ANCCCA/ATAD2 Overexpression Identifies Breast Cancer Patients with Poor Prognosis, Acting to Drive Proliferation and Survival of Triple-Negative Cells through Control of B-Myb and EZH2

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Abstract

Chromatin coregulators are important factors in tumorigenesis and cancer progression. ANCCA is an AAA+ ATPase and a bromodomain-containing nuclear coactivator for the estrogen and androgen receptors that is crucial for assembly of chromatin-modifying complexes and proliferation of hormone-responsive cancer cells. In this study, we show that ANCCA is overexpressed in >70% of breast tumors and that its high protein level correlates well with tumor histologic grades (P < 0.0001), highlighting ANCCA as a prognostic factor for poor overall survival and disease recurrence. Strikingly, high-level ANCCA correlated with triple-negative tumors that represent highly aggressive disease. Analysis of ANCCA transcript levels in multiple expression profiles of breast cancer identified ANCCA as a common signature gene, indicating that elevated transcripts also strongly correlate with tumor metastasis and poor survival. Biological and mechanistic investigations revealed that ANCCA is crucial for proliferation and survival of triple-negative/basal-like cancer cells and that it controls the expression of B-Myb, histone methyltransferase EZH2, and an Rb-E2F core program for proliferation, along with a subset of key mitotic kinesins and cell survival genes (IRS2, VEGF, and Akt1). In particular, ANCCA overexpression correlated strongly with EZH2 in tumors. Our results suggest that ANCCA may integrate multiple oncogenic programs in breast cancer, serving in particular as a prognostic marker and a therapeutic target for triple-negative cancers. Cancer Res; 70(22): 9402–12. ©2010 AACR.

Introduction

Functional genomics and gene-profiling studies of breast cancer continue to improve our prediction of clinical outcome and selection of therapeutics as well as our understanding of tumor biology, by subtyping the tumors on their molecular profiles. Among the subtypes, the luminal/estrogen receptor α (ERα)–positive tumors present themselves as more differentiated, less aggressive, and highly responsive to endocrine therapy. Tumors overexpressing human epidermal growth factor receptor 2 (HER2), although often more aggressive, are responsive to therapeutics targeting the growth factor receptors. The triple-negative [ER, progesterone receptor (PR), and HER2 negative] breast cancers (TNBC), however, remain a major challenge for the development of effective therapeutics and identification of risk factors (1). Recent expression array studies (2, 3) characterize most TNBCs as being basal-like due to an expression profile containing high levels of basal cell cytokeratins, similar to those of myoepithelial cells. Although poorly understood, one striking feature of TNBCs is the high expression of proliferation signature genes. Unlike ER-positive orHER2-high subtypes, no molecular markers have been defined that underpin TNBC development or effectively guide their clinical treatment. As a result, conventional chemotherapy remains the mainstay treatment for them. Therefore, there is an urgent need for the identification of risk factors and new treatment options for triple-negative tumors.

Chromatin coregulators, particularly in the forms of histone-modifying and chromatin-remodeling enzymes, have recently emerged as important players in tumorigenesis (4–6). We recently identified a previously uncharacterized gene product dubbed ANCCA (for AAA+ nuclear coregulator cancer associated) as both a direct target and an activator of the proto-oncogene AIB1 (also known as ACTR and SRC-3; refs. 7, 8). ANCCA, a novel member of the AAA+ ATPase
family, also possesses a bromodomain. We found that high levels of ANCCA are expressed in breast cancer and prostate cancer cells and that RNAi-mediated knockdown of ANCCA strongly inhibits hormone-dependent cancer cell proliferation (8, 9). We also showed that ANCCA acts as a coactivator of ERα and androgen receptor to mediate estrogen- or androgen-induced expression of specific subsets of genes involved in proliferation and survival of cancer cells. Both the AAA+ ATPase and bromodomains are required for ANCCA to serve as a transcriptional coregulator of the receptors (8). Our further study indicated that one major mechanism of ANCCA function is to facilitate the assembly of a histone-modifying protein complex at the chromatin (8).

