TBX3 Is Overexpressed in Breast Cancer and Represses p14ARF by Interacting with Histone Deacetylases

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Abstract

TBX3 is a transcription factor of the T-box gene family. Mutations in the TBX3 gene can cause hypoplastic or absent mammary glands. Previous studies have shown that TBX3 might be associated with breast cancer. Here, we show that TBX3 is overexpressed in malignant cells of primary breast cancer tissues by immunohistochemistry. TBX3 interacts with histone deacetylases (HDAC) 1, 2, 3, and 5. TBX3 interacts with HDAC1, 2, and 3 via two distinct binding sites. However, deletion of the repression domain (amino acids 566–624) of TBX3 completely abolishes its interaction with HDAC5. Endogenous TBX3 and HDACs interaction and colocalization are found in a breast cancer cell line by coimmunoprecipitation and immunofluorescence, respectively. The functional significance of the interaction between TBX3 and HDAC is also tested in a breast cancer cell line by coimmunoprecipitation and immunofluorescence. Some evidences suggest that TBX3 plays a role in breast cancer development. Previously, we have shown that TBX3 is overexpressed in a number of breast cancer cell lines (7). In the search for breast cancer biomarkers in blood, one study showed that serum TBX3 protein levels were abnormally high in patients with breast cancer (8). This suggests that serum TBX3 levels could serve as a biomarker and it warrants a more extensive evaluation of the role of TBX3 in breast cancer and the pathogenesis of this disease in humans. TBX3 has been shown to repress the expression of the tumor suppression gene p14ARF (2, 9–11) and the murine homologue p19ARF (11). p14ARF is a tumor suppressor shown to be aberrantly expressed and mutated in breast cancer. In addition, p14ARF inhibits human double minute 2–mediated degradation of p53. Therefore, TBX3 repression of p14ARF decreases the half-life of p53 (12). All these suggest that TBX3 plays a role in breast cancer development.

Introduction

TBX3 is a T-box transcription factor that consists of a DNA binding domain (1), a nuclear localization signal, and a repression domain (2). In humans, mutations in TBX3 lead to loss of function and result in the Ulnar-Mammary Syndrome (UMS), an autosomal dominant condition that is characterized by mammary gland hypoplasia and other congenital anomalies (1, 3), suggesting that TBX3 is required for normal breast development. In mice, TBX3 haploinsufficiency does not affect the mammary placode initiation, but the branching of ductal trees in adults is significantly reduced (4). Furthermore, in homozygous Tbx3 knockout mice, the mammary glands are absent (5, 6), suggesting that altered TBX3 function may play a role in the development of breast cancer (7).

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*2008 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-07-5012

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are mapped using different mutant TBX3 constructs. The physiology significance of the TBX3 interaction with HDACs was tested using a p14ARF promoter–driven reporter. Our results show that TBX3 expression is up-regulated in breast cancer cells where it interact and colocalized with HDACs, suggesting that HDACs could serve as effective therapeutic agents for the treatment of breast cancer.

Materials and Methods

Breast cancer samples. Forty-two breast cancer cases with pathology verification and biomarkers were used. The features of the population-based breast cancer tissue samples are shown in Table 1. These breast cancer cases were ascertainment and enrolled as part of a previous study. Tissue procurement was one component of the study and paraffin-embedded tissue blocks, containing tumor and adjacent normal tissue, were obtained from all cases. All the tissue blocks were sectioned and reviewed by an independent pathologist to verify the diagnosis and histology. For this project, unstained slides and H&E reference slides were used under a modified Institutional Review Board protocol (IRB# 2002-2587).

Plasmid construction. A full-length human TBX3 cDNA was purchased from Invitrogen. The cDNA was cloned into the EcoRI and XhoI sites of the pOTB7 vector. The expression vectors encoding TBX3-myc, TBX3 (1–361)-myc, TBX3 (362–723)-myc, and TBX3 (Delta66–624)-myc fusion proteins were constructed by subcloning the TBX3 coding region and TBX3 fragments into the ClaI and XbaI sites of the pcDNA3.1 vector. TBX3 and TBX3 fragments were PCR amplified with pfU Ultra polymerase (Stratagene). Sequences were verified using an automatic sequencer 3130xl.

