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Betel-quid chewing, but not smoking, has a significant impact on mitochondrial DNA sequence in buccal epithelial cells—implications for forensic DNA typing

Chung-Yen Pai,[1] Ph.D.;Su-Lien Chou,[1] M.S.;Chia Tien Chan,[2] M.S.; Yen-Yuh Cheng,[3] M.S.;
Chiou-Herr Yang,[1,*] Ph.D.

[1]*Department of Forensic Science, Central Police University, Taoyuan33304, Taiwan, ROC*
[2]*Nantou County Police Bureau, Nantou, Taiwan, ROC*
[3]*Criminal investigation Bureau, Taipei, Taiwan, ROC*

Abstract

Much is currently known that organic chemicals and toxic agents in betel quid (BQ) and cigarette smoke may cause extensive damage to human mitochondrial DNA (mtDNA) that has no protective histone and is replicated without proofreading as well as effective DNA repair systems. However, oral epithelial cells and blood samples usually serve as essential reference or known samples when typing the mtDNA of hair evidence. It is thus of interest to identify the extent of mtDNA sequence variation in regular smoking BQ-chewers'and smoking non-BQ chewers'oral epithelial cells. The hypervariable segments I and II in mtDNA between paired blood and buccal samples from 69 non-BQ chewing smokers, 60 BQ chewing smokers, and a control group consisting of 75 subjects without both BQ chewing and smoking were DNA sequenced and compared. Among the three groups, the alteration rates of 1.3% (1 out of 75), 1.4% (1 out of 69), and 10% (6 out of 60) were identified from the control, non-BQ smokers, and BQ smokers, respectively. Our and other's data also revealed that almost BQ chewers were smokers, and most started chewing after smoking, signifying that chewers in Taiwan were basically a subset of smokers. Based on statistical analyses, three pieces of important results were reached. First, there appeared to be high mtDNA stability in non-BQ smokers' oral epithelial cells (p value=0.503), and thus can be effectively used as a reference or a known sample in forensic and maternal typing. Second, once the non-BQ smokers pick up the habit of BQ chewing, there will be a substantial impairment on the mtDNA in oral cells, meaning a significant relation with the BQ-chewing factor (p value=0.034). In addition, result further demonstrated that the factor of BQ chewing would only have an impact on point alteration, but not on the poly-C track length alteration (p value=0.465). Our findings would be helpful for forensic typing using the mtDNA marker in hypervariable control region.

KeyWords:cigarette smoking, betel quid (BQ) chewing, mitochondrial DNA, DNA alteration, buccal epithelial cells

Introduction:

Up to now, cigarette smoking and betel quid (BQ) chewing are quite popular in Taiwan. It is reported that BQ compositions could generate reactive oxygen species, which may in turn cause DNA damage [1], and that about 80 % of oral cancerous patients in Taiwan are associated with the habit of BQ-chewing [2]. Cigarette smoking contains more than 4800 chemical materials such as the nicotine, tar, carbon monoxide, and a large number of free radicals and reactive oxygen species (ROS). In addition to BQ carcinogens, cigarette smoking is an important cause of oral cancer [3]. These organic materials and toxic agents in BQ and tobacco smoke might easily and extensively do harm to human mitochondrial DNA due to its nude genome, thus leading to mtDNA sequence mutation. Noticeably, the control region (HV-1 and HV-2) in mtDNA has become an important forensic marker. It is therefore of interest to know whether the content of mtDNA is altered in buccal cells of cigarette smokers and betel-quid chewers. In practice, blood and buccal scrape samples have long been used in forensic and maternal typing using mt

DNA markers and are often considered as a reference or known sample when comparing the mtDNA sequence of criminal hair shafts [4-8]. Presence of mtDNA sequence alterations between the questioned and known oral sample within an individual might falsely lead to exclude the person as the source of an evidential sample.