In this study, we investigated ANCCA expression at both protein and transcript levels in multiple sets of human breast cancer specimens and found that ANCCA is overexpressed in the majority of the tumors. High levels of ANCCA directly correlate with poor survival and disease recurrence in the patients. We also present results that high ANCCA is strongly associated with triple-negative tumors and that aberrant ANCCA expression in TNBC cells controls multiple oncogenic pathways for cancer cell proliferation and survival.

Materials and Methods

Cell culture, siRNA transfection, chromatin immunoprecipitation assay, RNA analysis, and Western blotting

Human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC) in 2002 and 2008. They were tested recently (in 2009) for authenticity by monitoring cell morphology under a microscope; growth rate through cell counting; response to factors or hormones for growth; and expression of genes/proteins, including the hormone receptors and members of the HER2/ErbB2 family, through Western blotting and reverse transcriptase-PCR (RT-PCR). These cell lines were confirmed to be in line with the ATCC descriptions and with the literature. Human breast epithelial cells (HMEC) were from Lonza/Clonetics. All cells were used within 5 months after initial receipt or thawing. RNA analysis and Western blotting were performed as previously described (10). For transfection of siRNAs, cells plated at 2.0 × 10⁵ per well in six-well plates were transfected using Dharmafect 1 (Dharmacon) according to the manufacturer’s instructions. siRNA targeting ANCCA or control siRNA were used at 100 nmol/L concentration. Details of cell lines and their culture conditions, chromatin immunoprecipitation (ChIP), antibodies used for Western blotting, and sequences for PCR primers are described in Supplementary Materials and Methods.

Cell proliferation and soft-agar colony formation assays

HCC1937 cells were seeded at a density of 1.5 × 10⁵ per well of a six-well plate and maintained in full growth medium for 24 hours before being infected with equal titers of adenovector adenoviruses (8). MDA-MB 468 cells were plated at a density of 2.0 × 10⁵ per well in six-well plates, and were transfected with siRNA 24 hours later as described above. The medium was changed every other day after transfection, and cell proliferation was measured by cell counting of coded samples in triplicates. For colony formation in soft agar, 5,000 cells were seeded in each well of six-well plates in medium containing 0.4% SeakPlate Agarose (Lonza) on top of a base medium containing 0.8% agarose. Colonies were stained 4 to 5 weeks later with MT (Sigma Aldrich) and counted using a light microscope.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

Cells were plated and transfected with siRNA 24 hours later as described above. Cells were plated 72 hours later on glass chamber slides (Nunc) and processed for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay using the In Situ Cell Death Detection kit (Roche Diagnostics) according to the manufacturer’s protocol. Slides were mounted with VECTASHIELD Mounting Media (Vector Laboratories). Images were acquired using Olympus BX6. For quantification of TUNEL-positive cells, 10 random fields of the same condition were counted and averaged.

Tumor specimens and immunohistochemistry

Archival paraffin-embedded primary tumor samples were from 225 women diagnosed with breast carcinoma and treated at UC Davis Medical Center during 1998 to 2004. Other information on the patients and tumors are in Supplementary Table S1. Five-micrometer sections of the tumor blocks were first subjected to deparaffinization and then antigen retrieval in 0.01 mmol/L sodium citrate buffer (pH 6.0) in a microwave oven at 1,000 W for 5 minutes and then at 100 W for 30 minutes. Breast tumor tissue microarrays (TMA; BR1002, BR961, and BR208) were obtained from US BioMax, Inc., baked at 60°C for 2 hours, and processed for immunostaining. TMA for EZH2 and ANCCA correlation study has been described (7). Non-specific immunoglobulin binding was blocked using 10% fetal bovine serum in PBS for 30 minutes at room temperature. Slides were then incubated at room temperature for 30 minutes with anti-ANCCA antibody (at 1:300), anti–Ki-67 (at 1:1,000; NeoMarker), and anti-EZH2 (AC22; at 1:50; Cell Signaling). Anti-ANCCA antibody was raised in rabbit (Covance) and affinity-purified by using GST-ANCCA (amino acids 2–264) expressed and purified from Escherichia coli. Its specificity for immunohistochemistry (IHC) was determined using a panel of cell lines and xenograft tumors (9) and is also shown in Supplementary Fig. S1. After incubation with the primary antibody, the sections were washed and incubated with biotin-conjugated secondary antibodies for 30 minutes followed by incubation with avidin DH-biotinylated horseradish peroxidase and developed using a diaminobenzidine substrate kit (Vector Laboratories) and counterstained using Gill’s hematoxylin. Images were acquired using an Olympus microscope with DPController software. The percentage of positively stained nuclei was scored as follows: 0% to 10%, score 0; 11% to 25%, score 1; 26% to 50%,
score 2; >50%, score 3. The immunoreactivity was evaluated by at least two different investigators with no prior knowledge of patient data.