Cell culture and transfection. COS-1 cells were grown in a complete medium (DMEM, 10% fetal bovine serum, and 1% Penicillin/Streptomycin). The cells were transfected with the control vector pcDNA3.1-myc or the expressing vectors pcDNA3.1-myc-TBX3 or pcDNA3.1-myc-TBX3 fragments using Lipofectamine according to the manufacturer’s instructions (Invitrogen). Cells were harvested 48 h after transfection, paraffin embedded, and subjected to immunohistochemistry. Cell lysates were used for immunoprecipitation and Western blot analysis with an anti-TBX3 or anti-myc antibody.

Immunohistochemistry. Breast cancer tissues or transfected COS-1 cells embedded in a paraffin block were cut into 6-μm thick sections. The sections were deparaffinized with a xylene substitute (Clear-Rite 3 Cardinal Health) and rehydrated using decreasing concentrations of 90:50:5 ethylene-methylisopropyl alcohol. Pretreatment was performed using the Dako Target Retrieval Solution (pH 6) in a pressure cooker for 5 min. Immunoperoxidase reactions were performed using a Dako Autostainer Plus automated immunostainer (Dako, Inc.) according to the manufacturer’s instructions. Briefly, the automated steps included blocking of endogenous peroxidase with DakoCytomation Dual Endogenous Enzyme Block. The sections were incubated with a 1:100 dilution of mouse anti-human TBX3; horse anti-mouse IgG diluted 1:100 was used as a secondary antibody. Incubation with DakoCytomation EnVision+ Dual Link System-horseradish peroxidase followed the primary antibody reaction. For all reactions, the chromogen was diaminobenzidine. Negative controls were treated identically, except the primary antibody was substituted with mouse immunoglobulin. The slides were examined using an Olympus BX41 light microscope and photographed using an Olympus C-5060 Wide Zoom Camera.

Scoring method for TBX3 expression. Images of immunostained breast cancer tissue slides were scored by two independent researchers not involved in any step of their preparation and without the clinical information regarding these slides. Cells of TBX3-stained tissues were scored using the criterion established by Sasaki et al. (ref. 22; Scoring method 1). Briefly, 20 mammary epithelial cells or tumor cells were randomly chosen and a score of 0 to 3 was given to reflect the intensity of nuclear staining, and another score of 0 to 3 was given to reflect the intensity of cytoplasmic staining. The sum of these two scores, 0 to 6, was also assigned to each cell to reflect overall intensities. Each tissue sample was given a score by adding the total scores of all 20 cells. The cells of TBX3-stained tissues were also scored using the criterion described by Leake et al. (ref. 12; Scoring method 2). Briefly, 20 epithelial or tumor cells were randomly chosen, and a score of 0 to 5 was given for the proportion of staining located in the nucleus 0 for no nuclear staining, 1% for <1% nuclear staining, 2% for 1% to 10% nuclear staining, 3% for 11% to 33% nuclear staining, 4% for 34% to 66% nuclear staining, and 5% for 67% to 100% nuclear staining. A second score was given for the intensity of the stain: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The sum of these two scored 0 to 8; the sum of the two separate scores was given to each cell to reflect both localization and intensities of staining. The tissue sample was scored by adding the total scores of all 20 cells.

Data analysis of immunohistochemistry. We compared expression of TBX3 in normal and breast cancer tissue of our patients’ samples using standard statistical methods. We also investigated the correlation between clinical variables and TBX3 overexpression. Correlations (Spearman or Pearson, depending on the normality of the data) were used to determine tumor stage and survival time, using expression as a continuous variable. TBX3 expression in cancer samples, relative to normal samples, was coded as a categorical variable (–1, less than in normal tissue; 0, same as in normal tissue; and +1, increased over normal tissue). The TBX3 expression levels were correlated with other clinical variables including tumor stage, estrogen receptor (ER) and progesterone receptor (PR) status, and menopausal status (23).

