

Expression of p53 in the Tumor and Oral Epithelium in Patients with Cancer of Mouth and Pharynx

Expressão da p53 no Tumor e no Epitélio Oral em Pacientes com Câncer de Boca e Faringe

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SUMMARY

- Introduction:** Expressive percent of patients with oral and oral pharynx carcinomas presents with overexpression of protein p53 induced by tobacco, alcohol and radiotherapy.
- Objective:** To describe the p53 expression in areas of the normal mucosa adjacent to the tumor and in mouth and pharynx carcinomas.
- Method:** Prospective study with clinical follow-up of one year of 24 patients with oral and oral pharynx spinocellular carcinoma. We performed biopsies in the neoplasm and areas of the normal mucosa adjacent to the tumor, before and 9 months following radiotherapy and an immunohistochemical study of p53 expression.
- Results:** Before radiotherapy, there was a change to the p53 expression in 20 out of the 24 biopsies made in the neoplasm and in 14 in those of normal mucosa adjacent to the tumor. Eleven patients died 1 year before clinical follow-up. From the 2 initial patients with increase of p53 after radiotherapy it remained increased in 7 in the neoplasm region and in 6 in the normal mucosa regions. We noticed association of p53 with smoking and tumor stage ($p < 5\%$), but not with the degree of cellular differentiation and alcoholism.
- Conclusion:** The increase to the p53 expression was viewed both in the neoplasm region and in the normal mucosa in most patients with oral and oral pharynx carcinoma before and after radiotherapy. There was a statistically significant correlation of the p53 expression with smoking and neoplasm stage.
- Keywords:** mouth neoplasms, pharynx neoplasms, p53 genes, cocarcinogenesis.

RESUMO

- Introdução:** Expressiva porcentagem de pacientes com carcinomas de boca e faringe apresentam superexpressão da proteína p53 induzida por tabaco, álcool e radioterapia.
- Objetivo:** Descrever a expressão da p53 em áreas de mucosa normal adjacente ao tumor e em carcinomas da boca e faringe.
- Método:** Estudo prospectivo, com seguimento clínico por um ano, de 24 pacientes com câncer espinocelular de boca e faringe. Foram feitas biópsias na neoplasia e em áreas de mucosa normal adjacente ao tumor, antes e 9 meses após a radioterapia, e realizado estudo imunohistoquímico da expressão da p53.
- Resultados:** Antes da radioterapia, houve alteração da expressão da p53 em 20 das 24 biópsias feitas na neoplasia e em 14 nas de mucosa normal adjacente ao tumor. Onze paciente morreram antes de 1 ano de seguimento clínico. Dos 2 pacientes iniciais com aumento da p53 após a radioterapia continuava aumentada em 7 na área da neoplasia e em 6 nas áreas de mucosa normal. Observou-se associação da p53 com o tabagismo e estágio do tumor ($p < 5\%$) mas não com o grau de diferenciação celular e alcoolismo.
- Conclusão:** O aumento da expressão da p53 foi observado tanto na área da neoplasia como em mucosa normal na maioria dos pacientes com carcinoma de boca e faringe antes e após a radioterapia. Houve correlação estatisticamente significativa da expressão da p53 com o tabagismo e estágio da neoplasia.
- Palavras-chave:** neoplasias bucais, neoplasias faríngeas, genes p53, cocarcinogênese.

INTRODUCTION

The head and neck cancer (HNC) has an incidence of two to three percent of all human neoplasms and takes the sixth place as the most frequent type of cancer worldwide. It occurs more typically in patients of the male sex between the fifth and the eighth decade of life and is less common in patients younger than 45 years old. Forty per cent of the head and neck cancer occur in the oral cavity, 25% in the larynx, 15% in the pharynx and 20% in other parts, including salivary glands. The HNC represents a significant health problem and causes almost 200,000 deaths all over the world yearly. In India, the oral spinocellular carcinoma (SCC) corresponds to approximately 50% of the new cases of cancer diagnosed and its incidence is growing in spite of the advances of prevention and new therapies (1,2). Nevertheless, the rates of morbidity and mortality have had a few improvements during the last 30 years, especially when it occurs in young patients, before the fourth and fifth decades of life (3, 4). Recent clinical observations have shown that oral cancer in young patients has a specific etiology and clinical progression in which the genetic factors prevail irrespectively of exogenous carcinogenic factors, such as smoking and alcoholism (5, 6, 7).

