Prognostic Significance of Bax and Bcl-2 Expressions in Adenoid Cystic Carcinoma of Major and Minor Salivary Glands of Nasal and Oral Epithelium

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Abstract: Two apoptosis-related proteins Bax and Bcl-2 in bcl-2 gene family were studied in thirty-one randomly selected patients with T1 to T4 adenoid cystic carcinoma of the major salivary glands and minor glands of oral and nasal mucosa to investigate their prognostic value. Sections from diagnostic, formalin-fixed, paraffin-embedded specimens were stained immunohistochemically to detect Bax and Bcl-2 protein. No correlation was found between Bcl-2 and Bax expression. Bax expression correlated negatively with N-classification (p=0.0132). Tumors with low Bax expression (p=0.0088) or high Bcl-2 expression (p=0.0054) correlated with a shorter disease-free period. The combined parameters Bcl-2 – Bax and Ki-67 – Bax, showed an enhanced prognostic significance (p=0.002 respectively). Our investigation revealed that Bax and Bcl-2 expression have prognostic value in ACC. The combined evaluation between these two proteins or between Ki-67 and Bax expression revealed an enhanced prognostic potential.

Keywords: Apoptosis, prognosis, treatment.

INTRODUCTION

Adenoid cystic carcinoma (ACC) is a relatively rare malignant tumor, which originate in major and minor salivary glands. The clinical behavior of this tumor is characterized by a long period of progression followed by continuing local recurrence and distant metastases over many years eventually with a fatal outcome.

Apoptosis, which involves in the deletion of cells in normal tissues as well as in malignant tumors, is a genetically predetermined mechanism that may be elicited by several molecular pathways [1-4]. Recently the proteins of bcl-2 gene family, which contain at least one Bcl-2 homology region, have been shown to play an important role in the regulation of apoptosis [5]. According to their function these proteins are divided into two groups, namely the anti- and pro-apoptotic proteins. The Bcl-2, Bcl-xL and Mcl-1 are considered as antiapoptotic proteins, whereas pro-apoptotic proteins such as Bax, Bak, Bad and Bcl-xS promote apoptosis [5-8]. The mechanisms by which these proteins regulate apoptosis is largely unknown. It has been suggested that the proteins of the bcl-2 gene family heterodimerize and homodimerize with each other and that the relative proportions of these dimers determine whether or not a cell becomes apoptotic [9, 10]. Experimental evidence indicates that proteins of the Bcl-2 family regulate apoptosis by controlling the function of mitochondria [11-14]. Pro-apoptotic proteins protect mitochondria against the mitochondrial membrane permeability, presumably by binding to and neutralizing other pro-apoptotic proteins from the Bcl-2 family, which on the contrary induce mitochondrial membrane permeabilization

Several studies have investigated the correlation between Bcl-2 expression and prognosis in ACC [15-18], but without establishing any information with regard to prognosis. The possible prognostic value of Bax expression in ACC has, to our knowledge, not been studied.

In the present study we have analyzed Bax and Bcl-2 expression in ACC in order to evaluate their possible prognostic significance. Furthermore, we tested the possible prognostic effect of a combination of these apoptotic proteins and the proliferative marker Ki-67 [19].

MATERIALS AND METHODS

The material consists of 31 randomly selected previously untreated patients with ACC admitted to the Department of Otolaryngology, Rikshospitalet, Oslo, Norway, in the period of 1983-1991. There were 15 women and 16 men ranging in age from 38 to 88 years (mean 70 years). Tumor location, TNM staging (UICC classification of 1987), treatment and follow-up has been recorded in a prospective manner. There were 17 T1-T2 and 14 T3-T4 tumors. Four patients had regional metastases at diagnosis (Table **1**). None of the patients had distant metastases at diagnosis. Four of the sinonasal tumors were locally advanced T4 and none T1 or T2.

