Expression of the adenocarcinoma-related antigen recognized by monoclonal antibody 44-3A6 in salivary gland neoplasias

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The monoclonal antibody 44-3A6 detects a cell-surface protein that has been shown to be a useful marker in distinguishing adenocarcinomas from other histologic tumor types in a variety of tissues. The objective of this study was to determine whether 44-3A6 could be used as a tool in the classification of salivary gland neoplasms. These complex tumors share overlapping pathologic features but distinct clinical outcomes. This study used 44-3A6 to immunohistochemically describe the pattern and frequency of this antigen in salivary gland neoplasms. Formalin-fixed, paraffin-embedded tissue sections of 22 benign and 26 malignant salivary tumors were evaluated. The patient population consisted of 25 (52.1%) women and 23 (47.9%) men selected from archival pathology files to reflect a range of salivary gland diseases. Normal surrounding salivary glands were found to have intense focal staining almost exclusively localized to ductal luminal cells. There was little staining of either myoepithelial or acinar cells. A wide spectrum of expression was found between and within tumor types, but a trend toward more expression of this antigen with decreasing differentiation was seen. A significant increase in staining was also seen in those tumors with ductal differentiation (n = 41) as opposed to those with predominantly acinar (i.e., acinic cell carcinoma) or myoepithelial (i.e., myoepithelioma; n = 8) differentiation (2.6 vs. 1.3, $p < 0.05$). No correlation was found between staining intensity and facial paralysis, pain, skin involvement, TNM stage, residual disease, or disease-free or total survival. Therefore this antigen appears to designate a duct luminal phenotype in normal and neoplastic salivary tissues. (Otolaryngol Head Neck Surg 1998;118:603-9.)

The classification of salivary gland neoplasms can be a diagnostic dilemma for several reasons. Neoplasms of salivary glands are comparatively rare disorders, with predominant histologic subtypes of salivary gland tumors accounting for only 1% of all neoplasms of the head and neck region.[1] This rare occurrence makes it unlikely that most pathologists would be involved with a sufficient number of cases to obtain extensive expertise in their classification. Second, these neoplasms exhibit one of the most complex arrays of histopathologic presentations of tumors arising in any organ within the body. Even the most common salivary gland tumor, the pleomorphic adenoma, can demonstrate histologic characteristics that closely mimic malignant salivary variants. Compounding this problem is the fact that salivary tumors with similar pathologic presentations may have dramatically different clinical outcomes. Third, the classification of salivary tumors has become increasingly complex. The 1972 World Health Organization (WHO)[2] classification schema had 5 categories of malignant and overall 11 different epithelial lesions, whereas the current WHO classification now recognizes 18 separate categories of malignant epithelial neoplasms alone.[3] A recent report speculated that the incidence of salivary gland neoplasms may be on the rise.[4] It is therefore important that investigations continue to explore ways to improve histopathologic diagnosis and to elucidate the pathogenesis of these tumors.

Electron microscopy and, more recently, immunohistochemistry have contributed significantly to our understanding of these complex neoplasms. With the use of these techniques, the bicellular (epithelial vs.

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myoepithelial) constitution of these tumors has been firmly established.[5-7] In select cases the immunophenotype of the neoplasm may be extremely useful in determining the correct diagnosis and predicting prognosis.[8,9]

Currently, work is under way to clarify the pathogenesis of these tumors, to identify factors that modulate the direction and extent of differentiation, and to predict their clinical behavior and/or response to treatment. Developments in monoclonal antibody (Mab) technology have offered an opportunity to expand our knowledge of the cellular and subcellular events that produce human disease. In accordance, increased application of Mab technology to salivary neoplasias has been seen in the literature. Antigens that have been examined include amylase, S-100 protein, actin, myosin, cytokeratin, glial fibrillary acidic protein, epithelial membrane antigen, vimentin, and others.[10-13]