When there is exposure to carcinogenic agents in cells like those of the mouth and pharynx, there may be the "cancerization" of such cells whose first stage is the appearing of atypical and dysplastic cells with several genetic and molecular changes with a final result of neoplasm (7, 8). Therefore, the genetically altered cells spread and expand gradually to adjacent parts that are still without neoplasm, but are already vulnerable to cellular mutations. These regions that are normal upon clinical exam and optical macroscopy are susceptible to evolve into neoplasms, which would explain many neoplasm recurrent situations and the occurrence of a second primary tumor (9, 10). The probability for this to occur in oral cancer is of 10 to 25%, especially because many of these patients keep the prior characteristics of exposure to carcinogenic agents, like the habit of smoking and drinking alcoholic beverages, now connected to therapeutic irradiation (11).

However, the concept of evolution from normal cells at optical microscopy into neoplastic cells is not very clear yet so far. The concept that is current today is that these normal cells adjacent to the tumor would only be in stages different from the dormant cellular cycle but already with potential nuclear mutations into neoplastic cells (12, 13).

Biomolecular researches indicate that these cells have certain genes undergoing mutations that depend or

not on the action of exogenous factors (14). The alterations of the genes and the expression of proteins they encode would make the cells lose the capacity to adjust their growth and cause the appearing of neoplasms (15).

One of the first genes that are more studied from this point of view is Tp53 and its expression is protein p53. When a cell is exposed to carcinogenic agents, there is an increase to the levels of protein p53 that connect to transcription factors and prevent the cell to get in phase S of the cellular cycle (16). The reversible stoppage of the cellular cycle at transition G1-S permits the DNA repairing and the establishment of the genome integrity. When the DNA alterations exceed the repairing capacity, there occurs cellular death by apoptosis and very often loss of cellular growth control. We notice evolving immature cells with a strong degree of aneuploidy, loss of heterozygosis and aberrant DNA expression (17, 18, 19, 20).

Currently, the importance of gene Tp53 would be that of the "guardian of the genome" able to control the DNA duplication quality when detecting an error at the sequence of the cellular cycle, which causes cellular apoptosis and would prevent the loss of control of cellular differentiation (16, 17).

However, there is no unanimity concerning such observations. Immunopathological studies of lesions to the mouth, pharynx and larynx did not find any statistically different alterations between the p53 altered percentage, severity of the epithelial dysplasia and neoplastic lesions (18, 19, 20, 21). An explanation to these dissentient findings is that the overexpression or lack of expression of p53 and other cellular markers may not always indicate the presence or absence of neoplasm. Another hypothesis is that the expression of p53 would be masked by gene deletion or even connected to virus such as hpv (22, 23).

A current interesting description is that the study of genetic alterations like those of p53 may be used for tumor resistance prognosis to radiotherapy and chemotherapy (24, 25, 26). Even today in the clinical practice we ascribe the tumor resistance of the treatment to the selective advantages of the neoplastic cells on normal cells for the loss of control mechanisms that regulate their growth and the cellular proliferation (27, 28). The clinical applicability of these concepts like that of obtaining morbidity and mortality predictive values of patients with oral and pharynx cancer would be a great advance (29, 30).

Unfortunately, today the therapeutic choice in patients with this type of cancer is still based on clinical staging and histological graduation of the neoplasms. Although current concepts of organs preservation connected

to chemotherapy and radiotherapy, considered so far to be secondary in head and neck cancer, have gained preference for the therapies of such cancer, which decreases the surgery extension and improves the quality of life, we still do not apply the molecular biology as an instrument for the choice of the treatment modality.

Thus, the objective of this work is to describe the gene alterations, specifically the p53 expression in neoplastic lesions of the oral and pharyngeal mucosa and areas close to the tumor, that are normal upon histopathology after radiotherapy in individuals with oral and pharyngeal spinocellular carcinomas.