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Table 1. Site of the Primary Tumor According to T-Classification in 31 Patients with Adenoid Cystic Carcinoma

Site	T-Classification				Total
	1	2	3	4	
Major salivary glands	4	6	1	5	16
Oral cavity glands	5	2	0	2	9
Sinonasal glands	0	0	2	4	6
Total	9	8	3	11	31

Our treatment of ACC has, through the years been combined surgery and radiotherapy. Radical removal of tumor manifestations was attempted whenever this was possible and compatible with preservation of an acceptable cosmetic appearance and function. Three of the patients, who due to medical contraindications were unfit for surgery received radiotherapy alone. The radiation dose delivered to the primary site ranged from 60 to 70 Gy in once-a-day fractions of 2 Gy, 5 days a week. The four patients with N+ status had radical neck dissection followed by radiotherapy (60 Gy) of the neck.

Since 1983 MB hold an authorization from the Norwegian Data Inspectorate to collect and evaluate data from patients admitted to our department. The local Ethical Committee and the Ministry of Health approved the study. There is no conflict of interest.

The outcome was defined as treatment failure if the primary treatment failed to eradicate the disease or if recurrence occurred locally, regionally or at distant sites (n=11). Site of reccurences and survival time according to site are summarized in Table **2**. A case was censored if death resulted from an unrelated diseases or if the patient was alive with no evidence of the original tumor at the last the follow-up consultation/contact (n=20). Complete follow-up was obtained by reviewing hospital charts, through direct contact with local hospitals, family physician or direct contact with patients or next of kin. Median follow-up for non-failures was 67(10-127) months.

Table 2. Site of Reccurences and Survival Time for 31 ACC According to Site

Primary Tumor Site	No of Patients	Site of Failure	Mean Survival Time (Months) (CI)
Major salivary glands	15	L:1, R:0, D:5	87(62-113)
Oral cavity glands	10	L:0, R:0, D:1	108(94-120)
Sinonasal glands	6	L:4, R:0, D:0	29(7-50)
Total/ overall mean	31		

Abbrevations: L=local, R=Regional, D=Distant.

Histopathology

Successive 5 μm sections were cut from formalin-fixed, paraffin-embedded pretreatment or operative specimens and mounted on gelatine coated slides. One section was stained with hematoxylin and eosin to verify the initial histopathological diagnosis, and two sections of each tumor were used for immunostaining for Bax and Bcl-2 proteins respectively.

Having verified the diagnosis of ACC, histopathological sub-classification was performed based on the relative amount of the different histological subtypes (WHO). This classification distinguishes three different tumor grades. Grade l includes the tubular and/or cribriform patterns with no elements of solid growth pattern. Grade 2 is composed of tubular and/or cribriform patterns with less than 30% of the solid growth pattern. Grade 3 has 30% or more of the solid growth pattern [20].

Immunohistochemistry

Bcl-2 and Bax immunostaining and the evaluation were performed according to methods described previously [21]. In brief, the sections were deparaffinized by 2 washes in xylene for 5 minutes each and then dehydrated in absolute ethanol. The sections were incubated in 3% (v/v) hydrogen peroxide in methanol (45 sec.) to block endogenous peroxidase, followed by incubation with 95% and 70% ethanol (15 sec. each), distilled water (1 min.), and phosphate-buffered saline (PBS) (5 min.). They were then heated in a pressure cooker for 5 minutes in 10 mM citric acid buffer (pH 6.0), followed by rinsing in lukewarm tap water. Thereafter the sections were then placed in Tris-buffered saline (TBS, pH 7.8) for 5 minutes, and blocked in TNK buffer (100 mM Tris 7.6 to 7.8, 550 mM sodium chloride, 10 mM potassium chloride) which contained 2% (w/v) bovine serum albumin (BSA), 0.1% Triton X100, and 1% normal goat serum. The rabbit anti-human Bax or Bcl-2 polyclonal antibodies (Santa Cruz Biotechnology, CA, USA; 1:20 dilution of 100 μg/ml stock made up in TNK buffer) was added and the sections were incubated overnight in a humidified chamber at 4^0 C. The sections were then washed once with PBS and incubated for 1 hour at room temperature in a humidified chamber with biotinylated goat anti-rabbit antibody (1:500) made up in TNK buffer, followed by washing with PBS, incubation for 30 minutes at room temperature with streptavidin horseradish-peroxidase (1:20) made up in TNK buffer, then in a development solution containing 0.06% diaminobenzidine (DAB) and 0.1% (v/v) hydrogen peroxide made up in TNK buffer (without goat serum, BSA, and Triton X100). The sections were finally counterstained with hematoxylin and mounted. For each batch of stained sections one positive and one negative control were included. Sections without primary antibodies were used as negative controls.

