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Original Article

High frequencies of vitamin B12 and folic acid deficiencies and hyperhomocysteinemia in Taiwanese male patients with oral submucous fibrosis

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Abstract *Background:* /purpose: Oral submucous fibrosis (OSF) is a betel quid chewing-caused oral mucosal disease with progressive collagen deposition. This study evaluated whether Taiwanese male OSF patients had high frequencies of vitamin B12 and folic acid deficiencies, hyperhomocysteinemia, and serum gastric parietal cell antibody (GPCA) positivity.
Materials and methods: The blood hemoglobin (Hb), serum iron, vitamin B12, folic acid, homocysteine, and GPCA concentrations in 62 male OSF patients were measured and compared with the corresponding data in 124 age-matched male healthy control subjects.
Results: We found that 5 (8.1%), 12 (19.4%), 32 (51.6%), 31 (50.0%), 22 (35.5%), and 6 (9.7%) of the 62 male OSF patients had Hb (<13 g/dL), iron (≤70 μg/dL), vitamin B12 (≤450 pg/mL), and folic acid (≤6 ng/mL) deficiencies, hyperhomocysteinemia (>12 μM), and serum GPCA positivity, respectively. Furthermore, OSF patients had significantly higher frequencies of Hb

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($P = 0.006$), vitamin B12 ($P < 0.001$), and folic acid ($P < 0.001$) deficiencies, hyperhomocysteinemia ($P < 0.001$), and serum GPCA positivity ($P = 0.030$) than 124 healthy control subjects. Of the 22 OSF patients with hyperhomocysteinemia, 4 had vitamin B12 deficiency only, 7 had folic acid deficiency only, and 11 had both vitamin B12 and folic acid deficiencies.

Conclusion: We conclude that Taiwanese male OSF patients have high frequencies of vitamin B12 and folic acid deficiencies, hyperhomocysteinemia, and serum GPCA positivity. The hyperhomocysteinemia in our OSF patients is predominantly due to deficiencies of either vitamin B12 or folic acid or both.

Introduction

Oral submucous fibrosis (OSF) is a gradually fibrotic oral mucosal disease characterized by a gradual collagen deposition in the subepithelial connective tissue and superficial muscle layer. The betel quid chewing and areca nut ingredients are the main etiological factors causing the OSF.[1,2] Areca nut contains alkaloids, flavonoids, and copper. Arecoline and arecaidine, the chief alkaloids in areca nut, are found to cause fibroblast proliferation and elevated collagen synthesis. Moreover, arecoline can augment collagen synthesis by upregulation of fibrogenic growth factors and profibrogenic cytokines, which subsequently result in fibrosis. Tannins and catechins, the main flavonoids in areca nut, can inhibit collagenase, stabilize the collagen fibrils, and in turn render collagen fibrils resistant to degradation by collagenase. Furthermore, arecoline can reduce the matrix metalloproteinase (MMP)-2 secretion and increase the tissue inhibitor of MMP (TIMP) level, finally resulting in elevated deposition of collagen in the extracellular matrix. The high concentration of copper in the areca nut has been reported to stimulate lysyl oxidase activity and increase the final cross-linking of collagen fibers.[1,2] In addition, arecoline can cause a significant inhibition of collagen phagocytosis by OSF fibroblasts, resulting in insufficient collagen degradation. Therefore, areca nut ingredients can cause the increased collagen synthesis and reduced collagen degradation in the oral tissues, finally leading to the OSF.[1,2]

Our previous studies demonstrated low serum vitamin B12 and folic acid levels as well as high frequencies of vitamin B12 and folic acid deficiencies and gastric parietal cell antibody (GPCA) positivity in Taiwanese male OSF patients.[3,4] However, the serum homocysteine level and the frequency of hyperhomocysteinemia in OSF patients have not been studied. In this study, 62 Taiwanese male OSF patients were prospectively collected from our oral mucosal disease clinic. We mainly wanted to assess whether Taiwanese male OSF patients also had a significantly higher mean serum homocysteine level and a significantly higher frequency of hyperhomocysteinemia than healthy male control subjects, and evaluated whether the hyperhomocysteinemia in Taiwanese male OSF patients was predominantly due to vitamin B12 and/or folic acid deficiencies.

