Detection of Field Cancerization in The Clinically Normal Oral Mucosa of Cannabis and Cigarette Smokers

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Abstract

Background: Head and neck cancer represents one of the most common cancer types. Numerous risk factors are implicated in the development of HNSCC. In our study we aimed to investigate the changes that occur in the oral mucosa of cannabis smokers and compare them to cigarette smokers and non-smokers.

Methods: Three groups of subjects were included in our study (33 in each group); cannabis and cigarette smokers, cigarette smokers and non-smokers. The biopsies were examined by routine H&E techniques and immunohistochemical expression of p53.

Conclusion: The present work demonstrated evident dysplastic and pre-dysplastic changes in H&E stained sections. The changes were detected histologically in both smoker groups. These changes were more pronounced in cannabis smokers’ group than cigarette smokers’ group. Moreover, p53 immunostaining was higher for cigarette smokers’ group than cannabis smokers’ group. It is evident that cigarette smoking and cannabis smoking results in field changes in the oral mucosa which are detectable in tissue sections. These changes, such as hyperplasia and dysplasia are more detectable in H&E sections of cannabis smokers than cigarette smokers. However, positive p53 immunoexpression was lower in cannabis smokers than cigarette smokers.

Key words: Head and neck cancer, oral cancer, cannabis smoking, cigarette smoking, field cancerization.

Introduction

Oral cancer is the 11th most common malignancy in the world and is the most common type amongst all head and neck cancers with an annual incidence of about half a million new cases and around 300,000 death each year (1%–2% of all cancer death). Among all oral cancers, oral squamous cell carcinoma (OSCC) accounts for 90% of them.

Field cancerization is quite a new terminology in the cancer field, it indicates that, when cancer develops in a tissue, group of genetically altered clones of cells in adjacent clinically normal tissues occur, which are prone to development of synchronous and metachronous tumors. The field cancerization theory emphasizes high probability of recurrences in head and neck squamous cell carcinoma patients.

Multiple factors have been involved in the etiology of oral cancer. Simultaneous cigarette smoking and alcohol consumption produce their synergistic effects and are by far the strongest factor in oral carcinogenesis. Cannabis can be used by smoking (combined with tobacco), within food or as an extract. Cannabis smoking releases its main chemical component which is the tetrahydrocannabinol (THC) which is then absorbed into the bloodstream via the lungs.

Cannabis is listed as the most commonly used illegal drug in the world. Estimated on the basis of
official statistics reported to the UN and WHO, there are many countries that demonstrate increased number of cannabis users. This list includes the United States, Canada, as well as Australia and New Zealand.\textsuperscript{7,8}

Worldwide cancer research has not been directed enough to look at the effect of cannabis on cancer. Cannabis smoking related carcinogens have usually been mixed with tobacco smoking ones. It has been suggested that this combination increases the risk of head and neck cancer. However, others claim that cannabis kills cancer cells in vitro. In addition, numerous cell cultures showed antitumor effect in various cancer types. However, tumor destruction in the laboratory is much easier than in a live person and this has to go through the full process of animals and in-vivo testing to make sure of its effect. The lack of profound safety, funding and effective clinical trials make it very difficult to assess the potential benefits and risk of using cannabinoids in many cases. Smoking is the most common way of marijuana use. However, this is medically unsuitable. Moreover, the antitumor effects of cannabis have to overcome their known immunosuppressive effects which can be potentially pro-tumorigenic.\textsuperscript{9}

All things considered, it is relatively evident that studies on the relationship between oral cancer and cannabis smoking independently lack the proper depth due to the overlapping carcinogens for tobacco and tobacco/marijuana smoking or due to community restrictions. However, it is of utmost importance to further investigate such a rapidly growing habit that might cause a burst in cancer related deaths within the next few decades.

