APRIL promotes breast tumor growth and metastasis and is associated with aggressive basal breast cancer

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Abstract

APRIL (a proliferation-inducing ligand) is a cytokine of the tumor necrosis factor family associated mainly with hematologic malignancies. APRIL is also overexpressed in breast carcinoma tissue lesions, although neither its role in breast tumorigenesis nor the underlying molecular mechanism is known. Here, we show that several breast cancer cell lines express APRIL and both its receptors, B cell maturation antigen (BCMA) and transmembrane activator and CAML-interactor (TACI), independently of luminal or basal tumor cell phenotype, and that the mitogen-activated protein kinases p38, ERK1/2, and JNK1/2 are activated in response to APRIL. The inflammatory stimulus poly I:C, a toll-like receptor (TLR) 3 ligand, enhanced APRIL secretion. Silencing experiments decreased cell proliferation, demonstrating that APRIL is a critical autocrine factor for breast tumor growth. Studies of 4T1 orthotopic breast tumors in APRIL transgenic mice showed that an APRIL-enriched environment increased tumor growth and promoted lung metastasis associated with enhanced tumor cell proliferation; BCMA and TACI expression suggests that both participate in these processes. We detected APRIL, BCMA and TACI in human luminal, triple-negative breast carcinomas and HER2 breast carcinomas, with increased levels in more aggressive basal tumors. APRIL was observed near Ki67+ nuclei and was distributed heterogeneously in the cancer cells, in the leukocyte infiltrate, and in the myoepithelial layer adjacent to the tumor area; these results imply that APRIL provides proliferation signals to tumor cells through paracrine and autocrine signaling. Our study identifies participation of APRIL signaling in breast cancer promotion; we propose impairment of this pathway as a potential therapeutic strategy.

Introduction

Breast cancer is the most commonly diagnosed tumor type, and its incidence grows annually (1). The last decade has seen much progress in our understanding of the biology of breast cancer, a complex and heterogeneous disease (reviewed in ref. 2). Based on molecular and genetic profiles, breast carcinomas were recently classified in six major subsets that include luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, basal-like, normal breast and claudin-low carcinomas (3). Luminal carcinomas express luminal markers, estrogen receptor and/or progesterone receptor (PR), account for 60–80% of diagnosed cases, and generally have a good prognosis (4). HER2-enriched carcinomas are HER2 positive and make up 15–20% of cases, whereas basal-like carcinomas express basal/myoepithelial markers, are frequently negative for estrogen receptor, PR...
and HER2 (triple negative), and represents 10–20% of all breast cancers. HER2-enriched and basal-like are usually more aggressive and have a poorer prognosis than the luminal subtype (5), although anti-HER2 treatment has substantially improved survival of HER2-enriched carcinoma patients in the last decade (6). Even so, 20–30% of all breast cancer patients develop metastatic disease that remains incurable, with a median survival between 2 and 4 years depending on subtype (7). Better understanding is thus needed for the molecular mechanisms and signaling pathways that govern the processes of breast carcinoma formation, maintenance and expansion and this knowledge must be translated into more effective therapeutic strategies.

Cytokines and other soluble factors are key molecules in breast cancer development and progression (8). They can be secreted by tumor cells and by cells in the tumor environment, and establish complex communication networks to promote tumor cell proliferation and survival, epithelial-to-mesenchymal transition, as well as tumor cell invasion and metastasis. They are also involved in tumor lymphocyte recruitment and modulate the type of immune response generated, which contributes to disease outcome (9). APRIL (a proliferation-inducing ligand) is a TNF family cytokine originally named for its ability to stimulate tumor cell proliferation in vitro (10). Cells of the immune system as well as epithelial and adipose-derived mesenchymal stem cells can secrete APRIL (11–13), which binds to two known receptors, the transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and the B cell maturation antigen (BCMA) (14,15). APRIL also binds heparan sulfate proteoglycans (HSPG) on the cell surface, which are highly expressed in tumor cells (16).

