ZNF217 Is a Marker of Poor Prognosis in Breast Cancer That Drives Epithelial–Mesenchymal Transition and Invasion

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Abstract

The Krüppel-like zinc finger protein ZNF217 is a candidate oncogene in breast cancer. In this study, we showed that high levels of expression of ZNF217 mRNA are associated with poor prognosis and the development of metastases in breast cancer. Overexpression of ZNF217 in breast cancer cells stimulated migration and invasion in vitro and promoted the development of spontaneous lung or node metastases in mice in vivo. ZNF217 also promoted epithelial–mesenchymal transition (EMT) in human mammary epithelial cells, and the TGF-β–activated Smad signaling pathway was identified as a major driver of ZNF217-induced EMT. In addition, a TGF-β autocrine loop sustained activation of the TGF-β pathway in ZNF217-overexpressing mammary epithelial cells, most likely because of ZNF217-mediated direct upregulation of TGFB2 or TGFB3. Inhibition of the TGF-β pathway led to the reversal of ZNF217-mediated EMT. Together, our findings indicate that ZNF217 mRNA expression may represent a novel prognostic biomarker in breast cancer. Therapeutic targeting of ZNF217 of the TGF-β signaling pathway may benefit the subset of patients whose tumors express high levels of ZNF217. Cancer Res. 72(14): 3593–606. ©2012 AACR.

Introduction

ZNF217 is a candidate oncogene located on chromosome 20q13.2, a region that is frequently amplified in many tumors, including those of the breast (1). The first direct evidence for a role for ZNF217 in oncogenesis was that ZNF217 could give rise to immortalized cells (2, 3). ZNF217 is a Krüppel-like zinc finger protein that localizes to the nucleus (4) and interacts with corepressors and histone-modifying proteins, suggesting that it may be part of a transcriptional repressor complex (5–7). However, the role of ZNF217 in transcriptional regulation is likely to be complex, as ZNF217 has also been shown to induce positive transcriptional regulation of target genes (8, 9). Previous studies have found that high levels of ZNF217 expression have been associated with resistance to chemotherapy and with deregulated apoptotic signals in breast cancer cells (10, 11). The precise molecular mechanisms involved in ZNF217 prosurvival functions are currently unknown, but activation of the Akt pathway (10), ErbB3 overexpression (12), Aurora-A overexpression (11), deregulated expression of several members of the Bcl-2 family (11), eEF1A2 overexpression (13), and interaction with the HIF pathway (14) have been proposed.

High-level amplification of 20q13 is found in 6.8% (15), 8% (16), and 18% (17) of breast cancers. Although amplification of 20q13 has been correlated with poor prognosis in breast cancer (15), contradictory data have emerged (16, 17). Although high levels of ZNF217 expression have been mainly attributed to amplification of ZNF217 (18), other mechanisms may be important in regulating ZNF217 expression in cancer cells. Indeed, high levels of ZNF217 expression have been observed in breast tumors and breast cancer cell lines in the absence of amplification (4, 18), and no statistical correlation between ZNF217 mRNA expression levels and ZNF217 amplification was found in breast tumors in which only the ZNF217 locus of the 20q13 region was amplified (16).

In this context, we thus sought to assess the potential value of ZNF217 mRNA expression levels (independently of ZNF217 amplification status) as a biomarker in breast cancer. Our study reports for the first time the strong prognostic value of ZNF217 mRNA expression levels in several independent breast cancer cohorts. We also show that ZNF217 overexpression confers invasive properties, both in vitro and in vivo, and...
promotes epithelial–mesenchymal transition (EMT). Finally, biochemical and transcriptomic investigations allowed us to identify the molecular pathways involved in the ZNF217-driven phenotype.

Materials and Methods

Breast tumor cohorts
Women with primary breast tumors who had not received any therapy before surgery and who did (Met+ group) or did not (Met- group) develop metastases while receiving chemotherapy and/or endocrine therapy were from the Centre Léon Bérard (CLB, Lyon) or from the Centre René Huguenin (CRH, St Cloud; Supplementary Materials and Methods, Tables S1 and S2). Total RNA was extracted from frozen tumor samples as previously described (19). Gene expression data from Ma and colleagues’ study (ref. 20; GSE1379) or Kaplan–Meier plotter (21) were also investigated.

