＊　統計的有意

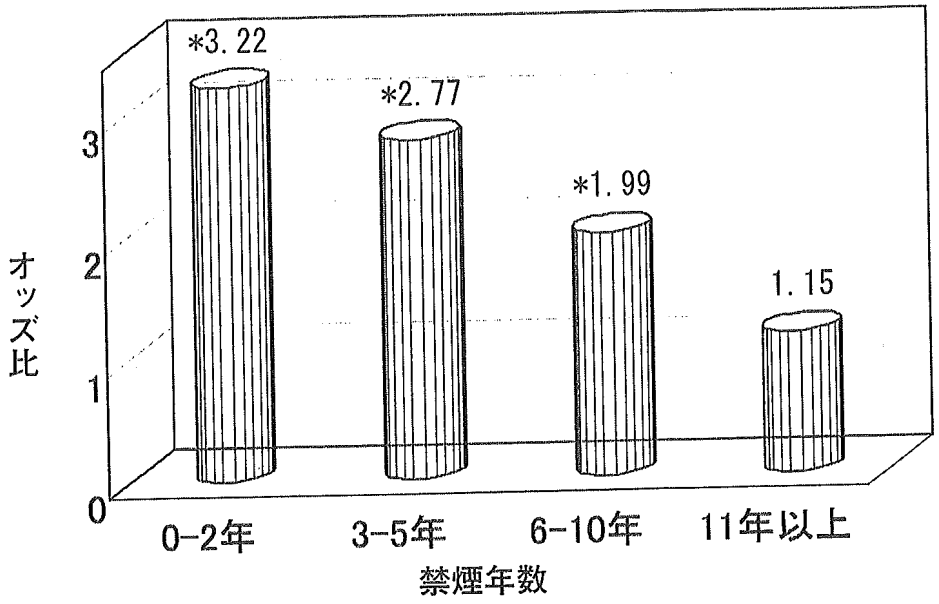

図10　禁煙による歯周病リスクの低下
歯周病の指標：アタッチメントロスと歯周ポケット深さ
(NHANES Ⅲ：Tomar & Asma，2000[8])

満足した結果を得るのは困難である。そして、ある程度の期間禁煙することにより、歯周病のリスクも低下し、歯周治療の効果も非喫煙者と変わらなくなる。したがって、歯周治療やインプラント治療を行う場合には、禁煙をする価値のあることを詳しく説明し、禁煙を奨める必要がある。一方、非外科的処置、組織誘導再生術やインプラント治療等を行う際には喫煙者に対して、感染予防として抗生物質を局所または全身的に使用することが奨められている[24]。

4．禁煙支援プログラム

健康日本21での歯周病予防の目標に、禁煙、節煙を希望する者に対する禁煙支援プログラムを全ての市町村で受けられるようにすることが挙げられている。このことは、市町村などの行政にまかせておいてすむことではなく、歯科医療を担う者に期待されているところも大きい。なぜならば、歯科診療所には、歯周病をもつ喫煙者が多く通院しており、歯周疾患指導管理が日常的に行われており、医科よりも歯科の方がより禁煙指導を行う環境が整っているといえるからである。しかしながら、歯科診療所でまだまだ日常的に禁煙指導が行われている訳ではない。それにはいくつかの理由が考えられる。ひとつの大きな理由として、日本では、近いうちにタバコをやめようと思っている人が欧米に比べて少ないといわれている[25]。喫煙者が禁煙に至るステージには無関心期（禁煙することに関心がない)、関心期（禁煙することに関心があるが、１ヶ月以内に実行する気がない)、準備期（１ヶ月以内に禁煙しようと思っている)、実行期（禁煙開始２週間以内)、維持期（禁煙後１週間以内、１ヶ月、３ヶ月）に分けられ、多くの人は短期的には禁煙に成功しても、何かをきっかけに喫煙を再開し、このプロセスを何回か繰り返したのち、長期的に成功するといわれている（図

11)。われわれの診療室で調べたところ、準備期の人は喫煙者の約20%ほどで、多くは無関心期か関心期の人々である。禁煙支援は準備期の人を実行期や維持期に至るよう支援するもので、禁煙プロセスの中心であるが、主として行動科学療法やニコチン代替療法などのカウンセリングを行うため、時間と費用が掛かる。したがって、対象になる患者さんも少なく、しかも、忙しい診療の合間に禁煙支援の時間がなかなかとれないのが実情である。また、歯科臨床において禁煙指導の効果に関する研究では（表2）、一般の臨床医の簡単なアドバイスなどであると禁煙率は約8～9%であるが[26]、ニコチン代替療法を含めると10%以上に上昇する[26,27]。また、歯周病専門医のアドバイスは効果が高いという[28]が、いずれも期待するほど効果はあがらない。

最近、名古屋大学大学院医学系研究科の浜島信之先生が提唱されている禁煙誘導が注目されている[29]。禁煙誘導とは、無関心期や関心期の喫煙者を準備期に誘導する点に重点をおいた禁煙指導の方法のひとつである（図12）。これは、禁煙支援とは異なり、短時間であまり費用もかからず、そして、簡便で、多数の喫煙者が対象となる方法である。禁煙の実行をサポートするのではなく、無関心期や関心期の喫煙者に対して、ビデオやパンフレットを見せたりして、禁煙意欲を高めて禁煙行動を誘発させる方法である。呼気中のCO濃度や唾液中のコチニンを測定し、喫煙の体への影響を示したり、タバコの影響を受けやすい遺伝子型や歯周病が進行しやすい遺伝子型の喫煙者にその情報を知らせることなども禁煙誘導といえ

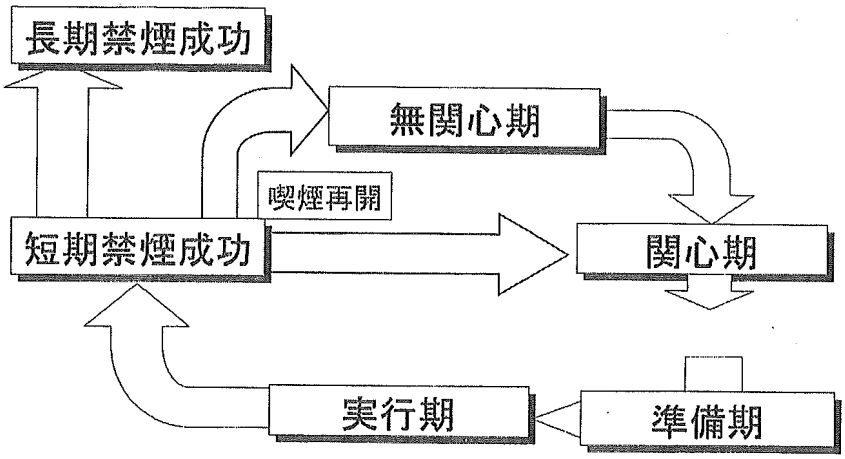

図11　禁煙成功へのプロセス

表2　歯科臨床での禁煙指導の効果

	対象者	方法	期間	禁煙率(%)
歯科開業医 (Cohen et al., 1989[26])	374	簡単なアドバイス	1年間	7.7
		簡単なアドバイス＋チャートを用いた指導	1年間	8.6
		簡単なアドバイス＋ニコチンガム	1年間	16.3
		全てを含む指導	1年間	16.9
歯科臨床医 (Smith et al., 1998[27])	154	簡単なアドバイス＋ニコチンパッチ	9ヶ月	11
病院歯周病医 (Macgregor, 1996[28])	98	歯周治療＋簡単なアドバイス	1年間	13.3
	38	歯周治療のみ	1年間	5.4

