Fatty Acid Binding Protein 7 Is a Molecular Marker in Adenoid Cystic Carcinoma of the Salivary Glands: Implications for Clinical Significance[1,2]

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Abstract

Adenoid cystic carcinoma (ACC) is an aggressive malignant neoplasm of the salivary glands. Its diagnosis is difficult due to overlapping features with other salivary tumors. Gene expression analysis may complement traditional diagnostic methods. We searched gene expression patterns in the Gene Expression Omnibus (GEO) database and in our tumor and normal samples. The biologic and prognostic potential of the identified genes was analyzed. The GEO data set of primary xenografted ACCs revealed that expression of five genes, *engrailed homeobox 1* (*EN1*), *fatty acid binding protein 7* (*FABP7*), *hemoglobin epsilon 1*, *MYB*, and *versican* (*VCAN*), was dramatically increased. mRNA expression of *EN1*, *FABP7*, *MYB*, and *VCAN* distinguished our sporadic ACCs from normal tissues and benign tumors. *FABP7* expression appeared to be regulated differently from *EN1* and *MYB* and was crossly correlated with poor prognosis in our ACC cohort. Immunohistochemistry showed that FABP7 protein was predominantly expressed in the nucleus of myoepithelial cells of both tubular and cribriform subtypes. In contrast, in the solid subtype, which is often associated with a lower survival rate, FABP7 protein was uniformly expressed in cancerous cells. One case with cribriform architecture and the highest level of *FABP7* mRNA showed strong FABP7 staining in both duct-type epithelial and myoepithelial cells, suggesting that diffuse expression of FABP7 protein might be related to aggressive tumor behavior and poor prognosis. We propose FABP7 as a novel biomarker in ACC. The molecule may be useful in diagnosis and for identifying more effective therapies targeting this protein or upstream molecules that regulate it.

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Introduction

Adenoid cystic carcinoma (ACC) is a high-grade malignant neoplasm of the salivary glands with unique histology and variable clinical behavior [1–5]. ACC has a propensity to metastasize extensively and the long-term prognosis is not favorable. Distant metastases can develop despite local and regional tumor control and can be delayed, sometimes occurring 10 to 20 years after diagnosis. Unfortunately, therapeutic options for ACC are limited and usually consist of surgery and postoperative radiation therapy. These interventions, however, have failed to affect long-term outcomes in ACC.

The diagnosis of ACC is another challenge. Existing imaging methods, including ultrasonography, computed tomography, magnetic resonance imaging (MRI), and radionuclide scanning, do not

provide a definitive diagnosis [6,7]. Evaluation of fine needle aspiration biopsy material is not always reliable diagnostically, due to the overlapping microscopic features between ACC and other salivary gland neoplasms [8–10]. Accurate diagnosis, however, is important to guide proper surgery and adjuvant treatment [1–5]. Gene expression analysis will likely be an important complement to traditional diagnostic methods in the diagnosis of ACC [11,12]. As an example, immunohistochemical staining for c-Kit is often used in conjunction with histology to aid in diagnosis of ACC. c-Kit, a proto-oncogene, is overexpressed in almost all ACCs but seldom increased in other head and neck tumors [3–5].

Other potential diagnostic markers for ACC have been reported. For example, t(6;9) chromosomal translocations involving genes encoding transcription factors *MYB* and *nuclear factor I/B* have been found in roughly half of ACCs [13,14]. In addition, a gene expression profile of ACC found elevated expression of a variety of extracellular matrix gene products, including *versican (VCAN)* [15]. More recently, *engrailed homeobox 1 (EN1)* was reported as a biomarker for ACC [16]. However, it is not clear whether these molecules were increased specifically in ACC or to what extent they contribute to its malignant growth, metastasis, and prognosis. The objective of this study was to identify a diagnostic molecular marker for ACC, which would be a predictor of the prognosis and a possible therapeutic target. With a biomarker, advances in ACC management may be possible [2].

We searched the Gene Expression Omnibus (GEO) database for potential diagnostic biomarkers of ACC. Expression microarrays of 11 primary xenografted ACCs revealed that levels of *EN1*, *fatty acid binding protein 7* (*FABP7*; also known as *brain lipid binding protein*), *hemoglobin epsilon 1* (*HBE1*), *MYB*, and *VCAN* were elevated compared to normal salivary tissues and were the five most elevated genes [17]. Similar findings were obtained from 27 tumor samples of sporadic ACCs in our archives. We found that expression of *EN1*, *FABP7*, *MYB*, and *VCAN* were considerably elevated. Expression of these genes distinguished ACCs from normal salivary tissues and benign tumors, including basal cell adenomas (BCAs) and pleomorphic adenomas (PAs). *HBE1* was not detectable with our extracts. We also observed a correlation between *FABP7* expression and overall survival in ACC, suggesting that *FABP7* might have prognostic value in patients with ACC.