Materials and Methods

1) Selection of Subjects:

The specimens were obtained from three groups of patients each group comprised 33 subjects:

1. Nonsmokers.
2. Cigarette smokers.
3. Smokers of both cigarette and cannabis.

All subjects had non-contributory medical history and demonstrated no obvious oral lesions; exclusion of subjects who had any systemic illnesses or were simultaneously exposed to any other carcinogenic substance was done.\textsuperscript{10}

A) Selection of cigarette smokers:

Cigarette smokers included in the study were frequent smokers (at least 10 cigarettes a day) for a period not less than 5 years.\textsuperscript{10}

B) Selection of simultaneous cigarette and cannabis smokers:

Simultaneous smokers in the study were frequent cigarette smokers (at least 10 cigarettes a day) for a time period not less than 5 years plus frequent cannabis smoker (at least 3 times per week) for a period not less than 5 years. They must fulfill both inclusion criteria.

Subjects were only involved if they were undergoing simultaneous oral surgical procedures after filling an informed consent form stating that they agree to participate in the study. Approval was obtained from the ethical committee of the Faculty of Dentistry, Cairo University before the beginning of the study.

An incisional biopsy was obtained from the buccal mucosa of subject (preferred due to rapid healing and minimal complications). The biopsies were an average size of 0.5 cm X 0.5 cm. The biopsies were fixed in formalin and embedded in paraffin. From each block, three cut sections were obtained at 4 μm thickness. One was mounted on a glass slide and stained by routine hematoxylin and eosin (H&E) stain. Positively charged slides were done for the other 2 sections for better adhesion during the immunohistochemical staining procedure.\textsuperscript{10}

Immunohistochemical staining was carried out using the automated immunostainer (AutostainerLink48, Dako, Denmark). Each section was stained using the ready to use, monoclonal mouse antihuman P53 antibody (Clone DO-7, Code IR616, Dako, Denmark). Examination of the stained sections was carried out using low and high-power light microscopy (Leica, Switzerland). Quantification of the cell count of the positive p53 immunoreaction was done using an image analyzer computer system applying the software Leica Quin 500 (Leica Microsystem, Switzerland). The most homogenous areas of reaction were evaluated to avoid edge artifacts. It was suggested that a positive
immunoexpression of p53 in 25% of the cells is a sufficient cut off value to indicate TP53 mutations 11.

A standard measuring frame of 10 μm per ten fields using a magnification x400 by light microscopy transferred to the monitor’s screen was used for automated cell counting. Five fields were measured per section. It was suggested that a positive immunoexpression of p53 in 25% of the cells is a sufficient cut off value to indicate TP53 mutations 11.

Values were presented as mean and standard deviation (SD) values. Kolmogorov-Smirnov test was used to check normality of the data which show that most of the data were normally distributed (parametric data). Accordingly, one way analysis of variance ANOVA test was used to compare between groups and different intervals within the same group, followed by Tukey’s post hoc test when the difference was found to be significant.

**Results and Discussion**

**Histopathological Examination of Hematoxylin and Eosin Stained Sections:**

During microscopic examination of H&E stained sections dysplastic and pre-dysplastic features (hyperplasia) were detected in all smoking groups. Hyperplasia was found in a greater percentage in cannabis smokers (78.7%) compared to cigarette smokers (60.6%). Pseudo-epitheliomatous hyperplasia was also detected more in cannabis smokers (36.4%) compared to cigarette smokers (24.2%). Furthermore, increased nuclear cytoplasmic ratio and prominent nucleoli were more pronounced in cannabis smokers. Moreover, in six cases of cannabis smokers (18%) frank dysplasia was actually evident in the clinically normal oral mucosa compared to three cases of cigarette smokers (9%). Dyskeratosis was evident in 12.1% of cannabis smokers, while it was found in 6% of cigarette smokers (table 1, figs 1, 2). Subepithelial inflammation was also detected in a few cases.

**Immunohistochemical expression of P53**

Positive nuclear p53 expression was mainly evident in the basal cells and to a lesser extent in prickle cells. The greatest mean number of p53 positive nuclei/high power field was observed in cigarette smokers, followed by cannabis smokers, with the lowest mean value was recorded in the non-smoking group. The difference between groups was statistically significant (p<0.0001) according to one-way analysis of variance. Tukey’s post hoc test revealed a significant difference between each two groups (table 2, fig 3).

When considering how rapidly cannabis smoking is a widely disseminated trend not only in our society but also worldwide, research should be directed towards this habit to try to uncover the relationship between oral cancer and cannabis smoking and to solve the big question mark whether cannabis aggravates or diminishes the harmful effects of cigarette smoking.