The association between APRIL and cancer has been studied mainly for leukemia and lymphoma, due to the initial description of APRIL receptors in B cells (17) and to the observation that APRIL transgenic mice develop lymphoid tumors (18). In these hematological malignancies, APRIL overexpression correlates with disease progression and is a poor prognostic factor for patient survival (19,20); it protects tumor cells from spontaneous and drug-induced apoptosis, promotes cell cycle progression and enhances cell survival and/or proliferation (21,22). In human solid tumors, APRIL overexpression has been detected in many cancer types (23–34). APRIL induces growth arrest in hepatocellular carcinoma cells (28), and promotes tumor growth and/or metastasis in glioblastoma (30), pancreatic cell lines (31), and in vivo colon carcinoma models (32). Data on APRIL relevance in breast cancer are very limited (23,33,34). APRIL is over-expressed in breast tissue lesions and is detected in cancer cells, but is associated mainly with the stroma and non-malignant structures (23,34). Its involvement in breast tumorigenesis and metastasis, its association with breast carcinoma subtypes, and the underlying molecular mechanisms are not known.

Here, we show that APRIL as well as BCMA and TACI are expressed in human luminal and basal-like breast carcinoma cell lines as well as in primary breast carcinomas. We found that APRIL signals through p38, ERK1/2 and JNK1/2 mitogen-activated protein (MAP) kinases, and show that Toll-like receptor (TLR3) activation induces APRIL secretion in breast cancer cells. Loss-of-function experiments demonstrated that APRIL sustains cell proliferation in an autocrine manner. In vivo studies using 4T1 orthotopic tumors confirmed that ectopic APRIL promotes breast tumor growth and lung metastasis. Moreover, we found higher APRIL and TACI expression in human triple-negative breast carcinomas (TNBC) compared with luminal breast carcinomas, which suggests association of this APRIL signaling pathway with tumor aggressiveness.

**Methods**

**Mouse, cell lines and human tumor samples**

B6-Tg(Lck-hAPRIL) mice were previously described by Michael Hahne’s laboratory (17) and backcrossed at least 10 times onto the BALB/c background to generate the BALB/c-Tg (Lck-hAPRIL) mice used in these experiments and termed APRIL-Tg for short. APRIL-Tg and wild type littermates were bred in-house under specific pathogen-free conditions. Animal experiments were supervised by the Centro Nacional de Biotecnología Ethics Committee, in compliance with national, institutional and EU guidelines. Human breast carcinoma cell lines MDA-MB231, MDA-MB468, MCF7, T47D (35) and the murine 4T1 cell line, derived from a spontaneous mammary tumor in BALB/c mice, were obtained from the American Type Culture Collection (ATCC, Manassas). ATCC ensures authenticity of these cell lines using short tandem repeat DNA profiling. Short tandem repeat authentication of cell lines was conducted by the Genomics Core Facility at the Instituto de Investigaciones Biomedicas ‘Alberto Sols’ and verified with the ATCC data base. A certificate of analysis was provided. Cells were kept in culture and used no longer than 3–4 months after authentication. Cells were cultured according to supplier’s recommendations; for culture media, see Supplementary Table 1 available at Carcinogenesis Online. For quantitative real-time polymerase chain reaction (qPCR) analysis, samples from breast cancer patients were collected from the University Hospital of Bellvitge (Barcelona, Spain), using protocols approved by the IDIBELL ethics committee and according to Declaration of Helsinki. Samples were collected immediately after surgery, frozen and stored at −80°C. For immunohistochemical studies, nine IDC tissue samples were obtained from the CNIO Tumor Biobank (Madrid, Spain), with anonymized patient data and consent (36). Molecular characteristics of human samples are provided in Supplementary Table 2 available at Carcinogenesis Online (samples used for molecular analyses) and S3 (samples for IHC analyses). We used the hAPRIL ELISA kit (e Bioscience) to quantify APRIL protein in 37 human serum samples from breast carcinoma patients with three carcinoma subtypes (luminal, n = 20; triple negative breast carcinoma, TNBC, n = 12; HER2-enriched, n = 5; obtained from Biobank HUB-ICO-IDIBELL, Barcelona, Spain).

