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Expression of p53 and bcl2 in squamous cell carcinoma of head and neck

Kanika Taneja[1], Sumiti Gupta[1], Ashok Chauhan[2], Rajnish Kalra[1], Aditi Arora[3], Sohrab Arora[4], Rajeev Sen[1]

ABSTRACT

Background: Head and neck squamous cell carcinoma (HNSCC) is the sixth-most common malignancy worldwide. Despite advances in radiotherapy and surgical treatment, survival rates have not changed significantly in the last 40 years. Molecular markers are currently being identified that can determine prognosis preoperatively by routine tumor biopsy, leading to improved management of HNSCC patients.

Aim: The aim of the present study was to demonstrate the expression of p53 and bcl2 proteins in squamous cell carcinoma of head and neck (HNSCC) and to correlate the expression of p53 and bcl2 with clinical staging (AJCC) and WHO histological grading of SCC.

Materials and methods: The study population comprised 50 cases of HNSCC. Tissue sections from these cases were subjected to hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining using p53 and bcl2, and a comparative analysis of the results was performed. Cases of colon carcinoma and benign lymphoid hyperplasia were used as positive controls for p53 and bcl2, respectively.

Results: Positivity for p53 was recorded in 30 cases (30/50), while positivity for bcl2 was recorded in 44 cases (44/50). Positivity for p53+/bcl2+ coexpression was seen in 28 cases (28/50). The frequency of p53 expression was associated with tumor histologic grade (p=0.02), increasing lymph node involvement (p=0.01), and clinical stage (p=0.038). The frequency of bcl2 expression was associated with histological grade (p=0.02) and increasing lymph node involvement (p=0.028), but not with clinical stage (p=0.242). Moreover, the combined p53+/bcl2+ expression was significantly associated with histological grade (p=0.02) and lymph node involvement (p=0.01).

Conclusion: Study of p53 and bcl2 expression may provide clinicians with more exact information in order to evaluate tumor aggressiveness and survival rates.

Key words: *Immunohistochemistry, p53, bcl2, head and neck squamous cell carcinoma*

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth-most common type of malignancy worldwide, and represents over 6% of the global cancer burden. HNSCC accounts for nearly 650,000 new cases of cancer worldwide, and over 35,000 deaths each year. Ninety percent of cancers in the head and neck area are due to squamous cell cancers arising from mucosal epithelium. The remainder includes adenocarcinomas (of salivary gland origin), mucoepidermoid carcinomas, melanomas, malignant mixed tumors, various carcinomas, and other varieties [1].

Author affiliations : *[1]Departmet of Pathology, [2]Department of Radiotherapy, PGIMS, Rohtak, India [3]Department of Pathology, Guru Teg Bahadur Hospital, UCMS, Delhi, India [4]Department of Urology, SGPGIMS, Lucknow, India*

Correspondence : *Kanika Taneja, Departmet of Pathology, PGIMS, Rohtak, India. e-mail: drkanikataneja09@gmail.com*

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Head and neck cancers are amongst the commonest cancers in India, accounting for 21% of all malignancies, with an annual incidence of around 1.8 lakh cases (Male:female ratio: 5:1), contributing to 7% of the annual worldwide incidence of 8 lakhs [2]. In Haryana, the northern state of India, these cancers accounted for 33% of all cancers, in a study conducted in the Department of Radiotherapy, Pt. B.D. Sharma Post Graduate Institute of Medical Sciences, Rohtak [3].

HNSCC is a heterogeneous group of cancers, with a variable, but usually poor, prognosis in patients. Despite advances in treatment, the mortality rate of HNSCC has not changed markedly over the last few decades [4]. Once dissemination to regional lymph nodes occurs, the probability of 5-year survival, regardless of the treatment rendered, is reduced to nearly half of that seen in early-stage patients. Therefore, the single most important prognostic factor in the treatment of patients with SCC of the head and neck is the status of cervical lymph nodes [5].

Since the mortality rates have not changed significantly in the last 40 years despite advances in radiotherapy and surgical treatment, molecular markers are currently being identified that can determine prognosis preoperatively by routine tumor biopsy, leading to improved management of HNSCC patients [6].