This study proposes that FABP7 is a biomarker that can be used to diagnose ACC, aid tumor screening, help delineate surgical margins, predict prognosis, monitor patients in remission, and is a starting point for more effective therapeutic options. We discuss a potential role of FABP7 in ACC based on its subcellular distribution and cell type–specific expression.

Materials and Methods

Tumor and Normal Samples

We obtained 27 ACCs, 4 BCAs, and 5 PAs, as well as 5 normal salivary tissue samples from the University of California, San Francisco (UCSF) Anatomic Pathology archives. Institutional review board (IRB) approval was obtained and UCSF guidelines for handling human tissue were followed. Representative normal salivary tissues were additionally chosen from ACC patients whose tumor samples were included in this study. Slides were reviewed to determine tissue suitability for gene expression analysis.

TaqMan Quantitative Polymerase Chain Reaction Assay

Gene expression was analyzed in triplicate with TaqMan quantitative polymerase chain reaction (qPCR). Total RNA was isolated using

RNAeasy kits (Qiagen, Valencia, CA) from formalin-fixed, paraffin-embedded tumor tissue sections composed of at least 70% tumor cells. cDNA from 500 ng of total RNA was synthesized with an RT first-strand kit (Life Technologies, Carlsbad, CA). cDNA (5 ng) was mixed with transcriptase (RT) qPCR master mixes, and aliquots were placed with gene-specific primer sets. The following TaqMan assays (all from Life Technologies) were used: *EN1* (Hs00154977_m1), *FABP7* (Hs00361426_m1), *HBE1* (Hs00362216_m1), *VCAN* (Hs00171642_m1), and *MYB* (Hs00920554_m1). Expression levels normalized to endogenous *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* were determined by real-time PCR and analyzed at the UCSF Comprehensive Cancer Center Genome Analysis Core Facility. Fold changes in gene expression were calculated as the ratio of expression in each sample to average expression in controls. Statistical analyses and graphs were made using Microsoft Office Excel and XLSTAT (Addinsoft, New York, NY). Statistical comparisons between data sets were made with two-tailed Student's t tests and log rank tests, and $P < .05$ was considered significant.

Immunohistochemistry

Immunohistochemistry was performed on unstained sections using antibody-based staining kits for FABP7 (AF3166; R&D Systems, Minneapolis, MN) or goat isotype control (AB-108-C; R&D Systems) at the UCSF Comprehensive Cancer Center Immunohistochemistry and Molecular Pathology Core Facility. The detailed procedure for staining has been described [3]. FABP7 staining was visually estimated by a head and neck pathologist (A.v.Z.).

Results

Tumor Characteristics

A total of 41 samples of salivary tissues from the UCSF archives was included in this study: 27 cases of ACC, 4 BCAs, 5 PAs, and 5 normal salivary gland samples. We included BCAs and PAs in the study because the microscopic features of these tumors often overlap with those of ACC, and distinguishing them is important for appropriate management of patients with salivary gland tumors. All tumors had arisen sporadically. Sixteen ACC tumors occurred in women. The median age at presentation was 58 years (range, 33-91 years). Tumors arose at the following sites: maxillary sinus (nine tumors), submandibular gland (six tumors), parotid gland (five tumors), sublingual gland (two tumors), and one each in the nasal cavity, mandibular mucosa, nasopharynx, base of tongue, and tongue. Tumors were classified by morphologic subtype: tubular (4 cases), cribriform (3), solid (1), combined cribriform and tubular (10), combined solid and tubular (8), and combined cribriform and solid (1). In the BCAs, all four tumors occurred in women and arose in the parotid gland. Median age at presentation was 60 years (range, 40-73 years). In the PAs, three tumors occurred in women. Median age at presentation was 46 years (range, 20-67 years), and four tumors arose in the parotid gland with one in the submandibular gland. Two of five normal salivary tissue specimens were from women. Median age at presentation was 62 years (range, 33-91 years). Three samples represented tissue excised from the submandibular gland; two were from the parotid gland.