Quantitative Real-time PCR
Quantitative Real-time PCR (qRT-PCR) measurements were carried out as described previously (22). All statistical analyses assessing ZNF217 prognostic value were carried out using SPSS Software. The data are divided at the median value of ZNF217 mRNA expression into 2 groups with either high or low expression. P value less than 0.05 was considered to be statistically significant.

Immunohistochemistry
Experiments were carried out as previously described (ref. 22; see Supplementary Materials and Methods).

Cell culture
MDA-MB-231, MCF10A, and HEK-293T cells were purchased from American Type Culture Collection (ATCC) and cultured as recommended. These cell lines have been routinely tested and authenticated by the ATCC. The identity of the MDA-MB-231 cells was also confirmed by sequencing KRAS and TP53 genes. The D3H2LN cells were purchased from Caliper and tested for invasive capacities by Boyden chamber and in vivo investigation.

ZNF217 stable transfectants
MDA-MB-231-pcDNA6, ZNF217-1, and ZNF217-2 cells have been described previously (11). Populations of MCF10A-pcDNA6 and MCF10A-ZNF217 stable transfectants were obtained using the same plasmid and selection process as described in Thollet and colleagues (11).

Western blots
The antibodies used were anti-ZNF217 (Abcam Ltd); anti-phospho-FAKY997 and phospho-FAKY577 (Invitrogen); anti-FAK and anti-ErbB2 (Santa Cruz Biotechnology Inc.); anti-E-cadherin, anti–α-catenin, anti–β-catenin, and anti-N-cadherin (BD Biosciences); anti-occludin (Zymed Laboratories); anti-vimentin (Dako); anti–α-tubulin (Sigma-Aldrich); and anti-ErbB3 (Cell Signalling).

Gene silencing
Cells were transfected with 5 nmol/L of Stealth siRNAs targeting ZNF217 (Invitrogen; ref. 11), siRNAs targeting Smad4 (Dharmacon; ref. 23) or scrambled control RNA (Invitrogen).

Soft-agar colony-formation assay
Experiment was carried out as previously described (24). Twenty days later, the cells were stained with 0.005% Cristal violet (Sigma-Aldrich) for 1 hour.

Wound healing assay
Cells were plated, the medium was removed, and replaced by FBS free medium 16 hours before wounding of the confluent monolayer by scratching.

Matrigel invasion analysis
A total of 105 cells were suspended in 1 μL Dulbecco’s Modified Eagle’s Medium and seeded in the middle of a Matrigel layer (BD Biosciences).

Boyden chamber invasion assay
A total of 5 × 104 cells were plated on top of a thick layer of Matrigel in Transwell chambers (Fluoroblock; BD Biosciences). Cell invasion (24 hours) and quantification were carried out as described in Vinot and colleagues (25).

In vivo experiments
Populations of 2 × 106 stably transfected MDA-MB-231-pcDNA6 and MDA-MB-231-ZNF217 cells were suspended in PBS/Matrigel v/v (BD Biosciences) and injected into the mammary fat pad of female Swiss nude mice (Charles River). After sacrifice, the presence of metastases in the lung and the lymph nodes was detected by histologic analysis of tissue sections stained with hematoxylin and eosin.

Luciferase stably transfected MDA-MB-231-ZNF217 or MDA-MB-231-pcDNA6 cell populations were produced. Female athymic Swiss nude mice (Janvier) were each injected with 106 cells suspended in PBS. Before each bioluminescence imaging (IVIS Kinetic; Caliper), anesthetized mice received an intraperitoneal injection of Luciferin (Promega).

All animal studies were conducted in accordance with European Union guidelines and approved by the regional ethics committee.

Transcriptomic analysis
Experiments were carried out as previously described (ref. 26; see Supplementary Materials and Methods).

Immunofluorescence assay
For E-cadherin staining (23), the E-cadherin antibody and the Alexa Fluor 488 anti-mouse antibody were used. For actin staining (23), cells were incubated with tetramethyl rhodamine isothiocyanate–conjugated phalloidin (Sigma-Aldrich). Images were acquired using an Axioplan 2 microscope (Carl Zeiss). Recombinant human TGF-β1 (5 ng/mL) was washed in PEP and SB431542 (10 nmol/L) from Sigma.
Luciferase assay

HEK-293T cells were transfected with 150 ng of CAGA-luc plasmid (with repeated CAGA Smad-binding elements controlling firefly luciferase gene transcription; ref. 27) and 10 ng of pTK-RL (Renilla luciferase plasmid). Twenty-four hours later, supernatants from MCF10A-pcDNA6 or MCF10A-ZNF217 cells were incubated for 24 hours with transfected HEK-293T cells and luciferase activities were assessed.