In recent years, there has been increasing interest in the role of apoptosis in tumorigenesis. Programmed cell death in eukaryotic cells, also called apoptosis, is an active process that depends on the expression of a specific set of genes. Among them, wild type p53 can induce apoptosis, and bcl-2 and mutant p53 can inhibit apoptosis [7].

A number of studies have been performed to examine the correlation between the anomalous accumulation of these proteins and prognosis of HNSCC, but conflicting results have been obtained. Possible explanations for the discordance between these studies may be due to a failure of immunohistochemical techniques to detect actual mutations. Nevertheless, p53 probably plays a significant role in the pathophysiology of HNSCC [8].

The available data in the literature (though still controversial) have shown that assessment of the regulators of apoptosis may provide crucial information regarding the prognosis of HNSCC. The HNSCC may be stratified into subtypes using a panel of immunohistochemical markers that may provide not only important information regarding prognostication and therapeutic management, but may also represent an inexpensive and readily available alternative to costly molecular classification. This study was carried out to assess expression of p53 and bcl2 in head and neck squamous cell cancers and their correlation with grading and clinical staging of SCC.

This approach may help us to assess which early-stage patients should have adjuvant neck dissection and radiotherapy, and whether late-stage patients with operable lesions would benefit from resection and reconstructive surgery, or whether clinicians should adopt a conservative approach for patients with poorer prognosis, regardless of treatment [7].

Materials and Methods

This study group comprised 50 cases of head and neck squamous cell carcinoma (HNSCC) biopsies pertaining to oral cavity, oropharynx, larynx, and hypopharynx. Patients with diagnoses other than HNSCC such as adenocarcinoma, melanoma, sarcoma, metastasis, etc., were excluded.

The tissue was fixed in buffered formalin (pH=7.0) and embedded in paraffin. The tissue block was sectioned at 4-5 μm, and the sections were stained for hematoxylin and eosin and examined. One section from each representative tumor block was subjected to p53 and bcl2. Tumor grade was assigned based on the World Health Organization criteria for HNSCC [9]. Head and neck biopsies received were categorized under four categories of AJCC site distribution criteria [10]. Each patient was assigned a clinical stage in the Radiotherapy Department according to the TNM (tumor size, node, metastasis) staging system of AJCC (American Joint Committee on Cancer) 2010 [11].

Immunohistochemistry

All immunohistochemical analyses were performed by the peroxidase-antiperoxidase method. Paraffin-embedded sections were cut at 4-5 μm and dewaxed with xylene, and rehydration was performed through decreasing concentrations of alcohol and rinsing in running tap water. Antigen retrieval using citrate or Tris EDTA was performed in a pressure cooker or microwave. Sections were rinsed in phosphate buffered saline

(PBS), and excess PBS was drained off. Endogenous peroxidase activity was blocked by using peroxidase block for 20 minutes. Sections were washed with PBS for 5 minutes. Samples were incubated with protein block for 5 minutes. We optimally diluted the primary antibody (bcl2 Dako, Denmark) in concentrated form to 1 in 60 dilution and kept it for 24 hr at room temperature and for p53 (Dako, Denmark) antibody, 1 in 50 dilution was done and the solution was kept for 1 hr at room temperature. Then, the sections in both the p53 and bcl2 samples were incubated with biotinylated secondary antibody from Dako kit in 1:200 dilution for 20 min, and finally incubated with the streptavidin peroxidases from Dako kit in 1:50 dilution.

The positive control for p53 staining was a section of colon carcinoma, while for bcl2, it was a lymph node section from a patient with benign lymphoid hyperplasia [1].

Dark brown nuclear staining was taken as positive for p53, while dark brown cytoplasmic staining for bcl2 was interpreted as positive. The areas containing the largest number of positive cells were selected, and the percentage of positive cells to total tumor cells was calculated. Scoring was done using the following criteria:[12]

Score 0: <10% of cells positive.

Score 1: 10-50% of cells positive.

Score 2: >50% of cells positive.

