

Immunohistochemical assessment of the effect of tobacco on a molecular gatekeeper in oral squamous cell carcinoma

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ABSTRACT

Background: It has been emphasized that the molecular gatekeeper p53 has a key role in carcinogenesis and its mutation is seen in more than 50% of the human cancers including head and neck carcinomas and tobacco is the most important etiological agent in head and neck cancer. **Aims:** To assess p53 mutations in relation to tobacco usage in oral squamous cell carcinoma (OSCC) to understand the role of tobacco in the complex process of carcinogenesis. **Materials and Methods:** Forty formalin-fixed paraffin-embedded archival tissue samples were taken, of which 20 cases were associated with tobacco and 20 were not, and assessed immunohistochemically. **Results:** Enhanced expression of p53 was found to be associated with tobacco. **Conclusions:** p53 mutations are etiologically associated with the development of head and neck carcinomas and are associated with exposure to specific carcinogens of tobacco.

Key words: Carcinogens, immunohistochemistry, oral squamous cell carcinoma, p53 mutation, tobacco

INTRODUCTION

Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all oral cancers.^[1] Oral cancer is one of the most common cancers representing 6% of all cancers in populations.^[2,3] In India, it is the commonest cancer among males and third most common cancer among females.^[4]

The known classic risk factor for oral cancer is the use of tobacco and other etiological factors including consumption of alcohol, infections, dietary factors, and chemical irritants.^[5,6] Oral cancer is seen to be associated specifically with the use of tobacco and alcohol and the prevalence of the disease in different forms depends on the extent of exposure to these etiological agents. In Europe, America,

Australia, China, and Japan, smoking of cigarettes, cigars and pipes are the main forms of tobacco use and in contrast in India, Sri Lanka, and Southeast Asia, chewing of tobacco is prevalent. Other forms of tobacco use which are also potent risk factors for oral cancer includes smoking of beedis, and reverse smoking.^[7]

Repeated exposure to specific carcinogens in cigarette smoke may cause multiple neoplastic lesions in the mucosa of the aerodigestive tract. The upper aerodigestive tract, the only area in the body in which the alimentary tract and the airways form a common conduit, is an ideal site for evaluating the independent and synergistic effects of tobacco and alcohol.^[8] Carcinogens may leave unique 'fingerprints' in the form of specific mutations that cause the initiation or progression of cancer.^[7]

Neoplastic growth is characterized by alterations of two known groups of genes: Oncogenes and tumor suppressor genes. The interaction between activated oncogenes and mutations that results in a loss of function of tumor suppressor genes appears to be the driving force directing normal cells to uncontrolled growth and invasion.^[9]

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The p53 gene and its protein products is an area of intensive study ever since it became clear that slightly more than 50% of human cancers contain mutations in this gene.^[10]

The nature of these genetic changes in cancer cells is most commonly a missense mutation in one allele, producing a faulty protein that is then observed at high concentrations in these cells.^[10]

Mutation of the p53 gene, the most common genetic alteration, has been linked to tobacco in various forms in squamous cell carcinoma of the head and neck, as well as esophageal, lung, and bladder cancer.^[8] The link between specific induction of p53 mutation and exposure to specific carcinogens in tobacco smoke has been further reinforced by recent studies demonstrating that benzopyrene induces specific p53 mutations at 'hotspot' locations invitro similar to those observed in human lung tumors *in vivo*. These studies suggest that mutational alterations in target genes may accumulate via highly specific events and carcinogenic pathways.^[11]

In this study, we examined the relationship between mutations and genotypes in conjunction with exposure to known environmental risk factors for oral cancer, that is, tobacco use to understand the interplay between the most common mutation and the most common associated etiological factor of OSCC.

MATERIALS AND METHODS

Forty samples of histopathologically diagnosed OSCC were taken retrospectively from the archival specimens. Case sheets were analyzed for tobacco use, of which 20 cases were patients who used tobacco and 20 who did not. Patients who used tobacco were taken as the tobacco group, and those who did not formed the non tobacco group. Patients with cancer in any organ were excluded from the study. Two sections were cut for each of the formalin-fixed paraffin-embedded specimens from the rotary microtome; one section with a thickness of 4 μ , stained with Harris hemotoxylin and the other sections of 4 μ thickness were taken in poly-L-lysine-coated slides for immunohistochemistry (IHC).

Immunohistochemical staining

Sections cut at 4 μ were floated on to poly-L-lysine-coated slides and incubated 58°C overnight. The sections were then deparaffinized in two changes of xylene for 15 minutes each. Dexterylinization was done by immersing the slides in two changes of absolute alcohol for one minute each. The sections were alcoholized by immersing the slides in 90 and 70% alcohol for one minute each and then washed for 10 minutes and 5 minutes each in tap water and distilled water, respectively.