Materials and methods

Study and control subjects

The study group consisted of 62 male OSF patients (mean age 46.2 ± 13.4 years, range 23—80 years). Regarding the normal control group, two age-matched (±2 years of each OSF patient's age) healthy male subjects for each OSF patient were selected. Thus, the normal control group included 124 healthy male control subjects (mean age 46.1 ± 13.3 years, range 23—80 years). All the OSF patients were prospectively collected from the Department of Dentistry, Far Eastern Memorial Hospital, New Taipei City, Taiwan from 2019 to 2020. Clinical diagnosis of OSF was made when patients showed characteristic features of OSF, including hypersensitivity to spicy foods, whitening and stiffness of the oral mucosa, bands of fibrous tissues in the buccal mucosa, and progressive mouth-opening limitation.[3,4] Because the clinical symptoms and signs were characteristic enough to make a precise clinical diagnosis, only 15 of 62 OSF patients received incisional biopsy of the buccal mucosa to further provide the histological evidence of OSF. The histological diagnosis criteria for the OSF have been described previously.[3,4] The oral mucosal sites of involvement (SOI, including soft palate, retromolar area, buccal mucosa, labial mucosa, floor of the mouth, and tongue) and the maximum mouth opening (MMO, the distance from the cutting edge of maxillary central incisor to the cutting edge of the mandibular central incisor) of OSF patients were recorded. The severity of OSF was determined according to the MMO and/or SOI; the less the MMO and the more the oral mucosal sites involved, the more severe the OSF.[3,4] In the study, no mild OSF patient was included, because all our OSF patients had at least three SOI. Moreover, the exclusion criteria for OSF patients and the inclusion and exclusion criteria for healthy control subjects have also been mentioned previously.[3,4]

Patients' oral habits including betel quid chewing, cigarette smoking, and alcohol drinking were recorded. In this study, of the 62 OSF patients, all (100%) were betel quid chewers, 56 (90.3%) were cigarette smokers, and 30 (48.4%) were alcohol drinkers according to the definitions described in our previous studies.[3,4]

The blood samples were collected from all 62 OSF patients and 124 healthy control subjects for determination of

Table 1 Comparisons of the mean corpuscular volume (MCV), mean blood hemoglobin (Hb) level, and mean serum iron, vitamin B12, folic acid, and homocysteine levels between 62 oral submucous fibrosis (OSF) patients and 124 healthy control subjects.

Group	MCV (fL)	Hb (g/dL)	Iron (μg/dL)	Vitamin B12 (pg/mL)	Folic acid (ng/mL)	Homocysteine (μM)
OSF patients (n = 62)	87.9 ± 9.3	15.2 ± 1.5	107.8 ± 38.3	468.4 ± 184.1	7.1 ± 3.3	12.5 ± 6.2
[a]*P*-value	0.007	>0.999	0.119	<0.001	<0.001	<0.001
Healthy control subjects (n = 124)	90.5 ± 3.7	15.2 ± 0.7	115.3 ± 26.3	653.9 ± 199.9	13.9 ± 5.6	8.8 ± 1.6

[a] Comparisons of means of parameters between 62 OSF patients and 124 healthy control subjects by Student's *t*-test.

complete blood count as well as serum iron, vitamin B12, folic acid, homocysteine, and GPCA concentrations according to the methods described in our previous studies.[3–21] The informed consents were signed by all OSF patients and healthy control subjects before entering the study. The Institutional Review Board at the Far Eastern Memorial Hospital reviewed and issued the permission of this study (FEMH No.: 107116-E).

