Original Research

Prognostic significance of cytoplasmic SOX9 in invasive ductal carcinoma and metastatic breast cancer

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Abstract

SOX9, a high mobility group (HMG) box transcription factor, is required for development, differentiation and lineage commitment. It is known to exert its effects through nuclear translocation, such as cell cycle changes in response to retinoic acid treatment in breast cancer cells. However, it is not known whether SOX9 has prognostic significance in human breast cancer. Over-expression and cytoplasmic sequestration of nuclear proteins are implicated in tumor progression. To determine whether SOX9 has any prognostic significance in human breast cancer, its expression and subcellular localization were analyzed in more than 200 human breast carcinomas (BCs). SOX9 mRNA expression data for human BCs were computed from microarray studies available in public databases and correlated with known poor prognostic parameters of BCs. SOX9 protein expression and its correlation with Ki-67 staining in human BCs were assessed using immunohistochemistry. Higher SOX9 mRNA levels were significantly associated with estrogen receptor negative ($P \leq 0.001$) and higher grade ($P \leq 0.01$) human breast tumors. Patients with higher SOX9 mRNA level had significantly shorter overall survival ($P \leq 0.0001$). SOX9 protein, which is normally nuclear, was instead localized in the cytoplasm of 25–30% invasive ductal carcinomas (IDCs) and lymph node metastases. Its cytoplasmic accumulation significantly correlated with enhanced proliferation in breast tumors (Kendall's tau = 0.337 with a P value < 0.0001). Cytoplasmic SOX9 can serve as a valuable prognostic marker for IDCs and metastatic breast cancer. Its significant correlation with breast tumor cell proliferation implies that SOX9 directly contributes to the poor clinical outcomes associated with invasive breast cancer.

Keywords: SOX9, prognosis, human breast cancer, Ki-67, progenitor cells, metastasis, biomarker

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Introduction

Invasive ductal carcinoma (IDC), the most common type of breast tumor, accounts for ~70% of all reported cases of breast cancer in women and ~80% of invasive breast cancers overall. They are histologically diverse and show little consistency in tissue expression of common biomarkers such as Her2 neu and progesterone or estrogen receptors (ERs). IDCs have the poorest prognosis of all human breast tumor types[1] and continue to lack specific diagnostic tests that can potentially guide selection of patient-specific treatment regimens.

IDCs arise from non-invasive tumor tissue and rapidly spread to the lymphatic system and other surrounding tissues, suggesting that genes involved in orchestrating the distinctive interactions between the tumor cells and the surrounding extracellular matrix may play a significant role in tumor progression.[2,3] Indeed, gene expression profiling studies have identified characteristic signature genes that can predict clinical outcome of poor prognosis patients in retrospective studies.[4,5] However, such profiling studies are primarily transcriptional readouts, and may not mimic protein–protein interactions that drive signaling pathways promoting metastatic growth.[6,7] Hence, despite the development of sophisticated molecular profiling techniques, histological and genomic heterogeneity among cases of IDC continues to complicate the rational development of effective treatment strategies.

Recent studies have indicated that cancer cell invasiveness may directly be linked to epithelial mesenchymal transition (EMT),[8,9] a process that is highly influenced by the host

microenvironment.[10] However, matrix remodeling features of tumor cells may not only depend on the action of stromal fibroblasts or diffusible factors in the tumor microenvironment, but also on the differentiation status of the carcinoma cell itself.[11,12] If the carcinoma cells possess stem-like features, it can switch gene expression profiles to that of a bone cell or an endothelial cell or undergo EMT to extravasate and intravasate target tissues to form micrometastasis. Thus, factors that govern stemness and EMT through their interactions with microenvironment-specific factors such as transforming growth factor-ß (TGF-ß), epidermal growth factor and Wingless and integration site growth factor (Wnts) may represent promising targets for therapeutic intervention of invasive breast cancer. Indeed, these pathways have been shown to play a critical role in breast cancer metastasis.[13,14] Some recent studies have reported that Twist and Foxc2 transcription factors play an important role in luminal and basal breast cancer cell metastasis, respectively.[15,16] However, since only 40–50% of human tumors express these markers, it is likely that additional transcription factors may be involved in the progression of the remaining 50–60% invasive breast cancer.