**RNA isolation and quantitative RT-PCR**

Total RNA was isolated from each tumor cell line and from 4T1 tumors and lungs of 4T1-orthotransplanted WT and APRIL-Tg mice, using TRI Reagent (Sigma–Aldrich). RNA (1–2 μg) was used for reverse transcription with the High Capacity CDNA Reverse Transcription Kit (LifeTechnologies). qPCR assay was performed on an ABI 7900 Fast Real-time PCR System using TaqMan Fast Universal PCR master mix (LifeTechnologies). β-Actin and Pum1 served as internal housekeeping genes depending on the experiment. RNA extraction and qPCR from human breast tumors were performed as described (37), and mRNA levels were normalized to the PP1A gene. Primer sequences (Sigma–Aldrich) are shown in Supplementary Table 4 (available at Carcinogenesis Online).

**Immunofluorescence**

Breast cell lines were plated on fibronectin-coated slides and incubated (3–6h, 37°C), then fixed with 2% paraformaldehyde solution, permeabilized with 0.1% Triton X-100 (5 min, 20°C) and incubated with primary antibody anti-hAPRIL ED2 (overnight, 4°C, Alexis Biochemicals) that recognizes both full length and processed APRIL. Then cells were incubated...
with a Cy2-conjugated secondary antibody followed by DAPI. Samples were mounted in Vectashield medium (Vector Laboratories) and images were recorded in a confocal microscope (Olympus Fluoview 10) with a ×20 objective lens, using FV10-ASW 1.6 software (Olympus). Brightness and/or contrast were adjusted with ImageJ software (National Institutes of Health).

Western blot

To evaluate APRIL expression, MCF7, T47D, MDA-MB231 and MDA-MB468 cells were lysed with lysis buffer (Roche), and proteins quantified and analyzed by Western blot following standard procedures (38). As positive control, we used HEK-293T cells transfected with APRIL (293T-APRIL). To study APRIL induction by TLR ligands, MDA-MB231 and MDA-MB468 cells were cultured with ultrapure LPS (2 μg/ml), flagellin (0.5 μg/ml) or poly I:C (20 μg/ml); all from InvivoGen) and supernatants were collected at 24 and 48 h. Blots were probed with APRIL anti-hAPRIL antibody (Alexis Biochemicals), which recognizes full-length and cleaved APRIL. To analyze APRIL and TLR3 signaling, cells were starved overnight, and then stimulated with hAPRIL (200 ng/ml; R&D Systems) or poly I:C (20 μg/ml) in a time course. We used anti-phospho-ERK1/2 (Thr202/Tyr204), -ERK1/2, -phospho-p38 (Thr180/Tyr182), -p38, -JNK1/2, -IκBα and phospho- IκBα antibodies (Cell Signaling Technology); anti-phospho-JNK1/2 (Thr183/Tyr185, Invitrogen, Life Technologies), and appropriate IRF-conjugated secondary antibodies (Dako).

Proliferation assays

We quantified cell proliferation at 48 h using a thymidine incorporation assay. Briefly, cells from each cell line were cultured (105 cells/well, 96 flat-well plates), alone or with 100 ng/ml hAPRIL (MegaAPRIL, Alexis Biochemicals). [3H]thymidine (1 Ci/C/ml) was added to the culture for the last 8 h and thymidine incorporation was measured in a liquid scintillation counter (Wallac Trilux 1450 Microbeta, Perkin Elmer). Three independent assays were performed, with at least 15 wells per condition.

To determine proliferation after APRIL silencing, similar experiments were performed with cells previously transfected with APRIL-specific and control small interference RNA (ON-TARGETplus SMARTpool, Dharmacon) and independent assays were performed, with at least 15 wells per condition.

Blots were analyzed by Western blot following standard procedures (38). As positive control, we used HEK-293T cells transfected with APRIL (293T-APRIL). To study APRIL induction by TLR ligands, MDA-MB231 and MDA-MB468 cells were cultured with ultrapure LPS (2 μg/ml), flagellin (0.5 μg/ml) or poly I:C (20 μg/ml); all from InvivoGen) and supernatants were collected at 24 and 48 h. Blots were probed with APRIL anti-hAPRIL antibody (Alexis Biochemicals), which recognizes full-length and cleaved APRIL. To analyze APRIL and TLR3 signaling, cells were starved overnight, and then stimulated with hAPRIL (200 ng/ml; R&D Systems) or poly I:C (20 μg/ml) in a time course. We used anti-phospho-ERK1/2 (Thr202/Tyr204), -ERK1/2, -phospho-p38 (Thr180/Tyr182), -p38, -JNK1/2, -IκBα and phospho- IκBα antibodies (Cell Signaling Technology); anti-phospho-JNK1/2 (Thr183/Tyr185, Invitrogen, Life Technologies), and appropriate IRF-conjugated secondary antibodies (Dako).