Statistical analyses and analysis of microarray gene expression data sets

Values of patient age and tumor size are presented as mean ± SEM. Association between ANCCA immunoreactivity and other clinicopathologic parameters was evaluated using Pearson’s χ² test. Survival curves were generated using the Kaplan-Meier method, taking into account censored data. The curves were compared using the log-rank test (Mantel-Cox). For other assays and analysis of gene transcripts in tumor data sets using Oncomine (http://www.oncomine.org), Student’s (or paired) t test was used for comparison of experimental groups. Statistical analysis was performed using the SPSS software (version 18; SPSS, Inc.). P values of less than 0.05 were considered significant. The method for analysis of microarray gene expression data sets is provided in the Supplementary Methods.

Results

ANCCA, a “signature” gene, is overexpressed in >70% of human breast carcinomas and its overexpression is associated with a triple-negative status

To investigate ANCCA expression in breast cancer, we first performed IHC analysis of ANCCA protein expression in a cohort of 225 primary human breast ductal carcinomas and three independent sets of TMAs containing a total of 131 tumor samples and 24 normal breast tissues. Immunoreactivity for ANCCA was readily detected in the nuclei of a large subset of tumor tissues, whereas little or no staining was observed in the normal breast tissues or tumor-adjacent stroma (Fig. 1; Supplementary Fig. S2). When compared with normal breast tissue, more than 70% of all tumor samples examined showed increased levels of ANCCA protein expression (Table 1; Supplementary Table S2). Interestingly, although a large proportion (63%) of ERα-positive tumors displayed ANCCA overexpression, a much stronger association for its overexpression in ERα- and PR-negative tumors was observed (Table 1). More importantly, high levels of ANCCA associate significantly (P = 0.0071) with ERα-, PR- and HER2-negative status as more than 88% of all triple-negative samples showed high expression of ANCCA protein. Moreover, compared with normal HMEC, elevated levels of ANCCA proteins (~170 kDa) was observed in all of the breast cancer cell lines examined (Fig. 2A; Supplementary Fig. S1).

We also interrogated multiple microarray gene expression data sets for ANCCA mRNA change in normal and tumor tissues. Interestingly, ANCCA, listed as pro2000, atad2 or other names, is one of the few genes that overlap between gene signatures identified by several gene-profiling studies for tumor classification and prediction of disease outcome (e.g., ductal carcinoma in situ to infiltrating ductal carcinoma, time to distant metastasis; Supplementary Table S3). Consistent with our IHC analysis, the ANCCA transcript examined in multiple studies displayed much higher levels in tumors than in normal tissues.

Figure 1. IHC analysis of ANCCA expression in normal or cancerous human breast tissues. Representative images are shown for IHC score 0 with less than 10% of nuclei stained positive, in a histologically normal breast tissue (A) and in a tumor (B); for score 1 with <25% of positively stained nuclei in a tumor (C); for score 2 with 25% to 50% of positively stained nuclei in a tumor (D); and for score 3 with >50% of positively stained nuclei in an ER-positive tumor (E) and an ER-negative tumor (F).
normal breast tissues (Supplementary Fig. S3), and its high transcript levels strongly associated with the ER-negative or triple-negative status ($P < 0.009$ and $P < 0.0001$, respectively; Supplementary Table S4).