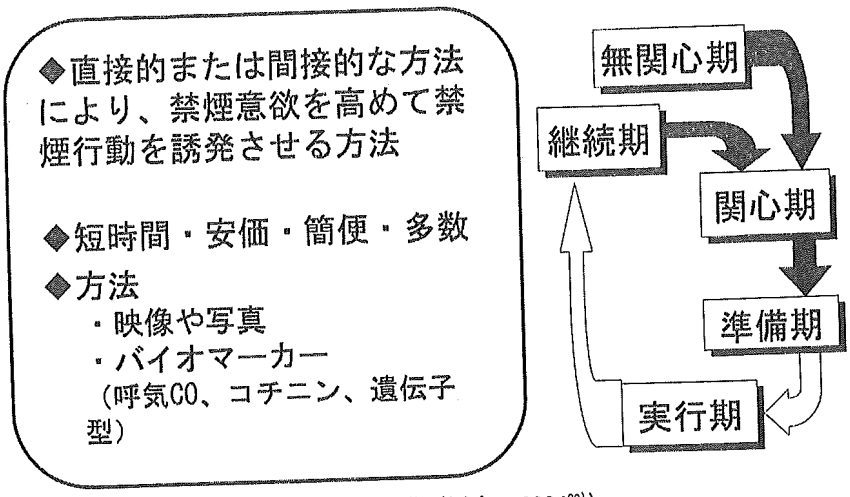

図12　禁煙誘導（浜島，2004[29]）

表3　歯科における禁煙誘導

禁煙誘導の内容	日常診療での禁煙誘導の機会
審美的障害と口臭	初診時の問診
口腔環境の変化	口腔診査と結果説明
粘膜の異常	保健指導
治療への影響	補綴物装着時
歯周病と歯の喪失	歯周治療実施前
子供への影響	インプラント診査
	抜歯実施後
	定期健診

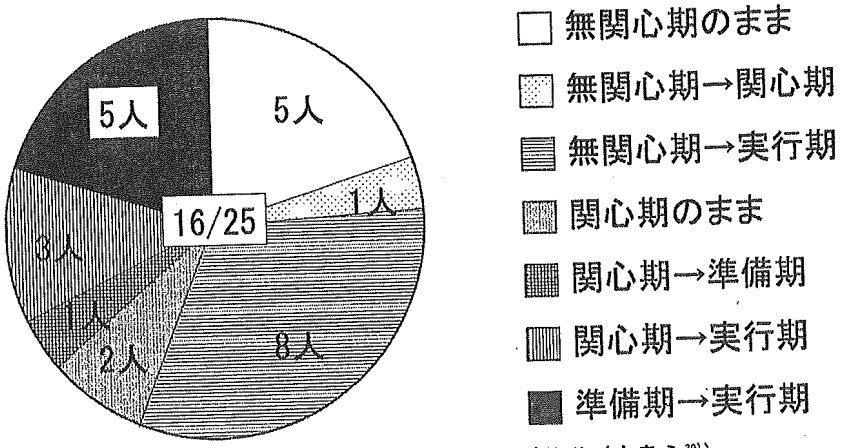

図13　禁煙誘導・支援前後のステージ移動（小島ら[30]）

る。また、歯科ではタバコの口腔への悪影響について、診療中に患者さんに見せたり話したりする内容は沢山あるし、日常の診療の流れの中で、それらのことについて繰り返し話す機会がある（表3）。われわれの禁煙外来で行った禁煙誘導の結果も（図13）、禁煙誘導前では、

無関心期15人、関心期５人、準備期が５人で、誘導後のそれらは、それぞれ、５人、２人、０人であった。関心期、準備期には、それぞれ１人づつが移動し、ステージ移動がみられたのは18人だった。無関心期と関心期の20人のうち11人が、準備期の５人は全員が禁煙を実行していた。このように歯科で行われる禁煙誘導は、大変効果的であることが明らかになった[30]。

おわりに

ここに示した多くの研究から分かるように、種々のバイアスがあることを考慮に入れても、喫煙が歯周病のリスクファクターであることは明らかであり、またそのリスクを取り除くことにより、歯周病の予防、歯周治療の効果の増強などが得られ、国民への有益性は非常に大きい。

歯科では、保健指導のひとつとして口腔清掃指導がよく行われているが、動機づけや習慣を変容するという点では共通点も多く、是非多くの歯科医や歯科衛生士の方々が禁煙誘導に取り組まれることを奨めたいと思う。

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Determination of Smoking and Obesity as Periodontitis Risks Using the Classification and Regression Tree Method

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Background: A model that focuses on personal risk factors associated with poor lifestyle has been proposed for the etiology of generalized periodontitis. Numerous investigations have linked individual lifestyle-related factors to periodontitis risk; however, a definite relationship among lifestyle-related factors remains unclear. The objective of this study was to determine which lifestyle-related factors demonstrated the greater impact on periodontitis risk.

Methods: The association of lifestyle-related factors, such as smoking status and obesity, with periodontitis was assessed in 372 Japanese workers via a self-administered questionnaire. Smoking status and obesity were evaluated in terms of pack-years and body mass index (BMI), respectively. Clinical periodontal examination included probing depth (PD). The effective impact on periodontitis risk was analyzed by the classification and regression tree (CART) method and multiple logistic regression analysis.

Results: Simple logistic regression analyses revealed that factors such as age, gender, alcohol consumption, smoking status, BMI, and frequency of toothbrushing were associated with periodontitis. CART results demonstrated a significant correlation between periodontitis and pack-years, BMI, and age; in contrast, alcohol consumption, gender, and toothbrushing frequency were not correlated with periodontitis. The strongest factor for periodontitis risk was pack-years of smoking. Additionally, both pack-years and BMI exhibited clear dose-response relationships with periodontitis. These relationships were maintained despite adjustment for known confounding factors.

Conclusions: Smoking displays the greatest impact on periodontitis among lifestyle-related factors. Both smoking and obesity are independent risk indicators for periodontitis; moreover, these parameters exhibit a dose-response relationship with respect to periodontitis risk. *J Periodontol 2005;76:923-928.*

KEY WORDS

CART; comparison studies; lifestyle; obesity/adverse effects; periodontitis/epidemiology; risk factors; smoking/adverse effects.

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Lifestyle-related factors including smoking, alcohol consumption and obesity are thought to contribute to local and systemic diseases, such as cancers, circulatory disease and other chronic diseases.[1] A model which focuses on personal risk factors associated with poor lifestyle has been proposed for the etiology of generalized periodontitis.[2] Smoking is recognized as a major risk factor for periodontitis.[3-7] Furthermore, alcohol consumption has been shown to increase the risk of periodontal disease, despite adjustment for other lifestyle factors including smoking.[8-10] Moreover, several researchers have documented a significant correlation between obesity and prevalence of periodontitis.[11-13] We have also demonstrated the association of lifestyle factors with periodontitis risk in previous publications.[14,15] Numerous investigations have linked individual lifestyle-related factors to periodontitis risk; however, a definite relationship among lifestyle-related factors remains unclear.

Various analytical techniques; i.e., multivariate regression and logistic models, have been employed for evaluation of periodontitis risk factors.[16] The classification and regression tree (CART) method[17] consists of an analytical process in which the relative significance of each factor is evaluated and an integral process involving identification of the optimal combination of independent variables over the dependent variable is utilized. Despite the obvious advantages offered

by the regression tree model, this analytical approach has not been applied to risk assessment of periodontitis. The objective of the present study was to establish which lifestyle-related factors display priority of impact on periodontitis risk via the CART method. In addition, the dose-response relationship between these factors and periodontitis risk was examined.