4T1 tumor transplantation model

Basal 4T1 cells (105) were injected into the 4th mammary gland of 8- to 10-week-old female APRIL-Tg and control mice (8-15 mice/group). After detection, tumors were measured weekly with calipers and volume calculated (length × width × depth)2. Mice were killed at different time points; tumors and lungs were excised, weighed and photographed, respectively, and optimal cutting temperature compound embedded for analysis.

Immunohistochemistry

Mouse 4T1 tumors

To detect hAPRIL and Ki67, a 5-μm-thick cryosections were fixed in neutral-buffered formalin (Sigma–Aldrich), then incubated overnight with mouse-on-mouse blocking reagent (Vector Laboratories). Sections were reblocked with 4% goat serum in TBS-Tween (blocking solution, 1h) and incubated with anti-hAPRIL antibody (overnight, 4°C; Alexis Biochemicals), followed by alkaline phosphatase (AP)-anti-mouse antibody (Sigma–Aldrich). We subsequently incubated the sections with anti-Ki67 antibody (overnight, 4°C, Invitrogen), followed by horseradish peroxidase (HPR)-anti-rabbit antibody (Dako). AP was developed with the Blue AP substrate kit (Vector Laboratories) and HP with 3-amin-9-ethylcarbazole (AEC, Sigma–Aldrich). All slides were counterstained in Mayer’s hematoxylin (20–60 s; Sigma–Aldrich) and mounted with Faramount aqueous mounting medium (Sigma–Aldrich).

Human breast carcinoma samples

Slides of 5-μm-thick formalin-fixed paraffin-embedded breast tumor biopsies were prepared and sequentially deparaffinized. Antigen was heat-retrieved in 10 mM sodium citrate buffer (pH 8.6, 30 min). Endogenous peroxidase was blocked with TBS 3% H2O2; sections were incubated with primary anti-hAPRIL and -Ki67, and antigen-antibody complexes were detected as above for mouse sections.

Image analysis and statistics

Tumor sections were imaged with a Microdigital Camera (Olympus DP70) mounted on an axioplan microscope (Leica). We analyzed the percentage of Ki67 stained area with Image Pro V6.0 software (Media Cybernetics) and GraphPad Prism 5 (GraphPad Software) to assess statistical significance. For Ki67 quantification, at least 7 fields per mouse and 5 mice per group were analyzed. P values indicate comparisons between two groups using unpaired, two-tailed Student’s t tests; ***P < 0.0005, **P < 0.005 and *P < 0.05.

Results

Breast carcinoma cell lines express APRIL and its receptors BCMA and TACI. APRIL production is induced after TLR3 activation

To study the relationship between breast cancer and APRIL, we first analyzed APRIL expression in four human breast carcinoma cell lines with different features. MCF7 and T47D cells have a luminal-like phenotype and are ERα/PR/HER2−, and MDA-MB468 and MDA-MB231 cells are basal A and B phenotypes, respectively, and all are ERα/PR/HER2+ (36). Basal TNBC are generally more aggressive and invasive than luminal carcinomas (5). Quantitative expression analysis (qPCR) of April mRNA showed April transcripts in all four cell lines, with a maximum 19-fold difference between MCF7 (lowest) and MDA-MB468 (highest) (Figure 1A).

Unique in its family, APRIL protein is processed at the Golgi apparatus and secreted through vesicular transport outside the cell (39). Both full-length APRIL (~30kDa) and processed APRIL (~17kDa) can be detected intracellularly while only the processed form is secreted outside the cells. Protein analysis confirmed mRNA results and showed that luminal and basal breast carcinoma cells expressed both APRIL forms (Figure 1B and Supplementary Figure 7, available at Carcinogenesis Online), with higher levels in MDA-MB468 and T47D cells compared to MCF7 and MDA-MB231 cells. Multiple bands for full-length APRIL have been previously described and might be due to post-translational modifications (40). Confocal analysis for protein visualization and localization within cells showed APRIL in cytoplasm, with a punctate pattern typical of the Golgi apparatus (Figure 1C), where this cytokine is cleaved (39). These data demonstrate that breast carcinoma cells express APRIL independently of their luminal/basal phenotype.