ANCCA overexpression correlates with tumor cell proliferation and disease progression

We next assessed the association of ANCCA expression with other clinical and pathologic variables (Table 1). Although no clear association was found between ANCCA level and lymph node status, we observed that high levels of ANCCA expression correlate strongly with higher histologic grades ($P < 0.0001$). Overall, more differentiated, low-grade tumors showed little to moderate expression of ANCCA, whereas less differentiated, high-grade tumors had markedly elevated levels of ANCCA protein (Fig. 2B and C; Supplementary Fig. S2). Similar results were obtained from analysis of ANCCA transcripts in a cohort of tumors used in a previous study by van’t Veer and colleagues (ref. 11; Fig. 2C). Consistent with the notion that basal-like/triple-negative tumors tend to

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**Table 1. Tumor characteristics of 225 cases of primary breast carcinoma analyzed by IHC**

<table>
<thead>
<tr>
<th>ANCCA score</th>
<th>0–1 N (%)</th>
<th>2–3 N (%)</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC all grade</td>
<td>49 (22)</td>
<td>176 (78)</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13 (45)</td>
<td>16 (55)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20 (27)</td>
<td>54 (73)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>12 (16)</td>
<td>63 (84)</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>12 (26)</td>
<td>34 (74)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>14 (20)</td>
<td>57 (80)</td>
<td>0.4</td>
</tr>
<tr>
<td>Hormone receptor status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER positive</td>
<td>31 (37)</td>
<td>53 (63)</td>
<td></td>
</tr>
<tr>
<td>ER negative</td>
<td>16 (14)</td>
<td>100 (86)</td>
<td>0.0004</td>
</tr>
<tr>
<td>PR positive</td>
<td>25 (35)</td>
<td>46 (65)</td>
<td></td>
</tr>
<tr>
<td>PR negative</td>
<td>17 (13)</td>
<td>112 (87)</td>
<td>0.0005</td>
</tr>
<tr>
<td>HER2 status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8 (20)</td>
<td>31 (80)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>39 (26)</td>
<td>112 (74)</td>
<td>0.63</td>
</tr>
<tr>
<td>TN</td>
<td>8 (12)</td>
<td>62 (88)</td>
<td></td>
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<td>NTN (all grades)</td>
<td>41 (31)</td>
<td>91 (69)</td>
<td></td>
</tr>
<tr>
<td>NTN (high grade)</td>
<td>27 (31)</td>
<td>59 (69)</td>
<td>0.01</td>
</tr>
<tr>
<td>KI67 (TN)</td>
<td></td>
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<tr>
<td>High</td>
<td>5 (7.5)</td>
<td>47 (70)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>3 (4.5)</td>
<td>12 (18)</td>
<td>$&lt;0.0001$</td>
</tr>
</tbody>
</table>

NOTE: IHC results are presented as frequency with the percentage of cases in parenthesis. Abbreviations: TN, triple negative tumors; NTN, non-triple negative tumors. *Pearson’s $\chi^2$ test, for association with grades and triple negative breast cancer was used.

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**Figure 2. ANCCA overexpression in breast cancer cell lines and its correlation with tumor grades.**

A. Western blot analysis of ANCCA in normal HMEC and different breast cancer cell lines. Top and middle, short and long exposure, respectively. B, distribution of tumors with low (yellow), moderate (green), and high (orange) expression of ANCCA in breast carcinomas (BC) of different grades ($P < 0.0001$). C, box-and-whisker plot of ANCCA mRNA expression in tumors of different grades analyzed using Oncomine (http://www.oncomine.org).
display high proliferation index (1), the majority (70%) of ANCCA-overexpressing triple-negative tumors also showed high Ki-67 staining ($P < 0.0001$; Table 1). IHC analysis of ANCCA and Ki-67 expression in adjacent sections of tumor specimens also revealed a high accordance in their expression in individual tumors (Supplementary Fig. S4).

**Highly elevated ANCCA predicts poor overall survival and disease recurrence**

We also examined whether strong ANCCA expression is predictive of disease progression by analysis of a cohort of 185 patients with up to 11 years of follow-up information. Tumors were divided into two groups (low ANCCA and high ANCCA) according to ANCCA IHC scores. As shown in Fig. 3A, patients whose tumors showed high ANCCA expres-