MATERIALS AND METHODS

Study Population

Four hundred fifty-three Japanese factory workers employed at a manufacturing company in Osaka were available for evaluation; in 1998, 409 (90.3%) of these individuals were surveyed via an oral examination and a self-administered questionnaire. Informed consent was obtained from all subjects. Three hundred seventy-two workers (290 males, 82 females, aged 20 to 59 years) completed all items of both the examination and the questionnaire. The mean (±SD) age of the participants was 40.5 ± 11.0 years. We introduced this study population in a previous report.[15] Permission for this study was obtained from the Ethical Committee for Clinical Research of Osaka University Graduate School of Dentistry.

Assessment of Lifestyle and Oral Health Behavior

Lifestyle behavior encompassed 103 items on a self-administered questionnaire that were evaluated in terms of eight categories (cigarette smoking, alcohol consumption, sleeping hours, breakfast, nutritional balance, working hours, physical exercise, and mental health) utilizing a protocol developed by Kusaka et al.[18] Questions were multiple choice in format (from two to six possible answers). Assessment of alcohol consumption involved information pertaining to drinking frequency, mean amount of alcohol consumption per occasion, and type of alcoholic beverage. There were 34 items related to oral health behavior (e.g., frequency of toothbrushing, method of brushing cervical teeth, use of an interdental brush).

Assessment of Smoking Status

Data corresponding to smoking habits (never, past, or current smoker) were derived from the self-administered questionnaire. Current smokers were also asked about the number of cigarettes smoked per day and the smoking years. Pack-years (number of cigarettes/20 per day × number of years smoking) were calculated to evaluate smoking status. Subjects were categorized into five groups according to their pack-years: 0.0, 0.1 to 9.9, 10.0 to 19.9, 20.0 to 29.9, and ≥30.0 pack-years.

Assessment of Obesity

Body mass index (BMI) was calculated as an indicator of overall adiposity with regard to obesity. BMI was computed from weight in kilograms divided by square height in meters. In order to analyze a dose-response relationship, participants were subdivided into six groups based on BMI: <20.0 kg/m^2, 20.0 to 21.9 kg/m^2, 22.0 to 23.9 kg/m^2, 24.0 to 25.9 kg/m^2, 26.0 to 27.9 kg/m^2, and ≥28.0 kg/m^2.

Assessment of Periodontitis

Probing depth (PD) measurements were performed with an automated probe‖ with a constant force (20 g) on all teeth present except for the third molars by two examiners. Each participant was examined for PD at six sites per tooth; the deepest was recorded for each. The percentage of teeth characterized by PD >3.5 mm was assessed as a periodontal parameter (%PD). Subjects were then classified into two groups, periodontitis or non-periodontitis, based on placement above or below the upper 20th percentile of the %PD, respectively. Calibrated examiners performed the periodontal examinations. The kappa value[19] for PD determined by the two examiners was 0.76, when a PD of 3.5 mm served as the cut-off point. Examiners were masked to subject smoking status.

Statistical Analysis

Data were analyzed with a statistical package.¶ The associations between periodontitis and surveyed lifestyle variables, which included smoking status and obesity, were examined using a simple logistic analysis. Classification and regression tree (CART)[17] analysis was employed to determine which variables demonstrated a significant independent effect on periodontitis. Subsequently, multiple logistic regression analyses served to clarify the dose-response between periodontitis and exposure to cigarette smoking or obesity. Odds ratios and their 95% confidence intervals (CI) were also calculated. Data, which were adjusted initially for age alone, were then adjusted for the following multiple covariates: age, gender, frequency of toothbrushing, alcohol consumption, BMI, or cigarette smoking. In addition, the multiple linear trends for risk were evaluated utilizing the mean values for each category of exposure to cigarette smoking and BMI, respectively. All reported P values are two-tailed; those P values <0.05 were considered statistically significant.

RESULTS

The distribution of the clinical parameters of the subjects is shown in Table 1. The median value for the subjects' BMI was 22.4. Since the smoking prevalence of the subjects was 44.6%, the median value for pack-year was 0. Table 2 presents the percentage and cumulative percentage of teeth characterized by PD ≥3.5 mm. %PD varied from 0 to 100%; median was 25.0%

‖ Vivacare TPS Probe, Schaan, Liechtenstein.
¶ Version 10.0J and Answer Tree, SPSS Inc., Chicago, IL.

Table 1.

Clinical Parameters

Parameter	Total (N=372) Median	IQR*	Male (N=290) Median	IQR	Female (N=82) Median	IQR
Age (years)	42.0	18.0	41.0	16.0	45.0	26.0
%PD >35 mm	25.0	42.1	29.6	42.0	17.9	31.9
BMI kg/m^2	22.4	4.0	22.6	3.9	21.1	4.2
Pack-years	0.0	15.0	4.0	19.0	0.0	0.0
Alcohol consumption (g/day)	22.0	15.4	22.0	22.0	0.0	22.0
Frequency of toothbrushing (times/day)	2	1	2	1	2	1

* Interquartile range.

Table 2.

Percentage and Cumulative Percentage of %PD ≥3.5 mm

%PD	N	Cumulative %
0.0	32	8.6
0.1-10.0	60	24.7
10.1-20.0	60	40.9
20.1-30.0	56	55.9
30.1-40.0	39	66.4
40.1-50.0	23	72.6
50.1-60.0	29	80.4
60.1-70.0	19	85.5
70.1-80.0	19	90.6
80.1-90.0	19	95.7
90.1-100.0	16	100.0
Total	372	

and the upper 20th percentile was 60.0%. Simple logistic analysis revealed that factors such as age, gender, alcohol consumption, smoking status, BMI, and frequency of toothbrushing were significantly associated with periodontitis (P <0.05, Table 3).

Table 3.

Association Between Periodontal Status and Variables

Independent variables	% Subjects in Upper 20th Percentile of %PD ≥3.5 mm		Odds Ratio (95%CI)
Age (years)			
20-39	11.1	*	1.0
40-59	26.3	*	2.85 (1.59-5.11)
Gender			
Female	9.0	*	1.0
Male	22.3	*	2.92 (1.28-6.66)
BMI			
<25.0 kg/m^2	15.1	*	1.0
≥25.0 kg/m^2	36.0	*	3.17 (1.79-5.61)
Alcohol consumption			
<33.0 g/day	15.6	*	1.0
≥33.0 g/day	34.7	*	2.87 (1.61-5.13)
Smoking status			
<15.0 pack-years	13.5	*	1.0
≥15.0 pack-years	36.2	*	3.62 (2.09-6.26)
Frequency of toothbrushing			
≥2 times/day	15.7	*	1.0
≤1 time/day	25.0	*	1.78 (1.06-3.02)

* P <0.05.

When these variables were tested by CART analysis (Fig. 1), the factor demonstrating the greatest impact on %PD was pack-years of smoking. Nodes 2 and 3 included those subjects displaying ≤7.8 pack-years (PY) and >7.8 pack-years, respectively. Node 2 was partitioned into nodes 4 and 5, based on BMI (4 = ≤25.1; 5 = >25.1). Node 5 could not be subdivided into two significantly distinct groups for any categories; consequently, it was a terminal node. Node 4 was divided by age into node 8 (≤31.5 years) and node 9 (>31.5 years). Nodes 8 and 9 were both terminal nodes. Node 3 was partitioned into nodes 6 and 7: node 7 contained subjects characterized by >26.3 pack-years and was a terminal node. Node 6 was subdivided by BMI into node 10 (BMI ≤25.5) and node 11, (BMI >25.5). These results suggested that pack-years of smoking exerted the greatest impact on periodontitis risk, followed by BMI. Cut-off points for BMI in this study were 25.1 or 25.5, which were nearly identical to those values of being overweight as defined by WHO.[20] Node 7 was not influenced by any other factor, except smoking. Factors such as gender, alcohol consumption, and frequency of toothbrushing were not significant variables in this analysis.