We also analyzed APRIL receptors BCMA and TACI by qPCR and detected Bcma and Tac1 transcripts in the four cell lines, with higher levels in MDA-MB231 and MDA-MB468 compared to T47D and MCF cells (Figure 1D), suggesting a direct correlation between APRIL receptor expression and cell aggressiveness. Flow cytometry analysis confirmed BCMA and TACI expression in the cell lines (Supplementary Figure S1, available at Carcinogenesis Online).

Given that APRIL is expressed in breast carcinoma cells, we studied the factors that regulate its expression. As TLR ligands induce APRIL secretion in hematopoietic and intestinal epithelial cells (12,40), we tested poly I:C (TLR3 ligand), LPS (TLR4), and flagellin (TLR5). APRIL measurement in MDA-MB468 and MDA-MB231 supernatants at various times post-stimulation indicated that poly I:C, but not LPS or flagellin, clearly enhanced APRIL secretion (Figure 1E and Supplementary Figure 8, available at Carcinogenesis Online). Poly I:C is a mimic of viral double-stranded RNA; interest in TLR signaling in breast and other cancers has increased, given its pro- and antitumor activities (41,42). TLR3 activation induces apoptosis in some breast cancer cell lines (43,44), but also stimulates tumorigenesis (42), probably by activating molecules associated with tumor cell survival and apoptosis resistance. Indeed, we found that poly
I:C activated the NFκB signaling pathway in MDA-MB231 cells. Effects of TLR3 activation on APRIL secretion and NFκB function might explain its tumorigenic activity.

APRIL activates MAP kinases ERK1/2 and JNK1/2 and sustains breast carcinoma cell proliferation

The finding that breast carcinoma cells express TACI and BCMA predicted that signaling through these receptors might promote tumor growth. We analyzed APRIL-mediated signaling via the MAP kinases ERK1/2, p38 and JNK1/2, which have been implicated in breast cancer tumorigenesis (45). Analysis of the phosphorylation kinetics in MDA-MB231 cells, which have the highest receptor levels in our cell panel, showed that APRIL rapidly induced JNK1/2 phosphorylation, followed by that of ERK1/2 (Figure 2A and Supplementary Figure 10, available at Carcinogenesis Online). Slight phosphorylation was also observed for p38, indicating that the three MAP kinases are activated after APRIL binding in MDA-MB231 cells.

To explore APRIL-mediated biological effects, we studied cell proliferation in response to exogenous APRIL in a [3H]thymidine assay. APRIL enhanced proliferation in the four breast carcinoma cells with a greater effect on MDA-MB231 (149%) and MDA-MB468 (139%) compared to T47D (118%) and MCF7 cells (111%) (Figure 2B, % relative to untreated cells). We performed silencing experiments to test whether APRIL promotes tumor growth through autocrine signaling. APRIL was efficiently depleted by specific small interference RNA (ON-TARGETplus; Figure 2C for MDA-MB468 and Supplementary Figures 3 and 11, available at Carcinogenesis Online).
At 48 h, cell proliferation was significantly reduced in all four APRIL-depleted cell lines. MCF7-APRIL-KO cell proliferation was reduced by 37% of control values, whereas that of MDA-MB231-APRIL-KO cells was reduced by 51% of control values. The greater MDA-MB231 response compared to MCF7 carcinoma cells coincides with increased APRIL receptor levels. These results show that autocrine APRIL stimulates proliferation of basal and luminal breast cancer cells, confirming APRIL signaling is functional in breast carcinoma, with greater activity in aggressive MDA-MB231 cells.

