Chromosomal alterations affecting the 1cen-1q12 region in buccal mucosal cells of betel quid chewers detected using multicolor fluorescence in situ hybridization

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Introduction

Epidemiological studies have shown that a high incidence of oral cancers is associated with chewing betel quid. Since chromosomal aberrations are involved in many types of cancers, we investigated whether increased frequencies of chromosomal alterations could be detected in the oral mucosa cells of betel quid chewers as compared to non-chewers. Due to the difficulty in culturing these epithelial cells, we used multicolor FISH with adjacent DNA probes to detect hyperdiploidy and breakage/exchanges affecting the 1cen-1q12 region in interphase cells. Buccal mucosa cells from 19 male betel quid chewers and 23 non-chewers were hybridized and 1000 cells per donor were evaluated. A highly significant increase in the frequency of breakage affecting 1cen-1q12 region was observed in the mucosa cells of the chewers as compared to the non-chewers. A good correlation was also seen between breakage and duration of chewing. A modest increase in hyperdiploidy for chromosome 1 was also observed among chewers who had used betel quid for many years. These results indicate that this FISH approach can be useful for human biomonitoring, particularly for detecting alterations in non-dividing cells.

Materials and methods

Subjects

Nineteen subjects with the habit of chewing betel quid with tobacco and 23 control subjects were recruited from workers at an enclosed vegetable market place in Hyderabad, India. Only male non-smokers or light smokers were selected for the study. All subjects were surveyed for personal information which included duration of betel quid chewing, number of quid used per day as well as lifestyle information such as age, smoking history, diet, consumption of coffee, tea, and alcohol, history of recent illness, and use of medication.

Betel quid

The main components of betel quid are raw betel leaf (Piper betle), betel nuts (Areca catechu), kivam (a gel form of tobacco extract, herbs, spices, flavors, glycerin, silver oils, saffron, and menthol), zarda (a mix of tobacco flakes, natural and synthetic flavors and aromatic spices), lime (calcium carbonate), and additional spices. Among the group studied, the practice was to smear the lime and kivam onto the betel leaf followed by zarda, betel nuts, and spices. The quid was then folded and chewed. According to questionnaire responses, the chewers did not consciously swallow any of these components.

Sample collection and slide preparation

Prior to sampling, the subjects were asked to rinse their mouth with water to remove betel quid components. All scrapings of the buccal mucosa were made with a wooden tongue depressor which was moistened with water prior to use. The surface cells were scraped from the buccal mucosa of both the right and left cheek of each individual. The cells were removed from the spatula by agitation in ice-cold 70% ethanol. After transport to a local laboratory, the cell suspension was centrifuged at 1200 rpm (300 g) for 10 min and the supernatant was discarded. The pellet was resuspended in fresh 70% ethanol, dropped onto a slide and air dried. All slides were stored under nitrogen at –20°C except during transport to the United States, during which time the slides were shipped at room temperature.

We have recently developed a multicolor fluorescence in situ hybridization (FISH*) procedure using tandem DNA probes which allows the detection of breakage and hyperdiploidy affecting the heterochromatin region of chromosomes 1 and 9 in interphase cells (11–13). Studies using cultured human lymphocytes have shown that the targeted heterochromatin regions frequently exhibit breakage following exposure to a variety of chemical and physical agents (14–16). The recently developed tandem labeling procedure provides a novel approach for chromosome analyses where the isolation of metaphase chromosomes is difficult or impossible. Recently, we have applied this procedure to compare the relative sensitivity of non-cultured granulocytes and Go lymphocytes with cultured interphase and metaphase lymphocytes to various doses of ionizing radiation, and observed that this tandem labeling procedure was effective at detecting aberrations in non-cultured interphase cells (17).

Since the buccal mucosa is in close contact with the bioactive components of betel quid and is the site affected by oral cancers, chromosomal alterations in this particular cell type may serve as early biomarkers of cancer predisposition and provide insights into oral carcinogenesis. Due to the difficulties in culturing this type of cell, we applied the tandem labeling procedure using multicolor FISH to study chromosomal aberrations affecting the 1cen-1q12 region in interphase oral mucosal cells.

Abbreviation: FISH, fluorescence in situ hybridization.
Fluorescence in situ hybridization

Prior to hybridization, slides were again fixed using Carnoy’s fixative (3:1 methanol:acetic acid) for 30 min at room temperature. The cells were then treated with pepsin (300 μg/ml in 0.01 N HCl, pH 2.3–2.6; Sigma Chemical Co., St Louis, MO) for 25–30 min, then rinsed in PBS twice, each for 2 min, and fixed in 4% paraformaldehyde for 5 min at room temperature.

