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TECHNICAL NOTE

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Evaluation of the DNA Stability of Forensic Markers Used in Betel-Quid Chewers' Oral Swab Samples and Oral Cancerous Specimens: Implications for Forensic Application

ABSTRACT: Chewed betel-quid (BQ) residues are often considered vital biological evidence at crime scenes, since the human DNA extracted from the residues is actually from buccal epithelial cells and can be associated with suspects. BQ-chewing is also a risk factor for oral diseases and/or cancers. Archived medical oral-specimens can be used to identify specific individuals under adverse conditions, although STR markers are known to be unstable in various tumor tissues. This study evaluates the DNA stability of forensic marker systems in BQ-chewers' oral epithelial cells, and in archived clinical specimens of oral cancer patients. The genotypes of oral and paired peripheral blood samples in 200 subjects were compared, using the commercialized typing systems of HLA-DQA1, PM (including LDLR, GYPA, HBGG, D7S8, and GC loci), and AmpFℓSTR™ markers (including 9 STR loci and the Amelogenin gene). The 100 healthy BQ-chewers had consistent oral swab and paired blood sample genotypes analyzed with both DQA1/PM and STR marker systems. In the 100 oral cancer patients, one discordant result at D7S8 was found in the 600 DQA1/PM-marker loci, and 25 allelic alterations with expansion or contraction were detected in the 900 STR loci. The findings herein suggest that when cancerous specimens were tested, the HLA-DQA1/PM system with point polymorphism appears more reliable than the STR system with length polymorphism. Our results also indicate that healthy BQ-chewers' oral cotton swabs containing buccal epithelial cells are useful for forensic purposes using the HLA-DQA1, PM, and STR marker systems.

KEYWORDS: forensic science, HLA-DQA1, polymarker (PM), STR, buccal epithelial cells, oral cancerous specimens, betel-quid (BQ) chewers

Betel-quid (BQ) chewing is widespread in Taiwan, South Africa, India, and many Southeast Asian countries. Approximately one hundred million people chew BQ worldwide (1). Chewed BQ residue is frequently taken as biological evidence at crime scenes in Taiwan (2), as the human DNA extracted from chewed betel residues is from transferred buccal epithelial cells, which can be linked to suspects. BQ-chewing has also been recognized as a risk factor for oral leukoplakia and submucous fibrosis, which can lead to oral cancers (3,4).

The genotype consistency between an individual's DNA samples constitutes a basic requirement for forensic identification. A recent study by Rubocki et al. (5), which assessed whether two separate tissue biopsies (a bladder cancer biopsy and a normal gastric tissue biopsy) were from the same person, observed a loss of heterozygosity (LOH) at the D13S317 locus when the nine STR markers of the AmpFℓSTR Profiler PCR amplification kit (PE Applied Biosystems, Foster City, California) were employed in typing experiments. Rubocki et al.'s study revealed that pathological tissues, when used as reference materials, might pose problems if the forensic applicability of archived samples is not well verified. Another investigation reporting a new tissue preservation solution for mass disaster DNA identification revealed several cases of peak imbalance as well as LOH at the commercialized STR loci, when compared to their respective control blood DNA (6). Further, archived medical oral specimens have been used to identify a specific individual in an airbus crash accident (7) or other mass disasters (8–10), when other reference materials are not readily available or are severely degraded. These investigations indicated that the types of reference samples potentially used for forensics and paternity determinations are plentiful and quite different (11–16).

AmpliType HLA-DQA1 and Polymarker (PM) (PE Applied Biosystems, Foster City, California) commercialized kits and several STR systems have been used in forensic applications and paternity determinations for many years (8,17–20). DQA1/PM and STR markers detect sequence polymorphism and length polymorphism, respectively, in the target template DNA. Both systems have undergone extensive validation experiments and are therefore reliable on laboratory mock samples as well as on evidentiary-type samples (21,22). While STR or minisatellites markers are unstable in certain tumor tissues, HLA-DQA1 and PM systems may be more

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stable. Artlett et al. (23), for example, confirmed scleroderma family membership using the HLA-DRB, -DQA, and -DQB markers. Giroti et al. (24) also employed HLA-DQA1/PM markers to identify carcinoma biopsy tissue; genotypes obtained from biopsy materials were consistent with those obtained from reference blood samples. However, few reports have addressed the DNA stability of BQ-chewers' buccal epithelial samples and clinical oral specimens of oral cancer patients, despite the fact that these samples are used in forensic human identification.