Chromatin immunoprecipitation

Experiments were carried out as previously described using anti-ZNF217 antibody or anti-rabbit IgG (Abcam Ltd) and Fig. S1C), supporting our previous results (11). Anchorage-independent growth is thought to be associated with aggressiveness and metastasis in malignant cells. We found that the number of colonies formed when ZNF217 was overexpressed was greater in ZNF217-1 and ZNF217-2 cells than in control cells (P < 0.001, Fig. 2A).

Results

High levels of expression of ZNF217 mRNA are associated with poor prognosis in breast cancer

To investigate the clinical relevance of ZNF217, we used qRT-PCR to explore ZNF217 mRNA levels in a test set of 47 samples of primary breast tumors (CLB1 cohort, Supplementary Table S1) from women who had (Met+ group) or had not (Met− group) developed metastases while receiving therapy. High levels of ZNF217 mRNA in the primary tumor were significantly associated with the subsequent development of metastases (significant overexpression of ZNF217 mRNA in the Met− group, P = 0.002, Supplementary Table S4) and shorter relapse-free survival (RFS; P = 0.003, Fig. 1A). These findings were verified in an independent cohort from a different geographic location, the CRH cohort (Supplementary Table S4 and Fig. 1B).

Retrospective analysis of gene expression array data from Ma and colleagues (20) showed that ZNF217 mRNA levels were still significantly higher in the Met+ group than in the Met− group (P = 0.0007, Supplementary Table S4), and that high levels of expression of ZNF217 were significantly associated with shorter RFS (P = 0.01, Fig. 1C). Finally, the most remarkable results arose from our analysis of publicly available microarray data for 2,414 breast cancer patients (21), in which we found that high levels of expression of ZNF217 were associated with poor prognosis, with a P value of 1 × 10−11 (Fig. 1D). Altogether, these data suggested that ZNF217 is a novel and powerful biomarker for poor prognosis in breast cancer.

Clinical relevance of ZNF217 mRNA levels for prognosis in an independent breast cancer cohort

In the independent CLB2 cohort (n = 100, Supplementary Table S2), ZNF217 mRNA was again significantly overexpressed in the Met− group compared with the Met+ group (P = 0.025, Supplementary Table S4), and high levels of expression of ZNF217 were significantly associated with shorter RFS (P = 0.02, Fig. 1E). No association was found between expression of ZNF217 with other clinical parameters (Supplementary Table S5). The prognostic value of ZNF217 mRNA was more informative than the ER, PR, or HER2 conventional biomarkers in this cohort (Log-rank test, Table 1). However, lymph node status was associated with shorter RFS (P = 0.013, Log-rank test, Table 1). Prognostic factors for RFS with a 0.05 significance level in univariate analysis were then entered in a multivariable Cox model. Both ZNF217 mRNA levels and lymph node status persisted in the model (P < 0.05), revealing that these 2 biomarkers are independent prognostic markers (Table 1).

We then constructed a signature based on ZNF217 mRNA expression levels and lymph node status by dividing patients into 3 groups: patients who expressed low levels of ZNF217 mRNA and had no or few (≤ 3) involved lymph nodes (group A); patients who expressed high levels of ZNF217 mRNA expression and no or 3 or less involved lymph nodes or those who presented with more than 3 involved lymph nodes and low expression of ZNF217 mRNA (group B); and patients who expressed high levels of expression of ZNF217 and had more than 3 involved lymph nodes (group C). The resulting Kaplan–Meier curves for RFS are shown in Fig. 1F (P = 0.004). Finally, we tested the ZNF217 mRNA levels/lymph node status signature with respect to RFS and found that the model associating ZNF217 mRNA levels and lymph node status was a better fit (likelihood = 199.83) than the model with ZNF217 mRNA levels only (likelihood = 208.16, P = 0.001) or the model with lymph node status only (likelihood = 207.74, P = 0.001; ref. 29), showing that, in this cohort, the ZNF217 mRNA level/lymph node status signature has the best prognostic value.