Evaluation: The sections were examined microscopically in conjunction by two of the authors (XX and OPFC) and scored according to the fraction of tumor cells stained and the staining intensity as described previously [21]. The scores for the percentages of positive tumor cells and the staining intensity were added to obtain a final score with 11 classes ranging from 0-5 (Table **3**). Muscular tissue and/or normal epithelium present in the sections served as internal controls for staining intensity and were given a score of 1. The intensity of the immunostaining was sometimes heterogeneous. Having examined the whole tumor area available in each section we classified the intensity

according to the dominant intensity [22]. Occasional disagreements regarding the classification were discussed and a consensus reached.

Table 3. Classification Used for the Evaluation of Immuno-Photochemical Staining of Bax and Bcl-2, in Addition, Calculation of Expression Scores

Percentage of Cells Stained	Score	Staining Intensity	Score
0	0	Neg.	0
1-30	1	Weak	0.5
31-70	2	Moderate	1
71-100	3	Very intense	2.0

The scores for the percentages of cells stained and the staining intensity are added resulting in 11 classes ranging from 0 to 5.

For all parameters, evaluated areas with pronounced inflammation, necrosis or artificial damage were avoided. The assessments were performed without knowledge concerning the clinical outcome.

The immunostaining and the evaluation of the Ki-67 expression has been presented previously [19]. The monoclonal antibody MIB1 (Immunotech, Marseille, France) was used and the results expressed as the percentage of positive nuclei among 1000 tumor cells in 400x fields evenly distributed through the largest part of the specimen.

Reproducibility: Six months after the initial evaluation eight sections were randomly selected and reevaluated for all parameters evaluated.

Statistics: The data were stored and analyzed by means of SAS 8 software (SAS Institute, Cary, NC, USA). The chi-square test was used for comparison of categorical parameters. Correlation between parameters was performed by the Pearson test. Kaplan-Maier plots and log-rank test were used to visualize and evaluate the significance of clinical and apoptosis-related variables in relation to treatment failure. A case was censored if death resulted from unrelated diseases or if the patient was alive with no evidence of the original tumor at the last follow-up consultation or contact. The DISCRIM analysis that computes various discriminant functions for classifying observations into groups on the basis of quantitative variables was used to screen possible combinations between two variables in relation to treatment failure. Prognostic significant parameters in the univariate analysis were further analyzed by means of Cox proportional hazards regression model. P-values <0.05 were considered statistical significance. Reproducibility was tested by means of the least squares regression analysis.

RESULTS

Bcl-2 expression was observed in all tumors, whereas Bax expression was seen in 26 of the tumors (84%). The mean score for Bcl-2 expression was 3.5 (range from 1.5 – 4.5), while the mean score was 2.5 (range from 0 – 4.5) for Bax expression.

No correlation was found between Bcl-2 and Bax expression. Bax expression correlated negatively with the N-classification (p=0.0132), but not with the T-classification. No correlation was found between Bcl-2 expression and the T- and N-classifications. Ki-67 correlated neither with Bcl-2, nor with Bax expression.

Patients having tumors with Bax expression below the mean (Fig. **1**) or Bcl-2 expression exceeding the mean (Fig. **2**) had an unfavorable prognosis (p=0.029 and p=0.024 respectively). Table **4** summarizes the univariate analyses for clinical and immunohistochemical parameters regarding treatment failures/non-failures. Sinosasal tumours had a worse prognosis in the univariate analysis but this significance disappeared in the multivariate analysis. Ki-67 expression and N-classification were also significant variables. Multivariate analysis revealed that Bcl-2 nodal status and Ki-67 expression were independent significant variables.