This research investigated the forensic usefulness of DNA extracted from oral cotton swabs containing buccal epithelial cells collected from healthy BQ-chewers, and oral cancer specimens. Their usefulness was evaluated using the HLA-DQA1/PM, and STR marker systems. Results in this study provide further insight into the limitations of forensic identification from these types of samples.

Materials and Methods

Sample Collection

Oral epithelial cells from BQ-chewers and oral cancerous patients' clinical tissues were used to investigate their forensic effectiveness. Two hundred subjects comprising 100 unrelated healthy BQ-chewers and 100 oral cancer patients were tested. BQ-chewers' oral cotton swab samples and paired blood samples were collected in Taitung, Taiwan, ROC; those with oral diseases or lesions were excluded from the investigation. The subject's mouth was vigorously cleaned three times by rinsing with mineral water, and then a cotton swab was used to collect oral epithelial cells. Blood samples were collected by blotting the blood with filter paper, which was then dried in the air. Age distribution of the 100 BQ-chewer subjects (78 males and 22 females) was from 21–80 years, with a mean of 45 years. BQ-chewing history ranged from 1–54 years, with a mean of 18.4 years.

The 100 oral cancer tissues (mostly oral cavity tissues) and their paired blood samples were confirmed histopathologically at the Chang-Gung Memorial Hospital (Taoyuan, Taiwan, ROC). Patients participating in this report were interviewed at the time of admission using a standardized questionnaire regarding their BQ-chewing history. Cancer patient age distribution was also from 21–80 years old, with a mean of 50.4 years old.

Sample Preparation and DNA Extraction

The 100 BQ-chewers' genomic DNA was extracted using the salt-chloroform extraction method (25). Oral swabs or bloodstained filter papers were cut into small fragments using sterilized forceps and scissors, and then placed into 1.5 mL sterilized microcentrifuge tubes. The swabs or bloodstained filter papers were soaked in a 1.5-mL microcentrifuge tube containing 1 mL sterilized water at room temperature for 1 h, and rotated with sterilized toothpicks for 2 min to agitate cells from the substrate. Substrates were then removed. Five hundred mL of SE buffer solution (75 mM NaCl, 25 mM EDTA, pH 5 8.0), 1% SDS (final concentration), and 200 mg/mL proteinase K (final concentration) were added to each sample, which was then incubated at 56°C overnight. A prewarmed saturated NaCl solution (6 M) was next added to a final concentration of 1.5 M, and gently mixed. The solution was agitated gently for 60 min after which an equal volume of chloroform was added. Subsequent procedures were then performed following the salt-chloroform protocol method (25).

Genomic DNA of oral cancer tissues and their paired peripheral blood samples were extracted according to the phenol/chloroform method (26).

Analyses of HLA-DQA1/PM and STR Markers

The AmpliType DQA1/PM PCR amplification and typing kit was used to amplify DNA extracted from samples. PCR was performed in a final volume of 45 mL, containing 20 mL of reaction mixture, 20 mL of primer set, and 20 ng of template DNA. PCR amplification was performed implementing a program of 32 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 30 s. Genotypes were analyzed with reverse dot-blot hybridization of PCR products, according to the protocol provided with the typing kit.

STR markers were analyzed using the AmpFℓSTR Profiler™ kit. Nine STR markers (D3S1358, vWA, FGA, TH01, TPOX, CSF1PO, D5S818, D13S317, and D7S820) and the Amelogenin gene were amplified according to a protocol provided by PE Biosystems. An ABI PRISM™ 377 DNA sequencer was used for genotype analysis and DNA sequencing.