SOX9 is a high mobility group (HMG) box transcription factor required for development, differentiation, lineage commitment and EMT during embryonic development.[17] The direct role for SOX9 in tumor growth has come from studies involving hormone refractory prostate tumors, colorectal cancer and in melanomas.[18–20] Interestingly, embryonic expression of SOX9 was observed in E14.5 and E17.5 mouse embryonic mammary bud (unpublished observation of the author GC, manuscript submitted and under review), and has been reported in the mammary primordium of marsupials.[21] It is also expressed in many human breast cancer cell lines where its expression is induced in response to retinoic acid treatment[22] and is regulated by Wnts in the intestinal crypts, hair bulge and the cartilage.[23–25] Together, these observations suggest that SOX9 may be an attractive candidate gene that may regulate stemness, EMT phenotype and proliferative potential of a subset of invasive breast carcinomas (BCs). Indirect evidence supporting this suggestion stems from the fact that multiple studies from other tissue types have identified SOX9 as a downstream target of major cellular signaling pathways (such as FGF, WT-1, Wnt, PTHrP, retinoic acid, TGF-ß1, Shh and RhoA), each of which has been implicated in promoting aggressive breast cancer.[26–31] Accordingly, to investigate the prognostic significance of SOX9 in human breast cancer, its expression, subcellular localization and association with other clinico-pathological characteristics across a range of human BCs is analyzed in this study.

Materials and methods

Differential expression of SOX9 with respect to ER status and grade was computed from data available from Oncomine[32] (http://www.oncomine.org). A summary of all the studies utilized in our analysis and their data links are included as supplementary information in Supplementary Table 1S. Briefly, Oncomine's gene search function was used to locate microarray studies for which gene expression data were publicly available. Studies were further queried to determine whether they also enlisted information on prognostic indicators of breast cancer such as histological grade and ER status in addition to the expression unit data for SOX9 in breast cancer. Data obtained for individual studies were processed and normalized by Oncomine and used directly for differential expression analysis of SOX9. The results were sorted based on each class of analysis and used to create box plots. Meta-analysis of these studies was not performed as some of these studies used different array platforms for hybridization and could not be combined. Data for the survival analysis were obtained from the ITTACA[33] website and can be accessed from http://www.rii.com/publications/2002/nejm.html.

Breast tumor tissue microarrays (TMAs) containing 206 cores of grade I–III breast tumors were purchased from Tissue Array Networks (Rockville, MD, USA). Specifically, the array contained 152 cores of BC (32 – lymph node metastasis, 68 – invasive ductal [IDC], 22 – invasive lobular [ILC], 22 – intraductal [DCIS], 4 – lobular carcinoma *in situ* [LCIS] and 4 – squamous cell carcinoma). There were an additional 40 cases of benign breast tissue that included 10 samples of adjacent-to-tumor normal breast parenchyma, six normal tissues, 16 hyperplasias, 16 inflamation and eight fibroadenoma.

The following primary and secondary antibodies were used at the specified dilutions: anti-SOX9 (1:250) from Chemicon (Temecula, CA, USA); anti-Ki-67 (1:200) from Biocare Medical (Walnut Creek, CA, USA); streptavidin horseradish peroxidase and biotinylated goat anti-rabbit IgG from Dako (Glostrup, Denmark).

Human adult skin sections were included as positive controls for SOX9 protein expression by immunohistochemical (IHC) detection method. Non-specific staining (negative control) was obtained by preadsorbing the antibody with the peptide antigen used to raise the antibody. However, due to limited availability of the peptide antigen, an additional negative control was obtained by omitting the primary antibody, and replacing it with normal rabbit serum from Dako.

Immunohistochemistry

SOX9 and Ki-67 IHC detection was performed on paraffin-embedded TMA with a rabbit polyclonal SOX9[34] and mouse monoclonal Ki-67 antibody using a Dako autostainer in accordance with the manufacturer's recommendations. CAT hematoxylin was used to counterstain the specimens. Blind IHC scoring was performed by our pathologist (KM), and the scoring was confirmed by two more 'blind' observers. Signals were considered positive when brown staining was observed either in the cytoplasmic or nuclear compartment. Intensity was scored as 0 (no signal), + (weak = 1), ++ (moderate = 2) and +++ (marked = 3).