Ectopic APRIL promotes tumor growth and metastasis in 4T1 orthotopic tumors through BCMA and TACI

APRIL is a soluble cytokine detectable in the serum of healthy donors that is increased in chronic lymphocytic leukemia patients (19). We hypothesized that in breast carcinoma patients, circulating APRIL can reach the tumor via the bloodstream and provide support to malignant cells. To determine the paracrine effect of APRIL on breast tumor promotion in vivo, we evaluated tumor growth and metastasis in a mouse model that overexpresses human APRIL (APRIL-Tg) (17). In these mice, hAPRIL is expressed under the lck distal promoter and is found in the circulation (Supplementary Figure 4A, available at Carcinogenesis Online). Mouse basal breast carcinoma 4T1 cells express preferentially TACI receptor and are APRIL-sensitive (Supplementary Figure 4B and C, available at Carcinogenesis Online). After 4T1 cells transplantation into the mammary fat pad, 4T1 tumors grew more rapidly and were larger in APRIL-Tg females than in the control littermates (Figure 3A). Significant differences in tumor volume were detected from day 25 (4T1Control, mean 71.3 ± 30.7 mm³; 4T1APRIL, 153.8 ± 38.7 mm³; day 25; P < 0.005) and in tumor weight from day 19 (4T1Control, 0.27 ± 0.03 g; 4T1APRIL, 0.39 ± 0.08 g; day 19; P < 0.05), until the end of the experiment.

Based on our in vitro results, we measured cell proliferation (Ki67) and human APRIL expression in tumor lesions by immunohistochemical analysis. 4T1 tumors from APRIL-Tg mice showed increased proliferation (measured as % Ki67-positive...
We examined the metastatic potential of 4T1 cells in the same type of assay, after cell transplantation into the mammary fat pad. At day 39, inspection of lungs and H&E analysis showed increased pulmonary colonization and larger metastases in APRIL-Tg mice than in wild type littermates (Figure 3D). To determine the role of APRIL receptors, we used qPCR to analyze...
BCMA and TACI in transplanted 4T1 tumors and lung metastases, and normalized mRNA levels to Pum1 gene. Results for both mouse genotypes showed a clear increase in Bcma and Taci transcripts in lung metastases compared to transplanted tumors (Figure 4), which suggests that both participate in the metastatic process. Bcma and Taci transcript levels were also significantly higher in lung metastases from APRIL-Tg compared to control mice (Figure 4), which was linked to increased endogenous April (Supplementary Figure 5, available at Carcinogenesis Online) and larger metastases in the transgenic mice. Transplanted 4T1 tumors showed no differences in Bcma, Taci and mApril mRNA between control and APRIL-Tg mice (Figure 4, Supplementary Figure 5, available at Carcinogenesis Online), suggesting that the pathway is active in both mouse groups and that enhanced tumor growth in APRIL-Tg mice is due to ectopic APRIL protein, that gives an additional ligand source to the tumors. These results provide evidence that APRIL contributes to breast carcinoma and enhances tumor growth and metastasis through BCMA and TACI receptors.

APRIL, BCMA and TACI are overexpressed in human basal-like breast carcinomas

To date, the APRIL pathway has not been analyzed in the distinct breast carcinoma subtypes. We measured BCMA and TACI by qPCR in a panel of ductal breast carcinomas containing luminal, TNBC and HER2 samples, the three most frequent subtypes (for sample information, see Supplementary Table 2, available at Carcinogenesis Online). We found April transcripts in most samples (13 of 14), with significantly increased levels in TNBC (0.037 ± 0.021) compared with luminal carcinomas (0.017 ± 0.012) (Figure 5A). For the receptors, we observed greater incidence of Bcma and Taci transcripts in TNBC and HER2 than in luminal samples (Figure 5B). Similar to April, Taci receptor levels were significantly higher in TNBC than in luminal carcinomas, suggesting direct correlation between the APRIL/TACI pathway and breast carcinoma aggressiveness.

We analyzed APRIL protein expression by IHC in tissue sections from nine ductal breast carcinomas (sample data in Supplementary Table 3, available at Carcinogenesis Online). APRIL staining in luminal, TNBC and HER2 carcinomas (Figure 6A, Supplementary Figure 6A, available at Carcinogenesis Online) confirmed our previous results; staining was detected at several sites within tumor sections, including epithelial cells of the basal layer near tumor areas, normal ducts, transformed tumor cell cytoplasm and the leukocyte infiltrate (Figure 6A, insets). Measurement of APRIL protein in the serum of breast carcinoma patients (n = 37; luminal, TNBC and Her2 subtypes) showed a concentration similar to that of healthy donor control sera (Supplementary Figure 6B, available at Carcinogenesis Online).