In previous studies, we have shown that there appears to be high stability of the STR markers in healthy BQ-chewers' buccal epithelial cells [9,10] and that the severity of mtDNA alteration in BQ-chewers' oral epithelial cells has a positive association with the history of BQ-chewing [11]. However, there is little information available on the extent of mtDNA sequence variation in smokers with/without BQ chewing. An assumption was thus proposed that the degree of mtDNA alteration between blood and buccal samples of BQ chewing smokers could be much higher compared to that of non-BQ smokers. Three groups of BQ-smokers, non-BQ smokers, and a control group (without BQ chewing and smoking habit) were compared. In each group, the mtDNA sequence alterations of the paired blood and oral samples were examined. The significance of the alteration of mtDNA sequence in oral epithelial sample from smokers with and without BQ chewing is also discussed.

2. Materials and methods

2.1. Sample and sample preparation

Paired buccal and its corresponding blood samples were collected from 60 BQ chewing smokers, 69 non-BQ chewing smokers, and 75 unrelated individuals as a control group (without the habit of BQ chewing and smoking). Subjects consenting to participate in the study were interviewed at the time of admission using a standardized questionnaire concerning their BQ-chewing and smoking history. The age distribution of the three groups (BQ chewing smokers, non-BQ chewing smokers, and the control) is from 18 to 62, 17 to 66, and 21 to 66, respectively. DNA was extracted from these samples using the QIAamp DNA kit (QIAGEN Inc, Valencia, CA, USA).

2.2. Sequencing of mitochondrial DNA

Two hypervariable regions (HVI and HVII) in mtDNA were PCR amplified. The following primer sets modified from the study [12], F15971: 5'-TTAACTCC ACCATTAGCACC-3', R16410: 5'-GAGGATGGT GGTCAAGGGAC-3', and F15: 5'-CACCCTATTA

ACCACTCACG-3', R389: 5'-CTGGTTAGGCTGG TGTTAGG-3', were used for amplifying the HVI and HVII, respectively. The PCR reaction consisted of 5μl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl2), 8 μl of 1.25 mM dNTP mixture, 5 μl of 1.5 μM forward and reverse primers, 2.5U of Taq DNA polymerase, 10 ng of DNA and distilled water to a final volume of 50μl. The PCR reaction conditions were as follows: 94°C for 30s, followed by 35 cycles of 94°C for 20s, 56°C for 10s, and 72°C for 30s. To detect the efficiency and fidelity of PCR, 5μl of PCR product were loaded on a 2 % agarose gel, electrophoresed at 100 V for 15 minutes and visualized using an UV-light illuminator. Prior to the sequencing reaction, the PCR products were purified using a QIAquick® PCR purification kit (QIAGEN Inc, Valencia, CA, USA).

Cycle sequencing was performed using 3.0 μl of ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction solution with AmpliTaq FS DNA polymerase (PE Applied Biosystems, Foster City CA, USA), 10 p mol of forward or reverse sequencing primer (F15971, R16410, F15, and R389), 2.3 μl of purified PCR product and sterile distilled water to final volume of 10 μl for each sample. The Perkin-Elmer 9600 thermal cycler was used for cycle sequencing reaction under the following conditions: 25 cycles at 96°C for 30s, 50°C for 15s, 60°C for 4 minutes. After sequencing, each sample was added to a sephadex-G-50 column (BioMax Inc. Odenton, MD, USA) and centrifuged at 1500×g for 3 minutes. Each purified sample was recovered at the bottom of the collection tube and dried in a vacuum centrifuge.

Automated DNA sequencing was carried out on an ABI 3730 or ABI 3100 DNA sequencer. For the ABI 3730 and 3100 sequencers, 4 μl of loading buffer (50 mM EDTA/deionized formamide 1:5) were added to the dried samples. The samples were vortexed, denatured at 95°C for 3 minutes and loaded on a long-range gel. Electrophoresis was run at 40 mA, 2500 V and 40°C for 6 h. For the ABI 3100 sequencer, 25 μl of ABI's template suppression reagent were added to each dried sample. The samples were vortexed, denatured at 95°C for 3 minutes, and then chilled on ice. Samples were analyzed using the suggested POP 6 E module of ABI Prism™ Collection software (PE Applied Biosystems). Analysis of mtDNA sequencing data was performed on a Macintosh computer using Sequence Navigator DNA and Protein Sequence Comparison software (Version 2.0, ABI). In order to rule out artifacts or to reduce

ambiguities, sequencing was carried out repeatedly for both the mtDNA strands and for those samples showing mtDNA alterations.