The DISCRIM analysis suggested a synergistic effect of Bcl-2 and Bax expression, and between Ki-67 expression and Bax expression in relation to treatment failures/non-failures. According to the distribution of the plots for combined parameters in relation to treatment failures/non-failures, we selected the mean value as cut-off levels to obtain two classes for Bcl-2 and Bax expression since the expression of these two proteins were quantified in the same way. Similarly we also selected 3-4 cut-off levels for Bax expression and Ki-67 expression, respectively, based on their distributions in order to obtain classifications for the new combined parameter Ki-67 – Bax. According to the type of prognostic association the scores for Bax expressions were subtracted from scores of Bcl-2 expression, or the score of Ki-67 expression to obtain the new combined parameters Bcl-2 – Bax, and Ki-67 – Bax respectively. Table **5** presents the log-rank analysis for individual parameter and the combined parameters in relation to treatment failures/non-failures. These results show that combined parameters were stronger prognostic indicators than single parameters. Figs. (**3**, **4**) show the co-operative effect between Bcl-2 and Bax expressions, and between Ki-67 and Bax expressions, respectively, in discriminating between treatment failures/non-failures.

The reproducibility of the apoptosis-related parameters was acceptable, with least square regression analysis resulting in the R^2-value of 0.81 for Bax expression and 0.78 for Bcl-2 expression.

DISCUSSION

The present study shows that low Bax expression correlate with poor prognosis, whereas high expression correlated with favorable clinical outcome. To our knowledge, we demonstrate for the first time a correlation between Bax expression and prognosis in ACC. This finding is in accordance with results from previous findings in head and neck cancer [21-25]. Previous studies have reported that no correlation has been found between Bcl-2 expression and prognosis in ACC [15-18]. Results from the present study have, however, shown that high Bcl-2 expression is associated with an unfavorable outcome. This finding is in agreement with the results on squamous cell carcinoma of the head and neck [22, 25-26] and contradict reports suggesting that there is no correlation The Different results

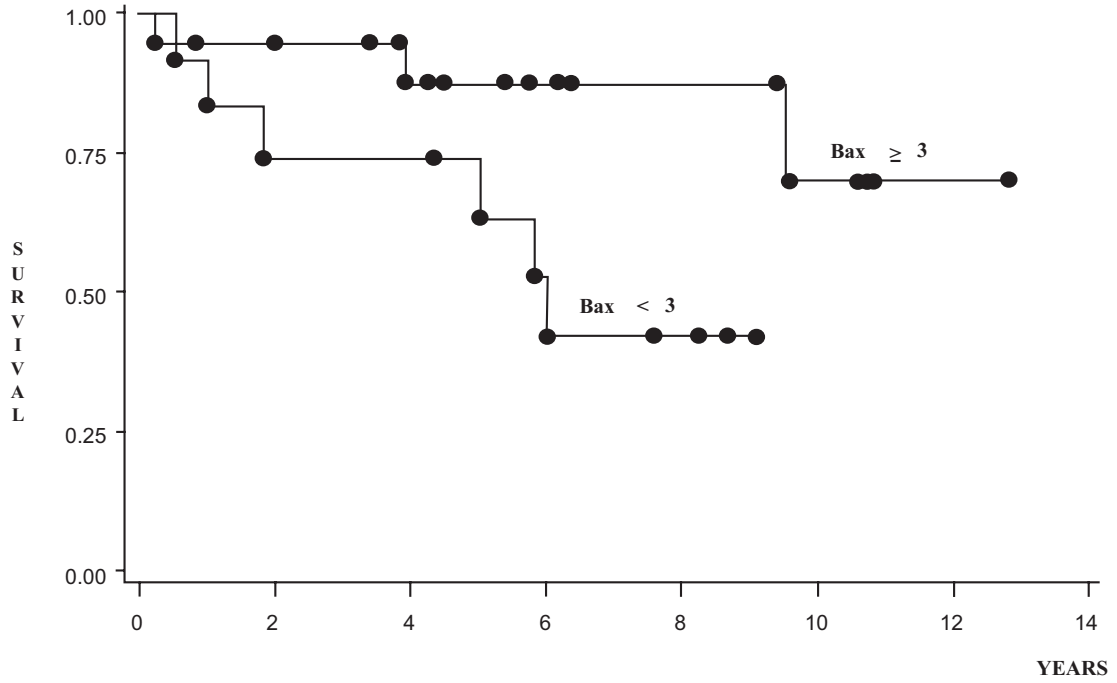

Fig. (1). Kaplan-Meier survival curves of treatment failure for Bax expression in 31 patients with adenoid cystic carcinoma (p=0.0088).