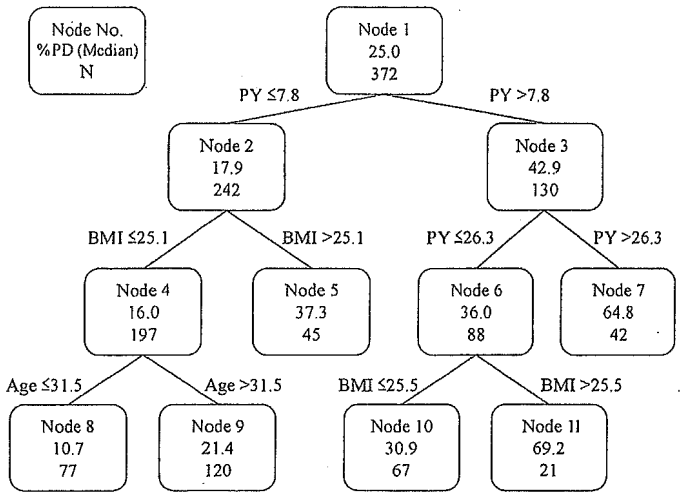

Figure 1.
Classification and regression tree (CART) methods for evaluation of periodontal status. The first divided factor (the most important factor) was pack-years, followed by BMI and age. Abbreviations: PY; pack-years, BMI; Body Mass Index.

Table 4.

Risk for Periodontitis by Smoking Status

	Pack-Years					*P* Value for Trend§
	0.0	0.1-9.9	10.0-19.9	20.0-29.9	≥30.0	
N	206	45	48	36	37	
Age, years, mean	40.9	30.6	37.8	45.0	49.6	
%PD ≥3.5 mm, median	18.2	18.2	32.7	42.9	60.7	
Pack-years, mean	0.0	4.8	14.5	23.6	44.4	
Odds ratio*	1.0	0.95	2.35	3.23	7.04	<0.0001
95% CI		0.34, 2.65	1.08, 5.10	1.41, 7.38	3.19, 15.55	
Odds ratio†	1.0	1.32	2.68	2.70	5.27	<0.0001
95% CI		0.45, 3.88	1.21, 5.94	1.16, 6.28	2.32, 11.98	
Odds ratio‡	1.0	1.15	2.00	2.27	3.96	0.0017
95% CI		0.38, 3.52	0.86, 4.66	0.92, 5.63	1.59, 9.82	

* Unadjusted.
† Adjusted for age.
‡ Adjusted for age, gender, BMI, alcohol consumption, and frequency of toothbrushing.
§ Calculated across increasing categories of smoking for current smokers only. The linear trends in risks were evaluated using the average value for each category of smoking status.

In order to assess exposure to cigarette smoking on periodontitis, subjects were categorized into five groups according to their pack-years (Table 4). Never or former smokers were categorized as 0.0 pack-years and current smokers were categorized into four groups. Following adjustment for age, the odds ratio were 1.32 (95% CI: 0.45 to 3.88) for 0.1-9.9 pack-years, 2.68 (95% CI: 1.21 to 5.94) for 10.0-19.9 pack-years, 2.70 (95% CI: 1.16 to 6.28) for 20.0-29.9 pack-years and 5.27 (95% CI: 2.32 to 11.98) for ≥30.0 pack-years (*P* for trend <0.0001) as compared with 0.0 pack-years. %PD increased with increasing pack-years in a dose-dependent manner.

Subjects were subdivided into six groups according to BMI to evaluate obesity (Table 5). Following adjustment for age, the odds ratios were 1.87 (95% CI: 0.68 to 5.15) for BMI of 20.0-21.9, 1.88 (95% CI: 0.68 to 5.18) for BMI of 22.0-23.9, 2.84 (95% CI: 0.99 to 8.16) for BMI of 24.0-25.9, 4.55 (95% CI: 1.48 to 14.03) for BMI of 26.0-27.9 and 4.57 (95% CI: 1.30 to 16.06) for BMI ≥28.0 (*P* for trend = 0.0018) as compared with BMI <20.0. Further adjustments slightly attenuated these correlations. Increasingly poor periodontal status corresponded to increased BMI in a dose-dependent manner.

DISCUSSION

The current study revealed a meaningful correlation between periodontitis and age, gender, BMI, smoking status, alcohol consumption, and frequency of toothbrushing via utility of simple logistic regression analyses. Furthermore, significant variables exhibiting greater risk for periodontitis were analyzed with the CART method. CART analysis affords numerous advantages;[17] CART results show independent variables in order of their impact on the dependent variable. The CART method also demonstrates the tendency with respect to earlier division (higher in the tree) as it relates to greater effect on severity of disease. Moreover, this approach stratifies for each variable and defines the cut-off points automatically. The most advantageous aspect of CART pertains to the graphical presentation of the results in an understandable manner despite their complexity.

The most potent factor identified by this investigation was smoking, followed by BMI and age (Fig. 1). Node 3 (Fig. 5) included those subjects who smoked more than 7.8 pack-years, considered as light smokers, suggesting that even light smoking status may affect periodontitis. Node 7 included those subjects who smoked in excess of 26.3 pack-years, heavy smokers characterized by high periodontitis risk. Node 11 subjects, who were moderate smokers displaying BMI >25.5, also exhibited great risk of periodontitis.

Table 5.

Risk for Periodontitis by Obesity

	Body Mass Index (kg/m^2)						*P* Value for Trend§
	<20.0	20.0-21.9	22.0-23.9	24.0-25.9	26.0-27.9	≥28.0	
N	73	96	91	57	34	21	
Age, years, mean	35.3	41.6	41.5	42.7	41.8	40.9	
%PD ≥3.5 mm, median	14.3	22.0	25.0	35.7	42.9	39.3	
BMI kg/m^2, mean	18.8	21.1	22.9	24.9	26.9	30.5	
Odds ratio*	1.0	2.25	2.34	3.64	5.33	5.33	0.0006
95% CI		0.83, 6.08	0.86, 6.33	1.30, 10.24	1.76, 16.13	1.66, 18.33	
Odds ratio†	1.0	1.87	1.88	2.84	4.55	4.57	0.0018
95% CI		0.68, 5.15	0.68, 5.18	0.99, 8.16	1.48, 14.03	1.30, 16.06	
Odds ratio‡	1.0	1.88	1.74	2.68	3.89	4.40	0.0057
95% CI		0.66, 5.37	0.61, 4.97	0.90, 7.98	1.20, 12.56	1.18, 16.44	

* Unadjusted.
† Adjusted for age.
‡ Adjusted for age, gender, pack-years, alcohol consumption, and frequency of toothbrushing.
§ Calculated across increasing categories of smoking for current smokers only. The linear trends in risks were evaluated using the average value for each category of BMI.

However, alcohol consumption did not demonstrate a significant effect on periodontitis according to CART, despite the significance determined by bivariate analysis. In persons of Asian extraction, alcohol sensitivity differs between individuals; in particular, this phenomenon depends on $ALDH_2$ genotypes.[21] We previously reported that alcohol consumption and $ALDH_2$ genotypes are important factors with respect to periodontal risk.[15] Thus, it may be necessary to add the factor of $ALDH_2$ genotypes to risk models in future studies in this population.