Immunoprecipitation. COS-1 cells were harvested when 60% to 80% confluent. The cell lysates were precleared by incubation with prewashed protein A beads for 2 h at 4°C. Next, the samples were incubated with 1 μg of anti-TBX3 or anti-Myc antibody for 2 h at 4°C. Next, the samples were incubated with prewashed protein A beads for 2 h at 4°C. Afterward, the beads were washed using lysis buffer and finally resuspended in SDS-PAGE loading buffer, heated to 100°C for 5 min, and analyzed by Western blotting.

Luciferase assay. Transfection was performed using lipofectamine according to the manufacturer’s protocol (24). Briefly, the day before transfection, 8 × 104 COS-1 cells were plated in each well. For each well, 1 l of Lipofectamine reagents were mixed with 300 ng of p14-Luc reporter.

### Table 1. Clinicopathologic variables in the patients entered into this study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>42 (100)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>9 (21.4)</td>
</tr>
<tr>
<td>&gt;50</td>
<td>33 (78.6)</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>6 (14.3)</td>
</tr>
<tr>
<td>Post</td>
<td>35 (83.3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td>SEER summary stage</td>
<td></td>
</tr>
<tr>
<td>Localized</td>
<td>20 (47.6)</td>
</tr>
<tr>
<td>Distant metastases</td>
<td>9 (21.4)</td>
</tr>
<tr>
<td>Regional, direct extension, and LN</td>
<td>8 (19.0)</td>
</tr>
<tr>
<td>Regional and LN only</td>
<td>5 (12.0)</td>
</tr>
<tr>
<td>Tissue type</td>
<td></td>
</tr>
<tr>
<td>Infiltrating duct ca</td>
<td>31 (73.8)</td>
</tr>
<tr>
<td>Intraductal ca</td>
<td>3 (7.1)</td>
</tr>
<tr>
<td>Lobular ca</td>
<td>3 (7.1)</td>
</tr>
<tr>
<td>Ductal and lobular</td>
<td>2 (4.8)</td>
</tr>
<tr>
<td>Intraductal and lobular Ca in situ</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td>Camycarcinoma</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td>Ca NOS</td>
<td>1 (2.4)</td>
</tr>
</tbody>
</table>

Abbreviations: SEER, surveillance epidemiology and end results; LN, lymph node; Ca, cancer; Ca NOS, cancer not otherwise specified.
Results

TBX3 overexpression in primary breast cancer tissues. Previously, we quantified TBX3 mRNA in 30 breast cancer cell lines and found that TBX3 was overexpressed in a subset of breast cancer cell lines. In addition, we showed that TBX3 was able to immortalize MEF cells. To translate what we discovered in breast cancer cell lines and MEF cells, we tested if TBX3 was overexpressed in primary breast cancer tissues. These experiments relied on the sensitivity and specificity of an anti-TBX3 antibody, which was raised against a recombinant TBX3 COOH-terminal fragment expressed in bacteria. To examine the quality of our anti-TBX3 antibody, we tested its performance using three different applications: immunohistochemistry, Western blotting, and immunoprecipitation. COS-1 cells were transfected with pcDNA-myc-TBX3 (Fig. 1, left). Next, the COS-1 cells were transfected with pcDNA-myc-TBX3 encoding vector (pcDNA3-myc-TBX3), and ~40% of the cells produced a very specific signal (Fig. 1, right).

Next, the anti-TBX3 antibody was tested by Western blotting. COS-1 cells expressing different TBX3 fragments fused with the myc epitope were used for Western blot analysis. The membranes were incubated with an anti-myc (Fig. 1B, left) or the anti-TBX3 antibody (Fig. 1B, right). As shown in Fig. 1B, both antibodies produced very similar patterns, except for lane 2, consistent with the fact that the TBX3 antibody was raised against the COOH-terminal fragment of TBX3 and can only recognize full-length TBX3 (lane 1), the TBX3 COOH terminal (lane 3), and full-length TBX3 with the functional domain deleted (lane 4), but not the NH2-terminal fragment. Significantly, there was no nonspecific background when the NH2-terminal fragment of TBX3 was used (lane 2). Endogenous TBX3 was detected in the MCF-7 breast cancer cell line and in a cell line ectopically expressing TBX3, by Western blotting (data not shown).