Statistical analysis

The full data set was entered in a Microsoft Excel master sheet and analyzed using SPSSv20 (IBM SPSS statistics for windows, version 20.0. Armonk, NY: IBM Corp.) software. The results obtained were interpreted, and descriptive statistics (mean, standard deviation, range, percentages) were applied wherever appropriate. The association of the immunohistochemical expression of individual proteins was established with patients' age and sex. Tumor site, tumor size, tumor grade, lymph node metastasis, and clinical stage were determined by chi square test. A value of $p < 0.05$ was taken as significant and < 0.01 as highly significant, whereas $p > 0.05$ was interpreted as non-significant. Pearson correlation coefficient was calculated.

Correlation of p53 and bcl2 with grade and stage was calculated by Pearson's coefficient of correlation. It yielded a value of r between -1 to +1. The significance of correlation was evaluated by using the critical values table for Pearson's coefficient of correlation (any value of $r > 0.273$, irrespective of sign, was significant with a p-value ≤ 0.05).

Results

The subjects' age ranged from 31 years to 90 years, with the mean age of 59.1 ± 11.6 years. Most patients (34%) belonged to the 51-60 years age group. The mean age of the males was 60.17 ± 10.73 years, while that of the females was 57.00 ± 16.51 years.

The male to female ratio was found to be 5.2:1. The largest group of males (35.6%) belonged to the 51-60 year age group, while the largest group of the females (50%) were in the 41-50 year group, which is a decade younger than the males.

The most common site of involvement was the oropharynx (62%), followed by the larynx (20%), hypopharynx (10%), and oral cavity (8%). Out of the 50 cases of HNSCC, 37 (74%) were grade 2, 10 (20%) cases were grade 1, as shown in Figure 1, and 3 (6%) were grade 3 tumors.

The size of the tumors ranged from 3 to 15 cm as assessed by clinical or radiological methods. The majority of the cases, i.e. 26 (52%), were T3 (4-6 cm), followed by 15 (30%) cases that were T4 (> 6cm). Only 3 (6%) were T1 (< 2 cm), and 6 (12%) were in the T2 (2-4 cm) category.

According to N stage, 38% were N0 stage (negative for lymph node involvement), 22% were N1 (< 3 cm single lymph node), 28% were N2 (> 3 cm single lymph node), and only 12% belonged to the N3 stage (> 6 cm lymph node size).

According to the AJCC staging of tumors, the largest group of cases, i.e. 25 (50%), belonged to stage IV, followed by 22 (44%) cases of stage III. Stage I and stage II comprised 2 (4%) and 1 (2%) cases, respectively.

Of the 50 total cases, 30 were positive for p53, the score being 2 in 25 cases, as shown in Figure 2, and 1 in 5 cases. The remaining 20 cases were negative for p53, with the score being zero. Of the 25 cases of p53 score 2, 20 (80%) cases were of grade 2, 15 (60%) cases were of T3 tumor size, 12 (48%) cases were of N2 lymph node status, and 14 (56%) cases belonged to stage IV. Highly significant associations were seen between p53 and tumor grade (p=0.002) as well as lymph node (p=0.01),

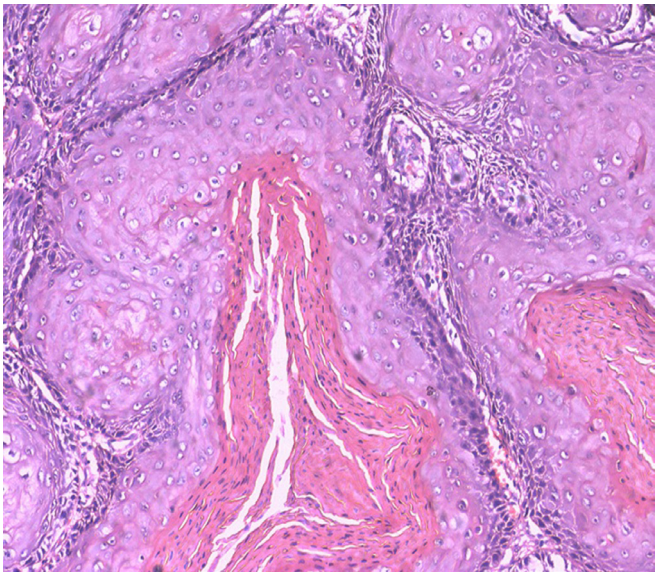

Figure 1. Photomicrograph showing well-differentiated squamous cell carcinoma of larynx (H&E; 200x).