We also investigated ZNF217 protein expression by immunohistochemistry in 10 ZNF217-mRNA–positive human breast tumors. ZNF217 expression was detected in all tumors and was mainly located in the nucleus of the stained tumoral cells (no staining was observed in the stromal cells). Moreover, the majority of the breast tumors investigated displayed a proportion of stained cells ranging from 50% to 80% and a positive but variable level of ZNF217-protein expression (Fig. 1G).

Overexpression of ZNF217 stimulates migration and invasiveness of breast carcinoma cells in vitro and in vivo

MDA-MB-231 cells, which have low endogenous levels of ZNF217, have been previously used by our group to establish 2 stable cell lines (ZNF217-1 and ZNF217-2) that constitutively overexpress the ZNF217 protein (ref. 11; Supplementary Fig. S1A). Overexpression of ZNF217 strongly simulates cell survival and/or proliferation (Supplementary Fig. S1B). Transient transfections with 2 ZNF217-targeted siRNAs resulted in a spectacular inhibition of cell proliferation/viability both in control and in ZNF217-overexpressing cells (Supplementary Fig. S1C), supporting our previous results (11). Anchorage-independent growth is thought to be associated with aggressiveness and metastasis in malignant cells. We found that the number of colonies formed when ZNF217 was overexpressed was greater in ZNF217-1 and ZNF217-2 cells than in control cells (P < 0.001, Fig. 2A).
A strong capacity for migration or invasion is another hallmark of tumor malignancy. We carried out wound healing experiments at short time points (15 and 24 hours) and in serum-free medium, to exclude interference attributable to the proproliferative action of ZNF217. Overexpression of ZNF217 in ZNF217-1 and ZNF217-2 cells stimulated cell migration compared with control cells (Fig. 2B), and this was entirely reversed in the presence of siRNA-A ZNF217 or siRNA-B.
ZNF217 (Fig. 2C and Supplementary Fig. S2). Matrigel invasion assays showed that ZNF217-overexpressing cells are also more invasive than control cells (Fig. 2D). Supporting data came from the Boyden chamber assay in vitro (P < 0.001, Fig. 2E). Focal adhesion kinase (FAK) signaling is crucial in mediating migration and invasion, in particular, in those induced by ErbB2 and ErbB3 receptor signaling in breast cancer cells (30, 31). We strikingly found increased levels of phospho-FAK/FAK total ratio associated with overexpression of ErbB2 and ErbB3 proteins in both ZNF217-overexpressing clones (Fig. 2F), suggesting that this molecular pathway could be involved in the ZNF217-driven phenotype.

We next explored whether deregulation of endogenous ZNF217 expression could be detected in a breast cancer cell model of invasion. The D3H2LN cell line is a clonal subline of MDA-MB-231 cells passaged twice in mice, and the resulting xenografts showed early and increased frequency of spontaneous lymph node metastasis in vivo (32). Strikingly, compared with controls, D3H2LN cells showed significantly higher endogenous expression of ZNF217 protein (Fig. 3A) and increased migratory properties (Fig. 3B). Moreover, the application of ZNF217-targeted siRNAs was sufficient to decrease the migratory and invasive properties of D3H2LN cells (Fig. 3B and C).

We then carried out in vivo investigations in nude mice. Xenografts were established by injecting populations of MDA-MB-231-pcDNA6 control cells or MDA-MB-231-ZNF217 cells into the mammary fat pads of female nude mice. A significant increase in the growth of MDA-MB-231-ZNF217 xenografts compared with control xenografts was observed and 2 of 4 control mice spontaneously developed metastases, either in the lung or in the lymph nodes (Figs. 3D–H). Supporting data came from intravenous injection of luciferase stably transfected MDA-MB-231-ZNF217 or MDA-MB-231-pcDNA6 cell populations in the tails of the nude mice. Interestingly, 2 of 5 MDA-MB-231-ZNF217–injected mice developed lung metastases, although none (0 of 5) of the control mice developed any metastases (Fig. 3I).

In summary, these data suggested that ZNF217 overexpression is associated with aggressiveness and invasion, both in vitro and in vivo.