![Figure 3. ANCCA overexpression correlates with poor outcomes. Kaplan-Meier analysis of ANCCA protein expression and disease-free survival (A) and disease recurrence (B). C, high level of ANCCA transcripts correlates with poor disease-free survival in tumor data sets from Ivshina and colleagues (26), and associates with metastasis at 5 years after diagnosis in tumor data sets from Wang and colleagues (19) and van’t Veer and colleagues (11). The data sets were analyzed for association by using Oncomine (http://www.oncomine.org).](http://www.oncomine.org)
ANCCA overexpression promotes anchorage-dependent and anchorage-independent proliferation and survival of TNBC cells

Because expression of ANCCA was particularly high in triple-negative tumors, we examined the role of overexpressed ANCCA in TNBC cells. As shown in Fig. 2A, ANCCA is highly overexpressed in the breast cancer cell lines relative to HMECs. Knockdown of ANCCA through one siRNA in MDA-MB-468 (Fig. 4A), MDA-MB-231 (Fig. 4C), and MDA-MB-453 (Supplementary Fig. S6) cells showed strong inhibitory effects on their proliferation either in regular two-dimensional culture or in a soft-agar, anchorage-independent growth assay. Knockdown of ANCCA by using a different siRNA gave a similar effect (Supplementary Fig. S7A and B). In the BRCA1-defective HCC1937 cells that express a moderate level of ANCCA, adenovirus vector–mediated, ectopic expression of ANCCA led to significantly increased proliferation when compared with cells infected with the adenovector control (Fig. 4B).

We next examined whether ANCCA was also important for survival of triple-negative cells. MDA-MB-231 cells were transfected with siRNA and processed for TUNEL assay. As shown in Fig. 4D and Supplementary Fig. S8, whereas mock- or control siRNA–transfected cells had very few cells positive for TUNEL staining (<3%), cultures treated with ANCCA siRNA showed a marked increase (~25%) of TUNEL-positive cells. Similar results were obtained with MDA-MB-453 and MDA-MB-468 cells (data not shown). Taken together, these results provide strong evidence that ANCCA overexpression promotes both proliferation and survival of TNBC cells.

ANCCA controls important regulators of cell proliferation and survival pathways, including EZH2 and B-Myb

Given the transcriptional coactivator function of ANCCA shown in our previous study (7–9), we analyzed the TRANSBIG Consortium gene expression data set (12), in which ANCCA serves as a prognostic signature gene, for genes having aberrant coexpression associated with overexpressed ANCCA. Strikingly, the analysis identified a group of genes highly enriched in control of cell proliferation and survival with the top 20 genes (25 gene probes) functioning primarily in mitosis (DLGAP5, BUB1, CEP55, KIF4A, KIF11, KIF15, KIF23, DSCC1, ECT2, SMC2, SMC4, GPSM2), DNA replication (MCM10 and Top2A), and cell cycle progression (Fig. 5A; Supplementary Fig. S9). Intriguingly, consistent with our IHC study, hierarchical clustering showed that high levels of the gene transcripts, including ANCCA, cluster primarily in ER-negative tumors (Fig. 5A, at the left side of the cluster where tumors are primarily ER-negative indicated by the red bars at the bottom). More importantly, results from ANCCA knockdown in two different TNBC cells showed that most of these genes are indeed controlled by ANCCA because their expression is downregulated upon ANCCA suppression (Fig. 5A, right; Supplementary Fig. S10A and B). Further analysis showed that additional key cell cycle and DNA replication regulators, including cyclin E1, cyclin B1, cyclin A2, E2F1, Cdc6, MCM7, and proliferating cell nuclear antigen, are also regulated by ANCCA (Fig. 5B, middle; Figure 4. Elevated ANCCA is required for proliferation and survival of TNBC cells. A and B, cells were either infected with equal titers of adeno-ANCCA or adeno-control (HCC1937) or transfected with siRNA targeting ANCCA or control sequence (MDA-MB-468). Western blot and cell proliferation assays were performed with cells harvested 48 hours later (for Western blot) or at the indicated days. C, MDA-MB 231 cells were transfected with siRNA-ANCCA or siRNA-control and plated in soft agar for colony formation or Western blotting. Colonies were stained with MTT 4 weeks later and counted. Representative images of colonies and their numbers from different treatments are shown. D, MDA-MB 231 cells were processed for TUNEL assay (left) or Western blotting, at day 3 after siRNA transfection. Paired-samples t test (A and B) or independent-samples t test (C and D) was used.
Figure A shows a heatmap of gene expression levels with relative transcript levels for various genes such as TOP2A, CDC2, CCNA2, DLGAP5, BUB1, CEP55, KIF4A, MCM10, KIF11, KIF15, RAD51AP1, NCPAG, KIF23, DSCC1, ECT2, SMC2, ANCCA, CCNE2, SMC4, GPSPM2, and TAF2. The heatmap is color-coded to indicate ER-positive or ER-negative status.