2.3. Detection threshold of heteroplasmy

Based on the guidelines described by Carracedo et al. [13], a detection threshold of 20% of proportions of electrophoretic peaks height was set for the counting of heteroplasmic variants. Any base point above this proportion was then considered as a presence of point heteroplasmy.

2.4. Statistical analysis

Statistical Analysis System (SAS) version 8.1 was used for the statistical analysis. The association between BQ-chewing and the frequency of mtDNA mutation was examined by the Fisher's exact test.

3. Results and discussion

Detailed results of sequence alteration in mtDNA hypervariable regions (HV1 and HV2) between the paired buccal / blood cells among the three groups are summarized in Table 1 and 2. The overall alteration

Table 1. Rate of subjects having sequence alteration of mtDNA in hypervariable region I and II between buccal samples and their corresponding blood cells among the control group, non-BQ smokers, and BQ smokers

Groups	Length alteration at poly-C302-309 and/or poly-C16184-16193	Point alteration	Total alteration	Risk of oral cancer*
Control	0%(0/75)	1.3 %(1/75)	1.3%(1/75)	1
Non-BQ smoker	0%(0/69)	1.4%(1/69)	1.4%(1/69)	18
BQ smoker	1.6% (1/60)	8.3 %(5/60)	10%(6/60)	90

* Data from reference [16].

Table 2. Data of subjects with point alteration of mtDNA in HVI and HVII among the control, BQ smoker, and non-BQ smoker group

Groups	Nucleotide position[#]	Anderson sequence	Sequence in blood cells	Sequence in buccal cells
Control				
1	215	A	A	A/G (2:1) *
BQ smoker				
1	16086	T	C	C/T (1:1) *
2	16093	T	T/C (3:2) *	T
3	16189	T	C/T (1:1) *	C
4	153	A	G	A/G (1:2) *
5	153	A	G	A/G (1:3.7) *
	150	C	C	T/C (1:4.7) *
non-BQ smoker				
1	16189	T	T	C/T (1:2)

according to Anderson [17]

* The numbers in parenthesis represent the peak height ratio of heteroplasmy.

rate of non-BQ smokers (1.4 %) appeared similar to that of the control group (1.3%) and much lower than that of BQ smokers (10%). The only one alteration, C (blood) to T (buccal) transition at np 16189 (figure 1), was identified from an aged male among the 14 non-BQ smokers having smoking age over 30 years. Interestingly, this transition site has been reported to be a mutation hotspot in human mtDNA [14]. Further investigation was performed using the AmpFlSTR Identifiler™ marker system. As expected originally, the outcome demonstrated that all the 15 STR and the sex markers are in consistence between the paired blood/ buccal samples. This result was in agreement with the conclusion of our previous study [9,10], emphasizing that there appeared to be high DNA stability of the HLA-DQA1, PM, and STR markers in regular BQ chewers' buccal epithelial cells. Statistical significance was not observed (p value=0.503), suggesting that the smoking behavior of non-BQ smokers didn't play an essential role in the mtDNA point alteration. Contrarily, the severity of mtDNA point alteration was significantly associated with the BQ-smoking history, with a frequency of 2.6% for BQ-smoking age less than 10 years, while to be 22.7% for more than 10 years (p=0.02 by 2-tailed Fisher's exact test). Moreover, the alteration rate further increased up to 80% (4 out of 5) when the BQ chewing and smoking period was over 30 years.
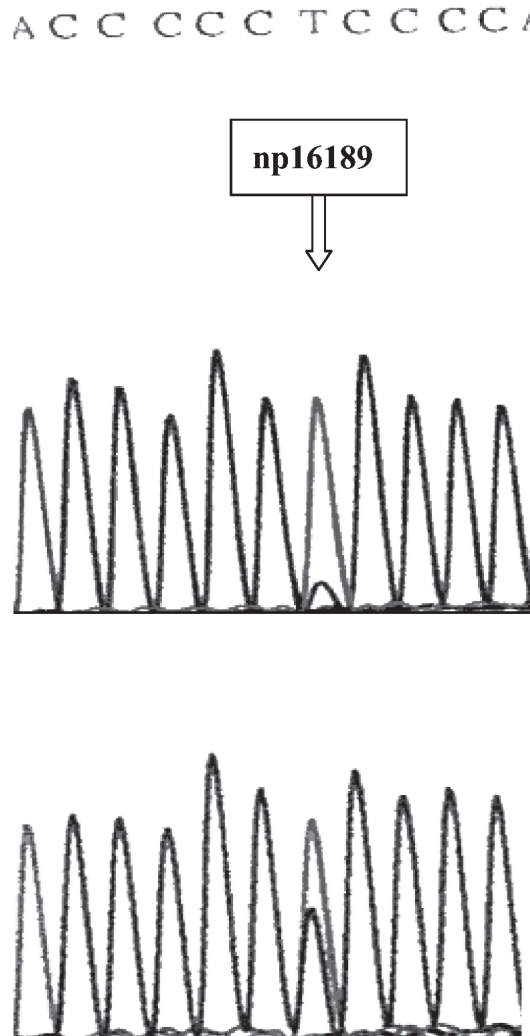