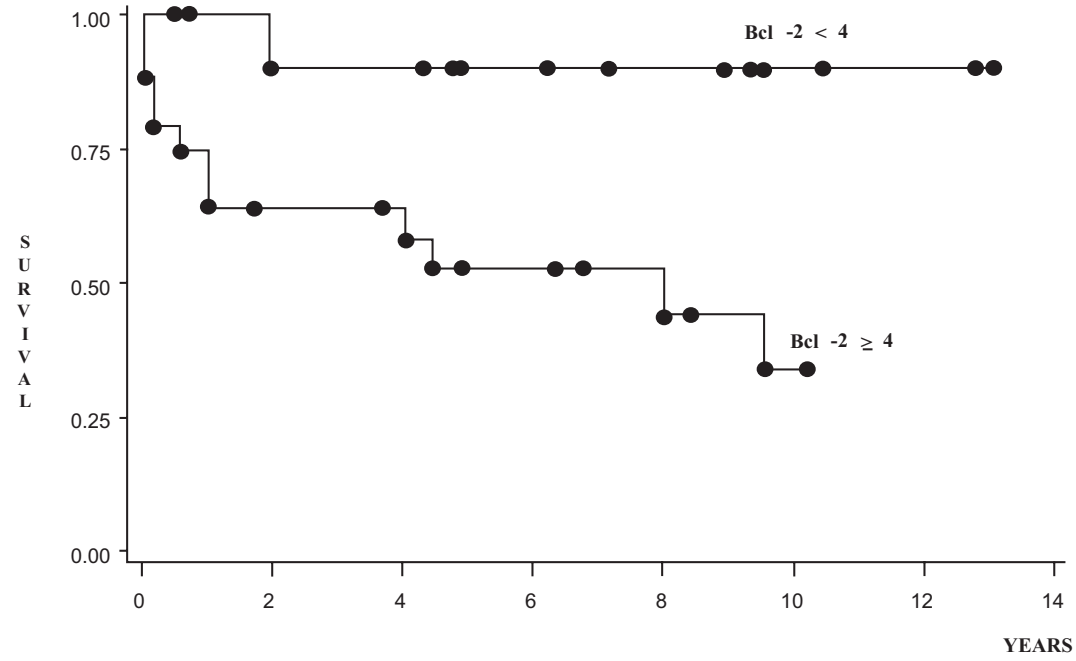

Fig. (2). Kaplan-Meier survival curves of treatment failure for Bcl-2 expression in 31 patients with adenoid cystic carcinoma (p=0.0054).

reported on the correlation between Bcl-2 expression and prognosis, may be explained by different methods regarding evaluation of Bcl-2 expression in ACC [15,18]. Our method is based on both the percentages of positive tumor cells and staining intensity. This method allows a more detailed evaluation and its reproducibility is acceptable [21, 22]. In previous studies only the frequency of positive cells were evaluated, and graded in two classes, either positive or negative grading system varied markedly from only 2 classes [17] to a percentage of total tumor cells counted [18]. Additionally, the materials of previous studies were somewhat different to our study. Carlifante *et al.* [18] studied only the palatal tumors that have a favorable prognosis, Norberg-Spaak *et al.* [15] and Jia *et al.* [17] did not include the sinonasal carcinomas.

The lack of correlation between the apoptosis related proteins and histological grade or tumor size or stage in the present study is in accordance with findings in earlier studies where sinonasal carcinomas were omitted [15, 17-18].

Table 4. Log-Rank and Cox Regression Analysis for Clinical Parameters, Histological Parameter, Proliferation- and Apoptosis-Related Parameters Ki-67, Bcl-2 and Bax Expressions in Relation to Treatment for 31 Patients with ACC

Prognostic Variables	Cut-Off Levels	Non-Failures (n=20)	Failures (n=11)	Log-Rank		Cox Method
				χ^2	p-Value	p-Value
T-class.	T1-2/T3-4	12/8	6/5	1.15	0.284	0.775
N-class.	N0/N+	19/1	8/3	4.69	0.030	0,009
Histol.-class	1/2/3	12/5/3	6/2/3	2.93	0.231	0.877
Site	Sinonasal/maj/min	2/9/9	4/6/1	7.47	0.024	0,195
KI-67 exp	<4%/≥4%	16/4	6/5	5.06	0.024	0.010
Bcl-2 exp.	<4/≥4	11/9	1/10	5.12	0.024	0.007
Bax exp.	<3/≥3	5/15	7/4	4.75	0.029	0.421

Ns: Not significant. Exp: expression.