One potentially significant antigen is recognized by Mab 44-3A6. It is a cell-surface protein with an apparent molecular weight of 40 kD, and which has been found to be related to glandular differentiation in pulmonary adenocarcinomas.[14-16] This tumor-related antigen has recently been cloned by our group and shown to have partial homology with human aspartyl β-hydroxylase (HAAH)[17] (unpublished data). In the breast the antigen recognized by Mab 44-3A6 is rarely expressed by normal breast ductal epithelium, but it is present in approximately 50% of ductal hyperplasias and most infiltrating ductal carcinomas.[18]

In light of these studies, we hypothesized that this antigen would be limited to select histologic subtypes of salivary gland neoplasias. We undertook this study to assess the pattern of Lab expression in these tumors and to correlate Lab expression with clinical and pathologic parameters. Furthermore, because the exact function of this antigen and its relationship to HAAH have yet to be fully elucidated, the study of this antigen in a complex exocrine group of tissues such as salivary gland neoplasms may yield insight about the biologic function of this adenocarcinoma-related marker.

METHODS AND MATERIAL

Patient Data

The protocol for this research was submitted to and accepted by Northwestern University's Institutional Review Board under exempt status. Forty-eight consecutive cases of benign and malignant salivary neoplasms were selected from the pathology files of Northwestern Memorial Hospital from the years 1972 to 1988. Criteria for exclusion from the study included lack of adequate pathology records, inadequate formalin-fixed, paraffin-embedded tissue for immunohistochemical analysis, and/or lack of available patient data. Patient charts were reviewed, and data were recorded on clinical presentation, including seventh nerve palsy, facial pain, skin involvement, TNM staging, grade, and follow-up data when available.

Mab 44-3A6

This commercially available IgG_1 Mab (Affinity BioReagents, Inc., Neshanic Station, N.J.) was produced by traditional hybridoma technology as previously described.[14,19] It has been demonstrated that this Mab is specific for a single protein by Western blot analysis.[14] The concentration of Mab used for immunohistochemical staining studies was approximately 1 μg/ml. Tissue specimens were formalin fixed and paraffin embedded, and hematoxylin and eosin–stained sections were classified according to the criteria previously described.[20] A single paraffin block was selected for each case, and 7 μm sections were cut and mounted on microscope slides. Individual sections were sequentially deparaffinized through a series of xylene, graded alcohol, and water emersion steps. Endogenous peroxidase activity was neutralized with 3% hydrogen peroxide. Slides were subsequently treated with normal horse serum in 1% bovine serum albumin/phosphate-buffered saline solution. Immunostaining was performed directly with Mab 44-3A6 and the avidin-biotin complex method (Vector Laboratories, Burlington, Calif.), as previously described.[21] The chromogen used was 3´-3 diaminobenzidine tetrahydrochloride. Slides were lightly counterstained with Harris's hematoxylin for 1 minute, dehydrated, and coverslipped. Slides were subsequently reviewed in a blinded fashion by the study pathologist (G. K. H.) and scored for both focal and overall staining intensity on a scale of 0 to 4+. Focal staining was defined as the maximum intensity of staining within a tissue. A set of slides stained with the omission of the primary antibody served as a negative control. Select slides were immunostained in duplicate to detect any variability in staining reaction.

Statistical Analysis

Statistical evaluation was performed with the SigmaStat statistical program (Jandel Scientific Software, San Raphael, Calif.). Mann-Whitney rank sums and Kruskal-Wallis analysis of variance on ranks were performed where appropriate. A p value of <0.05 was considered significant. Categories containing too few members to establish standard deviations for statistical evaluation were expressed by use of descriptive statistics. Survival was evaluated with the Kaplan-Meier method.[22] When provided, measures of variation of the mean are expressed as ± the standard deviation.