Figure B displays Western blots for ANCCA, ACTIN, IRS2, VEGFα, SGK1, Akt1, and pAkt1 under conditions of si-ANCCA and si-Chi. Additionally, the blots show expression levels of B-Myb, EZH2, and SUZ12.

Figure C presents a 3D bar graph indicating the number of cases with significance levels (P = 0.000026) for α-EZH2 and α-ANCCA.

Figure D illustrates the input (%) for various genes, including TOP2A, CDC2, CCNA2, DLGAP5, BUB1, KIF4A, MCM10, KIF11, KIF15, GAPDH, and ANCCA, showing differences between preimmune and anti-ANCCA conditions.
Supplementary Fig. S7C). Moreover, consistent with the critical function of ANCCA in TNBC cell survival, we found that expression of survival pathway proteins such as IRS2, SKG1, vascular endothelial growth factor α (VEGFRα), Akt, and phosphorylated Akt was also significantly affected by ANCCA knockdown (Fig. 5B, left).

Because overexpression of polycomb group protein (PcG) EZH2 and mitotic regulator B-Myb seems to correlate with high proliferation of basal-like tumors (13, 14), we examined whether ANCCA also controls their expression. Indeed, silencing of ANCCA markedly inhibited EZH2 and B-Myb expression at both the RNA and protein levels in TNBC cells (Fig. 5B, right), and the inhibition was also observed for Suzz12 protein, a critical component of the polycomb-repressive complex 2 (PRC2). Therefore, these data show that EZH2 and B-Myb are both downstream targets of ANCCA. To address the disease relevance of ANCCA control of EZH2, we examined by IHC the expression of ANCCA and EZH2 in a cohort of 48 invasive breast carcinoma specimens on a TMA. Consistent with previous findings (15), we found that EZH2 is overexpressed in 62% of the invasive tumors (Fig. 5C; Supplementary Fig. S11). Importantly, ANCCA overexpression was significantly correlated to the expression of EZH2 in the primary tumors (P < 0.000026). These results suggest that deregulated ANCCA may contribute to EZH2 overexpression in the tumors. Finally, to determine whether ANCCA directly controls the expression of any of the 20 genes identified, we performed ChIP assays. With PCR primers that can readily amplify the proximal promoter region of indicated genes, we found that ANCCA occupies the promoter region of Top2a, cdcl2, cyclin A2, DLGAP5, Bub1, MCM10, Kif-4A, Kif-11, and Kif-15 in MDA-MB-436 cells (Fig. 5D). Consistent with our data that ANCCA does not regulate glyceraldehyde-3-phosphate dehydrogenase (GAPDH), we did not detect any significant anti-ANCCA enrichment at the promoter of GAPDH. Similar ChIP results were obtained from MDA-MB-468 cells (Supplementary Fig. S10C). Together, our data indicate that ANCCA is directly involved in control of key components of cell proliferation and survival pathways in TNBC cells.

Discussion

TNBCs, particularly those with basal-like gene expression profiles, tend to show highly elevated mitotic and cell proliferation indices and poor prognosis for long-term survival (1). Aberrations of several key transcriptional regulators (e.g., B-Myb, EZH2 or c-Myc overexpression as well as pRb-E2F deficiency) may underlie the elevated expression of proliferation-associated genes (13, 16). Whether their abnormal functions represent independent events in different tumors or are integrated by other mechanisms is unclear. We initially identified ANCCA as a direct target of the oncogene AIB1/ACTR and as a transcriptional coregulator for E2F and AR to promote the expression of genes driving cancer cell proliferation and survival (8, 9). Recent studies from us and others suggest that ANCCA is overexpressed in many human cancers, including breast cancer, and may act as a coactivator of c-Myc (8, 9, 17). However, its role in breast cancer progression has been poorly understood. In this study, we examined ANCCA expression in several cohorts of human breast cancer specimens and found that its high levels were significantly associated with poor overall survival and disease recurrence. Interestingly, ANCCA overexpression correlated strongly with the triple-negative subtype of breast cancer. The results of in vitro experiments further showed that high levels of ANCCA were required to maintain proliferation and survival of TNBC cells.