Table 5. Log-Rank and Cox Regression Analysis for Individual Parameters for Apoptosis-Related Variables Such as Bax and Bcl-2, Proliferative Marker Ki-67, and Combined Parameter Bcl-2 – Bax, and Ki-67 – Bax in Relation to Treatment for 31 Patients with ACC

Variables	Cut-Off Levels	Non-Failures (n=20)	Failures (n=11)	Log-Rank	
				X^2	p-Value
Individual Parameters					
Bax exp.	<3/≥3	5/15	7/4	4.75	0.029
Bcl-2 exp.	<4/≥4	11/9	1/10	5.12	0.024
KI-67 exp	<4%/≥4%	16/4	6/5	5.06	0.024
Combined Parameters					
Bcl-2-Bax	≤0/>0	19/1	4/7	14.02	0.0002
Ki-67-Bax	≤0/>0	17/3	3/8	13.43	0.0002

A bewildering number of clinical (such as site of the primary tumor, T-, N- and M-classification)and histopathological factors (histological pattern/classification, resection margins, perineuralinvasion)determine the outcome of patients with ACC. The most important feature of ACC is its unique ability to generate systematic metastases which extends over a period of 20-30 years [27, 28].

In oral tongue squamous cell carcinomas, we have demonstrated that combined evaluation of two members of the bcl-2 family, such as Bcl-2 and Bax expression, Bax and Bak expression, Mcl-1 and Bad expression, has revealed stronger prognostic potential when compared with the single variables [22, 29, 30]. In the present study, such a combined evaluation between Bcl-2 and Bax expressions has shown to enhance the prognostic significance when compared with the single parameter. Although the mechanism by which apoptosis is regulated by proteins of bcl-2 gene family is still unclear, certain domains of homology between family members, termed Bcl-2 homology domains, are critical for various aspects of their activities, including the induction or suppression of cell death. Accumulated evidence has also revealed that these proteins hetero-dimerize and homodimerize with each other and that the relative proportions of these dimers appear to determine whether a cell becomes apoptotic [9, 10]. Thus, the combined evaluation of several proteins in the bcl-2 family might be more important than changes within single protein level for the understanding of the role-played by the bcl-2 family in the regulation of apoptosis.

Furthermore, combined evaluation of the expressions of proliferative marker Ki-67 and the apoptotic marker Bax has also been shown to be a consistent stronger prognostic parameter then individual variables in the present study. This is in agreement with our previous findings in glottic and oral tongue squamous cell carcinomas [21, 31]. Total tumor progression depends on both the proliferative activity and apoptosis, and many genes reflecting different biological aspects contribute to this process. Combined evaluation of several parameters might reflect more closely the tumor development and thus has stronger prognostic significance than individual parameters.

CONCLUSION

Our investigation revealed that Bax and Bcl-2 expressions have prognostic value in ACC. The combined evaluation of the expression of these two proteins, or between expressions of Ki-67 and Bax, respectively, has an enhanced prognostic potential when compared with single parameters. Because of the prolonged course of ACC, a longer observation period is needed to see if these results also cause a reduced survival.