As APRIL stimulated breast carcinoma growth in our experimental models, we analyzed the proliferative status of APRIL-positive carcinomas and tested for simultaneous expression of APRIL and Ki67. APRIL-positive areas were also enriched in Ki67-nuclei (Figure 6B), with cells double positive for both markers (Figure 6B, inset). These data suggest that APRIL expressed in the tumor lesions sustains proliferation of the malignant breast carcinoma cells; more extensive studies are under way to confirm this observation.

Discussion

A sustained proliferative signal is one of the signatures of cancer cells. After cell transformation, several mechanisms promote proliferation and provide tumors with independence from normal regulation of cell control (2). We show that APRIL is expressed by malignant luminal and basal breast carcinoma cells, as well as by other cells in the tumor environment, to generate autoimmune and paracrine signaling loops that enhance tumor cell proliferation and promote tumor growth and metastasis.

APRIL was initially described in the late 1990s based on its ability to induce proliferation of distinct types of cell lines (10). Initial data showed that APRIL is overexpressed in leukemia and lymphoma, and promotes tumorigenesis, but newer reports indicate a role for this cytokine beyond hematopoietic malignancies (23–34). There are currently three descriptive studies of APRIL in breast cancer (23,33,34). They found overexpressed APRIL protein (23,33) or RNA (34) in tissue lesions although none of them analyzed specific histological subtypes or the implication of APRIL pathway in breast cancer promotion.

BCMA and TACI are expressed mainly and abundantly in lymphoid cells, with few reports regarding solid tumors. Our group and others have nonetheless described BCMA and/or TACI expression in non-lymphoid cell linages such as adipose-related cells (13), renal carcinoma (25), squamous and basal cell carcinoma (26), hepatocarcinoma (28), glioma (29) and glioblastoma (30). Variation between studies is probably based on the distinct approaches, tools and sample types used to detect these receptors.

Here, we show that four breast carcinoma cell lines as well as primary breast carcinomas express APRIL, BCMA and TACI, and that their expression is not restricted to the basal/luminal phenotype. The mechanisms that induce the APRIL pathway during
breast malignant transformation remain undetermined. We found that TLR3 activation in cell lines promotes APRIL secretion and activates the NFκB transcription factor, which might also explain why some breast carcinoma cells such as MDA-MB231 are resistant to poly I:C-induced apoptosis (43,44). NFκB is associated with tumor cell survival and apoptosis resistance (46), is reported to be more active in basal than in luminal breast carcinomas (47), and is crucial in promoting transcription of the human April gene (48). Further studies are needed to determine the link between NFκB and other signaling pathways with APRIL in breast carcinoma and to establish whether TLR3 mediates APRIL production in vivo. There is evidence that endogenous

Figure 5. APRIL, BCMA and TACI expression is increased in TNBC breast carcinomas. mRNA expression of (A) APRIL and (B) BCMA and TACI in a panel of luminal (n=7), TNBC (n=5), and HER2 (n=2) breast carcinoma samples, measured by qPCR relative to PPIA. Left graphs show mean ± SD from triplicate determinations; right graphs show mean ± SD of data grouped by sample subtype (luminal and TNBC samples). *P ≤ 0.05.
or damage-associated molecular pattern (DAMP) molecules released from damaged/necrotic tissues activate TLR and promote tumor development (49); this could also be the case for APRIL.

Coexpression of APRIL and its receptors enables autocrine proliferation of tumor cells, as we confirmed in APRIL silencing assays. Basal MDA-MB231 and MDA-MB468 cells were more responsive to APRIL than luminal-like MCF7 and T47D cells, based on their higher receptor expression pattern; this suggests that the APRIL pathway is more active in aggressive carcinoma cells. APRIL also binds HSPG on the tumor cell surface, which is critical for the proliferation-mediated effect (16). We thus cannot exclude that HSPG in the breast carcinoma cells might contribute to the differences observed by concentrating the ligand or by direct signaling. We show that after binding, APRIL induces phosphorylation of JNK1/2, ERK1/2 and p38 in MDA-MB231 cells. These three MAP kinases are overexpressed in breast cancer and their activation enhances cancer cell survival and proliferation (45). Switching on the APRIL pathway provides malignant cells a mechanism to sustain activation of their MAP kinase signaling and promote tumor progression. Breast carcinoma cells expressed BCMA and TACI receptors; it is possible that both of these as well as HSPG contribute to this effect. We reduced BCMA and TACI expression in MDA-MB231 cells using small interference RNA but we did not detect consistent differences in cell proliferation, attributable to the poor efficiency of receptor silencing. Current experiments using CRISPR technology (clustered, regularly interspaced, short palindromic repeats) to