Statistical analysis

Comparisons of the mean corpuscular volume (MCV) as well as the mean blood Hb and serum iron, vitamin B12, folic acid, and homocysteine concentrations between 62 OSF patients and 124 healthy control subjects or between two different groups of OSF patients were performed by Student's *t*-test. The differences in frequencies of microcytosis, blood Hb and serum iron, vitamin B12, and folic acid deficiencies, hyperhomocysteinemia, and GPCA positivity between 62 OSF patients and 124 healthy control subjects or between two different groups of OSF patients were compared by chi-square test or Fisher exact test, where appropriate. The result was considered to be significant if the *P*-value was less than 0.05.

Results

The mean MMO of 62 OSF patients was 31.0 ± 7.6 mm. Of the 62 OSF patients, 31 had MMO ≤31 mm and the other 31 had MMO >31 mm (between 32 mm and 42 mm). The soft palate, retromolar area, and buccal mucosa were the three oral mucosal sites where were involved by the OSF in every OSF patient. Moreover, the labial mucosa was concomitantly involved in 50 patients (80.6%), the floor of the mouth in 32 patients (51.6%), and the tongue in 13 patients (21.0%). Furthermore, of the 62 OSF patients, 12 had 3 oral mucosal sites involved, 18 had 4 sites involved, 19 had 5 sites involved, and 13 had 6 sites involved by the OSF. Thus, 30 OSF patients had SOI ≤4 sites and the other 32 OSF patients had SOI >4 sites.

Comparisons of the MCV, mean blood Hb level, and mean serum iron, vitamin B12, folic acid, and homocysteine levels between 62 OSF patients and 124 healthy control subjects by Student's *t*-test are shown in Table 1. We found significantly lower MCV (87.9 ± 9.3 fL, $P = 0.007$), lower mean serum vitamin B12 level (468.4 ± 184.1 pg/mL, $P < 0.001$), and lower mean serum folic acid level (7.1 ± 3.3 ng/mL, $P < 0.001$) as well as a significantly higher mean serum homocysteine level (12.5 ± 6.2 μM, $P < 0.001$) in the 62 OSF patients than in the 124 healthy control subjects (Table 1).

We also discovered significantly lower mean serum vitamin B12 level (386.2 ± 117.8 pg/mL, $P < 0.001$), lower mean serum folic acid level (5.2 ± 3.0 ng/mL, $P < 0.001$), and higher mean serum homocysteine level (15.0 ± 7.8 μM, $P = 0.001$) in the 31 OSF patients with MMO ≤31 mm than in the 31 OSF patients with MMO >31 mm (Table 2). In addition, significantly lower mean serum vitamin B12 level (417.5 ± 110.5 pg/mL, $P = 0.023$), lower mean serum folic acid level (5.4 ± 2.8 ng/mL, $P < 0.001$), and higher mean serum homocysteine level (14.5 ± 8.0 μM, $P = 0.008$) were demonstrated in the 32 OSF patients with SOI >4 sites than in the 30 OSF patients with SOI ≤4 sites (Table 2).

In this study, the microcytosis was defined as having MCV <80 fL and the Hb deficiency or anemia was defined as having Hb < 13 g/dL for men.[22,23] Furthermore, the serum iron, vitamin B12, and folic acid deficiencies were defined as having the serum iron level ≤70 μg/dL for men, vitamin B12 level ≤450 pg/mL, and folic acid level ≤6 ng/mL as described previously.[24,25] Furthermore, hyperhomocysteinemia in OSF patients was defined as having the serum homocysteine level >12.0 μM (which was the mean serum homocysteine level of healthy control subjects plus two standard deviations). By the above-mentioned definitions, we found significantly higher frequencies of microcytosis (12.9%, $P < 0.001$), blood Hb deficiency (8.1%, $P = 0.006$), serum vitamin B12 deficiency (51.6%, $P < 0.001$), folic acid deficiency (50.0%, $P < 0.001$), hyperhomocysteinemia (35.5%, $P < 0.001$), and serum GPCA positivity (9.7%, $P = 0.030$) in the 62 OSF patients than in the 124 healthy control subjects (Table 3).