Age, smoking, and BMI displayed significantly independent effects on periodontal status in CART; consequently, we examined the dose-response relationship between severity of periodontitis and smoking or BMI in detail via adjustment for confounding factors including age. With regard to smoking status, subjects were classified into five groups according to their pack-years with former or never smokers categorized into 0.0 pack-years. Adjusted odds ratio of periodontitis, which increased with increasing pack-years, exhibited a dose-response association. This trend was significant despite adjustment for confounding factors such as age. Several reports pertaining to the dose-response relationship between smoking and periodontitis have been published.[3,22-25] We have also shown previously the dose-dependent manner between smoking and periodontitis; cigarettes/day represented smoking status and modified CPI score represented periodontal status.[14] Grossi et al. demonstrated the dose response between pack-years of smoking and periodontitis employing attachment loss[22] or bone loss[24] despite adjustment for confounding factors including age. The odds of more severe attachment and alveolar bone loss were 4.75 and 7.28 in heavy smokers (pack-years of 31.1 to 150.0), respectively. These data, which support our findings, suggest that smoking exerts cumulative detrimental effects on periodontal health.

When subjects were initially dichotomized into two groups according to BMI above or below 25.0 for bivariate analysis, the odds ratio of obesity for periodontitis risk was 3.17. When subjects were classified into six BMI categories, the odds ratio was less than 3.0 for BMI of less than 26.0; however, the odds ratio rapidly increased with a BMI of ≥26.0. This result is consistent with the cut-off points established by CART analysis. Wood et al. also documented the dose-response relationship between BMI and periodontitis, which was defined based on the presence of one or more periodontal sites characterized by both attachment loss of ≥3 mm and PD of ≥4 mm; however, the adjusted odds ratio for BMI ≥30 was 1.37.[12] Saito et al.[26] showed the dose response between periodontitis and obesity; subjects with BMI of 25.0 to 29.9 and >30.0 exhibited crude odds ratios of 3.4 and 8.6, respectively, based on PD ≥4.0 mm. Interestingly, in a later study, they noted that higher levels of BMI significantly increased the adjusted risk of periodontitis only in those subjects displaying high waist-hip ratio in comparison with subjects demonstrating low waist-hip ratio and the lowest category of BMI.[11] Thus, these findings, in concert with our data, suggest that obesity is associated with periodontitis risk.

Smoking and obesity are known to affect host immunity.[7,27,28] Both factors may also decrease blood flow in periodontal tissues of obese subjects, which promotes the development of periodontitis.[7,29] These immunological disorders or inflammation might be the reason that obese smokers tend to exhibit escalating poor periodontal status relative to non-obese and non-smoking individuals. Despite the primary limitation of

our findings obtained from a cross-sectional study, the results suggest that smoking exerts the strongest effect on periodontitis. Moreover, these data reveal that both smoking and obesity, which display a dose-response relationship with periodontitis severity, are potential risk indicators for periodontitis.

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Association of Melanin Pigmentation in the Gingiva of Children With Parents Who Smoke

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Association of Melanin Pigmentation in the Gingiva of Children With Parents Who Smoke

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ABSTRACT. *Objective.* **The association between gingival pigmentation and active smoking has been established. This investigation is the first to address the relationship between gingival pigmentation in children and passive smoking.**

Methods. **A case-control study was performed involving 59 nonsmoking children who were selected from patient records of a dental clinic in a rural town in Japan. The number of subjects was based on a power calculation. Two calibrated examiners independently observed labial gingiva via oral photographs.**

Results. **An interview determined that 61% of children had at least 1 smoking parent. Gingival pigmentation was observed in 71% to 78% of children. Interexaminer agreement was satisfactory ($\kappa = 0.73$). Percentage of smoking parents was higher in children with gingival pigmentation (70–71%) than in those who lacked pigmentation (35%). Odds ratios of parental smoking adjusted by age and gender were 5.6 (95% confidence interval: 1.5–20.0) and 5.4 (1.4–21.2) for the 2 examiners.**

Conclusion. **These findings suggest that excessive pigmentation in the gingiva of children is associated with passive smoking. The visible pigmentation effect in gingiva of children could be useful in terms of parental education.** *Pediatrics* **2005;116:e186–e190. URL: www.pediatrics.org/cgi/doi/10.1542/peds.2004-2628;** *parental smoking, melanin pigmentation, gingiva, child.*

ABBREVIATIONS. ETS, environmental tobacco smoke; OR, odds ratio; CI, confidence interval.

Brownish or black pigmentation in human gingiva has been reported in several countries. The prevalence rate of gingival pigmentation is diverse according to race and country[1–9]; hence, heredity may be a background factor. Intake of antimalarial drugs including chloroquine[10] and quinidine[11] is also associated with oral pigmentation.

Pigmentation in human gingiva derives from melanin granules, which are synthesized in melanosomes of melanocytes.[12] Melanocytes were identified as dendritic cells at the basal layer of gingival epithelium. Melanosomes, which are transferred via dendritic processes to keratinocytes by phagocytic activity, are degraded as they ascend to the surface. Melanin is synthesized from tyrosine and dihydroxyphenylalanine via dopaquinone as a result of the oxidation activity of tyrosinase.[13]

Melanin pigmentation in gingiva is correlated with active smoking: smokers displayed a greater propensity toward pigmentation than did nonsmokers.[3–7,9] A dose-response relationship with prevalence was detected.[4–7] Prevalence of pigmentation decreased in relation to the number of years after smoking cessation.[14] These findings indicate a causal association between tobacco smoke and melanin pigmentation in gingiva. Gingival pigmentation often occurred in the labial area of anterior teeth.[3,6,7,9] Excessive pigmentation in palatal mucosa as a result of tobacco smoke is a rare phenomenon, except in instances of reverse smoking.[15]

The prevalence of gingival pigmentation in smokers increased and approached maximum levels on slight exposure to smoking in minimal categories of duration of smoking and number of cigarettes smoked.[5,7] This characteristic is indicative of the sensitivity of gingival melanocytes to tobacco smoke. The prevalence of gingival pigmentation of workers was compared according to smoking status in 2 workplace locations.[7] The prevalence of pigmentation for current, former, and never smokers was 81%, 27%, and 15%, respectively, among 163 factory workers, whereas those rates among 154 office workers were 85%, 70%, and 37%, respectively. The apparent distinction in the prevalence rates of nonsmoking workers may be attributable to differences in environmental tobacco smoke (ETS) between workplace locations.

To date, few investigations have addressed the association between oral disease and exposure to ETS. On the basis of analyses of data derived from the Third National Health and Nutrition Examination Survey in the United States, the odds ratio (OR) of ETS exposure exceeding 1 hour to periodontal disease was 1.57 (95% confidence interval [CI]: 1.15–2.16)[16]; moreover, that of children who were aged 4 to 11 years and displayed serum cotinine levels of 0.2 to 10 ng/mL to untreated pediatric caries was 2.1 (95% CI: 1.5–2.9).[17] These findings suggest effects of passive smoking on oral condition. Parental smoking status was examined to estimate correlation between

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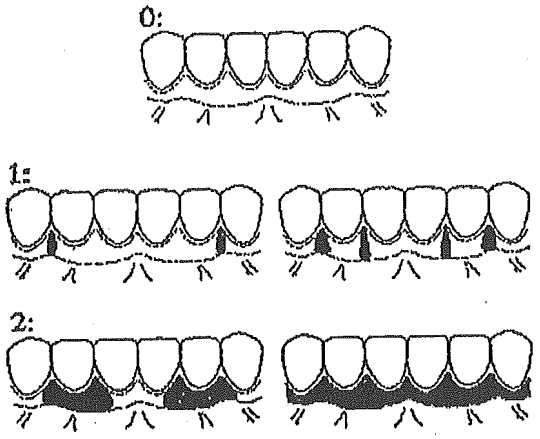

Fig 1. Classification according to extent of brownish or black pigmentation in labial gingiva of anterior teeth: 0, no pigmentation; 1, solitary unit(s) of pigmentation in papillary gingiva without extension between neighboring solitary units; and 2, formation of continuous ribbon extending from neighboring solitary units.

respiratory health and passive smoking in children.[18] The objective of the present study was to assess the relationship between melanin pigmentation in the gingiva of children and passive smoking.