Table 1. Staining intensity by pathologic diagnosis

Pathologic diagnosis	Focal intensity	Overall intensity
Benign tumors (*n* = 22)	2.3 ± 1.7	0.9 ± 0.8
Monomorphic adenomas (*n* = 8)	1.4 ± 1.8	0.5 ± 0.8
Basal cell adenoma (*n* = 5)	2.0 ± 2.0	0.8 ± 0.8
Myoepithelioma (*n* = 3)	0.3 ± 0.6	0.0 ± 0.0
Oncocytic tumors (*n* = 5)	3.8 ± 0.4	1.4 ± 0.5
Oncocytoma (*n* = 1)	3.0 ± 0.0	2.0 ± 0.0
Oncocytic papillary cystadenoma (*n* = 1)	4.0 ± 0.0	2.0 ± 0.0
Warthin's tumor (*n* = 3)	4.0 ± 0.0	1.0 ± 0.0
Pleomorphic adenoma (*n* = 9)	2.3 ± 1.5	0.9 ± 0.9
Malignant tumors (*n* = 26)	2.5 ± 1.7	1.3 ± 1.3
Acinic cell carcinoma (*n* = 5)	1.8 ± 1.3	1.0 ± 0.7
Adenoid cystic carcinoma (*n* = 7)	1.6 ± 2.0	1.0 ± 1.7
Carcinoma ex pleomorphic adenoma (*n* = 2)	4.0 ± 0.0	1.0 ± 1.4
Salivary duct carcinoma (*n* = 3)	2.0 ± 1.7	0.3 ± 0.6
Mucoepidermoid carcinoma (*n* = 7)	3.4 ± 1.1	1.7 ± 1.1
Intermediate grade (*n* = 4)	3.3 ± 1.5	2.0 ± 1.4
High grade (*n* = 3)	3.7 ± 0.6	1.3 ± 0.6
Polymorphous low-grade adenocarcinoma (*n* = 1)	4.0 ± 0.0	4.0 ± 0.0
Squamous cell carcinoma (*n* = 1)	4.0 ± 0.0	2.0 ± 0.0

RESULTS

The patient population consisted of 48 patients—23 men, with a mean age of 58.7 years (range, 36 to 78 years), and 25 women, with a mean age of 60.1 years (range, 30 to 85 years). The sites of primary tumors were as follows: 40 (83.3%) parotid, 5 (10.4%) submandibular, 2 (4.2%) minor salivary gland, and 1 (2.1%) sublingual gland. The cases comprised 13 different diagnoses (Table 1). The panel represented a spectrum of salivary gland neoplasms consisting of 9 pleomorphic adenomas; 7 mucoepidermoid carcinomas; 7 adenoid cystic carcinomas; 5 acinic cell carcinomas; 5 basal cell adenomas; 3 each of salivary duct carcinomas, myoepitheliomas, and Warthin's tumors; 2 carcinoma ex pleomorphic adenomas; and 1 each of oncocytomas, oncocytic papillary cystadenomas, polymorphous low-grade adenocarcinomas, and squamous cell carcinomas.

The staining intensity for these cases showed a broad range of staining between and within pathologic categories. Examples of staining for normal and pathologic tissue samples are seen in Fig. 1. Slides stained with omission of the primary antibody demonstrated no immunoreactivity. There was no significant variation in the staining reaction between sets of duplicate slides. Nonneoplastic salivary gland tissue was identified on the same histologic slide as tumor in 20 cases. These showed a mean focal Lab expression of 2+, which was limited almost exclusively to the duct luminal epithelial cells and was localized to the cytoplasm with or without membranous accentuation. No nuclear staining was observed. This same pattern of cellular localization was seen among the tumors.

The mean focal and overall staining intensity for each neoplastic category is summarized in Table 1. There was no difference in focal staining intensity between benign and malignant tumors (both 2.3). Among the 22 benign cases, those tumors showing oncocytic differentiation demonstrated the most expression of Lab, with a mean focal staining intensity of 3.8 out of 4. Both pleomorphic adenomas and basal cell adenomas demonstrated significantly higher levels of immunostaining (2.3 and 2.0 mean focal staining, respectively) than did the myoepitheliomas (0.3 mean focal Lab expression).

Among malignant tumors, polymorphous low-grade adenocarcinomas and squamous cell carcinoma demonstrated strong (4+) focal expression. Mucoepider-moid carcinomas also demonstrated high levels of immunoreactivity (3.4 mean focal intensity). Much lower staining was noted within the acinic cell and adenoid cystic carcinomas (1.8 and 1.6 mean focal staining intensity, respectively).