In our study, hyperplasia was detected more in cannabis smokers than cigarette smokers (78.7% for cannabis smokers versus 60.6% for cigarette smokers). This could be seen as a protective mechanism to the produced heat which is higher in cannabis smoking 12. Even though, basilar hyperplasia, nuclear hyperchromasia and prominent nucleoli are closely related to the amount of DNA in the cells and is closely related to their rate of growth, division and protein synthesis. This could be seen as the earliest dysplastic features in the spectrum of transformation of the normal oral mucosa into a dysplastic one. However, these changes are not exclusive to carcinogenesis, and may also be seen in reactive epithelium 13.

The presence of frank dysplasia in some of the subjects even though they presented normal mucosal appearance highlights the fact that histologic changes usually precede the clinical change and that dysplasia could be present in oral mucosa with no clinical detectable changes and that large areas of cells are affected by the carcinogenic insults that will be capable later on to develop multiple primary tumors 4.

The increase in the pre-dysplastic and dysplastic features in all cannabis smoking subjects more than cigarette smoking subjects could be attributed to the higher heat produced by smoking cannabis in a hand rolled cigarettes or joints (the commonest way), the habit of smoking joints without filters to the smallest butt size till the proximal end, this is added to the manner of cannabis smokers; they tend to hold their breath and inhale deeply, all this leads to higher concentration of the smoke inhaled.
The increased p53 positivity in cigarette smokers’ group was an expected result. Cigarette smoking has been linked to many cancerous and precancerous lesions specifically the oral and pharyngeal cancer with a higher incidence of p53 mutations in smoking linked carcinomas than the non-smoking linked carcinomas \(^{14}\).

p53 immunostaining relation with cigarette smoking was also confirmed by Mizobuchi, et al, 2000\(^{11}\); they examined p53 immunostaining in 74 patients with esophageal squamous cell carcinoma. They concluded that p53 was one of the main molecular targets of cigarette smoke in carcinogenesis.

On the other hand, the lower p53 expression in cannabis smokers could be linked to the anti-tumorigenic capability of cannabis that has been documented in the literature by Dariš et al, 2019\(^{8}\). It has been also verified in a study by Śledziński et al, 2018\(^{9}\). that cannabis could inhibit proliferation of cancer cells, and stimulate their autophagy and apoptosis. Moreover, Hermanson & Marnett, 2011\(^{15}\) concluded that cannabis can exert interesting effects on the cell line of many tumors, it can modulate cell proliferation, angiogenesis, reduce tumor growth, limit cellular migration and inducing apoptosis.

Moreover, this lower expression in cannabis smokers’ group compared to cigarette smokers’ group could be linked to the fact that the hand rolled cannabis joints are less densely packed compared to tobacco cigarette which can lead to lesser load of carcinogens smoked in a breath. Also, this could be linked to the CBD; another constituent of the cannabis sativa plant, which has been proven to be analgesic, sedating and anti-inflammatory\(^{16}\) and hence may play a role in decreasing the harmful side effects of THC; the major constituent of the plant cannabis sativa.

The lower p53 immunoexpression in the cannabis smokers’ group may support the ideological concern that being natural makes it harmless and better than pharmacological synthetic drugs. In 2013 Massi, et al.\(^{17}\) interestingly concluded that the anticancer effect of CBD seems to be cancer selective in vitro. The efficacy of CBD has been linked to its diverse ability to target numerous cellular pathways that control tumorigenesis. In 2018, Sultan et al.\(^{18}\) concluded that CBD inhibit cell survival and induced apoptosis in a dose dependent manner through down regulating of cyclin D and inducing DNA fragmentation. Comparably, Jeong et al., 2019\(^{19}\) concluded the same results on CBD induced apoptosis by regulating many pro- and anti-apoptotic proteins, among which is Noxa, which normally promotes activation of caspases and apoptosis. Noxa showed a significantly higher expression in human colorectal cancer cell line subjected to CBD thus confirming its role as a novel, reliable anticancer drug.

In line with our results, Zhang et al, in 2019\(^{20}\) examined the in vitro effects of CBD on human gastric cancer cells and concluded that p53 protein was highly expressed and p21 protein was downregulated which subsequently inhibit the levels of CDK2 and cyclin E, thus inducing cell cycle arrest at the G0–G1 phase.