METHODS

Determination of Sample Size

Preliminary documentation related to determination of sample size in the present case-control study is scarce. Furthermore, no published data pertaining to gingival pigmentation of children and parental smoking appear in the literature. Therefore, sample size was determined in accordance with the following reports: effect of active smoking on gingival pigmentation in adults[7] and the relationship between gingival pigmentation of children and their parents (Nakao Shimizu, DDS, written communication, 2002). Power analyses (Sample Power; SPSS Japan Inc, Tokyo, Japan) that were based on these reports indicated an appropriate sample size of 38 to 64 via consideration of overestimation with respect to the chance of exposure to tobacco smoke.

Subjects and Smoking Status

Oral photographs of 59 children (22 boys and 37 girls) aged 6 to 16 years (11.3 ± 2.5) were selected randomly from patient records of a private dental clinic in Yamagata, which is located in the northern region of Japan. Informed consent was obtained; subsequently, smoking status of children and parents was established via interview. Images of the frontal mouth, which were acquired in a standardized manner, were evaluated for gingival pigmentation. Oral images were obtained with a digital camera (EOS D30; Canon Inc, Tokyo, Japan) equipped with wide-angle conversion lens (C-AF1 2X TELEPLUS MC7; Kenko Co, Tokyo, Japan). Parental smoking status was recorded separately on the basis of oral photographs.

Evaluation of Melanin Pigmentation

Gingival pigmentation was assessed in the oral photographs, which were reproduced in a computer display. These reproductions exhibited size similar to that of the actual mouth. Brownish or black pigmentation in gingiva was classified according to extent of pigmentation unit in the labial aspect of anterior teeth (Fig 1). To date, no objective method for evaluation of gingival pigmentation has been developed. Gingival pigmentation was classified according to modification of melanin index categories[3]: 0, no pigmentation; 1, solitary unit(s) of pigmentation in papillary gingiva without formation of continuous ribbon between solitary units; and 2, at least 1 unit of formation of continuous ribbon extending from 2 neighboring solitary units. The current technique, which is subjective, was applied in children for the first time. Consequently, the reliability of this method was evaluated on the basis of interexaminer agreement: 2 examiners independently reviewed identical photographs. Examiners were trained and calibrated using typical photographs. Photographs in which visible pigmentation in the gingiva of children is apparent or lacking are presented in Fig 2. Gingival pigmentation of hemoglobin, melanoid, and carotene was obviously distinguishable from melanin pigmentation.[19] Several factors such as amalgam restoration adjacent to gingiva, melanoma, and long-term usage of antimalarial drugs[10,11] and minocycline[20] are potential confounders in terms of exposure to tobacco smoke; however, none of these parameters was applicable in this study. Status of parental smoking was withheld from the examiners. The current investigation was approved by the Ethics Committee of the Fukuoka Dental College.

Statistical Analysis

Interexaminer agreement was evaluated for the existence and the extent of pigmentation with the κ statistic. Additional analyses were performed using data sets that consisted of scores of gingival pigmentation assessed by the 2 examiners on the basis of parental smoking status, gender, and age. ORs of parental smoking with respect to gingival pigmentation were calculated via logistic regression analyses. Statistical analyses were conducted with software (SPSS; SPSS Japan Inc). The significance level was set at 5%.

RESULTS

No child reported active smoking. At least 1 parent of 36 (61%) children smoked. These parents smoked for 19.8 (±4.2) years on average (±SD), consuming 19.3 (±9.0) cigarettes per day. Two thirds of these parents smoked >20 cigarettes per day. Distribution of gender and age was similar between children in both parental smoking groups (Table 1). κ statistics for existence and extent of pigmentation were 0.73 and 0.68, respectively.

Gingival pigmentation was detected in 42 (71%) and 46 (78%) children by examiners A and B, respectively (Table 2). Prevalence of pigmentation was similar in boys and girls; 73% and 72% according to examiner A and 77% and 81% according to examiner B, respectively (data not shown). Solitary pigmentation was observed in 29% to 32% of the children. Continuous pigmentation, a form more distinct than solitary pigmentation, was noted in 42% to 46% of the children. Percentage of smoking parents was higher in children who displayed gingival pigmentation (70–71%) than in those who lacked pigmenta-

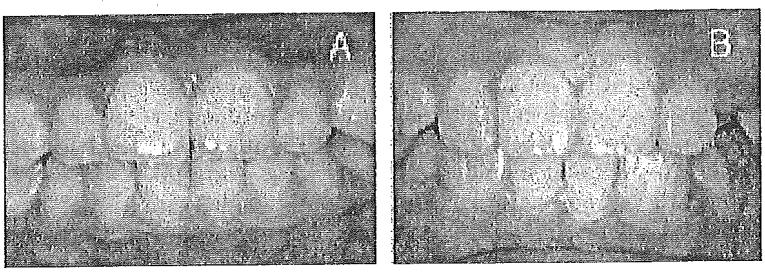

Fig 2. Typical photographs with (A) and without (B) visible pigmentation in the gingiva of children.

TABLE 1. Distribution of Gender and Age by Parental Smoking Status

Smoking Status of Parents	Gender, *n* (%)		Total	Age, Mean ± SD
	Male	Female		
Nonsmoking	9 (39)	14 (61)	23	11.4 ± 2.5
Smoking	13 (36)	23 (64)	36	11.3 ± 2.5
Total	22 (37)	37 (63)	59	11.3 ± 2.5

tion (35%). Percentage of smoking parents in children with solitary pigmentation (82%) was higher in comparison with those characterized by continuous pigmentation (64%).

Prevalence of gingival pigmentation in children with at least 1 smoking parent was 83% to 89%; in contrast, this condition was less prevalent (52–60%) in children whose parents did not smoke. Prevalence of gingival pigmentation in children was correlated significantly with smoking status of their parents (Table 3). Crude ORs of parental smoking were 4.6 (95% CI: 1.4–15.2, examiner A) and 5.1 (95% CI: 1.4–20.0, examiner B). Adjusted ORs relative to gender and age were 5.6 (95% CI: 1.5–20.0, examiner A) and 5.4 (95% CI: 1.4–21.2, examiner B).

DISCUSSION

Examiners detected gingival pigmentation in 71% to 78% of children. Percentages of parental smoking on the basis of gingival pigmentation score and ORs were similar between examiners. Interexaminer agreement for existence and extent of gingival pigmentation was satisfactory as indicated by the κ statistic. Thus, subjective evaluation of gingival pigmentation in children was sufficiently reliable so as to permit assessment of the association between gingival pigmentation in children and passive smoking.

Gingival pigmentation in Japanese children was described exclusively in an educational literature that introduced results of a survey regarding the prevalence among ~5000 children (age: 1–17 years) in metropolitan areas during 1982–1986.[21] Prevalence of gingival pigmentation increased and reached maximum levels in ~60% of children aged 1 to 6 years; moreover, prevalence continued at this level for nearly 7 years, followed by a gradual decrease to ~40% in children 17 years of age. No information related to smoking status was available; however, the value corresponding to prevalence of gingival pigmentation, which was smaller than that of the present investigation with respect to similar age group, may be attributable to differences associated with method of evaluation, eg, macroscopic versus photographic assessment. In adults, prevalence of melanin pigmentation was 15% to 37% for never smokers among Japanese workers.[7] Thus, prevalence in melanin pigmentation may differ between children and adults. Melanin pigmentation of oral mucosa was detected in 13.5% of Israeli children who were 6 to 10 years of age.[22] Melanin pigmentation varies in prevalence among different races and ethnic groups; for example, prevalence is higher in Asian populations (34.6%) in comparison with Ashkenazi (0.8%) and Sephardic (11.1%) Jews. In the present study, gingival pigmentation in the form of continuous ribbon was detected in 35% and 39% of children derived from the nonsmoking parent group. However, no comparable data exist regarding degree of pigmentation.