Results and Discussion

Genotypes of HLA-DQA1, five PM loci (LDLR, GYPA, HBGG, D7S8, GC), and nine STR loci of oral samples and their paired blood samples, which were collected from 100 healthy BQ-chewers and 100 oral cancer patients, were all determined. The genotypes of DQA1/PM loci of oral samples and paired blood samples were consistent in the 100 oral cancer patients, except at the D7S8 locus of one patient with a BQ-chewing history of 32 years (Table 1). The D7S8 genotype of the blood sample for this patient was A/B heterozygous, whereas the oral cancer tissue was B/B homozygous (Fig. 1). Triple analyses, including both positive and negative controls, were performed on the oral cancer sample, and each result confirmed the BB genotype. Additionally, when the AmpFℓSTR Profiler PCR amplification kit was used to further check the cancer patient's DNA, LOH was observed at D3S1358, FGA, CSF1PO, D5S818, and D13S317 (Table 1, Fig. 2).

The reverse dot-blot method of the HLA-DQA1 and PM kits was used to distinguish DNA sequence variations. Information on the difference between A and B allele sequences is not readily available. However, Horn et al. (27) reported a sequence difference be-

TABLE 1—*Results of the HLA-DQA1/PM typing and STR analysis (PE Applied Biosystems, Foster City, CA) by comparing the genotypes of cancerous tissue and the normal blood sample from a oral cancer patient. The numbers in parentheses represent the weakly detected alleles on the STR electropherogram, and the mutant allele for the D7S8 locus.*

Genetic Loci	Genotype of Oral Cancer Tissue	Genotype of Blood Sample
HLA-DQA1	4/4	4/4
LDLR	B/B	B/B
GYPA	A/A	A/A
HBGG	A/B	A/B
D7S8*	(B)/B	A/B
GC	A/C	A/C
D3S1358*	14/(16)	14/16
vWA	14/17	14/17
FGA*	(21)/22	21/22
Amelogenin	X/Y	X/Y
TH01	8/9	8/9
TPOX	8/10	8/10
CSF1PO*	(10)/12	10/12
D5S818*	(12)/13	12/13
D13S317*	8/(9)	8/9
D7S820	12/12	12/12

*Loci with allelic mutation at D7S8, and LOH at the five STR markers: D3S1358, FGA, CSF1PO, D5S818, and D13S317.

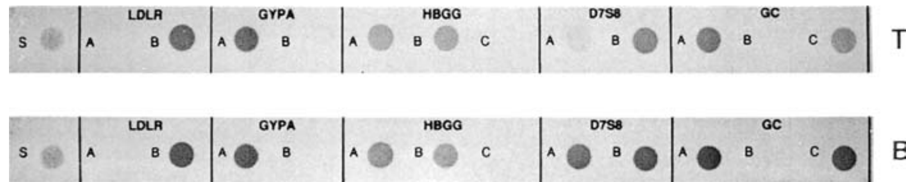

FIG. 1—*An inconsistent result at the D7S8 locus between the oral cancer tissue and paired blood sample from a cancer patient. PCR products from the HLA-DQA1/PM loci were analyzed by a reverse dot-blot technique using the "AmpliType DQA1&PM" PCR-amplification and typing kit (PE Applied Biosystems, Foster City, California). The result shows that the genotype of D7S8 locus of the oral tumor sample (T) was B/B, and that of the normal blood sample (B) was A/B.*