METHOD

This work was submitted and approved without limitations by the Ethics Committee in Research under number 435/2002-CEP, of our institution. The study is clinical, prospective and a one-year follow up of twenty-four patients with oral and pharyngeal (base of the tongue) carcinoma. The criteria for inclusion of patients were: individuals without diagnostic confirmation of cancer, who had no treatment, results of the histopathological exam of the neoplasm made in our hospital, confirmed to be spinocellular carcinoma and of the mucosa close to the tumor with absence of neoplasm. The treatment chosen was associated cobalt therapy and chemotherapy and the main authors did not take part in the choice of treatment. The variables studied were age, sex, smoking, alcoholic, tumor TNM classification, region of the tumor and presence or absence of expression p53.

This study included 24 patients with oral and pharyngeal cancer (base of the tongue) aged between 43 and 95 years (mean age of 69 years old), 22 (91.7%) of the male sex and 2 (8.3%) of the female sex (Table 1).

Fifteen patients were smokers, more than a package/day, on average for more than 10 years (positive), 2 were non-smokers (negative) and 7 had quit smoking before the entering our hospital. As for the use of alcohol, ten patients were alcoholic, four were non-alcoholic (negative) and ten reported having quit drinking and smoking when they were informed they had or could have cancer. These patients had been for 4 months on average without drinking alcoholic drinks or smoking.

As for the location of the neoplasm, twelve patients had lesion on the base of the tongue (50%), five patients on the floor of the mouth, two on the right side and three on the left side, six on tonsillar pillar (3 to the right and 1 to the left), and one on retromolar area. Four patients had clinical staging T1N0M0 (16.7%), six had T2N0M0 (25%),

Table 1. Results of the slides quantifying the protein p53 expression before and after radiotherapy.

No	ID	Pre-radiotherapy		Post-radiotherapy	
		ANM	T	ANM	T
1	43	08+91-	88+12-	Death	
2	69	87+13-	84+16-	M66+34-	M54+46-
3	77	35+65-	69+31-	Death	
4	46	02+98-	70+30-	Death	
5	65	16+84-	74+26-	M16+84-	M45+55-
6	71	14+86-	0+100-	M23+77-	M4+96-
7	62	2+98-	58+42-	Death	
8	58	17+83-	36+64-	M29+48-	M40+60-
9	95	7+93-	94+6-	M44+56-	M45+65-
10	48	14+86-	0+100-	Death	
11	51	64+36-	69+31-	M22+78-	M26+74-
12	51	6+94-	2+98-	M10+90-	M11+89-
13	56	7+/93-	18+/82-	Death	
14	53	25+/75-	55+/45-	M5+/95-	M16+/84-
15	49	3+/97-	49+/51-	M42+/58-	T33+/67-
16	52	17+/83-	77+/23-	Death	
17	89	20+/80-	56+/44-	Death	
18	83	3+/97-	0+/100-	M2+/98-	M9+/91-
19	47	5+/95-	74+/26-	Death	
20	49	44+/56-	95+5-	M10+/90-	T3+/97-
21	76	32+/68-	42+/58-	Death	
22	68	11+/89-	71+/29-	M0+/100-	M4+/96-
23	76	29+/71-	61+/39-	Death	
24	63	1+/99-	64+/36-	M9+/91-	M6+/94-

Subtitle: ANM: Adjacent Normal Mucosa; T: Tumor; Positive: +; Negative: - The expression is positive when presented 10 or more colored cells in a total of 100 cells.

seven had T3N2M0 (29.2%) and seven had T4N2M0 (29.2%) (Table 1).

Two initial biopsies were made on different areas, the first of which on the neoplastic lesion (A1) and the second biopsy on average one centimeter away from the neoplasm, on normal mucosa upon clinical and histopathological exam (A2). After the end of radiotherapy and chemotherapy treatment, two new biopsies were done again, one on the neoplasm or on the delimited area before starting the treatment and the other on the normal mucosa. The time interval between the first and the second biopsy was of 12 months.

Each of the tissue fragments was fixed in formalin, added to paraffin separately and colored by the hematoxylin-eosin technique. The histological analysis and histological graduation were made: slightly, moderately or well-differentiated. As regards to the degree of histological differentiation, we found 8 individuals with carcinomas moderately differentiated (Figure 1A), 14 well-differentiated (Figure 1B) and 2 slightly differentiated.

