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Cell-type dependent biomarkers expression in adenoid cystic carcinoma (ACC): biological and therapeutic implications

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Abstract

Adenoid cystic carcinoma (ACC), a rare and progressive salivary malignancy, is characterized by cellular, morphologic and clinical heterogeneity. We hypothesize that its dual cellular composition plays an important role in biomarker evaluation, tumor biological behavior and therapy response.

To investigate the differential localization and expression of c-kit and EGFR proteins, we performed immunohistochemical analysis on tissue arrays of 199 tumors and correlated the results with the clinico-pathological factors. The results show c-kit expression to be limited to the inner ductal epithelial cells and the EGFR expression mainly to the outer myoepithelial cells in the majority of tubular and cribriform patterns. In solid ACC, c-kit was uniformly positive while EGFR was consistently negative. Significant statistical correlation was found between c-Kit expression and a poor 3- year outcome, and EGFR expression with a better 3-year outcome. Our findings underscore the importance of cellular subtypes' localization of biomarkers in the clinical and therapeutic stratification of patients with this entity.

Keywords

adenoid cystic carcinoma; cellular localization; c-kit; EGFR

Introduction

Adenoid cystic carcinoma (ACC) is the second most frequent malignancy of the major and minor salivary glands and comprises approximately 15–25% of all carcinomas at these locations. 1–3 ACC is uniquely formed of dual epithelial and myoepithelial cells in the conventional tubular and cribriform patterns. Generally, these two forms pursue a protracted and progressive course, which can be attributed to the suppressive role of the myoepithelial cells 4–7. Further evidence for such function is sustained by the coincident association of the myoepithelial cell loss with the solid form transformation and aggressive behavior.

The role of myoepithelial cells in cellular polarization of the tubular and cribriform patterns and the biological behavior of ACC has been a focus of our research efforts. We hypothesize that the dual myoepithelial/epithelial structure of the majority of tubular and cribriform ACC and other salivary carcinomas with similar cellular composition underlie, at least in part, their protracted behaviour and may impact on their therapeutic management. Although several biomarkers have been studied in ACC including c-kit and EGFR expressions, the clinical relevance of the differential cellular localization and expression of these markers in ACC has not been addressed.

Studies of c-kit (CD117) protein, a member of class III receptor tyrosine kinase family, have reported overexpression in the majority of ACC 8–10. Elevated c-kit expression has also been detected in a variety of other tumors especially gastrointestinal stromal tumor (GIST), seminoma and malignant melanoma 11–16. Because c-kit is a target of the tyrosine kinase inhibitor imatinib mesylate (Gleevec™), to which significant treatment response has been achieved in chronic myelogenous leukaemia (CML) and advanced c-kit-positive GIST 17–19, an interest in its therapeutic potential in patients with ACC has been the focus of several groups. Although gain-of-function mutations in exon 9 and 11 of the c-kit and amplification were identified as the critical molecular alterations associated with its overexpression in GIST and seminoma, no such point mutation has been found in ACC 13, 19, 20.

Studies of the EGFR, a 170-kDa transmembrane glycoprotein receptor with tyrosine kinase activity in ACC, have also been conducted. EGFR, a member of importance in the signaling pathways, is implicated in proliferation, differentiation and survival of cancer cells. Overexpression of EGFR was found in a variety of human cancers, including salivary gland cancers. Numerous anti-EGFR strategies have been applied in retrospective and small prospective clinical trials, including patients with ACC 25–27.

To investigate the localization and the differential expression of c-kit and EGFR proteins and their biological significance in the assessment of adenoid cystic carcinoma, we performed immunohistochemistry analysis on 199 ACCs and correlated the results with the clinico-pathological factors.

Materials and Methods

Archival formalin-fixed paraffin blocks of 199 adenoid cystic carcinomas accessioned at the University of Texas M.D. Anderson Cancer Center between 1988 and 2006 formed this study. Donor blocks selected for the microarray construction were sectioned for H&E preparation and review. After marking spatially different regions for harvesting, two 1.0 mm diameter tissue cores were transferred to the recipient block. Pathologic patterns and the phenotypic expression of the c-kit and the EGFR were reviewed and scored independently along with traditional factors, such as gender, age, stage and clinical outcomes.