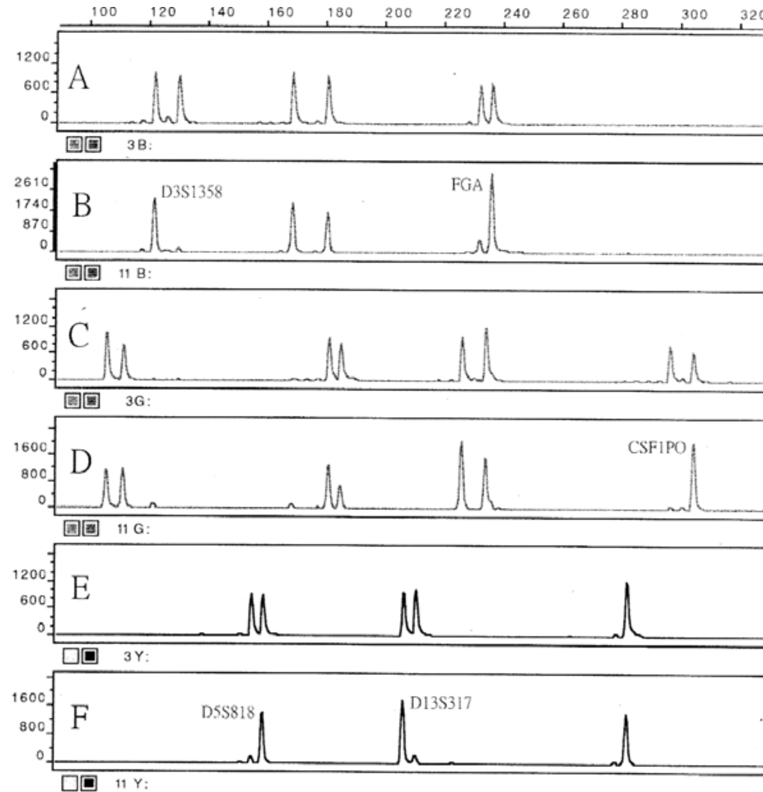

FIG. 2—*Electropherograms of AmpFℓSTR Profiler™ markers for the paired samples of blood and oral cancer tissue from a cancer patient. Panels (A), (C), and (E) represent the STR patterns of the blood sample, and panels (B), (D), and (F) show the result of DNA analysis of the cancerous sample. In the present paper, LOH is defined mathematically by the ratio of peak area of the shorter allele over the larger allele. The LOH value of 0.5 is recognized as a cutoff, because the ratio less than 50% is rare in normal samples under a regular manipulation. Note that the five STR loci demonstrate the LOH at D3S1358, FGA in panel (B), CSF1PO in panel (D), and at D5S818, D13S317 in panel (F), and the LOH values of the five loci are 0.10, 0.19, 0.04, 0.13, and 0.17, respectively. The LOH profile has been confirmed using another kit of AmpFℓSTR identifiler™ PCR amplification system (PE Applied Biosystems, Foster City, California).*

tween the two alleles occurring only in one nucleotide, at position 536 of D7S8 ("C" for allele A and "T" for allele B, accession number M37524, Genebank) (27). Interestingly, when the paired samples of this particular patient were DNA sequenced, a T/C heterozygote from blood sample and a T/T homozygote from tumor were identified at this polymorphic site. If this single point polymorphism was designed in the probe strip used, the D7S8 discrepancy (Fig. 1) would be explained.

When the STR markers were assayed in the 100 oral cancer patients, there were 25 occurrences in 17 patients of microsatellite mutational alterations (i.e., expansion or contraction), which would affect the STR allele type accuracy (Fig. 3). These allelic alterations occurred at 7 loci, including D3S1358, vWA, FGA, CSF1PO, D5S818, D13S317, and D7S820, with a frequency of 2, 3, 7, 5, 1, 4, and 3%, respectively. The current study of 100 oral cancerous specimens analyzed with the AmpFℓSTR system revealed that 17% of the cancerous samples had microsatellite alterations. In contrast, when HLA-DQA1 and PM marker systems were used on the 100-paired samples, only one discordance was found, at the D7S8 locus. This result indicated that cancerous clinical specimens should be employed with caution when used as forensic reference materials, as they can produce contradictory results, especially in the STR system. In the 100 healthy BQ-chewers, however, no microsatellite alteration was observed at any of the STR loci. Further, no contradiction was found between the oral swab sample and paired blood sample genotypes in the group of 100 healthy BQ chewers, using the commercial DQA1, and PM systems.