Tumor gene expression signatures have been instrumental in advancing our understanding of the molecular mechanisms underlying the diverse biological phenotypes of breast cancers and now have become a proven utility for prediction of clinical outcomes, such as tumor responsiveness to chemotherapy (2, 11, 18–23). However, only a few genes seem to be shared across all of the different signatures identified by various studies. Interestingly, ANCCA, which was listed as pro2000, ATAD2, or other undefined names, is one of the few genes that overlap frequently between different signatures. For instance, ANCCA is one of the 231 genes that gave rise to the 70-gene signature that predicts a clinical outcome of short interval to distant metastasis (11). It is also one of the 76 genes identified for prediction of distant metastasis of ER-positive, lymph node–negative, primary breast cancer, which was later validated in a multicenter study (12, 19, 24, 25). Consistent with our IHC results from an independent cohort of tumors showing that ANCCA protein levels alone predict tumor grades, a high level of ANCCA transcript is a constituent of the genetic grading signature that can reclassify histologic grades of breast cancer (26–28). We also found that ANCCA overexpression strongly correlated with elevated expression of proliferation-associated genes that are often components of the different gene signatures, thereby suggesting that overexpressed ANCCA may either collaborate with them or act upstream to stimulate their expression in the tumors. Intriguingly, ANCCA is not a signature gene for the core serum response signature, which largely overlaps with

Figure 5. ANCCA controls key regulators of cell proliferation and survival pathways including the oncogene EZH2. A, the top 20 genes (25 probe sets, not including ANCCA) found to be coexpressed with ANCCA in a set of 198 patients by unsupervised hierarchical clustering (12). Correlation coefficients (by Spearman’s test) for the similarity of the expression of each gene to that of ANCCA expression and other information are indicated in Supplementary Fig. S9. Tumor ER status is indicated below by blue bars for ER-positive tumors (a total of 134) or red bars for ER-negative tumors (a total of 64). Right, MDA-MB468 cells were transfected with siRNA targeting ANCCA (dark blue) or control sequence (yellow) and harvested 48 hours later for real-time RT-PCR analysis. Relative transcript levels were obtained by normalization of expression units for each gene with that of GAPDH. B, MDA-MB 468 cells were treated as above and analyzed by RT-PCR or Western blotting. C, the anti-ANCCA and anti-EZH2 immunoreactivity of a core in the TMA section was scored negative if <10% of the epithelial cells displayed staining with moderate to high intensity. The association between the different antibody staining was analyzed using Pearson’s χ² test. D, ChIP with control serum and anti-ANCCA antibody was performed with MDA-MB-436 cells. CHIP DNA was analyzed by real-time PCR. Anti-ANCCA enrichments are presented as percentage of input.
cell proliferation genes (29), but instead is part of the phospho-
ositide 3-kinase signature (30), suggesting that, in some cases,
elevated ANCCA may function primarily to promote cancer cell
survival. Our data from cell culture studies indicate that both
proproliferation and prosurvival genes are indeed controlled by
ANCCA. Thus, ANCCA overexpression may not only constitute
a part of the gene signature with prognostic value, but also act
as a driving force for the altered expression of the signature
genesis. Additional studies are needed to determine whether
ANCCA coordinates the tumor signature gene programs.