Immunohistochemistry and immunoreactivity analysis

Immunohistochemical analysis for c-kit and EGFR was performed using the BOND MAX IHC staining protocol by Vision Biosystems (Norwell, Massachusetts) on four-micrometer paraffin sections of the tissue microarray material. In brief, following dewaxing, washing and rehydration of the slides through xylene and graded alcohol concentrations, Tris-EDTA buffer was used for antigen retrieval. Slides were subsequently treated with 3% hydrogen peroxide to block endogenous peroxidase. Following incubation with the primary antibodies, c-kit (Dako, 1:100 dilution) and EGFR (Dako, 1:100 dilution), the secondary conjugate antibody was applied and followed by chromogen DAB and counterstaining with hematoxylin.

Membranous and cytoplasmic staining were scored for c-kit and EGFR expression in tumor cells. Complete lack of staining was considered negative, and staining was recorded as high (3+), intermediate (2+), low (1+). The intensity of staining compared to the normal EGFR staining in normal salivary duct control; cytoplasmic weak staining (low 1+) was combined with the negative. The expression of c-kit was recorded as high (3+) if more than 80%, intermediate (2+) if 30–80% and low (1+) if <10% were positive.

c-kit fluorescence *in situ* hybridization—Fluorescence *in situ* hybridization (FISH) was done on touch preps of fresh tissue from 27 patients. Hybridization and FISH analysis was done as described previously. To determine the deletion status, 200 individual interphase nuclei were analyzed for each case.

Statistical Analysis

Correlations within biomarkers and between biomarkers and endpoints were assessed by Pearson's Chi-square or, where there are fewer than ten subjects in any cell of a 2 × 2 grid, by the two-tailed Fisher exact test. Curves describing overall survival were generated by the Kaplan-Meier product limit method. The statistical significance of differences between the actuarial curves was tested by the log rank test. Follow-up time was the time from first appointment at the University of Texas M. D. Anderson Cancer Center for the primary tumor of concern until the date of last contact or death. Calculated P values < 0.05 were considered significant. These statistical tests were performed with the assistance of the *Statistica* (StatSoft, Inc., Tulsa, OK) and SPSS (SPSS for Windows, SPSS Inc., Chicago IL) statistical software applications.

Results

Demographic and pathologic findings

The study cohort comprised of 106 women and 93 men, who ranged in age from 15.9 to 81.9 years, with the median age 51.4 years. Tumor sites included 29 in the parotid gland, 28 in the hard palate, 26 in the maxillary sinus, 20 in the submandibular gland and 96 in various minor salivary glands sites. The AJCC staging comprised of 5 patients at stage I, 22 stage II, 7 stage III, 31 stage IV. For 134 patients staging was not available.

Histopathologic and Clinical Findings

Histopathologically, at least 2 distinctive patterns within and between cores of the same case tumor were found. A predominant pattern was determined based on the presence of more than 60% of a given pattern in tumor. Among the 190 tumors for which a predominant type could be ascertained, 57 were tubular (30.0%), 109 (57.4%) had predominantly cribriform patterns and 24 (12.6%) had the solid pattern (mostly devoid of myoepithelial cells). Overall survival plots for the three predominant patterns can be seen in Figure 3. At 5 years of presentation, the cumulative survival for the patients with tumors with predominant cribriform features was 73.6%, for tumors with tubular was 67.8%, and predominantly solid pattern was 57.2%.

EGFR and c-kit expression

Salivary gland tissue—C-kit and EGFR were highly expressed in membrane and/or cytoplasm of salivary ductal cells while moderate cytoplasmic expression was noted in epithelial cells of the intercalated and striated ducts. No immunoreactivity for c-kit and EGFR was noted in peripheral myoepithelial cells of the intercalated ducts.

ACCs—C-kit expression was limited to the inner ductal cells and was negative in the myoepithelial cells in both tubular and cribriform patterns of ACCs (Figure 1). Restricted c-kit expression to the inner ductal cells was found in 24.18% (22/91) of the cribriform, 22.22% (10/45) of the tubular patterns and 71.43% (15/21) of the solid form. Paradoxically, EGFR expression was mainly noted in myoepithelial cells and was negative or weakly cytoplasmic positive in ductal cells of both the tubular and cribriform patterns. EGFR immunoreactivity was restricted to the myoepithelial cells in 4.44% (2/45) of the tubular and 10.99% (10/91) of the cribriform. EGFR staining was negative in the solid type (Table 1).