Natural mutation rates of microsatellites are higher than those of expressed genes. The frequency of microsatellite and expressed

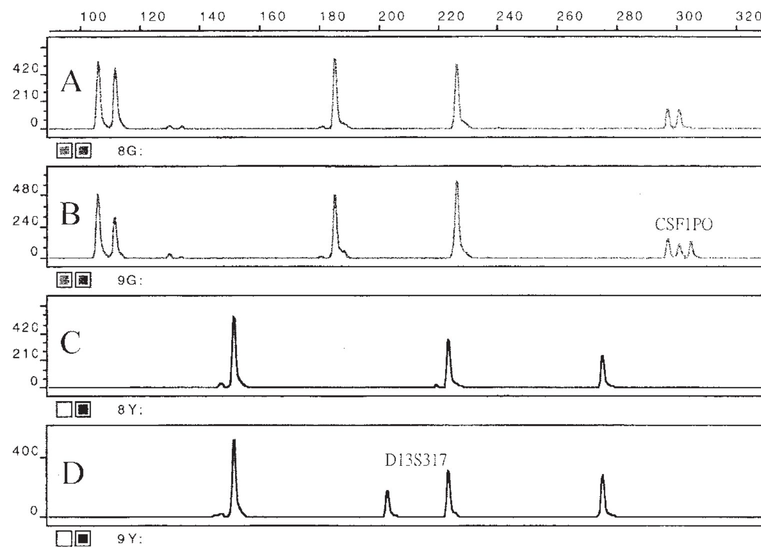

FIG. 3—*Representative electropherograms with allelic alterations from an oral cancerous tissue. Panels (A), (C) are the STR profiles of blood sample, and panels (B), (D) represent the paired cancerous sample from the same patient. An expansion (with an extra peak of one repeat unit) at the CSF1PO locus in panel (B) and a contraction (an extra peak with 5 repeat units lost) at D13S317 in panel (D) are shown.*

gene somatic mutation is estimated to be 10^{-2}–10^{-3}, and 10^{-4}–10^{-5} mutation/cell, respectively (28). The total number of mutations at microsatellite loci in tumor cells could be up to 100-fold than normal cells, reflecting the defect in the DNA mismatch repair genes (29). The STR mutations in cancer tissues have been commonly reported in literatures (30–36). These microsatellite alterations are particularly common in tri- and tetranucleotide repeats than in dinucleotides. Notably, most STR markers currently used in forensics are tetranucleotide repeats. A previous report, however, investigated twenty acute myeloid leukemia patients for microsatellite alteration by comparing buccal epithelial DNA and DNA from leukemia samples (37); out of 215 paired amplifications, no additional bands were found at any locus in any of the tumor samples. This concordant result could be due to either the leukemic etiology not involving any defect of mismatch repair genes, or the leukemia DNA with microsatellite alteration being severely diluted by the greater majority of normal leukocytes DNA.

As for the forensic applicability of oral cancer tissue, since the STR and HLA-DQA1/PM marker systems involve different mechanisms of mutation, it is not surprising they demonstrate different mutation rates. For the general biological evidences encountered at crime scenes, PCR-based STR markers analyzing length polymorphism are more powerful than the HLA-DQA1/PM markers with sequence polymorphism. However, for clinically preserved tumor tissues used for mass disaster DNA identifications, the HLA-DQA1/PM markers based on sequence polymorphism seem to be more reliable.

In conclusion, there appears to be high DNA stability of the HLA-DQA1/PM as well as STR markers in healthy BQ-chewers' buccal epithelial cells, but not in oral cancer patients. Results of this investigation suggest that oral swabs from healthy BQ-chewers could be effectively used for forensic identification. It should be cautious when using biopsy reference samples, particularly for STRs. For such clinical specimens, it may be better to use systems like DQA1/PM, which do not assay tandem repeats.

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