Furthermore, we also discovered significantly higher frequencies of serum vitamin B12 deficiency (37.1%, $P < 0.001$), folic acid deficiency (41.9%, $P < 0.001$), and hyperhomocysteinemia (29.0%, $P < 0.001$) in the 31 OSF patients with MMO ≤31 mm than in the 31 OSF patients with MMO >31 mm (Table 4). In addition, significantly higher frequencies of serum vitamin B12 deficiency (33.9%, $P = 0.043$), folic acid deficiency (38.7%, $P < 0.001$), and hyperhomocysteinemia (25.8%, $P = 0.028$) were identified in the 32 OSF patients with SOI >4 sites than in the 30 OSF patients with SOI ≤4 sites (Table 4).

Table 2 Comparisons of the mean corpuscular volume (MCV), mean blood hemoglobin (Hb) level, and mean serum iron, vitamin B12, folic acid, and homocysteine levels between 31 oral submucous fibrosis (OSF) patients with maximum mouth opening (MMO) $\leq$ 31 mm and 31 OSF patients with MMO >31 mm as well as between 30 OSF patients with site of involvement (SOI) $\leq$ 4 sites and 32 OSF patients with SOI >4 sites.

OSF patients	MCV (fL)	Hb (g/dL)	Iron (μg/dL)	Vitamin B12 (pg/mL)	Folic acid (ng/mL)	Homocysteine (μM)
MMO ≤31 mm (n = 31)	88.5 ± 9.2	15.5 ± 1.6	109.2 ± 36.3	386.2 ± 117.8	5.2 ± 3.0	15.0 ± 7.8
MMO >31 mm (n = 31)	87.2 ± 9.5	14.9 ± 1.4	106.5 ± 40.7	550.5 ± 202.7	8.9 ± 2.6	10.1 ± 2.2
[a]*P*-value	0.586	0.121	0.784	<0.001	<0.001	0.001
SOI ≤4 sites (n = 30)	88.0 ± 9.3	15.3 ± 1.2	112.5 ± 38.0	522.6 ± 228.7	8.8 ± 3.0	10.4 ± 1.9
SOI >4 sites (n = 32)	87.7 ± 9.4	15.1 ± 1.7	103.5 ± 38.6	417.5 ± 110.5	5.4 ± 2.8	14.5 ± 8.0
[b]*P*-value	0.900	0.597	0.359	0.023	<0.001	0.008

[a] Comparisons of means of parameters between 31 OSF patients with MMO ≤31 mm and 31 OSF patients with MMO >31 mm by Student's *t*-test.
[b] Comparisons of means of parameters between 30 OSF patients with SOI ≤4 sites and 32 OSF patients with SOI >4 sites by Student's *t*-test.

Table 3 Comparisons of frequencies of microcytosis, blood hemoglobin (Hb) and serum iron, vitamin B12, and folic acid deficiencies, hyperhomocysteinemia, and gastric parietal cell antibody (GPCA) positivity between 62 oral submucous fibrosis (OSF) patients and 124 healthy control subjects.

Group	Patient number (%)						
	Microcytosis (MCV <80 fL)	Hb deficiency (<13 g/dL)	Iron deficiency (≤70 μg/dL)	Vitamin B12 deficiency (≤450 pg/mL)	Folic acid deficiency (≤6 ng/mL)	Hyper-homocysteinemia (>12.0 μM)	GPCA positivity
OSF patients (n = 62)	8 (12.9)	5 (8.1)	12 (19.4)	32 (51.6)	31 (50.0)	22 (35.5)	6 (9.7)
[a]*P*-value	<0.001	0.006	0.626	<0.001	<0.001	<0.001	0.030
Healthy control subjects (n = 124)	0 (0.0)	0 (0.0)	19 (15.3)	25 (20.2)	7 (5.6)	4 (3.2)	2 (1.6)

[a] Comparisons of frequencies of parameters between 62 OSF patients and 124 healthy control subjects by chi-square test.