### Gene expression profiling of ZNF217-overexpressing cells

To provide new mechanistic insights into ZNF217-driven migration/invasion, we carried out global gene expression profiling on populations of MDA-MB-231-pcDNA6 and MDA-MB-231-ZNF217 cells. Focusing on significantly deregulated genes, we found that the 10 top-ranked biologic functions (P values from 10^-8 to 10^-11) included response to wounding, regulation of locomotion, locomotory behavior, cell motility, and anatomic structure morphogenesis, providing strong support at the molecular level for the cellular phenotype (migration/invasion) observed in ZNF217-overexpressing cells (Fig. 4A and Supplementary Table S6). We clearly identified deregulated expression of genes encoding adhesion proteins (Supplementary Fig. S3) and also discovered a deregulation in the focal adhesion pathway in ZNF217-overexpressing MDA-MB-231 cells (Fig. 4B), supporting the data presented in Fig. 2F.

### ZNF217 promotes EMT in mammary epithelial cells

EMT is known to be involved in the invasive capacity of transformed epithelial cells (33), and chromatin immunoprecipitation (ChIP) assays have previously shown that the ZNF217 complex is present on the E-cadherin promoter in breast cancer cells, driving downregulation of luciferase activity in a reporter gene assay (6). Compared with MCF10A-pcDNA6 control cells, stably transfected ZNF217 in human mammary epithelial MCF10A cells (34) was paired with invasion (Fig. 5A) and with significant decreased expression of CDH1/E-cadherin mRNA (Fig. 5B) and was sufficient to trigger some features of EMT, including fibroblastic morphology.

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**Table 1.** Univariate and multivariate analyses of the prognostic value of ZNF217 gene expression and clinical parameters with regard to RFS in the CLB2 cohort (n = 100)

<table>
<thead>
<tr>
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<th>Number of samples</th>
<th>Univariate (Log-rank test)</th>
<th>Multivariate Cox proportional hazard analysis</th>
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<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
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<tr>
<td>ZNF217 mRNA levels</td>
<td>100</td>
<td>2.71 (1.13–6.50)</td>
<td>0.020</td>
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<tr>
<td>Age (≤50 y old; &gt;50 y old)</td>
<td>100</td>
<td>0.81 (0.37–1.79)</td>
<td>NS (0.60)</td>
</tr>
<tr>
<td>Macrosopic tumor size (&lt;20 mm; ≥20 mm)</td>
<td>100</td>
<td>1.84 (0.25–13.63)</td>
<td>NS (0.54)</td>
</tr>
<tr>
<td>Lymph node status (&lt;3 involved; ≥3 involved)</td>
<td>100</td>
<td>2.72 (1.20–6.16)</td>
<td>0.013</td>
</tr>
<tr>
<td>Histologic grade (SBR1 + 2; SBR3)</td>
<td>100</td>
<td>0.64 (0.29–1.41)</td>
<td>NS (0.27)</td>
</tr>
<tr>
<td>Estrogen-receptor status (negative; positive)</td>
<td>100</td>
<td>0.60 (0.27–1.31)</td>
<td>NS (0.19)</td>
</tr>
<tr>
<td>Progesterone-receptor status (negative; positive)</td>
<td>100</td>
<td>0.63 (0.29–1.38)</td>
<td>NS (0.24)</td>
</tr>
<tr>
<td>HER2 status (negative; positive)</td>
<td>99</td>
<td>1.77 (0.78–3.99)</td>
<td>NS (0.16)</td>
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Abbreviations: NS, not significant; ND, not done.
is thus another important feature of the deleterious role of second model of human mammary epithelial cells (HMLE cells treated with TGF-β to stimulate EMT and cell migration (36, 38). TGF-β can elicit its cellular responses via TGF-β receptor sand Smad proteinsthat regulate the transcription of these genes (39). We thus sought to determine whether the TGF-β pathway is involved in ZNF217-induced EMT features.