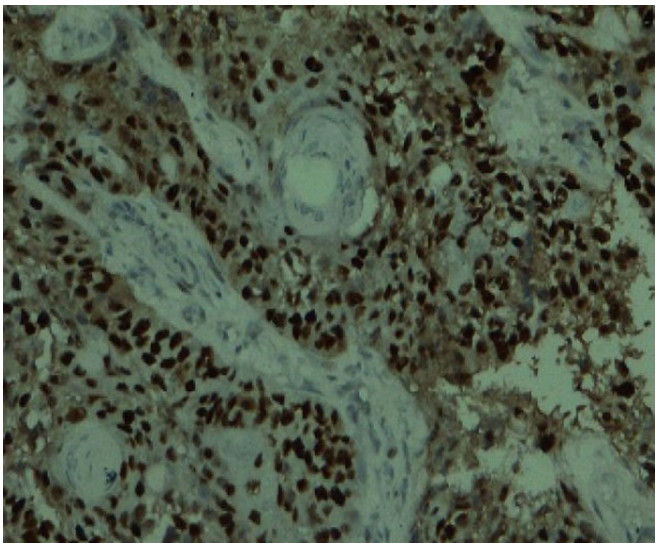

Figure 2. Photomicrograph showing p53 nuclear positivity score 2 (IHC; 200x).

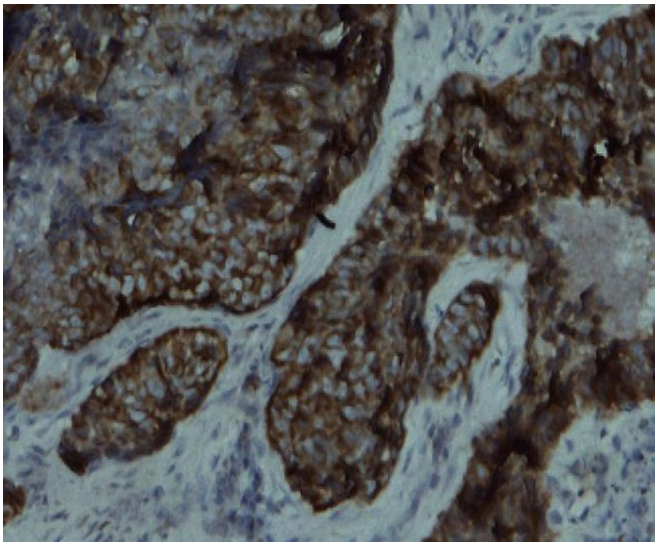

Figure 3. Photomicrograph showing bcl2 cytoplasmic positivity score 2 (IHC; 400x).

Table 1. p53 and bcl2 scoring of the cases.

IHC	No. of Cases of p53	No. of Cases of bcl2
Score 0	20	7
Score 1	5	7
Score 2	25	36
Total	50	50

while a significant association was seen with clinical stage (p=0.038), as shown in Table 2. However, no significant association was observed between p53 expression and tumor size (p=0.957).

A statistically significant direct association was observed between p53 and histologic grade, lymph node involvement, and clinical stage.

Although trends were observed between p53 expression and age, sex, site of the tumor, tobacco, alcohol, smoking, and tumor size, they were not statistically significant.

Of the 50 total cases, 43 were positive for bcl2, the score being 2 in 36 cases, and 1 in 7 cases. The remaining 7 cases were negative for bcl2, the score being zero.

Out of 36 cases of bcl2 score 2, 31 (83.7%) were of grade 2, as shown in Figure 3; 21 (58.3%) tumors were characterized by T3 tumor size, 13 (36.1%) tumors had of N2 lymph node status, and 19 (52.7%) were assessed as being stage IV. A highly significant association was seen between bcl2 expression and tumor grade (p=0.01). A significant association was seen in bcl2 expression with lymph node (p=0.028), while no significant association was observed with tumor size (p=0.595), nor with clinical stage (p=0.242) (Table 3).

A statistically significant direct association was found between bcl2, sex, alcohol consumption, histological grade, and lymph node involvement.