Expression of EN1, FABP7, MYB, and VCAN Distinguishes ACCs from Normal Tissues and Benign Tumors in the Salivary Glands

In our search of the GEO database, we obtained gene expression profiles of 11 primary xenografted ACCs and 3 normal salivary gland

tissue samples (Accession No. GSE36820). One of the most important applications of the GEO data set to ACC pathobiology is to identify diagnostic markers [18]. To find differentially expressed genes in the data set, we compared fold changes rather than the t-statistics because we considered that larger absolute changes in gene expression would be more practical for identifying diagnostic markers [19]. We found increased expression of the following genes in primary xenografted ACC tumors: *EN1*, *FABP7*, *HBE1*, *MYB*, and *VCAN*. Their $\log_2$ fold changes were 6.04 (*EN1*), 6.01 (*FABP7*), 5.96 (*HBE1*), 5.41 (*MYB*), and 5.24 (*VCAN*), meaning that these genes increased their expression by 65.8-fold (*EN1*), 64.4-fold (*FABP7*), 62.2-fold (*HBE1*), 42.5-fold (*MYB*), and 37.8-fold (*VCAN*).

We then investigated whether the expression of these genes would also be elevated in sporadic ACCs from our archives. mRNA was isolated from formalin-fixed, paraffin-embedded sections as described, and qPCR was performed. Figure 1 shows that mRNA expression of *EN1*, *FABP7*, *MYB, or VCAN* was elevated in ACC ($P < .05$; Figure 1, A–D). Our probe did not detect *HBE1* in these extracts. The relative expression of *EN1* (increased by 848-fold over the average of normal samples) and *MYB* (increased by 659-fold over the average of normal samples) distinguished ACC from normal tissues and benign tumors (Figure 1, A and C). *FABP7* mRNA in ACC was overexpressed relative to normal salivary glands ($P = 2.7 \times 10^{-10}$; Figure 1B), as well as BCA and PA ($P = 1.1 \times 10^{-2}$ and 5.0×10^{-3}, respectively). *VCAN*'s profile was similar to *EN1* and *MYB*, although the difference from normal salivary tissues and benign tumors was less than those in *EN1* and *MYB* (Figure 1D).

We concluded that expression of *EN1*, *FABP7*, *MYB, and VCAN* distinguished ACC from normal tissues and benign salivary gland tumors.

FABP7 Expression Is Poorly Correlated with Levels of EN1 and MYB in ACC Tumors

To distinguish one expression profile from another in our sporadic ACCs, we determined strengths of correlation between pairs of genes in all six combinations of all genes studied. We generated scatter plots with trend lines to show correlations (Figure 2, A–F). Trend line equations and R^2 values were calculated and are displayed atop each chart. Expression levels of *EN1* and *VCAN* (Figure 2A) and *EN1* and *MYB* (Figure 2B) were highly correlated. Their R^2 values were 0.5646 and 0.224, respectively ($R = 0.7514$ and 0.4732). There was also a moderate correlation between *MYB* and *VCAN* (Figure 2C; $R^2 = 0.1533$; $R = 0.3915$). In contrast, *FABP7* expression was poorly correlated with *EN1* or *MYB* expression (Figure 2, D and E); R^2 values were 0.0035 and 0.0126, respectively ($R = 0.05916$ and 0.1123). *FABP7* had a moderate correlation with *VCAN* (Figure 2F; $R^2 = 0.1011$; $R = 0.3180$). Overall, *FABP7* expression appeared to be regulated differently from *EN1* and *MYB* in ACCs.

