EXTENDED REPORT

Expression of apoptosis-related markers in malignant epithelial tumours of the lacrimal gland and their relation to clinical outcome

Diego Strianese, Gianluca Baldi, Stefania Staibano, Alfonso Baldi, Gaetano De Rosa, Fausto Tranfa, Giulio Bonavolontà

Br J Ophthalmol 2007;**91**:1239–1243. doi: 10.1136/bjo.2007.118661

See end of article for authors' affiliations

Correspondence to: Professor D Strianese, Dipartimento di Scienze Oftalmologiche, Università degli studi di Napoli "Federico II", Via Pansini, 5-80131 – Napoli, Italy; strianese@unina.it

Accepted 28 March 2007
Published Online First
12 April 2007

Objective: To investigate the correlation between the expression of apoptosis-related markers and prognosis in malignant epithelial tumours of the lacrimal gland.

Materials and Methods: Series of cases.

Participants: Twenty one cases with malignant epithelial tumours of the lacrimal gland. Histological diagnosis was re-examined and blocks selected were evaluated for the following parameters: incidence of apoptosis with TUNEL assay, expression of p53 and Bcl-2 using monoclonal antibody. Predictors factors for survival, local recurrence and cumulative probability of death were statistically evaluated.

Results: Re-examination of the 21 specimens was as follow: 11 adenoid cystic carcinomas, 4 mucoepidermoid carcinomas, 3 squamous cell carcinomas and 3 adenocarcinomas. Eleven of the 21 patients (53%) died during the follow-up period (4–192 months; mean 71). Bcl-2 staining >6% was significantly correlated with the death of patients. A statistically significant positive relationship for TUNEL and p53, and an inverse correlation for Bcl-2 staining, was demonstrated with overall survival.

Conclusion: The correlation with survival of apoptotic index, p53 and Bcl-2 expression suggest the more tumour cells go in apoptosis, upregulating p53 and down-regulating Bcl-2, the better the survival of patients. This study establishes a role of apoptosis-regulatory proteins in the pathogenesis of malignant epithelial lacrimal gland tumours, and supports the hypothesis that evaluation of the expression of apoptosis-related markers in these tumours may provide a prognostic tool.

Primary malignant neoplasms of the lacrimal gland are very rare, accounting for only 2% of biopsy specimens from expanding orbital masses.^{1,2} Histologically half of these carcinomas are adenoid cystics with the remaining divided between malignant mixed tumours and other carcinomas. These neoplasms have high morbidity and mortality, and the correlation of histological type to survival is still under discussion.^{3–11} The effect of different surgical procedures, such as local resection or wide cranio-orbital resection, as well as the use of the adjuvant radiotherapy on tumour recurrence remains uncertain.⁷

Since it has been proposed that defects in the apoptotic pathway may represent a critical element in the progression of neoplastic disease and may also determine treatment efficacy at the cellular level,^{12–14} we have studied the role of apoptosis in the progression of malignant tumours of the lacrimal glands in order to improve the knowledge of the prognostic factors of these tumours. Apoptosis is a form of cell death, which is regulated at the gene level and plays a central role in cell number control during embryonic development and organogenesis.^{15,16} Through TUNEL technique, apoptosis can be detected at single-cell level in formalin-fixed, paraffin-embedded tissue sections, thereby allowing the study of retrospective cases.¹⁷

In the present study, we analysed by TUNEL assay the incidence of apoptosis in a group of 21 cases of malignant epithelial tumour of the lacrimal glands and examined the expression of two apoptosis regulators—p53 and Bcl-2. We correlated these results with the pathological features of the neoplasms and the clinical outcome.

As these tumours are rare, very little information about the molecular changes leading to their development and progression has so far been reported.

MATERIALS AND METHODS

Patients

Tissues from 21 malignant epithelial tumours of the lacrimal glands were used for this study. All patients were diagnosed and treated at the "Federico II" University of Naples. Survival data were collected from hospital charts and from periodic interviews with patients and their relatives. The pertinent clinical and histopathological data of all the patients are listed in table 1.

Histology

The formalin-fixed, paraffin-embedded samples were sectioned at 5 µm and stained with H&E. The histological diagnosis was re-examined. In addition, the most representative blocks were selected to be cut into new 5 µm-thick sections for immunohistochemical studies.

Immunohistochemistry

Immunohistochemical staining was carried out using the standard avidin-biotin-peroxidase complex technique and the LV Dako LSAB kit (DAKO A/S, Carpinteria, CA, USA) after antigen retrieval by pressure cooking. After quenching in 3% hydrogen peroxide and blocking, the sections were incubated with primary antibodies overnight at 4°C, using the following dilutions: p53 monoclonal antibody (clone n° DO-7, DAKO) at 1:150 and Bcl-2 monoclonal antibody (clone n°124, DAXO) at 1:250. Biotinylated secondary antibody and streptavidin conjugated to horseradish peroxidase were subsequently applied with colour development with 3,3'-diaminobenzidine and haematoxylin counterstaining (figs 1 and 2). Negative control slides in the absence of primary antibody were included for each staining.