Similarly, trends were found between bcl2 expression and age, site of tumor, tobacco, smoking, tumor size, and clinical stage, but they were not statistically significant. Out of the 50 cases of HNSCC, 28 (56%) cases showed p53 and bcl2 coexpression (p53+/bcl2+), while in 5 cases (10%), expression of both p53 and bcl2 was negative (p53-/bcl2-). Fifteen cases (30%) showed only bcl2 positivity, while 2 (4%) showed only p53 positivity.

Out of the 28 cases showing p53+/bcl2+ coexpres-

Table 2. Correlation of p53 with various clinicopathologic parameters.

Clinicopathologic Parameters	Score 0	Score 1	Score 2	Total cases	p Value
Histologic Grade					
1	8	0	2	10	Pearson 'r' value=0.419 p=0.002
2	12	5	20	37	
3	0	0	3	3	
Total	20	5	25	50	
Tumor Size					
T1	1	0	5	6	Pearson 'r' value=0.008 p=0.957
T2	9	2	15	26	
T3	7	3	5	15	
T4	3	0	0	3	
Total	20	5	25	50	
Lymph Node Status					
N0	17	1	1	19	Pearson 'r' value=0.787 p=0.01
N1	3	2	6	11	
N2	0	2	12	14	
N3	0	0	6	6	
Total	20	5	25	50	
Stage					
I	2	0	0	2	Pearson 'r' value =0.294 p=0.038
II	1	0	0	1	
III	10	1	11	22	
IV	7	4	14	25	
Total	20	5	25	50	

sion, 24 (85.7%) tumors were of grade 2, 17 (60.7%) tumors were T3, 14 cases (50%) were N2, and 16 cases (57.1%) belonged to stage IV.

A statistically significant direct association was found between p53+/bcl2+ and both histological grade and lymph node involvement (Table 4).

However, no correlation was found between p53+/bcl2+ and age, sex, site of tumor, tobacco, alcohol, smoking, tumor size, and clinical stage.

Discussion

Head and neck cancers are characterized by genetic heterogeneity, exhibiting a wide variety of clinical presentations and of disease aggressiveness in different patients and ethnic populations. Despite aggressive and multidisciplinary treatment approaches, including preoperative or postoperative chemotherapy and/or radiotherapy with reconstructive surgery, there has been no significant improvement in 5-year survival over the past 20 years. Treatment failure still occurs in the forms of locoregional recurrence, distant metastasis, and/or second primary tumors. Currently, treatment strategies rely on clinical, radiologic, and histopathologic parameters to determine the stage of the disease. Although the grade of the tumor is not included in the staging of the tumor, it should be recorded. Grade is also a powerful predictor of distant metastasis (DM), and that fact adds important information to clinical and pathologic neck staging. It helps to identify patients at high risk of DM for whom an efficient systemic treatment is mandatory [13]. Although IHC-based assays do not provide as much biological insight into tumor biology as gene-based ones do, they are increasingly used as a surrogate for molecular gene profiling since they allow classification of tumors at affordable costs and in the absence of fresh tissue specimens [7].

p53

In the present study, it was observed that 25 cases (50%) were positive for p53 score 2, while 5 (10%) had

Table 3. Correlation of bcl2 with various clinicopathologic parameters.

Clinicopathologic Parameters	Score 0	Score 1	Score 2	Total cases	p Value
Histologic Grade					
1	6	2	2	10	Pearson 'r' value =0.623 p =0.01
2	1	5	31	37	
3	0	0	3	3	
Total	7	7	36	50	
Tumor Size					
T1	0	1	2	3	
T2	2	1	3	6	Pearson 'r' value=0.077 p=0.595
T3	4	1	21	26	
T4	1	4	10	15	
Total	7	7	36	50	
Lymph Node Status					
N0	5	2	12	19	
N1	2	3	6	11	Pearson 'r' value=0.312 p=0.028
N2	0	1	13	14	
N3	0	1	5	6	
Total	7	7	36	50	
Stage					
I	0	1	1	2	
II	1	0	0	1	Pearson 'r' value=0.169 p=0.242
III	4	2	16	22	
IV	2	4	19	25	
Total	7	7	36	50	

a score of 1, and 20 cases (40%) had score 0. While the role of p53 immunohistochemistry has been widely investigated, staining thresholds and cutoffs are not universally standardized, and different authors have used different cutoffs [12,13].