Plasmids and transient transfection of SOX9 cDNA

Full-length cDNA for SOX9 was cloned into a pCMV6 vector. Transient transfection studies were performed using Fugene

6 (Roche Diagnostics, GmbH, Mannheim, Germany) as per the recommendations of the manufacturer. Briefly, 293T cells were plated in six-well tissue culture dishes containing glass cover slips at 60–70% confluency. Cells were transfected with Fugene (2 μg of DNA and 6 μL of Fugene per well). Immunofluorescent detection was performed 24 h after transfection.

Statistical methods

Statistical significance of SOX9 expression was determined using non-parametric tests. One-tailed Wilcoxson's rank sum test was used for cancers versus normal cases, while the two-tailed Mann-Whitney *U* test was used for the ductal versus lobular ones. The two-sample unequal variance *t*-test was used to determine the significance of differential expression of SOX9 in ER− versus ER+ (raw normalized expression units) and in grade I versus grade III. Kendall's tau test was used to determine the correlation between cytoplasmic SOX9 expression and the Ki-67 expression. Follow-up data were measured from the date of diagnosis to the date of last news for live patients for the overall survival and a plot of the Kaplan–Meier estimate of the surviving fraction was generated. The two groups were compared with log-rank tests using the Graph Pad Prism 5 software.

Results

SOX9 expression is significantly associated with ER− and higher grade human breast tumors

To investigate if SOX9 was over-expressed in human breast tumors and to determine its relationship with ER status, tumor-specific SOX9 mRNA expression data were downloaded from Oncomine or ITTACA websites and analyzed to look for differential expression of SOX9 with respect to ER status and histological grade. Higher SOX9 expression (as detected with the probe set 202936_s_at) was significantly associated with ER− negative phenotype in three separate studies. Specifically, the mean SOX9 expression in ER+ tumors in the Bittner, Wang and Chin studies (Figure 1A and Supplementary Table 1S) was 8.12, 5.52 and 8.3 arbitrary units, respectively, whereas it was 11.32, 7.6 and 16.94 units, respectively, in ER− tumors, indicating a 40–100% increase in SOX9 expression in the ER− group. The comparison of mean SOX9 expression units with *t*-tests in the two groups of tumors further confirmed that SOX9 was significantly over-expressed in ER− tumors compared with the ER+ tumors ($P \leq 0.001$) in all three of these studies (Figure 1A). In our analysis of grade I, II and III breast tumors, at least two out of the three studies found significant increase in SOX9 expression with increase in histological grades ($P \leq 0.01$, grade I versus grade III, Figure 1B) even though all of these studies utilized different reporters (202936, 753184, NM_000346) to monitor SOX9 expression.

Higher SOX9 expression correlates with decreased overall survival

SOX9 expression values downloaded from a public database (http://www.rii.com/publications/default.html) of 259 patients with invasive breast cancers showed that over-expression of SOX9 also influenced a patient's overall survival. Although the data-set's top 10% SOX9 expressors and the bottom 10% SOX9 expressors shared the same characteristics until about 21 months, the survival probability among the top 10% SOX9 over-expressers began to drop considerably (Figure 1C). More specifically, the 50% survival probability in the top 10% group was lowered by approximately two years in this data-set, evident from our observation that the bottom 10% group had a 50% survival probability of seven years and six months as opposed to five years and eight months in the top 10% group. Kaplan–Meier curve comparisons using the log-rank (Mantel–Cox) test showed that this difference was statistically significant ($P = 0.0005$).