FABP7 May Predict Poor Prognosis in Salivary ACC Patients

To determine the biologic and prognostic significance of elevated expression of *EN1*, *FABP7*, *MYB, and VCAN* in ACC, we performed overall survival analysis by generating Kaplan-Meier plots with log rank testing (Figure 3, A–H). Log rank testing is a hypothesis
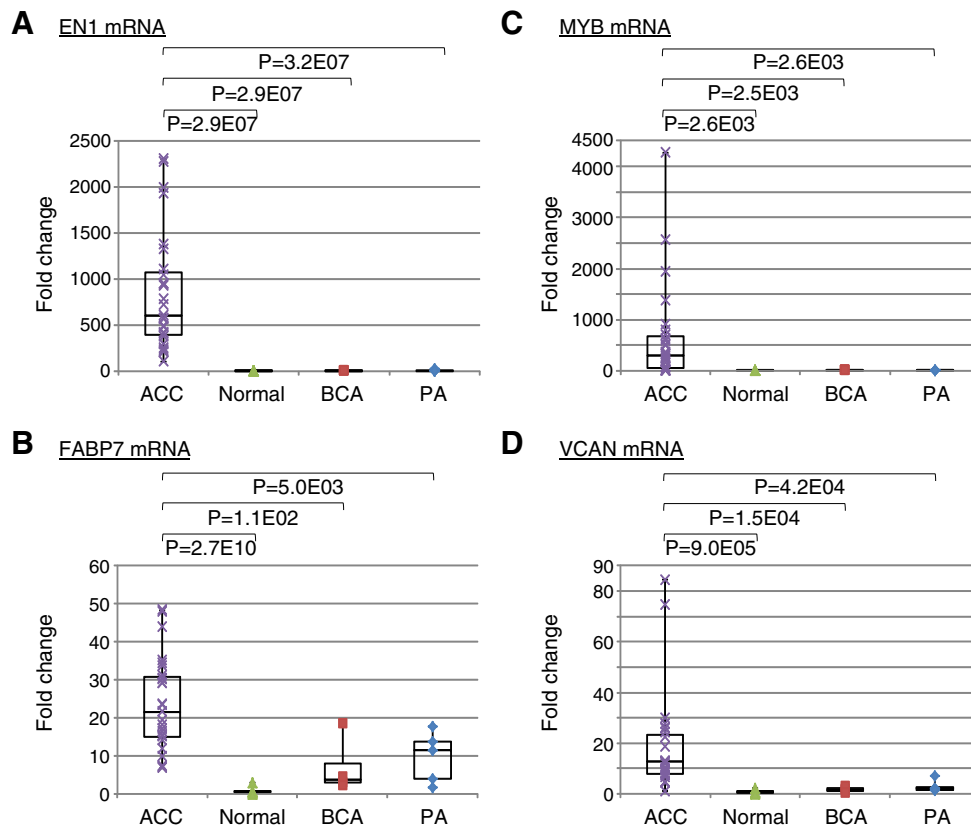

Figure 1. Expression of *EN1, FABP7, MYB, and VCAN* distinguishes ACCs from normal tissues and benign salivary gland tumors. (A–D) Box plot analysis of relative gene expression. Each box represents the quartile distribution range (25-75%). The median is shown by a black horizontal line. The whole range and individual cases are displayed as a black vertical line and scatter dots. The *y*-axis indicates fold change relative to the average of normal samples' gene expression. Statistical comparisons between data sets were performed with two-tailed Student's t tests. P values <.05 were considered significant.
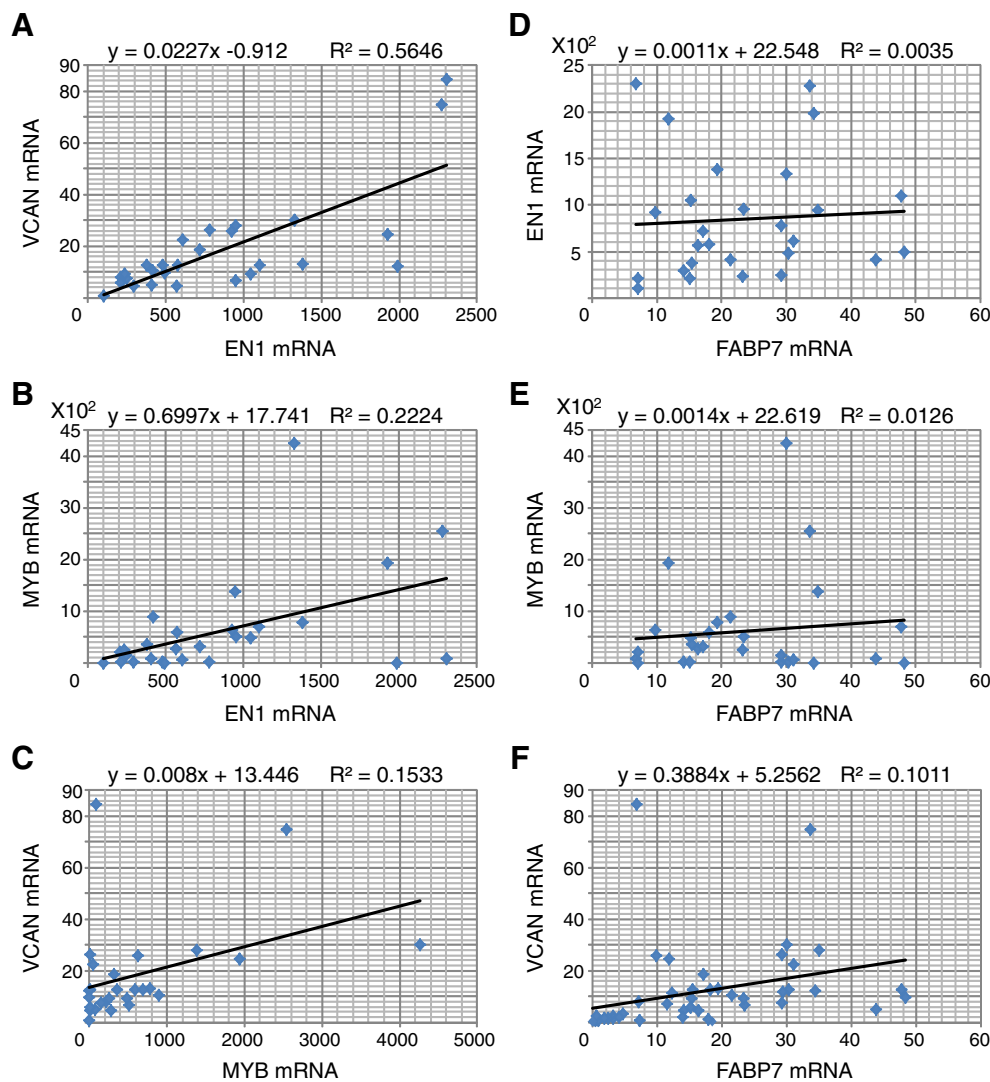
Figure 2. *FABP7* expression is poorly correlated with levels of *EN1* and *MYB* in ACC tumors. (A–F) Scatter plot analysis for the strength of a correlation in relative gene expression between two molecules from *EN1, MYB, FABP7, and VCAN*. Trend lines are included. Trend line equations and R^2 values were calculated separately.