TBX3 expression in primary breast cancer tissues could be examined at the mRNA or protein level. However, the primary tumor tissues represented a mixture of both normal and malignant cells, and therefore, only a subpopulation of tumor cells overexpressed
TBX3, which made the interpretation of mRNA levels challenging. To overcome this obstacle, we performed immunohistochemistry to allow better analysis of TBX3 protein levels and subcellular localization in individual cells. This method also allows us to monitor more sensitive protein levels. We found that TBX3 levels in the malignant cells (T) were much higher in nucleus compared with the normal breast epithelial cells (N; Fig. 1C). We used two different scoring systems to characterize the normal and tumor tissue of 42 patients. The two scoring systems produced very similar data. Results were scored with method one. We found that malignant cells of primary breast cancer tissue had higher TBX3 levels than normal breast epithelial cells in both nucleus and cytoplasm (P < 0.01).

**TBX3 is mislocalized in primary breast cancer tissues.** In addition to TBX3 expression levels, we noticed another striking difference between the malignant and control mammary epithelial cells. Malignant cells (T) displayed unusually high TBX3 levels in the cytoplasm compared with normal breast epithelial cells (N); little or no TBX3 was detected in cytoplasm of normal breast epithelial cells (Fig. 1C). We further investigated this observation by analyzing our immunohistochemistry scoring results in greater detail (data not shown). Both scoring methods indicated that the cytoplasmic TBX3 levels were increased in malignant cells compared with normal breast epithelial cells (P < 0.01). To determine if this observation was the result of an overall increase in TBX3 levels, we compared the levels of TBX3 in the cytoplasm to the levels of TBX3 in the nucleus in both cell types. We also found that the ratio of TBX3 levels in the cytoplasm/nucleus was also increased in malignant cells compared with normal breast epithelial cells (P < 0.01). Both of these findings indicate that TBX3 is mislocalized in the malignant cells of primary breast cancer tissues.

**TBX3 interacts with HDAC1, 2, 3, and 5.** Recently, it was shown that a TBX3 homologue, TBX2, specifically binds to HDAC1 and targets HDAC1 to the p21<sup>Cip1</sup> initiator (21). Another recent publication shows that HDAC1 specifically increases activation of the p14<sup>ARF</sup> promoter (20), a known downstream target of TBX3. Together, these studies suggest that TBX3, in concert with HDACs, regulates p14<sup>ARF</sup> gene expression. To test if TBX3 interacts with any member of the HDAC family, we performed TBX3 and HDAC cotransfection and coimmunoprecipitation assay. COS-7 cells were cotransfected with Myc-tagged TBX3 and Flag-HDAC1, Flag-HDAC2, Flag-HDAC4, Flag-HDAC5, Flag-HDAC7, or Flag-HDAC9. As shown in Fig. 2A and B, we found that TBX3 interacts with HDAC1, 2, 3, and 5.

**Identification of the TBX3 domain responsible for HDACs interaction.** To identify the region of TBX3 that is required for TBX3-HDAC interaction, we made deleted TBX3 constructs, which could be tested for HDAC interaction by coimmunoprecipitation. We started by testing the NH2-terminal fragment of TBX3 (amino acids 1–361), which includes the T-box domain. A second fragment starts at amino acid 361 and ends at the COOH terminus, without a T-box domain but has a repression domain. We also made a construct with a deletion of TBX3 repression domain (amino acids 566–624). The HDAC and the truncated TBX3 constructs were

![Figure 2. TBX3 interacts with HDAC1, 2, 3, and 5.](image)
coexpressed in COS-7 cells and tested for interaction by immunoprecipitation.