Antigen retrieval was done by placing the sections in citrate buffer and then pressure cooking for 10 minutes. The pressure cooker was then cooled for 20 minutes in the sink with water. The sections were rinsed with distilled water for 5 minutes and were then washed with two changes of Trisbuffer solution (TBS) for 5 minutes each. To block the endogeneous peroxidase enzyme activity, the sections were treated with peroxidase block for 10-15 minutes and then again washed with three changes of TBS for five minutes each. They were then treated with Power Block™ for 15 minutes to block nonspecific reaction with other antigens. The sections were drained and covered with primary antibody antihuman polyclonal p53 for one hour to identify tumor markers by antigen-antibody reactions and again washed with TBS as described earlier. To enhance the reaction between primary and secondary antibodies, the sections were then treated with Super Enhancer™ for 30 minutes and again washed with TBS. The enzymes were labeled by treating the sections with supersensitive secondary antibody and washed with TBS.

Chromogen was added to the sections for five minutes to give color to the antigens and the sections were again washed with TBS. The sections were then washed with tap water for five minutes and were counterstained with hemotoxylin for one minute and washed in tap water, dried, cleared in xylene, and mounted. The interpreted tumor marker was brown in color [Figures 1-3].

RESULTS

Of 40 samples, 22 samples showed positivity to p53;17 out of 22 samples of OSCC with tobacco usage showed p53 positivity whereas only five cases showed p53 positivity in OSCC without any tobacco usage [Figures 4 and 5]. Even intensity of the staining was shown to be more intense in the tobacco group when compared to positive cases of the non-tobacco group. So there is an increase in the p53 expression in the samples of the tobacco group showing 85% positive cases when compared to the nontobacco group which showed positivity only in 25% of the cases.

DISCUSSION

The role of p53 in the process of carcinogenesis is well established as playing a key role in the progression of cancer of various cell types.^[7] In particular, a high incidence of p53 mutations has been demonstrated in tobacco users in cancers involving the lung, larynx, renal, head and neck cancers.^[7]

High frequency of p53 mutation and protein accumulation have been reported previously in head and neck squamous cell carcinoma cell lines and tumor cells.^[9] The longer half-life

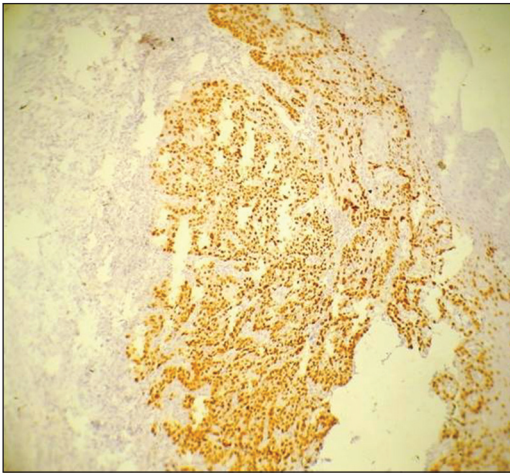


Figure 1: Positive p53 expression (×10)

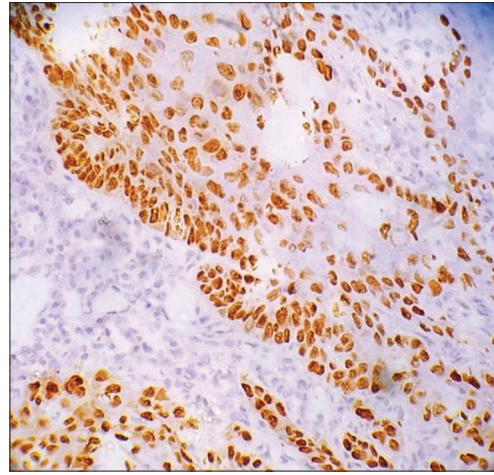


Figure 2: Positive p53 expression (×40)

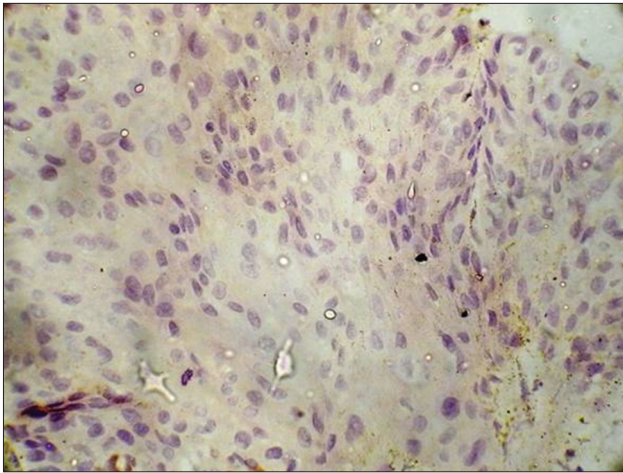


Figure 3: Negative p53 expression (×40)