assessment and compares survival distributions in two samples. We divided our ACC cohort into two groups according to gene expression scores. One group had expression values above the median, and the other half's values were below it (Figure 3, A–D). We also created groups whose expression values were in the highest or lowest quartiles (Figure 3, E–H). We observed a correlation between *FABP7* expression and survival (Figure 3, B and F). Specifically, the subset with the highest *FABP7* gene expression (top quartile) had the poorest survival (P = .006). In contrast, we found no apparent correlation between survival and expression of the other genes (Figure 3, A, C–E, G, and H). These observations may explain why *FABP7* expression was not strongly correlated with *EN1* and *MYB* expression in ACC tumors (Figure 2). We concluded that *FABP7* was the sole predictor of poor prognosis among the genes examined here.

FABP7 Protein Expression in ACC Tumor Specimens

We performed immunohistochemistry with an FABP7 antibody on normal salivary tissues and ACCs. Figure 4A shows that normal salivary glands expressed FABP7 consistently in the cytoplasm of serous acinar cells and occasionally in the nuclei of myoepithelial cells of the

intercalated ducts. Moderate cytoplasmic expression was detected in epithelial cells of the intercalated and striated ducts. Figure 4B shows a cribriform subtype ACC from the patient shown in Figure 4A. FABP7 protein was predominantly found in the nucleus of myoepithelial cells and, to a lesser extent, in the cytoplasm of duct-type epithelial cells. Similar observations were made in other cribriform (Figures 4C and S1A) and tubular pattern ACC tumors (Figures 4D and S1B).

