	0	1	+	−	−	−
5	Bilateral	4	3	0	1	−	−	−	−
6	Bilateral	2	2	1	1	−	+	−	+
7	Right	3	0	1	0	−	−	−	−
8	Right	4	0	1	0	+	−	+	−
9	Right	2	0	1	0	+	−	−	−
10	Left	0	7	0	1	−	+	−	+
11	Left	0	2	0	1	−	+	−	+
12	Left	0	2	0	1	−	+	−	−
13	Bilateral	3	2	0	1	−	+	−	+
14	Bilateral	2	2	1	1	+	+	+	+
15	Bilateral	2	2	1	1	+	+	−	−
16	Bilateral	5	5	1	1	−	−	−	−
17	Right	2	0	1	1	+	+	−	−
18	Right	5	0	1	1	−	+	−	+
19	Left	0	2	1	1	−	−	−	−
20	Left	0	2	1	1	+	+	−	−
21	Bilateral	4	4	1	1	+	−	+	−
22	Right	2	0	2	0	+	−	+	−
23	Right	2	0	2	0	+	−	−	−
24	Left	0	2	0	2	−	+	−	+
25	Left	0	2	0	2	+	+	−	+
26	Left	0	2	0	2	−	+	−	+
27	Left	0	5	0	2	−	+	−	−
28	Bilateral	2	2	1	2	+	−	−	−
29	Bilateral	2	2	1	2	+	+	+	+
30	Bilateral	2	2	2	1	−	−	−	−
31	Bilateral	6	2	2	1	+	+	+	+
32	Bilateral	3	2	2	2	+	+	+	+
33	Bilateral	2	2	2	2	+	+	+	+

Table II. Clinical parameters in relation to the degree of damage of parotid glands

Sialographic stage	*Disease laterality**		*Number of episodes†*	*Time of follow-up†*
	Unilateral	*Bilateral*	*Mean ± SD (range)*	*Mean ± SD (range)*
Stage 0 (n = 19)	89.5% (17)	10.5% (2)	3.0 ± 1.0 (2-4)	50.3 ± 29.3 (17-72)
Stage 1 (n = 33)	51.5% (17)	48.5% (16)	2.8 ± 1.3 (2-7)	64.7 ± 37.9 (7-148)
Stage 2 (n = 14)	42.9% (6)	57.1% (8)	2.6 ± 1.3 (2-6)	62.0 ± 39.7 (4-131)

*Statistical significance of the laterality condition per sialographic stage was assessed by the chi-squared test: significant ($P = .008$) for stage 0, nonsignificant for stages 1 and 2.

†Statistical significances of both the number of episodes and the time since diagnosis (months) per sialographic stage were assessed by the Kruskal-Wallis test. No differences were observed in both clinical variables ($P = .476$ and $P = .912$, respectively).

Logistic regression analysis was also used to determine associations between the presence of metalloproteinases MMP-2 or MMP-9 in parotid saliva, clinical parameters of the disease and sialographic stage of the glands. No statistical associations were observed between the presence of MMP-9 in parotid saliva and either laterality ($P = .692$; OR 0.755, 95% CI 0.188-3.033), number of episodes ($P = .544$; OR 0.848, 95% CI 0.498-1.444), or time of follow-up ($P = .455$; OR 1.007, 95% CI 0.987-1.028). In contrast, statistical association was found between presence of MMP-9 in parotid saliva and the sialographic stage of the glands

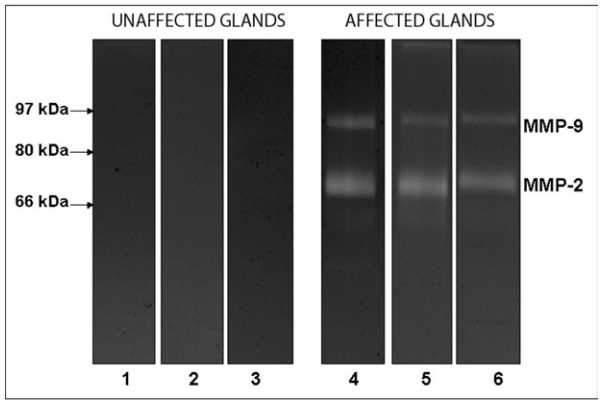

Fig. 2. Representative zymographies for MMP-9 (92 kDa) and MMP-2 (72 kDa) on parotid saliva from unaffected (lanes 1-3) and affected (lanes 4-6) glands of juvenile recurrent parotitis subjects. Lanes from different electrophoretic fractionations were aligned by matching molecular weight standards (*arrows*).

Table III. Occurrence of MMP-9 and MMP-2 in parotid saliva of juvenile recurrent parotitis patients in relation to the degree of gland damage

Sialographic stage	*MMP-9* +*	*MMP-2* +*
Stage 0 (n = 19)	10.5% (2)	0% (0)
Stage 1 (n = 33)	60.6% (20)	33.3% (11)
Stage 2 (n = 14)	85.7% (12)	71.4% (10)

*Statistical significance of the occurrence of either salivary MMP-2 or MMP-9 per sialographic stage was assessed by the chi-squared test. Differences ($P < .001$) were observed in both variables.