SOX9 protein expression is pronounced in BCs but undetectable in normal breast mammoplasty tissue

When a commercially available breast tumor TMA was monitored for SOX9 protein expression using the IHC detection method, SOX9 protein was detected in ductal epithelial cells of atypical ductal hyperplasia (ADH), ductal carcinoma *in situ* (DCIS), IDC and lymph node metastasis samples (Figure 2A and Table 1). However, with the exception of one sample that was classified as ductal ectasia, all other adjacent-to-tumor normal breast tissues and the six core biopsies containing normal breast mammoplasty samples were negative for SOX9 staining. Similarly, relative intensity of SOX9 in carcinoma samples ($n = 150$, two cores had no tissue) was significantly higher as compared with the normal breast specimens ($n = 15$, includes adjacent-to-tumor normal parenchyma [NAT] + normal mammoplasty specimens; one core biopsy was lost in the staining process). Particularly, the median intensity score for the carcinoma samples was 1, while it was zero for all the normal breast samples analyzed (Figures 2A and B). This difference was highly significant as determined using a Wilcoxon's signed-rank test ($P < 0.0001$). This was also true when ductal carcinoma samples ($n = 90$, but one biopsy had no tissue) were compared with the NATs ($n = 9$, one sample excluded from analysis because of low cellularity or complete loss, data not shown).

However, when lobular carcinoma samples (ILC + LCIS, $n = 26$) were compared with NAT parenchyma, this difference was lost as only 1/26 lobular carcinoma samples was positive for SOX9. By corollary, SOX9 expression was more pronounced in the ductal lineage specimens as compared with the LCIS, and the invasive lobular carcinoma (ILC) samples ($P = 0.008$, Mann-Whitney *U* test; Figure 2C). No staining was observed in the absence of the primary antibodies, or with non-specific immunoglobulin controls (Figure 2A[d]).

SOX9 expression is cytoplasmic in a subset of human BCs

In addition to the higher expression of SOX9 mRNA and protein in tumors, cytoplasmic expression of SOX9 was observed in DCIS (4/22 [18%]), IDC (18/68 [26%]) and lymph node metastases (3/32 [10%]) (Figure 3A and