Immunostaining was most intense in foci showing differentiation toward luminal ductal epithelium, particularly in cells showing oncocytic features. Staining was minimal in tumors with acinar differentiation (acinic cell carcinomas). With rare exceptions, myoepithelial cells expressed low levels of immunostaining. These findings are illustrated in Fig. 1. There was a statistically significant difference in expression between tumors showing predominantly ductal-type differentiation (n = 40; mean staining of 2.6) compared with tumors with predominantly acinar or myoepithelial differentiation (n = 8; mean staining 1.3, $p < 0.05$).

Otolaryngology–
Head and Neck Surgery
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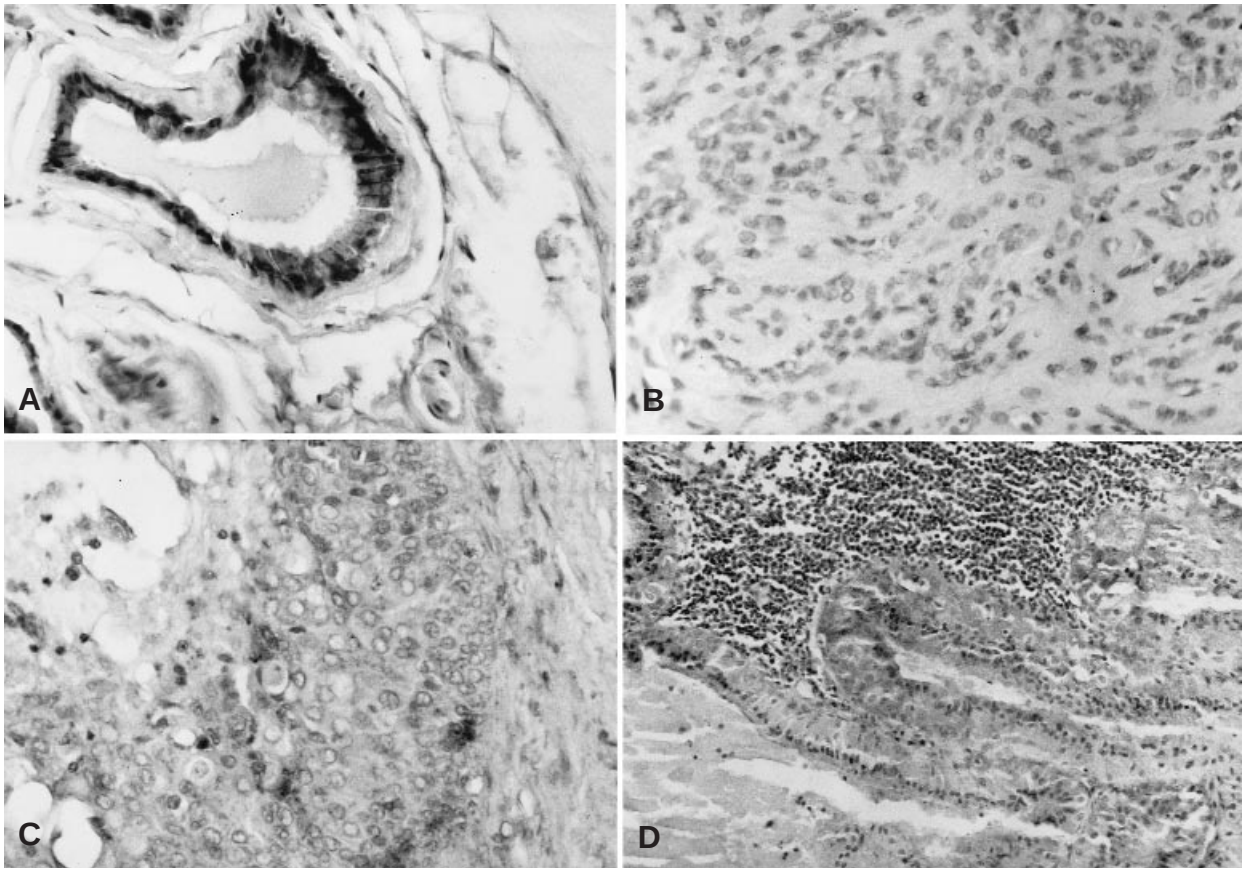