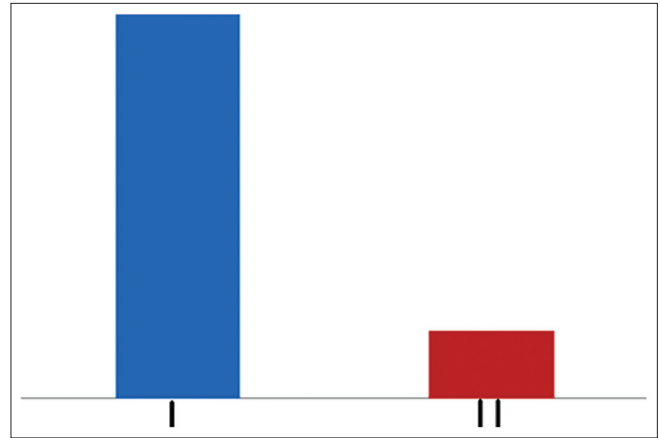


Figure 4: Indicating positive and negative percentages in patients with tobacco usage; I: Positive, II: Negative

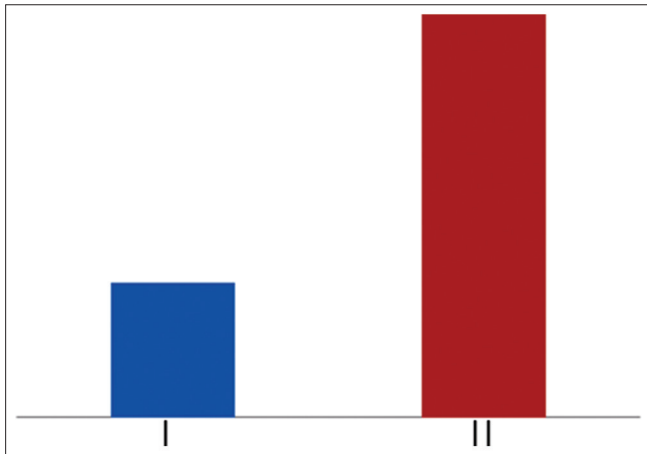


Figure 5: Indicating positive and negative percentages in patients without any tobacco usage; I: Positive, II: Negative

In this study, we found an increased incidence of p53 mutations in patients of the tobacco group when compared to patients belonging to the nontobacco group. In the tobacco group, 83.3% of the cases were positive whereas in the nontobacco group, only 25% of the cases were positive, showing an increased incidence of p53 mutations in relation to tobacco usage. Previous data suggests tremendous increase in p53 mutations in relation to tobacco usage.^[12-16]

In a study on bladder tumor, Moore *et al.*, showed increased frequency of mutations demonstrated by IHC and also showed that a distinct chemical mutational pattern with an increase in G-A mutation at CpG sites was observed in smokers with >20 pack/yr of exposure, and a mutational hotspot at codon 273 was associated with tobacco smoking.^[17]

of the mutant p53 protein results in the accumulation of this phosphoprotein in the nuclei, facilitating its detection by IHC analysis.^[9] Moreover, this increased incidence of mutation of p53 in OSCC shows tobacco association.^[12]

In colorectal neoplasia, the findings by Terry *et al.* suggest a positive association with alcohol intake and cigarette smoking which may have a relation with p53 early in the adenoma-to-carcinoma sequence.^[18]

A study on lung cancer by Rodin *et al.* also suggests the positive association between cigarette smoking and tobacco use and the p53 mutation and even an increase in the incidence of p53 mutation in environmental exposure to tobacco.^[19]

A study by Liu *et al.* on gastric mucosa suggests that metabolically activated carcinogens contained in tobacco smoke can directly affect the p53 tumor suppressor gene, which plays a pivotal role in the balance of cell proliferation and apoptosis, in the cellular response to various types of stress, and also explains that reactive oxygen intermediates generated from cigarette smoke interact with DNA damage and produce oxidative DNA damage with potentially mutagenic consequences leading to the activation of p53.^[20]

Several studies on OSCC has shown the association between tobacco and p53 mutations showing similar significant findings, like the above-mentioned studies.^[21-24]

The study by Basnaker *et al.* on oral smears evaluating the expression of p53 showed that some cases of epithelial dysplasias with tobacco usage showed the expression of p53 indicating that the tobacco insults on TSG before clinical alterations are evident and lend credence to the concept that inactivation of p53 protein occurs in the early phases of oral carcinogenesis and significant expression is seen in carcinomas of patients with tobacco habits, in specific more in tobacco chewing than smoking.^[25]

The findings of the present study are supported by the findings of the previous studies supporting the association of tobacco and p53 in the complex process of carcinogenesis.^[8,12,21,22]

Tobacco is the most important etiological factor in the development of OSCC and has specific mutagen carcinogens like benzopyrene and N-nitrosamine which induce a specific loci mutation in p53 which leads to the progression of the cancer.

CONCLUSION

The inactivation or mutation of the molecular gate keeper p53 by tobacco-specific carcinogens lead to progressive fatal disease and abstinence from the use of tobacco in various forms are important in the prevention of oral cancer.

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