The tissue samples fragments (A1) and (A2) were cut in blocks and the detection of the tumor suppressing gene TP53 was done by means of immunohistological reaction with the monoclonal antibody anti-p53 produced in mice (DAKO Code M7001).

The results were evaluated in common optical microscopy and the positive reaction was represented by deposition of the chromogen on connection sites antigen-antibody, with provision of nuclear marking to cells that presented the antigens studied. The analysis of the expression was considered to be positive upon optical microscopy when we detected the cell nucleus with a strong brown color (Figure 1C) and negative when light brown or transparent (Figure 1D). The positive criterion of the p53 expression was given by the counting of 10 or more cells (10%), in a total of 100 cells per reticulum, by counting the cells in at least three reticula (HAMILTON and ALLAN, 1995) (33).

We performed a comparative study of the p53 expression with the variables area of the tumor, alcoholism, smoking and staging. For statistical analysis, we used the Chi-square tests, the Fischer's exact test and McNemar's test. The significance level used was of $p < 0.05$.

RESULTS

We noticed an overexpression of protein p53 in the initial biopsy, before the treatment, in 14 of the 24 samples of adjacent normal mucosa (ANM) and in 20 of the twenty-four samples of the neoplastic lesions. After radiotherapy and/or chemotherapy treatment, out of fourteen ANM samples, with positive expression of the p53, 6 maintained the overexpressed p53 and 2 negatives. In the 20 neoplasm samples with increased p53 expression, they remained positive for seven patients, two of the cases had a negative expression of the p53. Eleven patients could not be evaluated for having died before the second immunohistochemical evaluation.

From the patients who died, one had staging T1, two had staging T2 (18.2%), six (54.6%) staging T3 and 2 staging T4 (18.2%) ($p < 0.05$). As regards to the histological graduation of these patients, we noticed that five cases were of well-differentiated, five were moderately differentiated, and one slightly differentiated of SCC. There was a statistically significant difference for the p53 overexpression relationship, both in the tumor and the normal adjacent mucosa, with the tumor staging, but not with any degree of the neoplasm differentiation (Tables 2 and 3). An important observation is that five patients with overexpression of the p53 were aged below 50 years old. The p53 positive expression association with

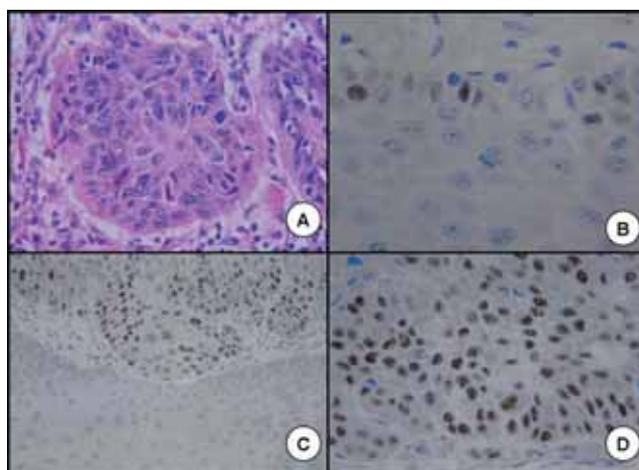


Figure 1. A. Spinocellular carcinoma moderately differentiated of pharynx. B. Spinocellular carcinoma well-differentiated of mouth. C. Positive expression of the p53 in oral spinocellular carcinoma. D. Negative expression of the p53 in oral spinocellular carcinoma.

Table 2. Staging of the patients and p53 expression in the neoplasm before and after radiotherapy treatment.

Permanence (TNM)	p53 Expression			
	Before radiotherapy		After radiotherapy	
	N	P	N	P
T1	1	3	2	0
T2	0	6	0	2
T3	1	6	0	3
T4	2	5	0	2
Total	4	20	2	7

Subtitle: N: negative; P: Positive *After one-year clinical follow up only 13 patients remained alive.

Table 3. p53 expression in the neoplasm and in the normal adjacent mucosa on the neoplasm.