Fig. 1 Point alteration at np 16189 between the blood sample and buccal cells within a non-BQ smoker. Top panel (blood), exhibiting a T homoplasmy, and bottom panel (buccal), a C/T (1: 2, peak height ratio) peak with a smaller C peak underneath.

Statistical significance appeared on the increase of alteration rate (p value=0.034) from non-BQ smokers (1.4%, one out of 69) to BQ smokers (10%, 6 out of 60), implying that once the smokers pick up the habit of BQ chewing, great impact on mtDNA will generate, leading to significant mtDNA alteration in buccal cells. Based on our questionnaire data of cigarette smoking and BQ chewing of the BQ smokers, we and other [15] found that almost all the smokers started cigarette smoking and then had the habit of BQ chewing. When they had the habit of BQ chewing, they smoked more cigarettes per day. It has been reported that oral cancerous patients risk from cigarette smoking is 18 times higher than that of non-smokers, and that when smokers possess the habit of betel quid, the risk of oral cancer is 90-fold higher than that of non-smoker [16]. Thus, it was not surprisingly to find that about 80 % of oral cancerous patients in Taiwan are associated with the smokers who also have the habit of BQ-chewing [2]. This inference meets the synergistic effect, reflective of the significant interaction between the BQ chewing and cigarette smoking behavior. This phenomenon incorporated with the statistical result suggested that the behavior of cigarette smoking only was not directly associated with the mtDNA mutation in the buccal cells. The inference hereby is also in line with one study [15], revealing that the risk of oral cancer for BQ smokers is 5-fold higher than that for non-BQ smokers. On the other hand, we further found that the impact of BQ chewing factor was generated only on mtDNA point mutation, but not on length alteration at the poly-C track (p value=0.465).

In the control group, however, there was no mtDNA alteration case found in the 42 subjects who are younger than 35-year-old. One point alteration at np 215 with a A in blood and a heteroplasmy A/G (A>G) in its paired oral epithelial cells was identified from the 33 subjects with age larger than 35 yrs. Statistical significance was reached (p=0.44, 0/42 vs. 1/33, by 2-tailed Fisher's exact test), suggesting that aging doesn't play an essential role in the mtDNA sequence alteration between the blood and their paired oral samples. Based on the interpretational guidelines [13], analyses of BQ smokers' buccal samples will produce a complicated result when rendering an interpretation in mtDNA sequence comparison. Taken together, these data suggest that the regular BQ-smokers' buccal epithelial cells could have a gradual mutation accumulation in mtDNA and thus should be employed with caution when used as a reference sample (i.e., the blood would be a better

choice), especially for those BQ-smokers with over 10 years of history.

4. Conclusion

In summary, three lines of important information were hereby obtained. First, non-BQ chewing smokers' oral cells (compared to the blood) do not have an accumulation of mtDNA variations, and thus can be used as a reference or a known sample in forensic and maternal mtDNA typing. Second, when the non-BQ smokers possess the BQ chewing habit, the severity of mtDNA mutation is positively associated with the BQ-chewing history. Third, this BQ chewing factor would affect the point alteration only, little influence on the poly-C track length. Our results might be valuable in forensic mtDNA analysis, especially for those countries where BQ-chewing is prevalent.

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