As shown in Fig. 3A to D, TBX3 interacts with HDAC1, 2, and 3 with NH2-terminal (amino acids 1–361) and the COOH-terminal region (amino acids 361–732), respectively, suggesting that the interaction is via multiple interaction domains, at least one in the NH2-terminal (amino acids 1–361) and another in the COOH-terminal region (amino acids 361–732). However, although HDAC1 still interacts with TBX3 that does not contain the repression domain (Fig. 3A), HDAC2 interaction is significantly decreased when this domain is removed (Fig. 3B). This suggests that the interaction domains between HDAC1 and 2 with TBX3 are different. In contrast to the data showing HDAC1 and 2 interaction, TBX3 NH2 terminal (1–361) is not sufficient for interaction with HDAC5. However, the COOH terminus mutant (361–732) strongly interacts with HDAC5. Furthermore, deletion of the repression domain (amino acids 566–624) completely abolishes TBX3-HDAC5 interaction (Fig. 3D, top). This suggests that TBX3-mediated repression is HDAC5 dependent. Our results are consistent with previous data showing that HDACs interact with their cofactors through multiple domains in different manners.

Interaction between endogenous TBX3 and HDAC in MCF-7 breast cancer cell line. Our results show that TBX3 interacts with HDAC 1, 2, 3, and 5 when they are coexpressed ectopically in COS-7 cells. To examine if this interaction occurs between the endogenous TBX3 and HDAC proteins in breast cancer cell lines, we performed immunoprecipitations with the anti-TBX3 antibody and analyzed the immunoprecipitants by Western analysis with anti-HDAC antibodies, using MCF-7 cells. MCF-7 cells were used because they are the most commonly used breast cancer cell line. It is also been previously shown that TBX3, HDAC 1, 2, 3, and 5 are expressed in this particular cell line. As shown in Fig. 4, HDAC 1, 2, 3, and 5 coimmunoprecipitates with TBX3. This result suggests that endogenous TBX3 interacts with HDAC 1, 2, 3, and 5. We further confirmed the endogenous TBX3-HDAC interaction in MCF-7 cells using immunofluorescence. Formaldehyde-fixed MCF-7 cells were probed using a mouse anti-TBX3 and a rabbit anti-HDAC antibodies. A nonspecific antiserum was used as a negative control (data not shown). The slides were visualized using isotype-matched Alexa Fluor 594 (Red)– and Alexa Fluor 488 (Green)–conjugated secondary antibodies. TBX3 colocalizes with HDAC1 and 5 (data not shown).

The interactions between TBX3 and HDACs are physiologically important for tumor repressor p14ARF expression. Our results suggest that TBX3 interacts with HDACs and represses its downstream gene expression. HDACs requirement for TBX3

Figure 3. Truncated TBX3 proteins interact with HDAC1, 2, 3, and HDAC5. COS-7 cells were cotransfected with vector only, Myc-TBX3, or various TBX3 deletion constructs, and the indicated HDACs. Forty-eight hours after transfection, the myc-tagged proteins were immunoprecipitated from the cell lysates using a rabbit anti-myc antibody and protein A agarose beads (Santa Cruz). To determine if the immunoprecipitated Myc-TBX3 proteins brought down the flag-tagged HDACs, we did Western blot analysis using a mouse anti-flag antibody (SIGMA). A to D, immunoprecipitations performed using a rabbit anti-myc antibody, followed by Western blotting of the precipitates using a mouse anti-Flag antibody (top). To confirm myc-TBX3 and HDAC expression, input cell lysates (5%) were tested by Western blotting with the rabbit anti-myc antibody and anti-flag antibody (middle and bottom), respectively.

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regulation of p14ARF was tested in a p14ARF promoter–luciferase reporter system. The p14ARF promoter with a binding site for TBX3 linked to a luciferase reporter was cotransfected with a Myc-TBX3 construct into COS-7 cells in the presence or absence of the HDAC sodium butyrate and Trichostatin A. The luciferase assay was carried out using the reporter system (Promega) according to the manufacturer’s instructions. After 48 h, luciferase activities were measured. The transcription was normalized to Rennilla luciferase activity. As shown in Fig. 5, TBX3 is able to inhibit p14ARF expression (Fig. 5, lane 2). When pcDNA-Myc-TBX3 is cotransfected with the p14ARF promoter–luciferase reporter in the presence of increasing concentrations of the HDAC, sodium butyrate (A), or Trichostatin A (B). The inhibition of TBX3 to luciferase activities is reversed in a dosage-dependent manner, indicating that TBX3 inhibition to p14ARF promoter–luciferase is HDAC dependent.