Concurrent c-kit and EGFR immunoreactivity in ductal and myoepithelial cells respectively was found in 62.22% (28/45) of tubular, 61.54% (56/91) of cribriform, and 4.76% (1/21) of solid type. Negative immunoreactivity to both c-Kit and EGFR was found in 11.11% (5/45) of the tubular, 3.30% (3/91) of the cribriform and 23.81% (5/21) of the solid forms of ACC (Table 1). Not all the data points were available for every sample. Only the samples for which the type was known and both markers in both types of cells scored were included in this tabulation (this accounted for 157 samples).

C-kit amplification—Fluorescence in situ hybridization for c-kit specific probe performed on touch preps from 27 tumors (16 cribriform, 6 tubular and 5 solid patterns) revealed gains in 13 patients (10 cribriform, 2 tubular and one solid), amplification in one case (cribriform) and normal signal in 13 patients (6 cribriform, 4 solid and one tubular). All specimens had variable immunoreactivity for c-kit in the epithelial cells (Figure 2).

Clinicopathologic Correlations

The cumulative proportional survival analysis of patients at 3 years from presentation was 82.8%, 71.7% at 5 years, and 50.9% at 10 years. At last contact, 27 (14%) of the patients were alive with cancer, 123 (62%) patients had no evidence of disease and 49 (25%) patients were lost to follow-up. Distant metastases were detected in 74 patients (37%). The median follow-up time was 62.1 months (range, 1.3–282.7 months).

Concomitant EGFR and c-kit positivity in ACC patterns with myoepithelial cells correlated with a better outcome with 3 years survival and 5 years survival of 85.4% and 75.1% respectively, regardless of histological type ($p=0.048$ by log rank test). Ductal c-Kit positivity was associated with 3 and 5 years survival in 68.9% and 58.5% respectively, regardless of histological type ($p=0.048$ by log rank test) (Table 2 and figure 4).

Discussion

Our study, the first to address differential cellular localization of biomarkers in ACC, shows c-kit expression to be limited only to inner epithelial cells. The EGFR expression, however, was mainly detected in myoepithelial cells, in both tubular and cribriform phenotypes, it was also found in inner ductal cells in some tumours. In the solid form where myoepithelial is lost, uniform positivity for c-Kit and lack of EGFR staining were found. These findings lend credence to the hypothesis that the histogenetic differences between myoepithelial and epithelial cells may underlie the biological heterogeneity and indirectly influence therapy response of patients with these tumors.

The overall incidence of c-kit expression in our study is in agreement with those reported in the majority of previous studies of ACC. In only one of these studies, was the localization of c-kit to ductal cells and the diffuse expression in the solid pattern of ACC reported. Although the underlying mechanism for the epithelial cell expression of c-kit in ACC still remains to be incicated. In this study, c-kit expression in the epithelial cells of ACC was

associated with a poor 3-year outcome of patients and was independent of the histological patterns of these tumors.

Studies of c-kit in GIST and seminoma, tumors with uniform cellular composition, have demonstrated that gain-of-function mutations of the c-kit locus resulting in an overexpression of the protein 13-24. C-kit overexpression in the absence of activating point mutation has also been described in various other tumor types including neuroblastoma, uterine sarcoma, and thymic carcinoma 30-31. These findings indicate that other genetic, epigenetic, biological events, and gene copy number, as in glioblastoma and small-cell lung cancers, may be involved in c-kit regulation. We have also reported amplification of 4q11-12 and increased copy number of this gene in some cases of ACC.

Although strong EGFR expression in the myoepithelial cells of the tubular and the cribriform patterns was noted, the ductal cells also expressed this marker in some tumors. The incidence of EGFR expression in studies of ACCs ranged from negative to 70%. In these studies, however, the localization of the EGFR expression and other markers were not reported. Only a study of lung adenoid cystic carcinoma reported the dominance of the localization to the myoepithelial component 35. Correlation between the EGFR- and/or the c-kit expression and clinicopathologic factors were inconclusive.

That a differential cellular expression of c-kit/EGFR in ACC was noted, in our study, underscores the biological differences between ductal and myoepithelial cells and the potential influence of such finding on the stratification of patients for target response to imatinib therapy. We contend that such cellular heterogeneity may have played a role in the inconclusive effect of imatinib on the clinical course of ACC. A recent study reported that the treatment of two initially unresectable ACC with imatinib in a neoadjuvant therapy resulted in a significant tumor regression 36. In contrast, the results of a phase II study of imatinib therapy in the treatment of advanced ACC showed no objective response in all 16 patients after two cycles 37-38. We, however, reason that the lack of response can be due to the cellular heterogeneity where response may be limited to c-kit expression in epithelial but not the myoepithelial cell component.