Table 1 Clinical and histopathological characteristics of patients

Patient	Age	Histological type	Therapy	Outcome
1	55	Mucoepidermoid	Surgery	Dead
2	44	Adenoid cystic carcinoma	Surgery/ radiotherapy	Dead
3	68	Adenocarcinoma	Surgery	Dead
4	45	Adenoid cystic carcinoma	Surgery/ radiotherapy	Dead
5	41	Squamous cell carcinoma	Surgery/ radiotherapy	Alive
6	59	Adenoid cystic carcinoma	Surgery/ radiotherapy	Dead
7	55	Adenoid cystic carcinoma	Surgery/ radiotherapy	Dead
8	52	Adenoid cystic carcinoma	Surgery/ radiotherapy	Alive
9	60	Mucoepidermoid	Surgery/ radiotherapy	Alive
10	13	Adenoid cystic carcinoma	Surgery	Alive
11	55	Squamous cell carcinoma	Surgery/ radiotherapy	Dead
12	25	Adenoid cystic carcinoma	Surgery/ radiotherapy	Alive
13	59	Adenocarcinoma	Surgery	Dead
14	55	Adenoid cystic carcinoma	Surgery	Alive
15	52	Adenoid cystic carcinoma	Surgery/ radiotherapy	Dead
16	66	Mucoepidermoid	Surgery/ radiotherapy	Alive
17	81	Adenoid cystic carcinoma	Surgery	Alive
18	53	Squamous cell carcinoma	Surgery	Dead
19	60	Adenocarcinoma	Surgery/ radiotherapy	Alive
20	44	Adenoid cystic carcinoma	Surgery/ radiotherapy	Dead
21	45	Mucoepidermoid	Surgery	Alive

TUNEL assays

TUNEL assay was carried out essentially as described previously.¹⁸ After removal of paraffin with xylene and rehydration in ethanol solutions of decreasing concentrations, sections were digested for 10 min with proteinase K (20 mg/ml), washed in distilled water, and exposed briefly to 3% hydrogen peroxide to inactivate endogenous peroxidase. The TUNEL reaction was performed using the peroxidase-based Apoptag kit (Oncor, Gaithersburg, MD, USA). TUNEL positive cells were detected with diaminobenzidine and H₂O₂ according to the supplier's instructions. Finally, stained sections were lightly counterstained with haematoxylin. Cells were defined as apoptotic if they were TUNEL positive (figs 3 and 4)

Figure 1 Expression of Bcl-2 in a case adenoid cystic carcinoma. **Figure 2** Expression of p53 in mucoepidermoid carcinoma.

Scoring and quantification of the immunoreactivity

Two observers (AB and GB) estimated the staining pattern of TUNEL, p53, and Bcl-2 separately, and scored each specimen for the percentage of positive cells ($\leq 6\%$ and $>6\%$ of cells TUNEL positive or expressing Bcl-2 and $\leq 10\%$ and $>10\%$ of cells expressing p53 or Bcl-2). The level of concordance, expressed as the percentage of agreement between the observers, was 90% (19 out of 21 specimens). In the remaining specimens, the score was obtained after collegial revision and agreement. Analysis of the data using such necessarily arbitrary cut-off was highly statistically significant and, therefore, functionally operative.

Statistical analyses

Spearman's rank correlation or Fisher's exact test were used to assess relation between ordinal data (correlation matrix between immunostaining indicators). The immunostaining variables were stratified as follow: TUNEL: $<$ or $>6\%$; Bcl-2: $<$ or $>6\%$; p53: $<$ or $>10\%$. Predictors for survival or local recurrence were evaluated either by an univariate model or multivariate analysis. The multivariate survival analysis was evaluated according to the Cox proportional hazards model. The cumulative probability of death was calculated for each group according to Kaplan–Meier analysis and compared using log rank test (figs 5, 6 and 7). A p value of <0.05 was regarded as statistically significant in two-tailed tests. SPSS software (version 9.00, SPSS, Chicago, IL, USA) was used for statistical analysis.

Figure 3 High expression of TUNEL assay in a case adenoid cystic carcinoma.

Figure 4 Low expression of TUNEL assay in a case adenoid cystic carcinoma.

RESULTS
Patients

There were 11 females and 10 males; median age was 51 years (range 14–81 years). The mean follow-up was 71 months—the shortest follow-up was 4 months (for patient 1, who had a dramatic spread of the disease and developed liver and bone metastasis), up to a maximum of 192 months. Among the group of patients alive the minimum follow-up was 24 months to a maximum of 192 months.