Discussion

Mutations in TBX3 cause UMS with features of mammary gland hypoplasia (1, 3). We found that TBX3 was overexpressed in malignant cells, compared with the matched normal mammary epithelial cells. Our results are consistent with previous studies showing that TBX3 was overexpressed in breast cancer cell lines. We also found high levels of TBX3 in the cytoplasm. The elevated cytoplasmic TBX3 protein levels in our result suggest that TBX3 “leaks” into the cytoplasm and, subsequently, enters the blood stream from the tumor tissue, which results in elevated serum levels in cancer patients (8). This result further suggests that TBX3 is involved in breast cancer and can serve as a biomarker for breast cancer.

Previously, it was shown that TBX3 combined with Myc or oncogenic Ras can lead to efficient transformation of MEF (9). In vitro, TBX3 can immortalize and transform MEF cells (7), probably by repressing p14ARF gene expression (10, 11). Overexpression of TBX3 may affect the degradation of p53 via p14ARF and allow breast epithelial cells to undergo additional rounds of cell division and even develop into breast cancer cells, suggesting that TBX3 may promote breast cancer by inhibiting p14ARF. However, the mechanism is unknown. In our study, we showed that TBX3 interacted with HDAC1, 2, 3, and 5. Because TBX3 is overexpressed...
in breast cancer cell lines and HDACs were also found to be overexpressed in many malignant tissues, including breast cancer tissues, HDACs and TBX3 may work together and play an important role in breast cancer development. There is increasing evidence that each HDAC plays a specific role (16, 25–27). It was found that HDAC1 was expressed at significantly higher levels in HER2 negative and ER-α/PR positive. HDAC1 mRNA levels correlated with prognosis (19). The specific roles of HDAC2, 3, and 5 in breast cancer is still unknown. HDACs may form a complex and interact with TBX3. HDACi have recently been tested for their ability to serve as therapeutic agents for the treatment of cancer, showing significant activity against tumors. HDACs can selectively alter genes expression (25, 28). HDACs may execute the anticancer effect partially through the TBX3-HDAC interaction. Indeed, we showed that TBX3 regulation of gene expression is HDAC dependent, and HDACi can reverse the regulation of TBX3 on p14ARF, further suggesting that the TBX3-HDAC interaction is important in breast cancer development.

TBX3-HDAC interaction can explain why T-box genes share a very similar T-box DNA-binding domain and bind to the same or similar synthetic oligonucleotides in vitro. Our result suggests that the functions of T-box genes are diverse by interaction with their cofactors, such as HDACs. Elucidation of TBX3-HDAC interaction enhances our understanding of TBX3 function, particularly in breast cancer and mammary gland development. Finally, we would like to point out that although our result showed that TBX3 represses p14ARF expression by interacting with HDACs, there is no evidence to suggest that p53 or p14ARF knockout is able to rescue the embryonic lethality of the homozygous TBX3 mutation (4). This suggests that TBX3 function is more diverse than we expected. TBX3 might regulate other pathways involved in embryoid development.

Acknowledgments


Grant support: NIH (RO3CA110034 and T32 CA121676), the California Cancer Research Coordinating Committee (CRC-3338), and The Susan G. Komen Breast Cancer Foundation (BCTR-133006; T. Huang). T. Barrientos was supported by NIH institutional training grant T32HD07029.

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We thank the patients for participating in our study and the physicians for referring the patients to us, and Drs. Eric Olsson and Rhonda Bassel-Duby, Department of Molecular Biology, University of Texas Southwestern Medical Center, for their stimulating discussion and several of HDAC expressing vectors.

We thank Drs. Edward Seto (H. Lee Moffitt Cancer Center & Research Institute) for providing HDAC3 construct and Bogi Andersen for critical reading of this manuscript, and Tracey Kingsley who performed the immunoperoxidase reactions. The immunohistochemistry was performed at the University of California, Irvine Chao Family Comprehensive Cancer Center Translational Pathology Core Facility.

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