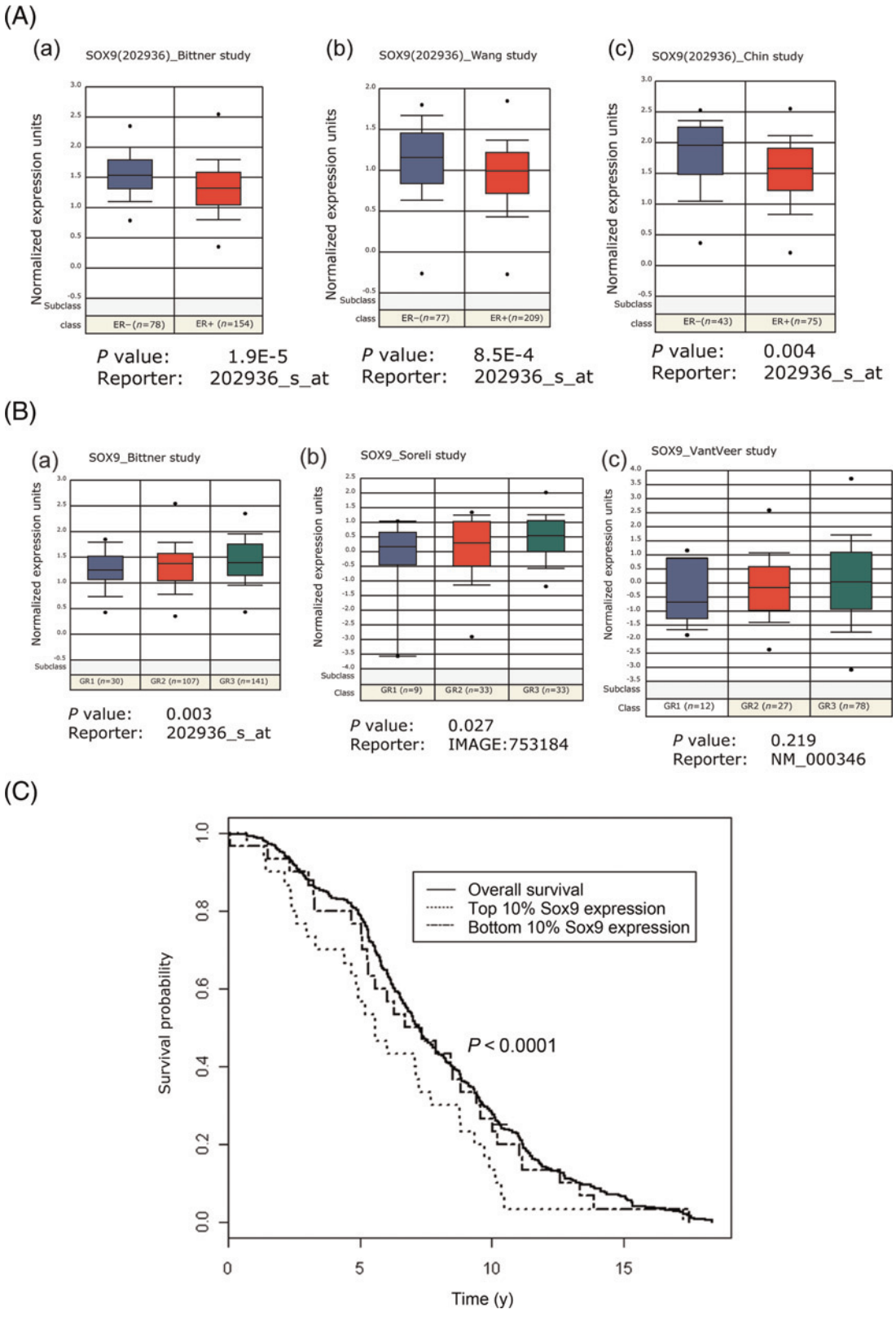

Figure 1 Higher SOX9 mRNA level was significantly associated with ER −, higher grade breast tumors and significantly correlated with decreased overall survival: Oncomine-generated box-and-whiskers plots for SOX9 expression in (A) ER − (blue) and ER+ (red), (B) grade I (blue), II (red) and III (green) breast cancer using breast cancer gene expression data-sets summarized in Supplementary Table 1S (supplementary information). *P* values were generated using Student's *t*-test through Oncomine. For ease of referencing, the total numbers of samples analyzed in each data-set are listed below the box plots of the respective groups. As the platforms and cut-offs used for analysis varied between the original studies, results are presented separately for each study as per the clinicopathological variables analyzed. (C) Kaplan–Meier plot for top and bottom 10% SOX9 expressors and association with patient survival using data obtained from the van de Vijver study.[44] ER, estrogen receptor (A color version of this figure is available in the online journal)