We have selected a value of 10% nuclear expression as positive since it represents a figure that is in excess of that determined for normal and is also described by Solomon et al. [12].

The positivity of p53 score 2 increased from oral cavity (0%) to hypopharynx (8%), to larynx (24%), and oropharynx (68%). No significant association was seen between p53 and tumor site (p>0.5). Similarly Gallo Oresto et al. [12], our results also showed no statistical difference in p53 and tumor site (p=0.333).

Our study showed no statistically significant association between p53 gene mutations and tobacco chewing (p=0.203), alcohol consumption (p=0.720), or smoking exposure (p=0.779). The difference from previous studies' findings may be due to geographic distribution and difference in prevalence of risk factors.

In the present study, p53 expression had a significant correlation with histologic grade of the tumor (p=0.02). While only 8% of grade I tumors were p53 score 2 positive, the corresponding values for grade II and III tumors were 80% and 12%, respectively. Our findings are in accordance with the previous studies in the literature [13, 14]. In 1996, Erber et al. [14] showed strong association of p53 overexpression with histological grading (p=0.021). Boslooper et al. [15] in 2007 also showed correlation between p53 and differentiation of the tumor.

When p53 was correlated with stage, all cases in stage I and stage II were p53-negative, while in stage III and stage IV, p53 was positive in a majority of the cases, and this association was found to be statistically significant (p=0.038). Similar to our study, Mojtahedi et al. [15] also reported that the p53 mutation genotype was

Table 4. Correlation of coexpression of p53 and bcl2 with various clinicopathologic parameter.

Clinicopathologic Parameters	Coexpression Present (n=28)	Coexpression Absent (n=22)	Total (n=50)	p Value
Histologic Grade				
1	1	9	10	Pearson 'r' value=0.486 p=0.02
2	24	13	37	
3	3	0	3	
Total	28	22	50	
Tumor Size				
T1	0	3	3	Pearson 'r' value=0.651 p=0.066
T2	4	2	6	
T3	17	9	26	
T4	7	8	15	
Total	28	22	50	
Lymph Node Status				
N0	1	18	19	Pearson 'r' value=0.802 p=0.01
N1	7	4	11	
N2	14	0	14	
N3	6	0	6	
Total	28	22	50	
Stage				
I	0	2	2	Pearson 'r' value=0.268 p=0.060
II	0	1	1	
III	12	10	22	
IV	16	9	28	
Total	28	22	50	

significantly increased in stage IV (30.8%) when compared with stage I-III (11.1%), and that this association was statistically significant (p=0.01). Also, a study by Ashraf et al. reported a significant correlation of p53 expression with tumor stage [16].

bcl2

It was observed that 36 cases (72%) were positive for bcl2 score 2, while 7 cases (14 %) were negative, and 7 (14%) had a score of 1.

The analysis of bcl2 protein expression in relation to tobacco chewing did not show any statistically significant difference (p=0.306). Conversely, we found that patients with bcl2 protein expression were heavier smokers than those with bcl2-negative cancers, although this difference is merely suggestive, rather than statistically significant (p=0.651). However, bcl2 expression was significantly associated with alcohol consumption (p=0.041) in our study.

bcl2 expression had a significant correlation with histologic grade of the tumor (p=0.02). The incidence of bcl2 score 2 positive tumors changed significantly from only 2 (5.5%) in grade 1 to 31 (86.1%) in grade 2, and 3 (8.4%) in grade 3. This finding is in concordance with studies by Wilson et al., [17], Yao et al., [18] and Solomon et al. [11]. Although 31/37 (86.1%) tumors of grade 2 had bcl2 scores of 2 in our study, the percentage dropped to only 18/182 (9.8%) in grade 2 bcl2-positive cases. In contrast, a study by Gallo Oresto et al. [12] did not show any statistically significant association (p=0.840) between bcl2 expression and histologic grade of head and neck tumors.

These differences could be caused by difference in sample size, distribution of tumor site, and different scoring criteria used by different authors.

A finding of variable bcl2 positivity with increasing T stage was also observed in our study. The positivity increased from 1 (5.5%) in T1 tumors through to 3 (8.4%), 21 (58.4%), and 10 (27.7%) in the progression

to T2, T3, and T4 respectively. Using a Pearson correlation coefficient for trend analysis, p value for increasing bcl2 positivity with increasing T stage was 0.595.