All patients underwent a lateral orbitotomy with complete removal of the mass and lateral orbital wall, except for one case (patient 1) who had replacement of the bone. Thirteen cases received radiotherapy of 60 Gy.

Information about survival time was available for all patients; 11 of the 21 patients (53%) died during the follow-up period.

One patient (patient 8), alive during follow-up, had a local recurrence of the disease 21 months after the first treatment and underwent surgery for a second time. Out the 11 patients who did not survive, 10 cases died from distant metastasis (lymphatic, liver, bone, lung, brain) and one case from a local

Figure 5 Cumulative probability of death for p53.

Figure 6 Cumulative probability of death for Bcl-2.

aggressive relapse of the disease. The death of these patients occurred from 4–72 months after the first treatment with a mean follow-up of 39.9 months.

Histology

We evaluated 21 specimens: 11 adenoid cystic carcinomas, 4 mucoepidermoid carcinomas, 3 squamous cell carcinomas and 3 adenocarcinomas (table 1).

Immunohistochemistry and TUNEL assays

TUNEL and p53 immunostainings were always nuclear, with a low to absent background, while Bcl-2 positivity was always cytoplasmatic (fig 1). The number of positive cells varied in different specimens. Using Spearman’s rank correlation matrix test, a significant correlation was found between all the molecular markers (table 2).

Figure 7 Cumulative probability of death for TUNEL assay.

Table 2 Spearman's rank correlation matrix (and statistical significance) for molecular markers

	TUNEL	Bcl-2	p53
Bcl-2	r=-0.901 p<0.0001	–	–
p53	r=0.714 p<0.0001	r=-0.567 p=0.007	–

In particular TUNEL index was positively correlated with p53 staining and inversely correlated with Bcl-2 staining; moreover p53 and Bcl-2 levels were inversely correlated. In multivariate analysis, according to the Cox proportional hazards model, neither histological type (TUNEL, p53 and Bcl-2 staining) or therapy type (surgery vs surgery plus radiotherapy) were predictors of local recurrence (table 3).

The same statistical analysis was used to investigate the efficacy of these parameters as predictors of survival.

As shown in table 4, in multivariate analysis only Bcl-2 staining >6% was significantly correlated with patient outcome. Finally we looked at the relation between TUNEL, p53 and Bcl-2 staining and overall survival of the patients affected by epithelial malignant tumours of the lacrimal glands. Indeed, a statistically significant positive relation was found for TUNEL and p53, while an inverse correlation was found for Bcl-2 (table 5).

All the histological types were not statistically correlated with overall survival of the patients (data not shown). To support the validity of these data, survival curves were constructed using product-limit survival analysis. The cumulative probability of death was calculated for each group according to the Kaplan-Meier product-limit method and compared using log-rank test (p<0.001).

DISCUSSION

Malignant epithelial tumours of the lacrimal gland have high morbidity and mortality. In the series reported here 53% of patients died during the follow-up period which ranged from 4 months to 192 months. Factors that influence the prognosis of these tumours are still uncertain.

Age of onset seems to be relevant. Tellado reported that young patients with adenoid cystic carcinomas have a better prognosis than adult patients.¹⁰ Wide surgical removal versus local resection, results insignificant for the prognosis. Wright has reported that the disease-free survival after treatment of lacrimal gland carcinoma appears unaltered by cranio-orbital resection.⁷ The effectiveness of adjuvant radiotherapy is also a doubtful point.⁸ Although in this study the histological types were not statistically significant predictors of local recurrence and mortality, Zimmerman³ and Forrest⁴ have demonstrated the significance of histological features for the prognosis. Recently Khalil¹¹ demonstrated that it is essential to differentiate a basal cell adenocarcinoma of the lacrimal gland from the solid basaloid type of adenoid cystic carcinoma, because the former has a better prognosis.

In the present study, we evaluated the apoptotic index and the immunohistochemical expression of p53 and Bcl-2 in a group of 21 surgically resected epithelial malignant tumours of the lacrimal gland to clarify critical events in the pathogenesis of these tumours, and to evaluate the impact of these events on patient outcomes. We choose the immunohistochemical method for our investigation. This method avoids the contamination by non-neoplastic cells that constantly affects both western blot analyses and nucleic acid-based approaches. In addition, immunohistochemistry on formalin fixed, paraffin embedded sections is a relatively easy method, which is suitable for routine evaluations.