These findings agree with previous reports that ACC is biphasic, composed of duct-type epithelial and myoepithelial cells, and shows cell type–specific gene expression. For example, c-Kit and EN1 expression have been limited to duct-type epithelial cells, whereas epidermal growth factor receptor (EGFR), p63, VCAN, and MYB have been found exclusively in more differentiated myoepithelial cells [14,16,17,20–22]. Nonetheless, one cribriform case with the highest level of *FABP7* mRNA (47 times normal) showed strong positive immunoreactivity in both duct-type epithelial and myoepithelial cells (Figure 4E). This observation suggests that diffuse expression of FABP7 among the two cell populations might be a factor in ACC cases with poor prognosis. Consistent with this idea, ACC cases of the solid subtype had uniform and strong positive immunoreactivity with the
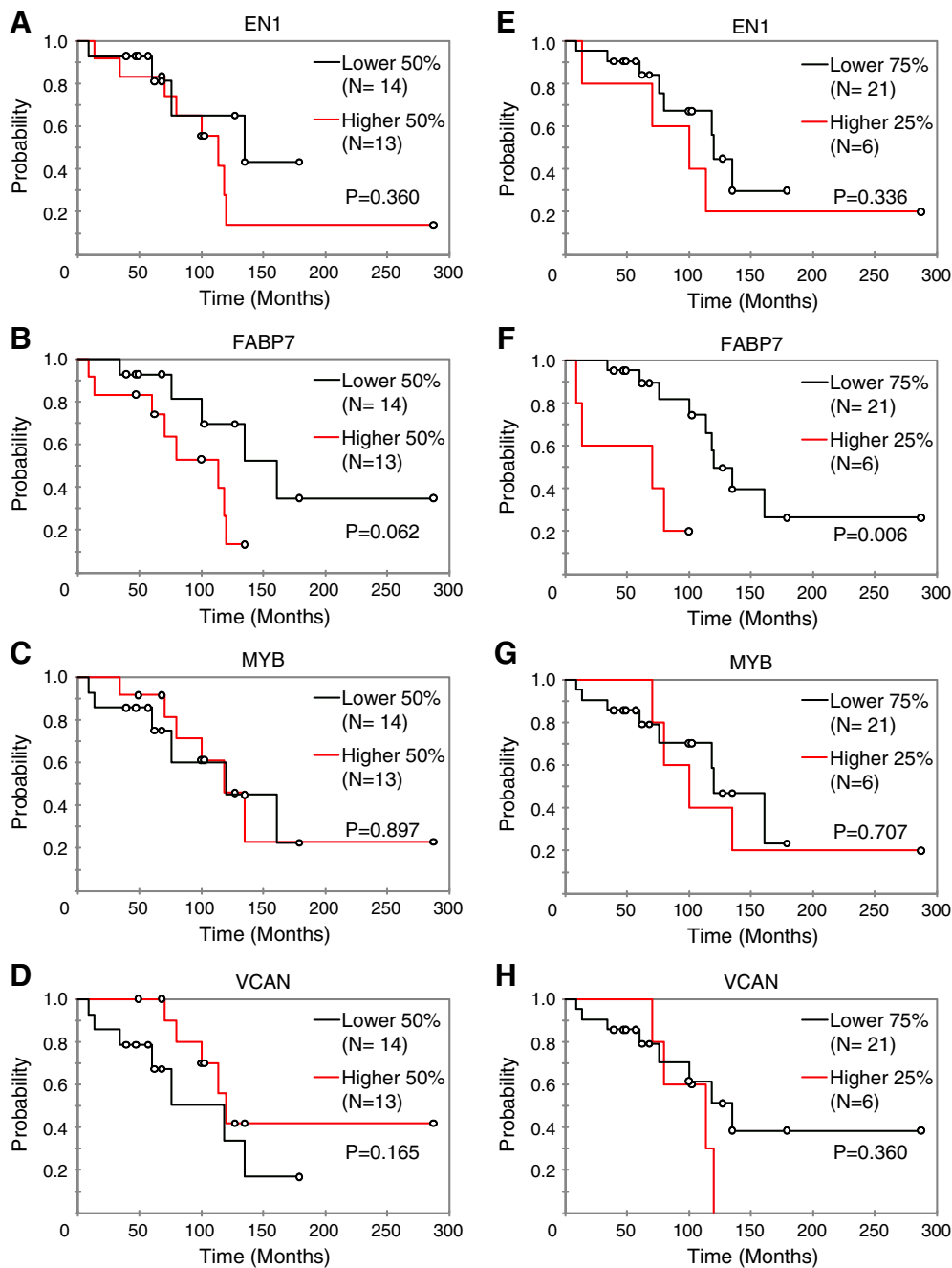
Figure 3. *FABP7* may predict poor prognosis in salivary ACC patients. (A–H) Kaplan-Meier curves showing overall survival rate of patients with sporadic ACC tumors with a higher or lower mRNA expression of *EN1* (A and E), *FABP7* (B and F), *MYB* (C and G), or *VCAN* gene (D and H). The ACC cohort was divided into two groups according to relative gene expression. Panels A to D compare patients with expression in the top half overall (red line) and the lower half (black line). Panels E to H compare expression in the higher quartile (red line) and lower three quartile (green line) subsets. Circles identify censored data. Statistical comparisons between data sets were made with log rank tests, and each *P* value is displayed. *P* < .05 is considered significant.

FABP7 antibody (Figures 4*F* and S1*C*). These solid tumors are mostly devoid of myoepithelial cells and often associated with aggressive behavior and poor prognosis. We concluded that increased *FABP7* mRNA was linked to increased protein expression and was clinically significant in ACC.