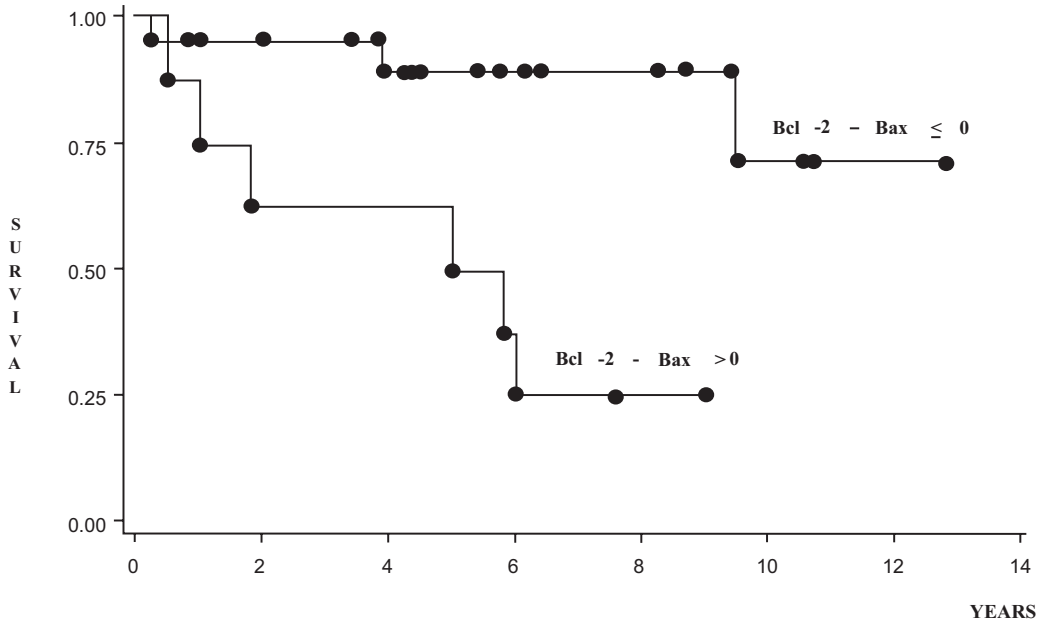

Fig. (3). Kaplan-Meier survival curves of treatment failure for Bcl-2 – Bax combination score in 31 patients with adenoid cystic carcinoma (p=0.0002).

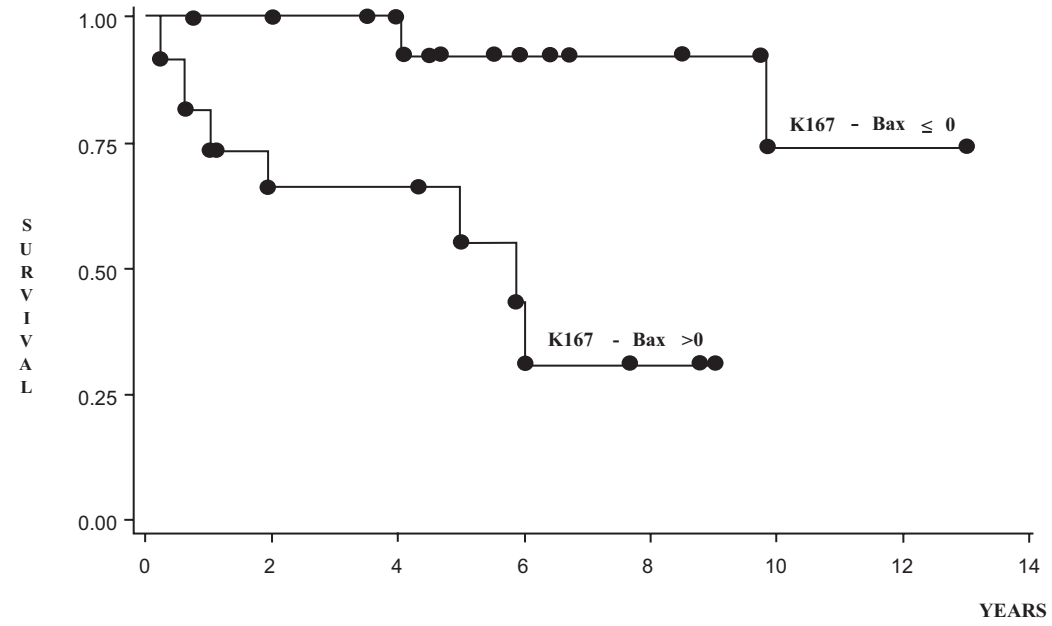

Fig. (4). Kaplan-Meier survival curves of treatment failure for Ki-67 – Bax combination score in 31 patients with adenoid cystic carcinoma (p=0.0002).

The evaluation of Bcl-2, Bax and Ki-67 should be made from preoperative biopsies when possible for treatment planning or from finally resected specimens to help in the decision regarding the need for radiation or for evaluating the radiation dose.

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