The significant association between parental smoking and gingival pigmentation in children suggests the presence of an ETS effect, which originated from parental smoking. No data corresponding to the exact amount of time that smoking parents and their children spent together were available. However, most parents smoked moderately or heavily; thus, their children may have been exposed to passive smoking for certain hours. In the 1999 report of the National Survey for Smoking and Health, 63.2% of Japanese were aware of the effect of ETS on asthma of children. Thus, a few parents in the smoking group may not smoke in the presence of their children. A second factor that skews the determination of the effect of ETS of parental origin is the impact of ETS from additional sources. ETS other than that of parental origin is likely to influence gingival pigmentation in the children. Passive smoking, other than that of parental origin, potentially may be similar or slightly higher in the smoking parental group than in the nonsmoking parental group. In both cases, the effect of ETS of parental origin on gingival pigmentation of children would be underestimated. National law restricting smoking in public areas went into effect in Japan on May 1, 2003.

Active smoking of participants was verified by interview. Parents or children who smoke might fail to report this activity. The smoking rate of male adults at ages corresponding to participating parents was ~60% in Japan. Because ~15% of women smoke, the smoking rate of parents (61%) was reasonable. In Japan, a national survey conducted in 1996 revealed that 0.7% of boys and 0.4% of girls at the age of ~13 years smoked. Therefore, we believe that the influence of smoking parents and children who failed to report is minimal. Future investigations that use determination of cotinine in urine[23] for validation of passive smoking in children could confirm direct association between passive smoking and gingival pigmentation. The present study suggested this relationship indirectly.

To date, a few reports appear in the literature regarding the potential sources that could stimulate melanin production in gingiva. An already high prevalence of gingival pigmentation was enhanced excessively by active smoking.[4] The stimulatory effect could be explained by high-affinity activity of polycyclic amines such as nicotine[24] and benzpyrene[25] in tobacco smoke on melanin; noxious substances in the epithelial cells were eliminated.

Two pathways by which stimulatory substances in ETS enter melanocytes in gingiva of children exist. One route involves penetration through oral mucosa; the second route is characterized by delivery via the bloodstream. Stimulatory agents of pigmentation in ETS can be introduced to saliva and might reach melanocytes through gingival epithelium. Gingival pigmentation was often observed in labial areas,[3,6,7,9] where ETS may not overlap directly. Furthermore,

TABLE 2. Distribution of Smoking Parents by Score of Melanin Pigmentation in the Gingiva of Their Children According to Observations of Two Examiners

Examiner	Score of Pigmentation*	Smoking Status of Parents, *n* (%)		Total
		Nonsmoking	Smoking	
A	0	11 (65)	6 (35)	17
	1	3 (18)	14 (82)	17
	2	9 (36)	16 (64)	25
	1 and 2	12 (29)	30 (71)	42
B	0	9 (65)	4 (35)	13
	1	6 (18)	13 (82)	19
	2	8 (36)	19 (64)	27
	1 and 2	14 (30)	32 (70)	46
	Total	23 (39)	36 (61)	59

* Scores of pigmentation: 0, no pigmentation; 1, solitary unit(s) of pigmentation in papillary gingiva without extension between neighboring solitary units; 2, formation of continuous ribbon extending from neighboring solitary units.

TABLE 3. OR and 95% CI of Gingival Pigmentation to Parental Smoking Status

Examiner	Smoking Status of Parents	OR (95% CI)	
		Crude	Adjusted*
A	Nonsmoking	1.0	1.0
	Smoking	4.6† (1.4–15.2)	5.6‡ (1.5–20.0)
B	Nonsmoking	1.0	1.0
	Smoking	5.1§ (1.4–20.0)	5.4‖ (1.4–21.2)

* Based on multiple logistic-regression analysis controlling for gender and age.
† $P = .013$.
‡ $P = .010$.
§ $P = .016$.
‖ $P = .015$.

the majority of ETS is aspirated through the nose. Thus, indirect stimulation by nicotine and benzpyrene in ETS of gingival pigmentation via the bloodstream may afford a more plausible explanation.

The effect of parental smoking on gingival pigmentation in children was apparent; however, because the percentage of smoking parents of children who displayed solitary pigmentation was higher than that of children who presented with the more distinct form of continuous pigmentation, the effect in terms of extent of pigmentation was not clear. Additional studies using quantitative analyses with respect to effect of ETS and gingival pigmentation could establish greater detail regarding the association between melanin pigmentation in human gingiva and passive smoking.

Gingival pigmentation might be suggestive of parental smoking; however, gingival pigmentation was frequently observed in children, although prevalence of the symptom was higher in children with smoking parents in comparison with nonsmoking counterparts. Melanocytes normally occur in the gingiva of all humans.[19] Therefore, clinicians should not use gingival pigmentation as an indicator of parental smoking. The present investigation suggested an association between excessive pigmentation in the gingiva of children and passive smoking.

The impact of graphic warning labels of cigarette packages on adult smoking behavior was demonstrated in Canada[26]: two images depicting a diseased mouth and a lung tumor were identified as most effective at discouraging smoking; furthermore, the image of the mouth was selected by more smokers, female individuals, and young adults than its counterparts.[27] Gingival pigmentation in the mouth of a child is visible to parents and practitioners; as a result, melanin pigmentation in gingiva should be introduced to lists that pertain to children's health in relation to ETS,[28] which can be used by pediatric practitioners to educate parents with respect to the dangers of ETS.

CONCLUSIONS

The oral diseases periodontitis and pediatric caries are related to passive smoking. The stimulatory effect of tobacco smoke on melanin pigmentation in gingiva was strong. This study is the first to describe the relationship between excessive pigmentation in the gingiva of children and parental smoking. This result is suggestive of the third effect of ETS exposure on oral symptoms: melanin pigmentation in gingiva of children. Future research may be necessary to confirm this finding such that the visible condition can be used in the education of parents.

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Association of Melanin Pigmentation in the Gingiva of Children With Parents Who Smoke

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Association between passive and active smoking evaluated by salivary cotinine and periodontitis

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Abstract

Aim: This study attempted to determine the relationship between passive and active smoking on the basis of salivary cotinine levels and periodontitis severity.

Methods: Japanese workers ($n = 273$) were surveyed via an oral examination, a self-administered questionnaire and collection of whole saliva. Probing pocket depth (PPD) and clinical attachment level (CAL) served as periodontal parameters. Periodontitis was defined as the presence of two or more teeth with PPD $\geqslant 3.5$ mm and CAL $\geqslant 3.5$ mm. Salivary cotinine was determined using ELISA. Statistical methods included Wilcoxon's rank-sum test and multiple logistic regression analysis.

Results: Based on the results of receiver-operating characteristic plots for cotinine-level classification derived from self-reported smoking status, non-, passive and active smokers were defined as those subjects exhibiting cotinine levels of 0, 1–7 and $\geqslant 8$ ng/ml, respectively. Numbers of teeth displaying CAL $\geqslant 3.5$ mm in passive and active smokers were significantly higher than those in non-smokers. Multiple logistic regression analysis revealed significantly higher periodontitis odds ratios in passive and active smokers relative to non-smokers following adjustment for other lifestyle factors; odds ratios were 2.87 [95% confidence interval (CI); 1.05–7.82] and 4.91 (95% CI; 1.80–13.35), respectively.

Conclusion: These findings suggest that passive smoking classified in terms of salivary cotinine level may be an independent periodontitis risk indicator.