Discussion

Obtaining an accurate diagnosis is a major challenge for an individual who has a rare cancer [23]. It is inevitable that most physicians will only encounter individual rare diseases a few times during their careers. Unfortunately, as a result, many patients are not diagnosed until the disease has become advanced. ACC of the salivary glands is such a rare disease [1–5]. The differential diagnosis of ACC is difficult in the clinic because of the absence of unique clinical symptoms, diagnostic imaging, or microscopic features. Nonetheless, therapies and long-term outcomes are very different for ACC and other salivary neoplasms. The accurate diagnosis of ACC is essential for improving patient outcomes.

We believe that gene expression analysis can complement traditional methods of ACC diagnosis. We found that mRNA
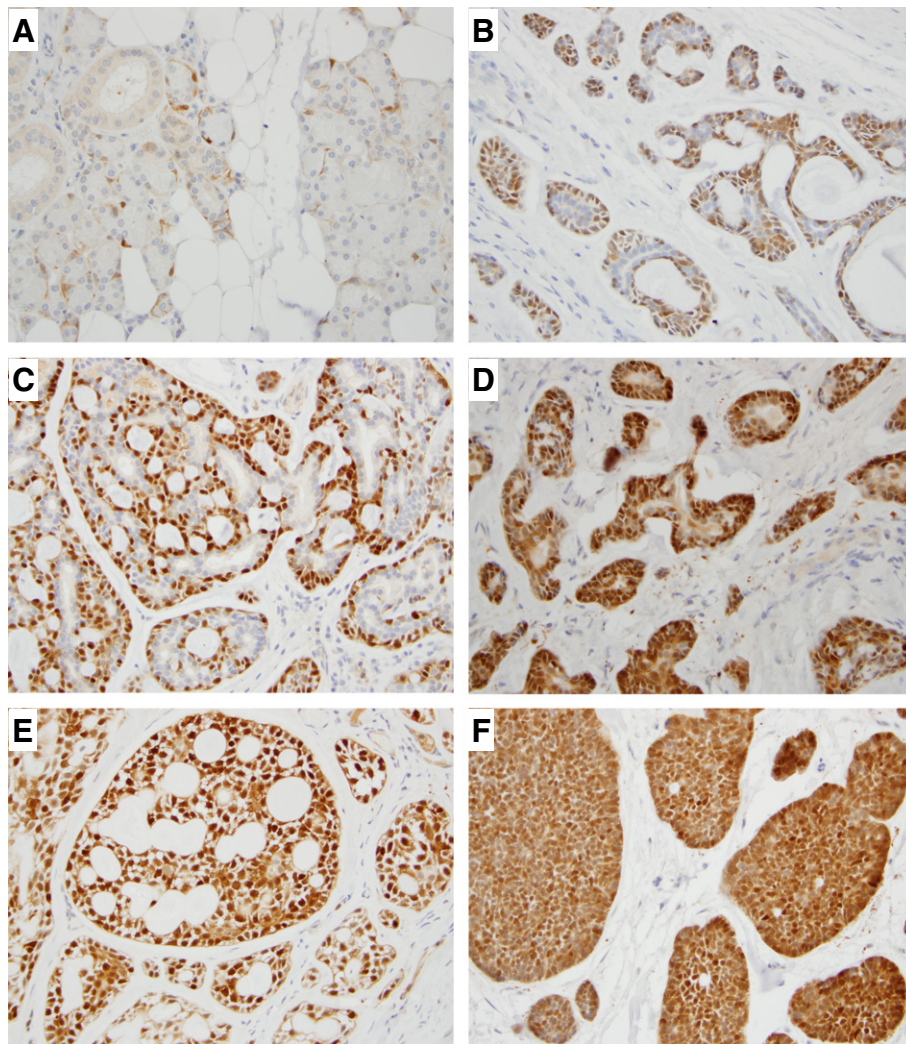
Figure 4. FABP7 protein expression in ACC tumor specimens. (A–F) Immunohistochemistry using an antibody to FABP7. (A) Normal salivary gland tissue. (B–F) ACC tumor specimens: cribriform (B, C, and E), tubular (D), and solid (F) patterns. (B) ACC tumor specimen from the patient in A. (E) The cribriform case with the highest level of *FABP7* mRNA. (B–D) FABP7 protein was predominantly expressed in the nucleus of myoepithelial cells and, to a lesser extent, in the cytoplasm of duct-type epithelial cells (E and F). Diffuse expression of FABP7 protein was associated with aggressive tumor behavior and poor prognosis.

expression of *EN1, FABP7, MYB, and VCAN* distinguished ACC from normal tissues and benign salivary gland tumors (Figure 1). Additionally, we propose that FABP7 may be a multipurpose molecular biomarker, because its expression was correlated with poor prognosis (Figure 3). Expression of FABP7 may be useful for diagnosing ACC, predicting prognosis, and searching for more effective therapeutic options. These options may include targeting FABP7 protein itself or upstream molecules that regulate it.