It is worth noting that in one of the microarray data sets
examined (12), the 20 genes most tightly associated with
overexpressed ANCCA (i.e., based on correlation tests) play
important roles in mitosis and/or cell proliferation, the
majority of which were found overexpressed in breast cancer
or other types of cancers. When deregulated, Top2A, cdc2,
and cyclin E, as well as the other targets of ANCCA identified
in this study (e.g., cdc6, B-Myb and EZH2), can display onco-
genic activities. Intriguingly, four members of the human
kinesin family (a total of 45 genes) were co-overexpressed
with ANCCA and were validated as ANCCA targets. These
kinesins function in different stages of mitosis for spindle
assembly and chromosome segregation (31, 32). The aberrant
functions of kinesins in cancer render them attractive therapeu-
tic targets (31, 33), or in certain circumstances, may
promote cancer cell resistance to taxane-based drugs (34).
Together with the role of ANCCA in mediating expression of pro-
survival genes (VEGF, IRS2, SGK, and Akt), our data suggest
that overexpressed ANCCA may function as a dominant node
for integrating and/or eliciting multiple gene expression pro-
grams to promote breast cancer progression. Deregression
of the multiple pathways identified here could constitute a part
of the molecular basis for the observed association between
highly elevated ANCCA protein and poor patient outcome.

Despite our initial identification of ANCCA as a hormone-
induced gene (8, 9), we show here that high levels of ANCCA
protein associate most strongly with TNBCs. We also show
that high transcript levels of ANCCA tend to associate with
the ER-negative or triple-negative status in several data sets
from studies of multiple cohorts of tumors (19, 26, 35). More-
over, our unbiased analysis of tumor gene expression data
sets points to the association of high levels of ANCCA with
the overexpression of other proliferation genes occurring
primarily in ER-negative tumors. How ANCCA is deregulat-
ed in ER-negative breast cancers is currently unclear.
Results obtained by Ciro and colleagues reported that
ANCCA is regulated by the pRb-E2F pathway in fibroblasts
and osteosarcoma cells (17). Whether this is the major mech-
nanism for ANCCA overexpression in breast cancer awaits
further analysis. Given that one salient feature of triple-
negative/basal-like tumors is their high proliferation index
and functional loss of the pRb-E2F pathway (1, 16, 35, 36),
deregulation of ANCCA through loss of pRb-mediated con-
rol is an attractive possibility. On the other hand, ANCCA
may not be merely a downstream target of pRb-E2F because
ANCCA itself mediates expression of multiple Rb-E2F target
genesis critical for TNBC cell proliferation.

Several transcriptional coregulators, including the ones
involved in steroid hormone signaling, have been strongly
implicated in human malignancies (37–42). AIB1 (also known
as ACTR/SRC-3) was initially identified as a gene amplified in
breast cancer and a coactivator for ERs (43). Later studies
revealed that its overexpression does not correlate with ER
and PR status and that its aberrant function may include
stimulation of tumor growth of both hormone-dependent
and hormone-independent breast cancers through ER-
dependent and ER-independent pathways (44–48). One
distinction between ACTR and ANCCA is that whereas ACTR
overexpression correlates strongly with high HER2 expres-
sion, high levels of ANCCA do not, but instead associate with
the triple-negative status. Although such distinction implies
a different mechanism for their aberrant expression, our re-
cent studies suggest that ANCCA and ACTR may regulate the
expression of each other in certain circumstances (7, 8). EZH2,
the histone methyltransferase subunit of the PRC2 complex,
is frequently overexpressed in multiple types of
cancer (15, 49). Like ANCCA, EZH2 overexpression in breast
cancer correlates with high proliferation and basal-like phen-
type and tumor invasiveness (14). ANCCA, however, is
unique in that it seems to play a critical role in the control
of expression of proliferative genes including EZH2 and
B-Myb in triple-negative cancers. Thus, our data suggest that
ANCCA may act as an integrator of several oncogenic pro-
grams. Given our finding that ANCCA is a transcriptional
coregulator, it is reasonable to predict that a major mecha-

ism of overexpressed ANCCA in breast cancer is alteration of
multiple gene networks, including those of EZH2, pRb-E2F
(this study), or c-Myc (17). Our previous study showed
that ANCCA is an AAA+ ATPase protein and that its ATPase
activity is required for its transcriptional stimulation
function (8). ANCCA also possesses a bromodomain that
may recognize a distinct histone modification. Given these
structure-function features of ANCCA and its aberrant
expression in multiple types of human cancers, further stud-
ies are warranted to exploit the potential of aberrant ANCCA
as a new prognostic marker and a therapeutic target.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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