Key words: active smoking; cotinine; passive smoking; periodontitis; saliva

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Smoking is generally accepted as a major environmental risk factor of periodontal diseases. The majority of previous investigations examined the association between periodontitis and active smoking (Johnson & Hill 2004). A meta-analysis of six studies concluded that active smokers were nearly three times more likely to exhibit severe periodontitis in comparison with non-smokers (Papapanou 1996). Approximately 40% of periodontitis cases are thought to be attributable to active smoking based on data from the National Health and Nutrition Examination Survey (NHANES) III (Tomar & Asma 2000). We previously demonstrated that active smoking displays the greatest impact on periodontitis among lifestyle-related factors (Nishida et al. 2004, 2005). Recently, Arbes et al. (2001) reported that adjusted odds of periodontal disease were 1.6 times greater for persons exposed to passive smoke than for persons not exposed via evaluation of self-reported environmental tobacco smoke (ETS) exposure. This result suggested the presence of a harmful effect in connection with passive smoking with respect to periodontal disease.

In most investigations, smoking status was evaluated exclusively via a self-administered questionnaire. The validity of self-reported smoking is often questioned because of the widespread belief that smokers are inclined to underestimate the amount smoked or to deny smoking altogether (Patrick et al. 1994). In addition, self-reported exposure to ETS may require detailed questionnaire items (Jarvis et al. 1984). Cotinine, a major metabolite of nicotine in body fluids, is considered an accurate indicator of current smoking or of exposure to smoke. Nicotine possesses a very short half-life in the blood, approximately 2 h; in contrast, cotinine exhibits a longer serum half-life, approximately 19 h (Haley et al. 1983). Thus, cotinine has been employed as a chemical

marker of nicotine exposure in numerous studies relating smoking to disease (Istavan et al. 1994). However, few reports have documented an association between cotinine level in body fluids and periodontitis (McGuire et al. 1989, Gonzalez et al. 1996, Chen et al. 2001). Furthermore, a correlation between passive smoke exposure determined with respect to cotinine level and periodontitis has not been used. The objective of the present study was to characterize the relationship between passive and active smoking on the basis of salivary cotinine levels and severity of periodontitis.

Subjects and Methods

Study population

Three hundred and sixty Japanese factory workers employed at a manufacturing company in Osaka were available for evaluation. In 2003, 273 (75.8%) of these individuals (236 males and 37 females, aged 18–62 years) were surveyed via an oral examination, a self-administered questionnaire and collection of whole saliva. Oral status was not examined in 61 subjects because of reasons corresponding to their work; additionally, 26 participants refused to provide saliva. Two hundred and fifty-six workers (221 males and 35 females, aged 18–62 years) completed all items of a self-administered questionnaire. Informed consent was obtained from all subjects. Permission for this study was obtained from the Ethical Committee for Clinical Research of Osaka University Graduate School of Dentistry.

Assessment of lifestyle-related factors

Lifestyle behaviour was evaluated in terms of eight categories (cigarette smoking, alcohol consumption, sleeping hours, breakfast, nutritional balance, working hours, physical exercise and mental health) utilizing a protocol developed by Morimoto (Kusaka et al. 1992, Shizukuishi et al. 1998). Questions were presented in multiple-choice format (from two to six possible answers). Each answer was dichotomized as a "good" or "not good" health practice. Body mass index (BMI) was calculated as an indicator of overall adiposity with regard to obesity. BMI was computed from weight in kilograms divided by square height in metres.

Assessment of smoking behaviour

Data corresponding to smoking behaviour (never, past or current smoker) were derived from a self-administered questionnaire. Moreover, individual passive smoking situation was probed in the self-administered questionnaire: "Are you currently exposed to tobacco smoke from other people within a week?" Five independent locations were examined: home, workplace, restaurants, recreation halls and traffic stations. Additionally, the frequency of tobacco exposure at four levels with respect to each of the aforementioned locations was surveyed: almost every day, sometimes, not at all and uncertain. The questionnaire was based on the guidelines of the Survey of Smoking and its Effect on Health in Japan (Ministry of Health, Labour and Welfare, Japan, 1999). ETS score was calculated on the basis of this self-reported questionnaire to evaluate passive smoking status as follows: the score for "almost every day" was 2, the score for "sometimes" was 1, the score for "not at all" was 0 and the score for "uncertain" was 0.5. Scores for the five locations were totalled and the individual ETS score was obtained. Subjects with or without ETS exposure were defined as those participants displaying ETS scores >2 or $\leqslant 2$, respectively.

Assessment of salivary cotinine level

Subjects received a piece of paraffin gum at the annual health check-up; subsequently, following chewing, saliva samples were obtained by expectoration. First, participants were asked to chew a piece of paraffin gum for 30 s. Then, they were asked to spit approximately 2.0 ml of saliva into a test tube. Saliva samples were collected between 9 and 12 am. Samples, which were stored at -80°C until use, were delivered to the laboratory for cotinine analysis. Cotinine levels were measured via a competitive enzyme-linked immunosorbent assay (ELISA). ELISA plates (Nunc A/S, Roskilde, Denmark) were coated (0.1 ml/well) with a solution of rabbit polyclonal anti-goat IgG (10 μg/ml) (Dako Cytomation A/S, Glostrup, Denmark) in tris-buffer, pH 8.4, and incubated overnight at 4°C. The plates were blocked with 0.2 ml of 10 mM phosphate buffer, pH 7.5, containing 0.1% BSA (phosphate-BSA buffer); subsequently, plates were incubated for 1 h at room temperature and stored at 4°C. A standard inhibition curve was generated by serial dilution (1:2) of a solution consisting of cotinine (160 ng/ml) in phosphate-BSA buffer to obtain seven dilutions of known concentration. Each dilution was tested in duplicate via addition of 50 μl of cotinine solution, 50 μl of (1/10,000) goat polyclonal anti-cotinine reagent (Affiniti Research Product Ltd, Exeter, UK) and 50 μl of cotinine conjugated with horseradish peroxidase, which was derived from carboxyl-cotinine (Aldrich Chem Co., Milw., WI, USA) and horseradish peroxidase (Sigma Co., St Louis, MO, USA), as described previously by Grabarek and Gergely (1990). Each unknown sample was also tested in duplicate with 50 μl of saliva at 1:2 dilution and 50 μl of anti-cotinine reagent and horseradish-conjugated cotinine reagent. Following a 1-h incubation at 25°C, plates were washed three times with 0.3 ml of distilled water. A substrate solution (100 μl) containing tetramethylbenzidine (TMB) was then added, and plates were incubated for 30 min at 25°C in the dark. Colour development was terminated upon the introduction of 100 μl (1 M) of phosphoric acid. The optical density of each well was determined with a microplate reader at 450 nm. The minimum limitation of the measurement for salivary cotinine was 1 ng/ml in this study. The coefficients of variation of the assay were 5.8% within batch and 9.6% between batches.

Assessment of periodontitis

The periodontal condition, measured as probing pocket depth (PPD) and clinical attachment level (CAL) in millimetres, was recorded using an automated probe (Vivacare TPS Probe™, Schaan, Liechtenstein) involving a constant force (20 g) by three examiners. Probing was performed at six sites per tooth for all teeth (excluding the third molar); moreover, the deepest reading was recorded for each. In two selected quadrants – one maxillary and one mandibular – CAL was calculated based on the probed distances (in millimetres) from the free gingival margin to the cemento-enamel junctions and the base of the sulcus; the greatest CAL was recorded for each tooth. Subsequently, subjects were classified into two groups, periodontitis or non-periodontitis, based on placement above or below each two teeth characterized by PPD $\geqslant 3.5$ mm and CAL