($P = .017$; OR 6.5, 95% CI 1.4-30.4). Likewise, no statistical associations were observed between the presence of MMP-2 in parotid saliva and either laterality ($P = .167$; OR 2.746, 95% CI 0.656-11.498), number of episodes ($P = .850$; OR 0.948, 95% CI 0.549-1.634), or time of follow-up ($P = .225$; OR 0.987, 95% CI 0.967-1.007). In contrast, statistical association was found between presence of MMP-2 in parotid saliva and the sialographic stage of the glands ($P = .009$; OR 6.1, 95% CI 1.6-23.7).

DISCUSSION

In a recent report we showed that MMP-2 and MMP-9 are present in a significantly higher frequency in saliva of JRP patients than in saliva of paired healthy controls.[19] However, it remained to be seen whether the occurrence of MMP-2 and MMP-9 in parotid saliva of JRP patients is associated with the degree of salivary gland involvement. Reports on suitable objective parameters for assessing gland damage among JRP patients were not available. Despite its invasiveness, sialography still represents a widely used procedure for assessing the status of the salivary gland tissue in this and other health conditions.[10,15] However, other clinical parameters of the disease might also be associated with gland involvement. In the present study we performed association analyses between the occurrence of MMP-2 and MMP-9 in parotid saliva, sialographic assessment of the glands, and various clinical parameters of the disease. Sialographic assessment of the glands is usually performed following some standard classifications, such as that of Blatt.[25] However, in the present study all of the altered sialograms corresponded to the lowest score of that classification, namely, punctate.[25] Therefore, we further graded the level of sialographic commitment of the glands displaying punctate sialectasis by assessing the involvement of the ductal system. This sialographic staging was taken as the gold standard of parotid gland involvement in this study. Disease laterality assessed clinically, time since the diagnosis, and number of recurrent episodes of JRP per gland were the clinical parameters included in these analyses. In effect, at the beginning of the study it was not clear whether bilateral patients were affected by a more severe type of the disease, and so their glands could be more severely affected. At that time it was not clear either whether patients who had been under a prolonged follow-up presented healthier glands because of the extent of the medical care or whether the glands of those patients were more severely damaged because of an eventual refractoriness to the treatment. Likewise it was also unclear whether patients with a short time of follow-up displayed glands with a higher level of damage because of a recent activation of the disease or, alternatively, with a lower level of damage because of a short period of disease and fewer recurrences. The wide range of distribution of the follow up time periods in the study population was a major advantage to approach these questions (Table II). Finally, because each recurrence of the disease might result in cumulative damage to the affected glands, the number of episodes was considered as another variable which might be related to gland damage.

The data presented here indicate that the levels of gland damage showed no association with disease laterality, time of follow up, or number of episodes. In contrast, the results showed that the level of damage of the affected parotid glands in JRP patients, as assessed by sialography, is associated with the ocurrence of MMP-2 and MMP-9 in the parotid saliva, that is, both MMPs are more frequently present in the saliva produced by the more severely affected parotid glands in these patients. The study was restricted, therefore, to assess either presence or absence of these MMPs with

no measurement of their enzyme activities.[19] Recent studies with Sjögren patients have shown that marked increases in MMP-9 were associated with dramatic alterations in the acinar integrity of minor salivary glands, that both MMP-2 and MMP-9 were markedly elevated in parotid saliva, and that both MMP-9 and the ratio of MMP-9 to its inhibitor tissue inhibitor of metalloproteinases 1 are increased in whole saliva of those patients.[22-24] Altogether, those studies strongly suggest that both enzymes could be associated with the destructive process that occurs in the affected glands of Sjögren patients. This association lends strong support to our view that the occurrence of MMP-2 and MMP-9 in parotid saliva of JRP patients would be a consequence of the gland damage put in evidence by sialography imaging. However, we have not discarded the possibility that the presence of both MMPs in the glandular saliva is also an expression of tissue remodeling occurring simultaneously in the damaged glands. Whatever the meaning of the presence of metalloproteinases in parotid saliva of JRP patients may be, their association with the damage experienced by the affected glands is especially important in considering that direct evidence from the parotid gland can be difficult to obtain because of the restricted accessibility of this tissue for biopsy owing to the close proximity of the facial nerve complex. In this regard, our present contribution offers data in support of the use of parotid saliva as a complementary and noninvasive source of information on the status of parotid glands among JRP patients. Parotid saliva can be readily obtained by using a variety of *ad hoc* collection devices, and therefore each analysis can be repeated as many times as necessary with minimal stress of the patient.[26,27] Thus, the assessment of MMP-2 and MMP-9 in parotid saliva collected from the affected parotid glands of JRP patients could eventually be used advantageously for a molecular monitoring of the evolution or involution of the disease, to evaluate the efficacy of alternative medical trials, to conduct studies on the metabolism of MMPs in the normal and ill salivary tissue which might be relevant for getting insight into the etiopathogenesis of JRP, and for the development of novel therapeutic approaches for JRP.

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