Fig. 1. Typical staining of normal salivary gland and neoplastic tissue. **A,** Normal glandular tissue demonstrates staining of the luminal ductal cells with little or no staining of the myoepithelial or acinar cells. **B,** Myoepithelioma does not stain for Lab expression. **C,** High-grade mucoepidermoid carcinoma with intense focal staining for Lab. Note absence of keratin pearls or dyskeratosis. **D,** Warthin's tumor with intense focal staining of epithelium. Note lack of staining for Lab in lymphoid stroma. (**A-D**, Original magnification ×40.)

We found no statistical difference in the expression of this adenocarcinoma marker and patient outcome based on the following criteria: presence or absence of seventh nerve palsy, facial pain/numbness, skin involvement, or local extension (perineural invasion, diffuse lymphatic involvement, or positive surgical margins); TNM category; disease-free survival; overall survival; or final patient status. Among malignant tumors, there was a trend toward increased expression in less differentiated tumors. The low-grade tumors demonstrated a mean focal staining intensity of 2.0, whereas the high-grade carcinomas were found to have a mean focal staining intensity of 4.0 (Table 2). A trend was also noted in examination of the Kaplan-Meier curves of disease-free survival, with less expression being found in those patients with longer disease-free survival (Fig. 2).

DISCUSSION

The antigen recognized by Mab 44-3A6 is a plasma membrane protein with an apparent molecular weight of 40 kD. It was first identified in the A549 human lung adenocarcinoma cell line (ATCC-CCL 185). It shares homology with HAAH, as assessed by gene-bank analysis. The function of the antigen recognized by Mab 44-3A6 is currently unknown because it does not have the domain credited of HAAH activity, and there are significant other differences between the known data of these two reported gene products. It is known that the expression of the antigen recognized by Mab 44-3A6 in A549 can be modulated by changes in pH (manuscript in preparation, 1996) and intracellular Ca^{2+} concentrations.[23]

The antigen recognized by Mab 44-3A6 has been shown to be a useful marker for pulmonary adenocarci-

nomas, where it is found to be more abundantly expressed in well-differentiated tumors.[15] Immunostaining for this antigen has also been reported to parallel the degree of differentiation in adenocarcinomas arising in the breast.[18] In both normal lung and breast tissue, comparatively little immunoreactivity is expressed. This is in contrast to normal salivary gland tissue, in which a significant amount was noted in normal tissue. We hypothesized that normal salivary tissue would show minimal immunoexpression and that it would be restricted largely to those tumors demonstrating a glandular phenotype. We found comparatively more immunoexpression in nonneoplastic salivary than normal lung or breast tissues. Furthermore, in normal salivary gland tissue, it was localized to luminal ductal epithelium, with little localization in myoepithelial and acinar cells.

We also found that those tumors showing ductal-type differentiation demonstrated higher levels of immunostaining than tumors without ductal differentiation. Within individual tumors, expression was highest in luminal duct epithelium, particularly where it had undergone oncocytic differentiation. Paradoxically, expression of this antigen within salivary neoplasms does demonstrate a trend toward increased expression with decreasing differentiation. These results are in contrast to those seen in pulmonary and breast neoplasms,[14,18] in which its expression increased with glandular differentiation. The correlation of its expression with glandular differentiation in salivary neoplasias was not as significant as noted in lung or breast carcinomas.

We subsequently reviewed those pathologic categories having a distinct myoepithelial component. Although not exclusively, epithelioid areas within pleomorphic adenomas demonstrated a trend toward more expression than the spindle cells in myxoid areas. Furthermore, in areas with tubular differentiation, staining predominated in luminal cells. This staining characteristic did not hold for other tumor types. In the adenoid cystic carcinomas demonstrating tubular patterns, no differences in staining were noted between luminal and abluminal cell layers. In contrast to the general pattern, one case of basal cell adenoma immunostained almost exclusively in the abluminal layer. These unique staining patterns underscore the complexity of salivary neoplasms.