Figure 6. Luminal, TNBC and HER2 carcinomas express APRIL protein heterogeneously and near proliferating areas. (A) Immunohistochemistry study of APRIL expression in paraaffin-embedded IDC. Representative images of APRIL staining (brown) are shown. Inset, magnifications of indicated areas showing APRIL at distinct sites, (a) basal epithelial layer, (b) ‘normal’ ducts, (c, d) tumor cell cytoplasm, and (e) inflammatory infiltrate. (B) Representative images of three IDC showing immunostaining of APRIL (blue) and Ki67 (brown). Numbers correspond to sample ID (see Supplementary Table 3, available at Carcinogenesis Online)
silence receptor genes will provide new data about the specific function of BCMA and TACI signaling in breast cancer.

In solid tumors, it has been described that APRIL triggers different signaling pathways depending on the cellular and tissue context inducing pleiotropic functions. APRIL binding to BCMA activates a JNK2-FoxO3-GADD45 pathway in HCC cell lines and promotes cell growth arrest (28). In addition, APRIL binding to BCMA in transfected 293 cells (50) and to TACI in B cell lines (15) activates NFkB and JNK pathways that are related to cell survival and proliferation. In breast cancer, knock down studies for BCMA and TACI are needed to clarify their specific function.

In addition to its autocrine role as a tumor-promoting factor, APRIL paracrine signaling is described in cancers such as leukemia or glioblastoma (reviewed in ref. 11). Our results indicate that this is probably to be the case in breast carcinoma, as we and others found APRIL expression in tumor cells, in tumor-infiltrating leukocytes, and in ‘normal’ epithelial cells near the tumor area (33). APRIL is detected in serum (17) and could reach tumor lesions via the circulation. Our data using orthotopic 4T1 tumor transplants in APRIL-overexpressing mice indicate that high serum APRIL levels promote tumor growth and lung metastasis and suggest that both BCMA and TACI have an important role in the metastatic process, as receptor expression was higher in the lungs compared to 4T1 transplanted tumors. In pancreatic cancer patients, serum APRIL levels are proposed as diagnostic and prognostic references, alone or in combination with other conventional markers (27). Our results nonetheless indicate that breast carcinoma behaves differently, as we observe no differences in serum APRIL levels.

There are currently no data regarding APRIL, BCMA and TACI expression in different breast carcinoma subtypes. We detected expression of all three in luminal, TNBC and HER2 carcinomas, and higher levels of APRIL and TACI mRNA transcripts in TNBC than in luminal samples, indicating that overexpression of APRIL and TACI in human breast cancer cells increases their aggressiveness and may result in poorer clinical outcome. Two reports have evaluated APRIL association with tumor grade in breast carcinoma, with contrasting results (33,34). This disparity might reflect the different experimental approaches used (protein versus mRNA), but also sample heterogeneity, as no specific subtypes were analyzed nor were histopathological features provided (hormone receptor, HER2 or Ki67 expression). We found higher APRIL expression in grade 3 TNBC than in grade 3 luminal samples (not shown), which underlines the importance of subtype analysis. Moreover, we and others found APRIL expression in normal epithelial structures (33) and in the inflammatory infiltrate of breast carcinoma tissue lesions, which implies a role for this cytokine in mammary tissue beyond cancer pathology and also in the inflammatory response. It would be interesting to investigate the function of APRIL in mammary gland development, in which cell proliferation and apoptosis are key events.

In summary, we identify an APRIL signaling pathway that functions and participates in human mammary tumorigenesis. Breast carcinoma cells express APRIL, BCMA and TACI to sustain their proliferation. Studies with human breast carcinoma samples and with mice indicate that APRIL signaling is linked to tumor cell aggressiveness, growth and metastasis.

Supplementary Data

Supplementary Tables 1–5 and Supplementary Figures 1–11 can be found at http://carcin.oxfordjournals.org/

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