The findings in the current work may support the claim that cannabis could play a context dependent role in cancer development. However, being mixed with tobacco and being smoked is still problematic and may mask the potential anticancer effect of cannabidiol on malignant proliferating cells. Moreover, our observations are not conclusive and should not be taken without further investigation. p53 was the only marker used in this research and although positive cases confirm a field change effect, negative cases couldn’t be excluded from having different forms of genetic mutations. A panel of markers and genetic analysis should be done. Social, educational and ethical background limited the number of sample size and recruited cases. Moreover, it was extremely difficult to investigate the field effect of cannabis smoking without tobacco effect, this overlapping of carcinogens requires higher stratification and a bigger sample size.
Figure 1 (A&B): A. bar chart demonstrating the different levels of dysplastic and pre-dysplastic changes detected in the various groups. B. Column chart showing mean number of p53 positive nuclei/high power field in different groups.

Figure 2:

Figure 2 (A&B): A. Photomicrograph of H&E stained section showing moderate epithelial dysplasia in a cannabis smoker (X200). B. Photomicrograph of H&E stained section showing epithelial hyperplasia in a cigarette smoker (X100).

Figure 3:

Figure (3): Photomicrograph showing positive nuclear expression in basal cells of a cannabis smoker (X100)

Table (1): The frequency of different dysplastic and pre-dysplastic changes assessed in the different groups.
<table>
<thead>
<tr>
<th></th>
<th>Cannabis smoker</th>
<th>Cigarette smoker</th>
<th>Non-smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>33</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>26 (78.7%)</td>
<td>20 (60.6%)</td>
<td>12 (36.4%)</td>
</tr>
<tr>
<td>Peudo-epitheliomatous</td>
<td>12 (36.4%)</td>
<td>8 (24.2%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basilar Hyperplasia</td>
<td>12 (36.3%)</td>
<td>10 (30.3%)</td>
<td>3 (9%)</td>
</tr>
<tr>
<td>Dyskeratosis</td>
<td>4 (12.1%)</td>
<td>3 (9%)</td>
<td>0</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>6 (18%)</td>
<td>3 (9%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Table (2): Number of p53 positive nuclei/high power field in different groups and significance of the difference using ANOVA test:

*significant at p<0.05

<table>
<thead>
<tr>
<th></th>
<th>Control (Non-smokers)</th>
<th>Cigarette smokers</th>
<th>Cannabis smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.33a</td>
<td>84.67b</td>
<td>73.22c</td>
</tr>
<tr>
<td>SD</td>
<td>0.37</td>
<td>11.79</td>
<td>11.89</td>
</tr>
<tr>
<td>Min</td>
<td>0</td>
<td>62</td>
<td>54</td>
</tr>
<tr>
<td>Max</td>
<td>3</td>
<td>97</td>
<td>99</td>
</tr>
<tr>
<td>F value</td>
<td></td>
<td>218.258</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>&lt;0.0001*</td>
<td></td>
</tr>
</tbody>
</table>

**Conclusions**

1. Cigarette smoking and cannabis smoking results in field changes in the oral mucosa which are detectable in tissue sections.

2. Hyperplasia and dysplasia are more detectable in H&E sections of cannabis smokers than cigarette smokers.

3. Positive p53 immunoexpression was detected in clinically normal oral mucosa of both cigarette smokers’ group and cannabis smokers’ group indicating field cancerization.

4. p53 immunoexpression was highly significant between the control group and both cannabis smokers’ group and cigarette smokers’ group.

5. p53 immunoexpression was lower in cannabis smokers than cigarette smokers which was statistically significant at p<0.05.

**Acknowledgement:** I would like to express my deep gratitude to the residents of the oral and maxillofacial surgery department, faculty of dentistry, Cairo university for their efforts in collecting of the subjects and taking the tissue specimens.
The authors declare that they have no conflict of interests

Source of Funding: There are no financial fundings to disclose

Ethical clearance: Taken from Research Ethics Committee, Faculty of Dentistry, Cairo University.

References
2- Stenson KM, Brockstein BE, Ross ME. Epidemiology and risk factors for head and neck cancer (2016).