Our initial tumor panel was compiled on the basis of the original surgical pathologic diagnosis. Within this group we encountered six cases of epidermoid carcinomas. All six of these cases had positive immunostains for Mab 44-3A6, which was in stark contrast to previously reported data in pulmonary squamous carcinomas, which were uniformly negative. One (16.7%) case stained 1+, one stained 3+, and four (66.7%) stained very intensely (4+). Subsequent staining for epithelial mucins confirmed that five of these six cases represented mucoepidermoid carcinomas. The remaining mucin-negative case demonstrated keratin pearls and dyskeratotic cells, consistent with a squamous cell carcinoma. The staining of all mucoepidermoid cases revealed an average focal staining of 3.4, but the overall staining remained 1.7.

Table 2. Staining intensity by prognostic indicators

Prognostic indicator	Focal staining	Overall staining
Presenting symptoms		
No seventh nerve palsy (*n* = 36)	2.4	1.0
Seventh cranial nerve palsy (*n* = 9)	2.8	1.3
No facial pain/numbness (*n* = 37)	2.6	1.2
Facial pain/numbness (*n* = 9)	1.6	0.7
No skin involvement (*n* = 41)	2.4	1.1
Skin involvement (*n* = 4)	2.5	0.8
Tumor size		
<2 cm (*n* = 17)	1.8	0.9
2-4 cm (*n* = 16)	3.0	1.3
4-6 cm (*n* = 10)	2.3	0.7
>6 cm (*n* = 4)	3.3	1.8
Lymph node status		
Negative lymph nodes (*n* = 39)	2.3	1.1
Positive lymph nodes (*n* = 7)	3.0	1.1
Metastases		
No metastases (*n* = 41)	2.5	1.1
Distant metastases (*n* = 5)	1.8	0.4
Differentiation		
Benign disease (*n* = 22)	3.0	1.0
Low grade (*n* = 13)	2.0	1.0
High grade (*n* = 13)	4.0	1.0
Local extension		
No local extension (*n* = 30)	2.4	1.0
Local extension (*n* = 15)	2.5	1.2

Our patient group represented almost equal populations of male and female patients, with a glandular incidence reflecting the general site incidence of salivary tumors. This study also represented a broad spectrum of tumor types, all of which were found to demonstrate at least some focal expression by Mab 44-3A6. A statistically significant increase in staining was found in those tumors thought to be of luminal ductal differentiation. No correlation was found between the expression of the antigen recognized by Mab 44-3A6 and tumor type, clinically relevant presenting characteristics, TNM staging, or local extension.

In salivary gland neoplasms, a wide spectrum of staining characteristics was seen within and between pathologic classifications. Although the expression of this adenocarcinoma-associated antigen did not correlate with prognostic indicators examined, such as clinical presentation, TNM staging, local extension, or total