The TGF-β pathway is a major driver of the ZNF217-induced features of EMT

In late stages of breast cancer tumorigenesis, TGF-β is known to promote cancer progression and contribute to the acquisition of metastatic phenotypes, partly through its ability to stimulate EMT and cell migration (36, 38). TGF-β canonically elicits its cellular responses via TGF-β receptors and Smad proteins that regulate the transcription of target genes (39). We thus sought to determine whether the TGF-β pathway is involved in ZNF217-induced EMT using SB431542, an ATP analog and inhibitor of the kinase activity of the TGF-β type 1 receptor, and a siRNA-based strategy targeting Smad4. Control experiments showed that exposure to SB431542 or Smad4 silencing of TGF-β1–treated MCF10A-pcDNA6 cells potently inhibited TGF-β1–induced features of EMT (Fig. 6A and B). Strikingly, treating with SB431542 or silencing Smad4 was also sufficient to reverse the ZNF217-induced EMT phenotype in MCF10A-ZNF217 cells (Fig. 6A and B). Similar data were obtained using HMLE-ZNF217 cells (Supplementary Fig. S6A and B), SB431542 treatment also prevented ZNF217-promoted migration of ZNF217-overexpressing MCF10A cells (Fig. 6C). Overall, these data thus strongly suggested that, firstly, ZNF217-induced EMT and migration in mammary epithelial cells is reversible; and secondly, that ZNF217-induced EMT is TGF-β type 1 receptor dependent and Smad4 dependent.

Supporting data showed that the expression of TGFβ1, TGFβ2, and TGFβ3 mRNA was significantly higher in MCF10A-ZNF217 cells than in the control cells, and the most striking increase was observed for TGFβ2 and TGFβ3 mRNA levels (Fig. 6D). Similar data were obtained using HMLE-ZNF217 cells (Supplementary Fig. S7A), suggesting that the upregulation of TGFβ2 and TGFβ3 mRNA levels is independent of the cellular context in ZNF217-overexpressing mammary epithelial cells.

We then carried out ChIP experiments on several ZNF217-binding sites (6, 8) found in the −5 kb to +1 kb promoter region of the TGFβ1, TGFβ2, and TGFβ3 genes (Supplementary Table S3). PCR amplification of the TGFβ2-P4 and the TGFβ3-P1 regions was observed in the MCF10A-ZNF217 cells and not in control cells (Fig. 6E), suggesting that ZNF217 can associate with TGFβ2 or TGFβ3 promoters and would directly upregulate the transcription of these genes. In the TGFβ1 gene promoter, only one ZNF217-binding site exists (Supplementary Table S3), but no ZNF217 association could be detected in any cells (data not shown), suggesting the involvement of other regulatory mechanisms. Similar data were obtained using HMLE-ZNF217 cells (Supplementary Fig. S7B).

To investigate whether TGF-βs autocrine production was paired with TGF-β pathway activation, supernatant from MCF10A-ZNF217 cells was used to analyze the transcriptional activity of a CAGA (Smad binding elements)-luciferase reporter plasmid in HEK-293T cells. We found that this supernatant induced a significant increase in CAGA-driven luciferase activity (in comparison with supernatant from MCF10A-pcDNA6 cells; Fig. 6F).

In conclusion, increased expression of TGF-βs may contribute, via an autocrine mechanism, to the constitutive activation of the TGF-β pathway, and the TGF-β pathway is a major driver of ZNF217-induced EMT features.