Discussion

This study discovered blood Hb, serum vitamin B12 and folic acid deficiencies, hyperhomocysteinemia, and serum GPCA positivity in 8.1%, 51.6%, 50.0%, 35.5%, and 9.7% of the 62 male OSF patients, respectively. In addition, significantly higher frequencies of anemia, serum vitamin B12 and folic acid deficiencies, hyperhomocysteinemia, and serum GPCA positivity were found in the 62 OSF patients than in the 124 healthy control subjects. Moreover, the 31 OSF patients with MMO ≤31 mm and the 32 OSF patients with SOI >4 sites also had significantly higher frequencies of serum vitamin B12 and folic acid deficiencies and hyperhomocysteinemia than 31 OSF patients with MMO >31 mm and the 30 OSF patients with SOI ≤4 sites, respectively. These results suggest that the serum vitamin B12 and folic acid deficiencies and hyperhomocysteinemia in our OSF patients are significantly associated with the severity of OSF characterized by the MMO ≤31 mm and the SOI >4 sites.

First, we discussed why the OSF patients were prone to have the vitamin B12 and folic acid deficiencies. In this study, 44 OSF patients had vitamin B12 and/or folic acid deficiencies. Of these 44 OSF patients, 13 had vitamin B12 deficiency only, 12 had folic acid deficiency only, and 19 had both vitamin B12 and folic acid deficiencies. The GPCA can destroy gastric parietal cells, resulting in lack of intrinsic factors, reduced absorption of vitamin B12 from mucosal epithelial cells of the terminal ileum, and finally the deficiency of vitamin B12.[26,27] Thus, we counted how many OSF patients with vitamin B12 deficiency were serum GPCA-positive and further found that only 3 OSF patients had serum GPCA positivity in the 32 OSF patients with the vitamin B12 deficiency, indicating that the vitamin B12 deficiency in our OSF patients is not mainly due to the serum GPCA positivity. As mentioned before, our results suggest that the serum vitamin B12 and folic acid deficiencies are significantly related to the severity of OSF. In this study, all our OSF patients had moderate or severe OSF with the mean MMO being 31.0 mm and the mean SOI being 4.5 oral mucosal sites. Moreover, 50 (80.6%) of the 62 OSF patients had 4 to 6 oral mucosal sites involved by the OSF. Moreover, OSF patients frequently have particular signs of stiffness of the oral mucosa, fibrous bands in the buccal mucosa, and severe attrition of the teeth as well as characteristic symptoms of mouth-opening limitation, intolerance to spicy foods, burning sensation of the oral mucosa,

Table 4 Comparisons of frequencies of microcytosis, blood hemoglobin (Hb) and serum iron, vitamin B12, and folic acid deficiencies, hyperhomocysteinemia, and gastric parietal cell antibody (GPCA) positivity between 31 oral submucous fibrosis (OSF) patients with MMO ≤31 mm and 31 OSF patients with MMO >31 mm as well as between 30 OSF patients with site of involvement (SOI) ≤ 4 sites and 32 OSF patients with SOI >4 sites.

OSF patients	Patient number (%)						
	Microcytosis (MCV <80 fL)	Hb deficiency (<13 g/dL)	Iron deficiency (≤70 μg/dL)	Vitamin B12 deficiency (≤450 pg/mL)	Folic acid deficiency (≤6 ng/mL)	Hyper-homocysteinemia (>12.0 μM)	GPCA positivity
MMO ≤31 mm (n = 31)	3 (4.8)	1 (1.6)	5 (8.1)	23 (37.1)	26 (41.9)	18 (29.0)	4 (6.5)
MMO >31 mm (n = 31)	5 (8.1)	4 (6.5)	7 (11.3)	9 (14.5)	5 (8.1)	4 (6.5)	2 (3.2)
[a]*P*-value	0.707	0.345	0.748	<0.001	<0.001	<0.001	0.671
SOI ≤4 sites (n = 30)	4 (6.5)	2 (3.2)	4 (6.5)	11 (17.7)	7 (11.3)	6 (9.7)	4 (6.5)
SOI >4 sites (n = 32)	4 (6.5)	3 (4.8)	8 (12.9)	21 (33.9)	24 (38.7)	16 (25.8)	2 (3.2)
[b]*P*-value	>0.999	>0.999	0.401	0.043	<0.001	0.028	0.418