DNA probes

The details of the probes and hybridization procedures have been reported elsewhere (12). Briefly, two chromosome 1-specific DNA probes were used for tandem labeling: an α-satellite probe specific for the centromeric region purchased commercially (Oncor, Gaithersburg, MD) (18) and the adjacent classical satellite probe, pUC17.7 (19), specific for the pericentric heterochromatin at 1q12 which was labeled with digoxigenin-11-dUTP (Boehringer-Mannheim, Indianapolis, IN) by nick translation in our laboratory according to the protocol of the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD).

The hybridization mixture containing 1 μl of digoxigenin-labeled classical satellite probe (5–20 ng), 1 μl biotin-labeled α-satellite probe (5–20 ng), 1 μl sonicated herring sperm DNA (1 mg/ml) and 7 μl of MM 2.1 hybridization mix (final concentration of 55% formamide/1× SSC/10% dextran sulfate) was denatured at 70°C and applied to slides which previously had been denatured in 70% formamide/0.3 M NaCl plus 0.03 M sodium citrate (pH 7.0) (2× SSC) and dehydrated. The slides and the hybridization mixture were incubated overnight at 37°C and washed in 60% formamide/2× SSC, three times for 5 min each and once in 2× SSC, all at 45°C. The slides were then rinsed twice in PN buffer (0.1 M phosphate buffer, pH 8.0, containing 0.5% NP-40) at room temperature. The digoxigenin-labeled classical satellite probe was detected using a mouse anti-digoxigenin antibody (3.2 μg/ml in PN buffer) and dehydrated. The slides and the hybridization mixture were incubated for 30 min, then rinsed in PBS twice, each for 2 min, and fixed in 4% paraformaldehyde for 5 min at room temperature.

No. of subjects

Chewers

<table>
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<tr>
<th>Age in years</th>
<th>38.4 ± 11.3</th>
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<tbody>
<tr>
<td>Occupation</td>
<td>Vegetable market workers</td>
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<tr>
<td>No. of quids per day</td>
<td>9.49 ± 4.2</td>
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<tr>
<td>Duration of use in years</td>
<td>12.7 ± 7.7</td>
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<tr>
<td>Smoking</td>
<td>Yes 2 5</td>
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<tr>
<td>No. of years</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>Diet</td>
<td>Vegetarians 3</td>
</tr>
<tr>
<td>Non-vegetarians</td>
<td>16</td>
</tr>
<tr>
<td>Coffee</td>
<td>No 3 4</td>
</tr>
<tr>
<td>Yes 16 19</td>
<td></td>
</tr>
<tr>
<td>Cups per day</td>
<td>Yes 9 9</td>
</tr>
<tr>
<td>Alcohol</td>
<td>No 19 24</td>
</tr>
<tr>
<td>Yes 0 0</td>
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</table>

aValues are for smoking individuals.

Table 1. Characteristics of the betel quid chewers and controls

No. of subjects

<table>
<thead>
<tr>
<th>No. of subjects</th>
<th>19 males</th>
<th>23 males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
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<td>35.4 ± 7.9</td>
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<tr>
<td>Occupation</td>
<td>Vegetable market workers</td>
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<td>No. of quids per day</td>
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<td>Yes 0 0</td>
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Results

Buccal mucosal cells were obtained from 19 male betel quid chewers and 23 male non chewers from the city of Hyderabad in Southern India. Key characteristics of the chewers and non chewers are shown in Table 1. The subjects’ ages ranged from 18 to 61 years with an average age of 38.4 for the chewers and 35.4 for the non chewers: This difference was not statistically significant. The chewers averaged 9.5 quid per day (range 0.3–20) and had chewed for an average of 12.7 years (range 2–31 years). As indicated in Table 1, the dietary and chewing habits were similar between the chewers and the controls. The incidence of illness within the past 12 months was also comparable among the two groups and consisted primarily of colds and the flu. Of note, two of the chewers had had malaria, one chewer and one non-chewer reported thyroid problems, one nonchewer had a history of diabetes and another of asthma.