FABP7 is a member of a large family of hydrophobic proteins and is expressed in the brain, heart, testis, and adipose tissues [24–26]. It binds to fatty acids and other lipids and functions as a cytoplasmic chaperone for lipid metabolism. Recent studies have found that high levels of FABP7 were also in the nucleus of glioblastoma and melanoma cells, and its expression was associated with reduced survival [27–30]. We have discovered that FABP7 protein was predominantly expressed in the nuclei of myoepithelial cells and, to a lesser extent, in the cytoplasm of duct-type epithelial cells in both tubular and cribriform subtypes of ACC (Figures 4, *B–D*, and S1, *A*

and *B*). These findings suggest a potential role of FABP7 in the nucleus of myoepithelial cells for genesis, development, proliferation, and maintenance of ACC tumors. In contrast, the solid subtype of ACC, which is often correlated with a lower survival rate, had uniform FABP7 protein expression over the malignant cells (Figures *4F* and S1*C*). A similar staining pattern was seen in one cribriform tumor harboring the highest levels of *FABP7* mRNA and was in the lowest quartile for survival. This result suggested that diffuse expression of FABP7 protein was likely to be associated with aggressive tumor behavior and poor prognosis (Figure *4E*).

FABP7 binds to docosahexaenoic acid (DHA), an omega-3 fatty acid, with the highest affinity of all FABPs [31]. In a study of U87 malignant glioma cells, DHA inhibited FABP7-mediated tumor progression by blocking the binding of arachidonic acid to FABP7 [32]. Arachidonic acid is an omega-6 fatty acid. As a result, FABP7 translocated DHA to the nucleus, where it was transferred to the peroxisome proliferator-activated receptor gamma (PPARγ) nuclear receptor and transactivated its downstream target genes, causing

attenuations of proliferation and promigratory genes. Humans naturally make small amounts of DHA but must get more from food or supplements [33].

We do not know if DHA supplementation increases risk or benefit in patients with ACC. If it inhibits FABP7-mediated tumor progression or increases health benefits in ACC similarly to the case in malignant glioma cells, DHA-rich foods such as cold water fish (e.g., salmon) or DHA supplements may benefit patients. Of course, a randomized double blind trial is necessary before such a recommendation can be made [34].

FABP7 is a Notch target gene [35,36]. Notch signaling is activated in ACC [37,38]. Recent genome-wide sequencing of ACC revealed activating mutations in genes within the Notch signaling pathway. One study performed whole exome sequencing in a series of 24 ACC tumors [37] and found two cases of missense and frameshift mutations in the *NOTCH1* gene and a single case of two truncating mutations in *NOTCH2*. Furthermore, it identified that *SPEN*, a negative Notch signaling regulator, had six truncating mutations in six ACC cases. Another report described the exome or whole-genome sequences of 60 ACC tumor-normal pairs [38]. That report demonstrated that components of the Notch signaling pathway including *CNT2, NOTCH1, FOXP2, DTX4, FBXW7, MAML3, and MYCN* were mutated in 13% of their cohort. That report also analyzed ACC samples with altered *NOTCH1* genes using a gene set enrichment analysis and showed a tendency toward enrichment of Notch signaling.

Given these findings, it is possible that FABP7 expression is regulated by the Notch signaling pathway in ACC. These results are intriguing in light of another study showing that NOTCH1 immunostaining was similar to what we observed here for FABP7 [39]. If this is the case, FABP7 may be a useful biomarker to measure response to Notch inhibitors in salivary tumors. This approach is both plausible biologically and attractive, in that Notch-targeted therapeutic antibodies are currently under clinical evaluation for other diseases [40]. This idea warrants studies addressing the links between Notch signaling and FABP7 expression in ACC.

This report proposes FABP7 as a biomarker that can help diagnose ACC, predict prognosis, and form a basis for more effective therapeutic options. Establishment of FABP7 as an ACC biomarker may aid the study of ACC in its earliest stages and will provide an opportunity to determine the contribution of normal salivary gland stem cells to the development of ACC.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2014.10.003.

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