[a] Comparisons of frequencies of parameters between 31 OSF patients with MMO ≤31 mm and 31 OSF patients with MMO >31 mm by chi-square test or Fisher exact test, where appropriate.
[b] Comparisons of frequencies of parameters between 30 OSF patients with SOI ≤4 sites and 32 OSF patients with SOI >4 sites by chi-square test or Fisher exact test, where appropriate.

dry mouth, and impaired tongue mobility.[3,4] These OSF-specific signs and symptoms may interfere with eating and chewing functions of OSF patients and lead to insufficient food intake and difficulty in digestion and absorption of nutritional elements from the ingested food stuffs, finally resulting in hematinic deficiencies, including iron, vitamin B12, and folic acid deficiencies, in our OSF patients.[3,4] Moreover, in this study, 62 (100%) and 56 (90.3%) OSF patients were betel quid chewers and cigarette smokers, respectively. Thus, the carcinogenic substances in betel quids and tobacco may cause DNA damage in oral epithelial cells, and the coarse fibers of betel nuts may also cause multiple microtraumas of the OSF oral mucosa, resulting in the need of frequent cell proliferation and DNA replication to mend DNA damage in oral epithelial cells and to repair microtraumas of the OSF oral mucosa.[28,29] Actually, our previous study also demonstrated a higher labelling index of proliferating cell nuclear antigen (PCNA) in OSF epithelial cells than in normal oral mucosal epithelial cells, suggesting a higher cellular proliferation rate in the OSF epithelium than in normal oral mucosal epithelium.[30] Because a higher cellular turnover rate may consume a great amount of vitamin B12 and folic acid for DNA replication, this may in turn cause a significantly higher frequency of vitamin B12 and folic acid deficiencies and a significantly lower serum vitamin B12 and folic acid levels in OSF patients than in healthy control subjects.[31,32] Patil and Joshi[33] also reported a significantly lower mean serum vitamin B12 level in 40 OSF patients than in 25 healthy controls. Moreover, Abidullah et al.[34] also demonstrated a significantly lower mean serum vitamin B12 level in 50 OSF patients than in 50 healthy controls. In addition, Ramanathan[35] found folic acid deficiency in 6 of the 6 OSF patients.

Second, we tried to discuss why our OSF patients had a high frequency of hyperhomocysteinemia. This study found that all the 22 OSF patients with hyperhomocysteinemia had serum vitamin B12 and/or folic acid deficiencies. Of these 22 OSF patients with hyperhomocysteinemia, 4 had vitamin B12 deficiency only, 7 had folic acid deficiency only, and 11 had both vitamin B12 and folic acid deficiencies. High blood homocysteine levels can cause the oxidative stress that subsequently damage endothelial cells, accumulation of platelets at the injury site, and finally lead to thrombosis of blood vessels.[36–40] In normal condition, the excessive amount of blood homocysteine is converted into methionine through the aid of vitamin B12 and folic acid acting as co-enzymes.[40,41] Therefore, OSF patients with deficiencies of either vitamin B12 or folic acid or both can result in hyperhomocysteinemia. Vanjani et al.[42] also discovered a significantly higher mean serum homocysteine level and lower mean serum vitamin B12 level in 30 OSF patients than in 30 healthy control subjects. Our previous studies have shown that a supplement therapy of vitamin B12, folic acid, and vitamin B complex for burning mouth syndrome or atrophic glossitis patients can reduce the high serum homocysteine level to a significantly lower or normal level.[24,25] These findings also indirectly prove that the hyperhomocysteinemia in our OSF patients is predominantly due to deficiencies of either vitamin B12 or folic acid or both.

We conclude that Taiwanese male OSF patients have significantly higher frequencies of vitamin B12 and folic acid deficiencies, hyperhomocysteinemia, and serum GPCA positivity than healthy control subjects. In addition, the hyperhomocysteinemia in our OSF patients is predominantly due to deficiencies of either vitamin B12 or folic acid or both.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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