In addition to the c-kit, EGFR expression in ACC indicates that this marker can be an attractive target for therapy in selected patients with ACC. EGFR has been shown to contribute to tumorigenesis in several human carcinomas by blocking apoptosis and promoting angiogenesis. Anti-EGFR targeted therapies, including cetuximab and erlotinib, have been used in clinical trials in solid tumors 23-26. That EGFR expression was mainly expressed in myoepithelial cells and correlates with better survival in ACC may in part associate with tumor suppressive effect and protracted clinical course of tumor with myoepithelial involvement. Moreover, such pattern of EGFR expression may also impact on the selection of patients for anti-EGFR therapy. In that context, patients with solid pattern tumor may be excluded along with tumor with tubular and cribriform pattern composed of only epithelial cells from such treatment.

In conclusion, our study provides evidence for a new therapeutic strategy in ACC based on the differential expression of markers in ductal and myoepithelial cells where combined therapy may benefit patients with tubular and cribriform tumors and the solid form may be responsive to a single agent, e.g. anti-c-kit agent. We, therefore, attribute the lack of progress in clinical trials of ACC, in addition to including other phenotypic carcinomas, to the differential response of myoepithelial and epithelial tumor cells in ACC to therapy. This is supported by evidence for a less aggressive and modulating role of myoepithelial cells in mammary and salivary carcinomas and the generally low malignant nature of salivary and

breast carcinomas with myoepithelial participation. Future therapeutic trials of these tumors may be better based on biomarker stratification and the cellular composition of these tumors.

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Figure 1.
H&E stained image of solid, tubular and cribriform adenoid cystic carcinoma.
Strong c-kit immunopositivity (b) and no EGFR immunopositivity (c) and in a solid type
ACC. Dual immunoreactivity for c-kit (ductal epithelial cells) (d) and EGFR (myoepithelial
cells) (e) in tubular and cribriform type ACC.

Figure 2. Interphase in-situ hybridization of touch preparations of Adenoid Cystic Carcinoma c-kit containing BAC clone-RP11-586A2: normal (a) and increased copy number for c-kit copies (b –gain, c- amplification).

Figure 3.
Overall survival plots for the three predominant patterns.

Figure 4.
EGFR positivity in myoepithelial cells correlated with a better outcome.

Table 1

Differential EGFR and c-kit expression in Adenoid Cystic Carcinoma patterns

Marker	Pattern			Totals (%)
	Cribriform	Tubular	Solid	
EGFR-m	10 (10.99)%	2 (4.44)%	0 (0.00)%	12 (7.65)%
c-kit-d	22 (24.18)%	10 (22.22)%	15 * (71.43)%	47 (29.94)%
EGFR-m + c-kit-d +	56 (61.54)%	28 (62.22)%	1 ** (4.76)%	85 (54.14)%
Totals -	3 (3.30)%	5 (11.11)%	5 *** (23.81)%	13 (0.083)%

EGFR, epidermal growth factor; m, myoepithelial cells; d, ductal cells

* The proportion of samples that expressed c-kit-d was statistically higher among cases of solid ACC (p = 0.00003).

** The proportion of samples that expressed both EGFR-m and c-kit-d was statistically significantly lower among cases of solid ACC (p < 0.00001).

*** The proportion of samples that did not express either EGFR-m or c-kit-d was statistically significantly higher among cases of solid ACC (p = 0.017).

Table 2

Three- and five-year survival of patients whose samples expressed the various markers.

	Pattern						Outcome	
	Cribriform		Tubular		Solid			
	3 Years	5 Years	3 Years	5 Years	3 Years	5 Years	3 Years	5 Years
Marker	100.00%	83.30%	100.00%	50.00%	None	None	100.00%	76.20%
EGFR-m								
c-kit-d	72.70%	62.30%	70.00%	60.00%	60.50%	50.40%	68.90%	58.50%
EGFR-m + c-kit-d	79.80%	75.90%	96.20%	69.50%	100.00%	100.00%	85.40%	75.10%
Neither	1.0*	66.70%	80.00%	80.00%	80.00%	40.00%	86.70%	62.00%

EGFR, epidermal growth factor; m, myoepithelial cells; d, ductal cells