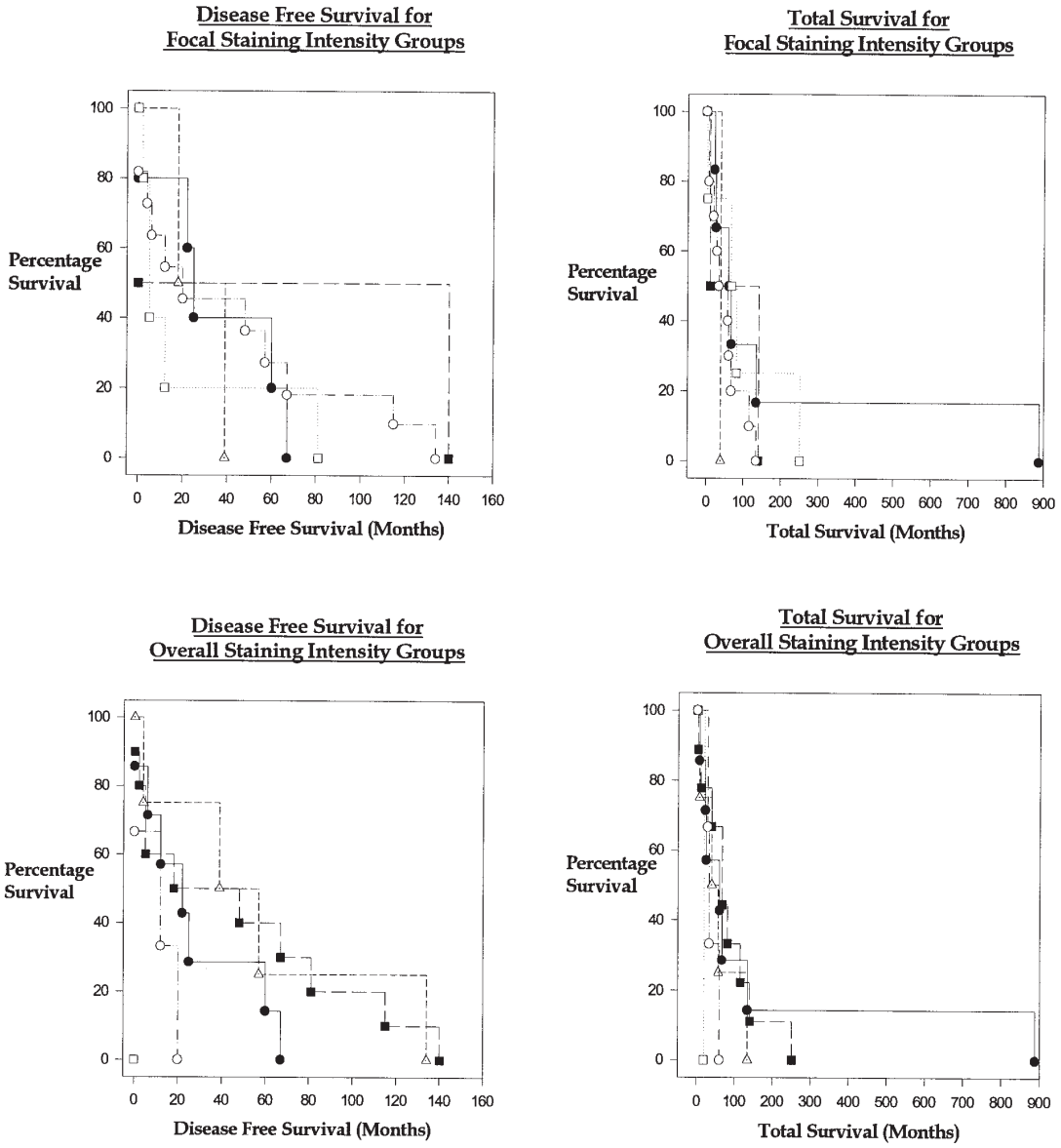

Fig. 2. Disease-free survival and overall survival for focal and overall staining intensity categories. Staining groups are as follows: 0+ *(solid circles)*, 1+ *(solid squares)*, 2+ *(dotted triangles)*, 3+ *(open squares)*, and 4+ *(open circles)*. Although no category reached statistical significance, note that in contrast to overall survival, which did not demonstrate differences between staining groups, there is a trend toward increased disease-free survival with less focal and overall staining.

Table 3. Staining intensity by patient status

Status of patient on last follow-up	Focal staining	Overall staining
Alive (n = 11)	2.2	1.3
No evidence of disease (n = 6)	2.0	1.0
Alive with disease (n = 3)	4.0	2.7
Alive, unknown disease status (n = 2)	0.0	0.0
Dead (n = 13)	2.6	1.4
Dead of unknown causes (n = 1)	4.0	3.0
Dead of other causes (n = 6)	2.7	1.2
Dead of disease (n = 6)	2.3	1.3

survival, it did correlate with luminal ductal differentiation as opposed to acinar or myoepithelial differentiation ($p < 0.05$). Furthermore, these data demonstrated a trend toward increased expression because these tumors became less differentiated and exhibited shorter disease-free survival.

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