Discussion

The key findings of our study were that high levels of expression of ZNF217 mRNA are associated with poor prognosis and with the development of metastases in breast...
Figure 3. ZNF217 is involved in cell migration/invasion. A, Western blot analysis of ZNF217. Histograms represent quantification of Western blot signals (mean ± SD of independent experiments). B, wound healing assay in D3H2LN cells transfected or not with either scrambled RNA, siRNA-A ZNF217, or siRNA-B ZNF217 (representative of independent experiments). C, Boyden chamber assay carried out in triplicate (mean ± SD). D, growth curves of MDA-MB-231-pcDNA6 xenografts (n = 4) and MDA-MB-231-ZNF217 xenografts (n = 4; mean ± SD). Histologic views of lung metastasis (E) and the matched murine primary tumor (F); lymph node metastasis (G) and the matched murine primary tumor (H). N, normal tissue; T, tumoral tissue. I, representative bioluminescent images of mice intravenously injected with luciferase stably transfected MDA-MB-231-pcDNA6 or MDA-MB-231-ZNF217 cell populations. Red circled areas indicate lung metastases. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student t test).
cancer patients. Our findings were powerfully validated in 3 independent cohorts and in 2 retrospective analyses of publically available transcriptomic data. To our knowledge, this is the first article that ZNF217 mRNA levels have prognostic value in cancer patients, except for a very recent study that found elevated expression of ZNF217...
Figure 5. ZNF217 induces invasion, EMT, and mammosphere formation in MCF10A cells. A, representative images of cell-invasion Matrigel assay (3 independent experiments). B, gene expression of CDH1/E-Cadherin measured by qRT-PCR (means ± SD of 3 independent experiments). C, representative images of MCF10A-pcDNA6 and MCF10A-ZNF217 cell morphology. D, Western blot of ZNF217, epithelial markers, and mesenchymal markers. Signal quantifications are presented in Supplementary Fig. S9. E, qRT-PCR measurement of transcription factors known to be major drivers of EMT (mean ± SD of 3 independent experiments). F, MCF10A-pcDNA6 and MCF10A-ZNF217 cells were grown in nonadherent culture conditions and mammospheres were counted (mean ± SD of 3 independent experiments). **, *P* < 0.01; ***, *P* < 0.001 (Student t test).
The TGF-β pathway is crucial for ZNF217-induced EMT and migration. Immunofluorescence acquisition showing E-cadherin and actin subcellular localization in TGF-β1–treated MCF10A-pcDNA6 and in MCF10A-ZNF217 cells under SB431542 exposure (A) and after Smad4 silencing (B, concomitant Western blot of Smad4 expression is presented in Supplementary Fig. S10A). C, wound healing assay in MCF10A-pcDNA6 and in MCF10A-ZNF217 treated or not with SB431542 (representative of 3 independent experiments). Histograms representing the distance between edges of the wound are presented in Supplementary Fig. S10B. D, qRT-PCR measurement of expression of TGF-β family members (mean ± SD of 3 independent experiments). E, ChIP assays using anti-ZNF217 antibody or human IgG as a control on TGFB2 and TGFB3 promoter regions. Immunoprecipitated ChIP DNA was analyzed by PCR using ZNF217 binding site–specific primers (Supplementary Table S3). F, HEK-293T cells were cotransfected with the CAGA-Luc plasmid and the pTK-RL plasmid. HEK-293T cells were then incubated with supernatant (1:1 and 1:2 dilutions) from MCF10A-pcDNA6 and MCF10A-ZNF217 cells. The luciferase activity results are means ± SD of 3 independent experiments. ***, P < 0.01; ****, P < 0.001 (Student t test). G, model for ZNF217-driven EMT.
to be associated with poor survival in patients with glioma (14).

We were thus able to classify breast tumors on the basis of ZNF217 mRNA expression as those with "good prognosis" or those with "poor prognosis". This stratification could be refined in the CLB2 cohort using a signature based on both ZNF217 mRNA expression levels and lymph node status, which defined 3 prognostic classes of breast cancer patients (good, intermediate, or poor). In the CRH cohort, although lymph node status was associated with shorter RFS, we found that ZNF217 mRNA expression is an independent prognostic marker that is more informative than lymph node status in this cohort (P = 0.01, multivariate analysis, data not shown). In the Ma and colleagues cohort (20) and in the CLB1 cohort, lymph node status, ER, PR, and HER2 had no prognostic value (data not shown), showing that the prognostic value of ZNF217 was again superior. Overall, ZNF217 mRNA expression is a prognostic marker that possesses an added value to current biomarkers. Assessing ZNF217 mRNA expression levels (alone or associated with lymph node status) would thus allow the restratification of patients with breast cancer into outcome-dependent subclasses.

Other major findings of our study were that overexpression of ZNF217 protein strongly stimulates migration and invasion in several in vitro independent breast cell models and is associated with the development of spontaneous lung or node metastases in mice. Interestingly, levels of ZNF217 mRNA in the ZNF217-overexpressing cells used in this study were in the same range as endogenous levels of ZNF217 mRNA detected in ZNF217-positive breast tumor samples (data not shown). This supports the idea that similar levels of ZNF217 mRNA are associated with poor prognosis and the development of metastases in breast cancer patients and with an aggressive and invasive phenotype in vitro or in mice experiments. New immunohistochemical data also revealed heterogeneity in ZNF217 staining in human ZNF217-positive breast tumors, and ZNF217 positively stained cells thus seem to represent a specific subpopulation of tumoral cells within the breast tumor. On the basis of the aggressive ZNF217-associated phenotype identified in vitro and in mice and the fact that ZNF217-positive breast cancer patients are prone to develop metastases, we suggest that high levels of ZNF217 expression in a specific subset of breast tumoral cells could provide these cells with a selective advantage for tumoral escape and generation of metastases. Remarkably, we also showed that ZNF217 enhances mammosphere formation, indicating that ZNF217 expression stimulates the growth of the cancer stem cell population in ZNF217-overexpressing cells.