Histological graduation	p53 Expression Neoplasm	
	N	P
BD	2	12
MD	1	7
PD	1	1
Total	4	20

Subtitle: N: negative; P: positive; BD: well-differentiated; MD: moderately differentiated; PD: slightly differentiated.

exogenous factors was statistically evident for smoking ($p = 0.005$) and not significant for the use of alcohol ($p = 0.22$).

DISCUSSION

The data of our work confirmed the literature, that is, the incidence of oral and pharyngeal spinocellular carcinoma is predominant in men, older than the fifth decade of life and having smoking and alcoholism as their risk factors (1, 2). These works report that smoking separately increases the risk of cancer development in six times and alcohol in 3.5 times. When associated, such risk indicators can increase in up to 15 times the possibility to develop oral and pharyngeal cancer, and the induced malignant transformation potential by these agents is durable and the risk is dose-dependent (7, 11). In our study, such observations were not different; 90% of the patients with oral cancer were smokers and 75 to 80% were alcoholic, taking into account the individuals that reported having quit alcohol and smoking a few weeks before definitive diagnosis of cancer.

However, the observation of oral and pharyngeal spinocellular carcinoma in five patients younger than 50 years, with aggressive neoplastic clinical characteristics, shows us the establishment of the pathology of cancer in these individuals could be associated to other risk factors, such as the genetic inherited characteristics and not only induced cellular mutation by exogenous carcinogenic agents (3, 4, 5, 6). In these cases, the ideal for diagnosis and life prognosis is that we should more and more use methods that compute not only histological findings, like types and degrees of the neoplasms cellular differentiation, but also the molecular characteristics and genetic susceptibility of the oral and pharyngeal carcinoma neoplastic cells (15, 24, 25, 26). In the last two decades, the descriptions of TP53, K167 genes, amongst others, with cellular growth control functions presented new perspectives as regards to the treatment and prognosis of this type of cancer (30).

These new concepts are far interesting for us to understand whether the normal and/or dysplastic cells progression for cancerization, especially in young individuals, occur by means of carcinogenic genetic mutations alterations that could already be present and not yet visible through conventional histology (27, 28, 29). This concept that the cancer genetic theory is very timely today was what led us to research genetic changes not only in the tumor but also in the normal adjacent mucosa on the neoplasm (12). In our findings, the p53 increased expressions occurred both in the area of the tumor and in the normal mucosa. For REGESI et al. (1995) (19) and AGUIAR and ARAÚJO (1997) (20) such changes could have a value of prediction as regards to the appearing of a neoplasm or of resistance upon radiotherapy and chemotherapy. Despite not conclusive, our data seems to confirm such information because most patients who had overexpression of the p53 had large

tumors and reacted negatively to the radiotherapy. In these individuals, the persistence of overexpression of the p53, both in the tumor and in the normal mucosa could have an indication of a higher resistance to radiotherapy treatment, and consequently affect the survival of patients with oral and pharyngeal cancer (30, 31).

However, such observations are not conclusive. The clinical pathological correlation we described between the size of the tumor and the p53 overexpression indicates that possible genetic and molecular changes, between them, of the p53, when detected in individuals with oral and pharyngeal carcinomas can be a bad prognosis factor, especially when in the case of the p53 expression and of other proteins in areas of the normal mucosa adjacent to the tumor (2, 20, 26). Such findings would show a possible multicentric origin of the oral and pharyngeal cancer and the examiner must make biopsies and exams in other sites close to the lesion and not only on the neoplasm; molecular lesions could be present in the epithelium adjacent to the tumor but not visible in optical microscopy (12). This meets the field cancerization theory (12), and explains the appearing of a second primary tumor and of many neoplastic recurrences (9, 10, 11). Finally, the exact molecular characteristic of the genetic susceptibility of this changed tissue field is not clear yet, but tumor suppressing genes, like Tp53 may be the key factor in the initial stages of the cancer, by opening several possibilities to predict short time better results for treatment and life prognosis.

CONCLUSION

The findings of this study show the p53 expression was increased in 20 of the 24 biopsies in the area of the neoplasm and in 14 biopsies of the normal mucosa adjacent to the tumor before beginning of the radiotherapy, and biopsy was maintained after the end of radiotherapy treatment. There was a statistically significant correlation between the increase of the p53 with the size of the neoplasm and smoking, but not with the degree of cellular differentiation.

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