Using this approach the frequencies of hyperdiploidy and breakage (including exchanges) affecting the 1q12 region were determined in the chewers and non chewers. A highly significant increase in the frequency of breakage and exchanges affecting this region was seen in the group of chewers as compared to the non-chewers (P = 0.0001). The median frequency of breakage among the chewers was 0.002 with an interquartile range (IQR) of 0.001–0.003. In comparison, the median breakage frequency in the controls was 0.000 with an IQR from 0–0 (Figure 1b). The frequency of hyperdiploid cells among the chewers (median 0.000, IQR 0–0.00075) was not significantly elevated as compared to the non-chewers (median 0.000, IQR 0–0) (Figure 1b). However, it appeared that those who had chewed betel quid for the longest period of time had a somewhat higher incidence of hyperdiploidy. This was confirmed by using Poisson regression which indicated that there was a significant association between the frequency of hyperdiploid cells and duration of chewing (P <0.01). The frequencies of nuclei containing 0 and/or 1 hybridization regions did not differ significantly between the chewers and non-chewers.
was used to detect alterations affecting the 1cen-1q12 region in non-cultured buccal mucosa cells of betel quid chewers and non-chewers. A significant increase in breakage/exchanges was observed in the chewing population compared to controls. There was also a significant association between breakage/exchange frequencies and both duration of chewing and the number of quids used per day. This provides strong supportive evidence that the observed alterations were induced by the use of betel quid. The association between duration of quid chewing and breakage/exchanges is intriguing in that it indicates that continued exposure of the buccal mucosal cells to quid and its components may have induced persistent alterations in the oral mucosa. These results also indicate that this multicolor FISH approach may be an effective biomarker of early effect, as a similar relationship has been seen between the incidence of oral cancer and the duration of chewing as well as the number of quids used per day (21).

Previous studies using the micronucleus assay have compared the frequencies of chromosomal alterations in the oral mucosal cells of betel quid chewers with those in non-chewers (22,23). These studies have demonstrated that chewers have significantly higher frequencies of micronuclei than do non-chewers. Additional studies have also demonstrated that supplementation of the chewers with vitamin A and β-carotene yielded a substantial decrease in the frequency of micronuclei in the mucosal cells (24). This decrease could be detected within several months indicating a rapid turnover of damaged cells in this tissue. Related studies in which the formation of micronuclei was studied in the oral mucosal cells following treatment of patients with ionizing radiation for cancer of the head and neck showed that the frequency of micronucleated cells increased within a week following radiotherapy and returned to baseline levels within one month of the cessation of treatment (20,25). These results also support the transitory nature of chromosomal breaks originating in the mucosal stem cells.

In contrast, our results using multicolor FISH with the tandem probes showed a strong association between the frequency of breakage/exchanges and quid chewing which took place over periods encompassing many years (Fig. 2). These results suggest that a portion of the alterations that
are being detected using this FISH assay reflect persistent aberrations such as translocations and inversions which have occurred in the mucosal stem cell. This is consistent with previous in vitro studies conducted in our laboratory in which 30–50% of the alterations detected using the tandem FISH assay in irradiated cultured metaphase lymphocytes represented translocations, inversions or other complex rearrangements, which would be likely to persist in the affected cells (12). Alternatively, the increase in the frequency of alterations affecting 1cen-1q12 could be a consequence of damage resulting in genetic instability, which has been reported to occur following breakage in the centromeric heterochromatin (26,27).

The detection of breaks in buccal cells using the tandem labeling procedure indicates that cell culture is not required for the detection of breakage/exchanges using this FISH approach. Furthermore, the ready availability of this tissue which can be collected using noninvasive techniques, indicates the potential for the use of this tissue for routine biomonitoring. However, it should be mentioned that there are some characteristics of buccal mucosal cells that increase the challenges in performing this type of study. Oral mucosal cells have a high degree of keratinization which can interfere with efficient probe penetration. Although the use of proteases can be used to facilitate penetration, these treatments can also affect nuclear morphology resulting in diffuse signals and increasing difficulties in scoring. Considerable donor to donor variability was also observed in the quality of the preparations and in hybridization efficiency. Furthermore, preparations often contain a significant number of pyonuclei and debris which can make scoring more difficult. In addition, the low frequency of aberrant cells increases the difficulty of statistical analysis. In spite of these limitations, these results demonstrate that FISH techniques can be used to detect structural and numerical alterations occurring in this cell type.

Conclusions

In summary, we have observed a high frequency of alterations affecting 1cen-1q12 region in the buccal mucosa cells of betel quid chewers which showed a significant association with duration of chewing and number of quids used per day. Breakage affecting the heterochromatin regions of human chromosomes 1, 9, 16 has been seen in many cancer types and may contribute to tumor development (28–30). Consequently, the ability to detect chromosomal aberrations in the heterochromatin of epithelial cells may be a potentially powerful tool for monitoring individuals at increased risks for cancers occurring in epithelial tissues.

Acknowledgements

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References

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