In the present study, out of 2 cases in Stage I, neither had negative bcl2 expression, while each (50%) of these cases had a positive bcl2 score of 1 and 2, respectively. All cases in stage II were bcl2-negative. The majority of cases (16 cases, or 44.4%) in stage III and 19 (52.7%) in stage IV were bcl2 score 2, but this association was not found to be statistically significant (p=0.242).

p53+/ bcl2+ Coexpression

The alteration (loss of function) of p53 possibly causes the aberrant bcl2 expression. The overexpression of bcl2 and loss of function of p53 (which is also represented by its expression) play an important role in the tumorigenesis of oral cancers by resulting in defective apoptosis and subsequent tumor progression [19].

p53 expression in the present study did not have a significant relationship with bcl2 (p value=0.412). The majority of p53-positive cases (28 cases, or 56%) were positive for bcl2 expression, while 2 (4%) of the p53-positive cases were negative for bcl2 expression. Similarly, 5 (10%) of the bcl2-negative cases were negative for p53 expression, while 15 (30%) of the bcl2-positive cases were negative for p53 expression. However, this correlation was not statistically significant.

Our results were well in accordance with various other studies [1]. Pena et al. [1] confirmed that there was no evident correlation between the presence of p53 staining and bcl2 expression.

The observed association of p53 and bcl2 expression respectively with tumor histologic grade, lymph node stage, and TNM stage revealed the possibility that the simultaneous absence or presence of bcl2 and p53 expression may more precisely represent the favorable and unfavorable biological characteristics of HNSCC. Therefore, we further correlated the combined pattern of bcl2+/p53+ (coexpression present) and three other categories categorized as coexpression absent (bcl2+/p53-, bcl2-/p53+, bcl2-/p53-) with clinical parameters, risk factors, and pathological factors.

Significant differences among IHC subtypes were observed regarding histologic grade (p value 0.02). Amongst all subtypes, the majority of grade 1 tumors 9/10 (90%) had no coexpression of p53+/bcl2+, while all grade 3 tumors (3/3, or 100%) had coexpression of p53+/bcl2+. Thus, there was a variable trend of p53+/bcl2+ coexpression with increasing grades of the tumors with 1 (3.5%) of grade 1, to 24 (85.7%) of grade 2, to 3(10.8%) of grade 3. This association was also statistically significant (p=0.02). Statistically significant association of histologic grade with various IHC subtypes is well in accordance with the study of Yao et al.,[18] while Oresto et al. failed to demonstrate a significant correlation [12].

The present study also demonstrated that were significant differences (p=0.01) in p53/bcl2 positivity with increasing N stage. The positivity rate varied from 1 case (3.5%) in node negative tumors through to 7 (25%), 14 (50%), and 6 (21.5%) in the progression to N1, N2, and N3, respectively. Lymph node status has a controversial association with p53/bcl2 expression. In our study, the comparison of bcl2 with this important prognostic variable revealed a statistically significant association, and our result complements various studies in the literature [18], but is at slight variance with the data of Oresto et al. who suggested no significant association of p53+/bcl2+ coexpression with lymph node metastasis [12].

In the present study, none of the stage 1 and stage II cancer had a positive p53+/bcl2+ coexpression. A large proportion of cases, 12 (42.8%) in stage III and 16 (58.2%) in stage IV, had p53+/bcl2+ coexpression, and this association was statistically not significant (p=0.214). This is in accordance with most of the studies that also concluded no statistical significance between p53+/bcl2+ coexpression and stage [12,18].

Conclusion

Head and neck carcinoma is a heterogeneous entity in terms of location, and biological or clinicopathologic behavior determined by numerous factors. The present study demonstrated the variable expression of p53 and bcl2 in HNSCC. In this study, we demonstrated a positive correlation of p53, bcl2, and p53+/bcl2+ with histological grade and lymph node status, which are the most important prognostic markers of HNSCC. Thus, associations of these markers with other well-established prognostic markers need to be assessed for any variable outcome.

Hence, the study of p53 and bcl2 expression may

provide clinicians with more exact information in order to evaluate tumor aggressiveness and survival rates.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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