Previous studies have shown that FAK signaling mediates migration and invasion, particularly in those induced by ErbB2/ErbB3 receptor signaling in breast cancer (30, 31). Biochemical and transcriptomic analyses undertaken in our study both indicate that the ErbB2/ErbB3/FAK signaling pathway is deregulated in ZNF217-overexpressing breast cancer cells. Our transcriptomic investigations also found high ERBB3 mRNA expression levels in ZNF217-overexpressing MDA-MB-231 cells, supporting the recent finding that ZNF217 regulates ErbB3 expression at the transcriptional level (12).

During oncogenesis, epithelial tumor cells undergo EMT and display enhanced migratory capacity and invasiveness (33, 40). Sustained TGF-βR signaling can be required for the maintenance of EMT for metastasis in mouse models (41). Another major finding of our study is that ZNF217 promotes EMT in independent models of human mammary epithelial cells. We thus propose a model for ZNF217-driven EMT that incorporates the direct transcriptional downregulation of E-cadherin expression and/or the constitutive activation of the TGF-β-activated Smad signaling pathway (Fig. 6G). Indeed, we showed that ZNF217 overexpression in MCF10A cells promotes downregulation of endogenous mRNA levels of E-cadherin, supporting for the first time at the endogenous cellular level the observations made by Cowger and colleagues (6). E-cadherin is known to be mainly functionally inactivated by transcriptional repression at the promoter level via several transcription factors (known to be themselves directly regulated at the transcriptional level by TGF-β signaling; refs. 40, 42, 43). ZNF217 could thus represent a new important transcription factor promoting EMT and belonging to the family of zinc finger factors (such as Snail or Slug). Our results also show constitutive activation of the TGF-β pathway in MCF10A-ZNF217 or HMLE-ZNF217 cells. Supporting data showed overexpression in MCF10A-ZNF217 cells of transcriptional factors known to be induced by TGF-β signaling (refs. 40, 42, 43; Fig. 5E). In addition, sustained activation of the TGF-β pathway was the consequence of a TGF-β autocrine loop (increased expression and secretion of active TGF-βs), and direct binding of ZNF217 to ZNF217-binding sites located within the TGFB2 or TGFB3 promoters may contribute to the upregulated expression of these TGF-βRII ligands. Strikingly, inhibition of the TGF-β pathway leads to the reversal of ZNF217-dependent features of EMT, supporting the finding that the TGF-β-activated Smad signaling pathway is a major driver of ZNF217-induced EMT. Finally, the close cross-talk existing between ZNF217 and the TGF-β signaling pathway was also validated by our transcriptomic data in a third cellular model (MDA-MB-231 cells; Supplementary Fig. S8).

Overall, our new findings have important medical applications: (i) the ZNF217 mRNA expression level of a breast tumor is informative and provides a novel and powerful biomarker of poor prognosis that could aid clinicians in therapeutic decisions; (ii) clinical strategies to counteract ZNF217-mediated effects, either by targeting ZNF217 directly and/or by targeting FAK or TGF-β signaling using clinically tested inhibitors (44, 45), could be a potentially valuable approach to the management of breast cancer, particularly for the subpopulation of breast tumors we have identified that possesses high levels of ZNF217 mRNA expression and poor prognosis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank the Centre de Ressources Biologiques of the CLB and of the CRH and also thank Dr. Mattock for edition, S. Léon-Goddard for technical support, and Dr. Nguyen for fruitful discussions.

Grant Support
This work was supported by PIRS/Lyon Science Transfert (LST607, Université de Lyon) and the Ligue Nationale Contre le Cancer (Grants 2009/2010, Comité 71) and the CLARA (Grant 2012/Oncostarter). Nhan T. Nguyen was supported by the USTH PhD fellowships program.

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Received September 28, 2011; revised May 2, 2012; accepted May 3, 2012; published OnlineFirst May 16, 2012.

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