We found that the expression of p53 was directly correlated with the apoptotic index, while expression of Bcl-2 was

Table 3 Analysis of histological, molecular and therapeutic predictors for local recurrence

	Univariate analysis		Multivariate analysis	
	Relative risk (95% CI)	p Value	Relative risk (95% CI)	p Value
Histology				
Adenoid cystic carcinoma	1	–	1	–
Adenocarcinoma	0.635 (0.365 to 1.07)	NS	0.9 (0.67 to 1.45)	NS
Mucoepidermoid/squamous cell carcinoma	2.1 (1.1 to 4.3)	0.05	1.7 (0.9 to 2.3)	NS
TUNEL >6%	0.7 (0.38 to 1.29)	NS	1.1 (0.59 to 1.96)	NS
Bcl-2 >6%	4.0 (1.23 to 11.23)	0.007	2.1 (1.1 to 13.4)	NS
p53<10%	1.2 (0.9 to 1.99)	NS	1.44 (0.44 to 1.6)	NS
Therapy (surgery + radiotherapy)	1.1 (0.87 to 1.28)	NS	0.9 (0.67 to 1.6)	NS

Table 4 Analysis of histological, molecular and therapeutic predictors of survival

	Univariate analysis		Multivariate analysis	
	Relative risk (95% CI)	p Value	Relative risk (95% CI)	p Value
Istology				
Adenoid cystic carcinoma	1	–	1	–
Adenocarcinoma	0.5 (0.25 to 1)	NS	0.4 (0.31 to 1.12)	NS
Mucoepidermoid/squamous cell carcinoma	0.333 (0.108 to 1.03)	NS	0.5 (0.56 to 0.18)	NS
TUNEL >6%	0.244 (0.69 to 0.871)	0.003	0.99 (0.56 to 1.78)	NS
Bcl-2 >6%	3.850 (1.3 to 14.378)	0.0015	2.1 (1.67 to 12.56)	0.011
p53<10%	4.950 (1.89 to 16.6)	0.001	1.87 (1.2 to 2.23)	0.05
Therapy (surgery + radiotherapy)	0.943 (0.48 to 1.85)	0.872	0.78 (0.44 to 1.89)	NS

Table 5 Survival and immunohistochemical parameters

	Median survival (95% CI)	p Value
TUNEL		
<6%	24.4 (15.5 to 30.5)	0.001
>6%	33.7 (31.56 to 35.4)	
Bcl-2		
>6%	17 (11.7 to 35.84)	0.024
<6%	33 (11.7 to 30.15)	
p53		
<10%	24.4 (15.83 to 30.3)	0.004
>10%	33.8 (30.90 to 34.95)	

inversely correlated. This expression pattern has been already described in other tumours.¹⁹⁻²¹ Although several studies have confirmed genetic point mutations as a frequent cause of p53 accumulation,²² environmental conditions may also influence protein stability or gene transcription and therefore alter the cellular content of p53 protein.²³⁻²⁴ Presumably, in our case, the correlation between apoptotic index, p53 and Bcl-2 indicates that tumour cells have lost or down-regulated their survival gene Bcl-2, and up-regulated the expression of p53, presumably to attempt damage repair. If this is unsuccessful, apoptosis would occur. The fact that in multivariate analysis only Bcl-2 staining >6% was significantly correlated with patient outcome suggests that Bcl-2 expression is an important molecular event correlated with the progression of these tumours. The correlation we found with survival of apoptotic index, p53 and Bcl-2 expression strengthens this functional hypothesis. The more tumour cells go into apoptosis, up-regulating p53 and down-regulating Bcl-2, the better the survival of the patients.

Malignant lacrimal gland tumours are histologically related to those lesions that arise in the intra-oral minor salivary glands.⁹ Similar abnormalities have been found in these tumours, suggesting possible common molecular mechanisms involved in their neoplastic proliferation.²⁵ Indeed, studies on apoptosis and apoptosis-related factors in salivary gland tumours have shown similar correlation as that found by us in lacrimal gland tumours.²⁶⁻²⁷

This study establishes a role of apoptosis-regulatory proteins in the pathogenesis of malignant epithelial lacrimal gland tumours and suggest that the expression of apoptosis and correlated proteins may provide a tool for evaluating the prognosis of these tumours. Further studies are needed to better define the involvement of these proteins and prove the clinical utility of targeting this pathway.

Authors' affiliations

D Strianese, F Tranfa, G Bonavolontà, Department of Ophthalmology – Orbital Section, University of Naples, Naples, Italy
G Baldi, U. O. of Ophthalmology, Hospital “Umberto I” Nocera Inferiore, Salerno, Italy

S Staibano, G De Rosa, Department of Biomorphological and Functional Science – Pathology Section, University of Naples, Naples, Italy
A Baldi, Department of Biochemistry – Pathology Section, Second University of Naples, Naples, Italy

Competing interests: None declared.

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