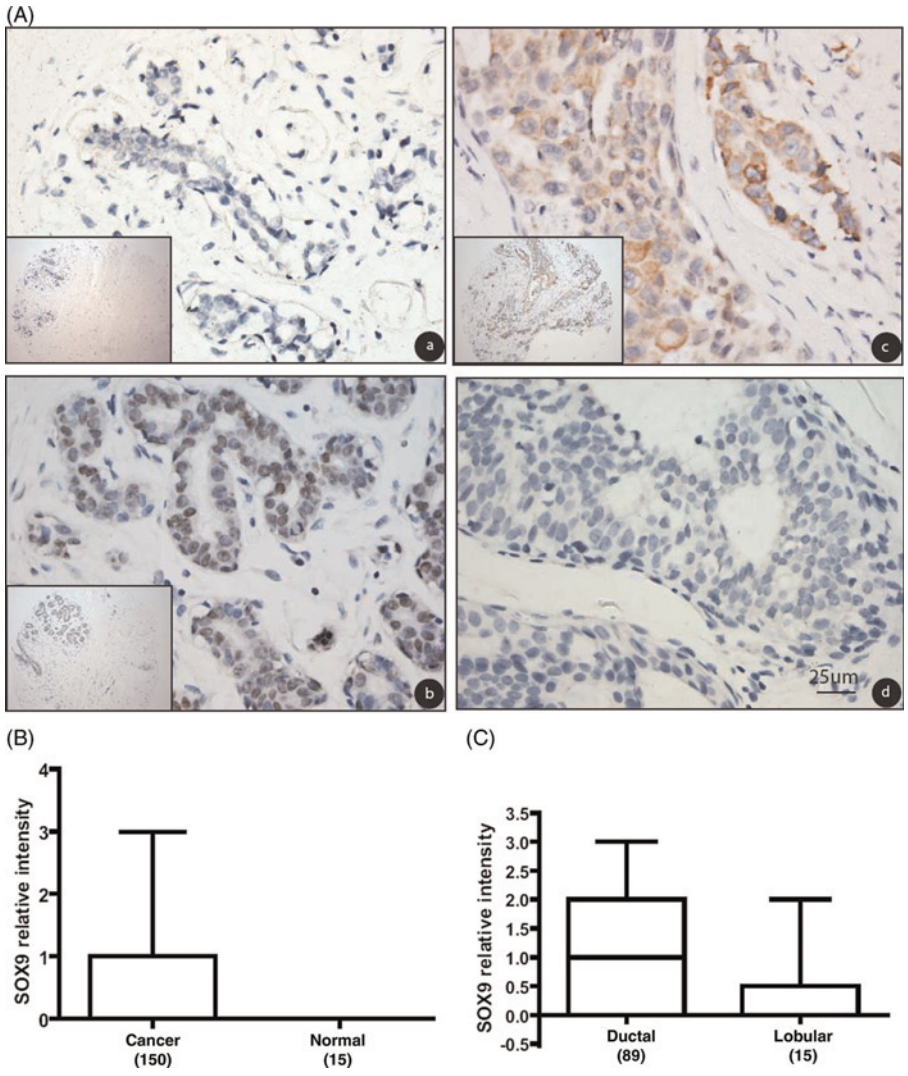

Figure 2 SOX9 is differentially expressed in normal, hyperplasia and invasive ductal carcinomas. (A) Immunohistochemical localization of SOX9 expression on a tissue microarray containing both normal (a), atypical ductal hyperplasia (b) and invasive ductal carcinoma sample (c). Please note that SOX9 protein is undetectable in normal adult breast tissue, is nuclear in the hyperplasia specimen and is over-expressed in the cytoplasm of invasive ductal carcinoma. The insets in a–c are low magnification images of the specimens in a–c. An additional IDC was stained with rabbit IgG to serve as negative control (d). It is important to mention here that because of the very low cellularity of the six normal mammoplasty samples, photographic comparisons were carried out with adjacent-to-tumor normal parenchyma (NAT) specimens. (B) Box plots of SOX9 expression intensities across all breast cancer versus normal breast tissue. (C) Box plots of gene expression intensities in ductal versus lobular carcinomas. Statistical significance of differential SOX9 expression in cancer and normal and in ductal and lobular was determined using one-tailed Wilcoxson's rank sum test and two-tailed Mann-Whitney *U* tests ($P \leq 0.0001$ and $P \leq 0.0001$), respectively. Numbers in brackets indicate the total number of specimens analyzed in each category (A color version of this figure is available in the online journal)

Table 1). The two-tailed Fisher's exact test showed that when invasive specimens (IDC + LN) were compared with all other specimens including NAT and normal, cytoplasmic SOX9 expression was significantly associated with invasive cancers ($P = 0.01$). To ensure that the antibody was not cross-reacting with non-specific cytoplasmic proteins, HEK 293T cells were transfected with a full-length SOX9 cDNA construct and immunostained with the SOX9 polyclonal antibody. Transfected HEK 293T cells exhibited strong nuclear staining with no staining in the non-transfected cell nuclei or cytoplasm (Figure 3B). It was intriguing to note that some nuclei in the merged image visually appeared unicolor (blue), suggesting incomplete merger. However, pixel-wise examination of the red (R), green (G) and blue (B) colors within the nuclei clearly indicated that this was not so. The average distribution of these colors in the most intense regions within the nuclei showed complete merger. While the blue and green colors were predominant in the blue and green fields, respectively, the blue and green colors showed almost equal intensities in the merged image (Figure 3B, Supplementary Figure 2S and Supplementary Table 4S), thus showing that the merger is complete, and that SOX9 is indeed localized in the nucleus of these cells.

The above observations were also substantiated by data from the human adult skin section, used in our study as positive control for SOX9 staining and stained in parallel with the TMA. As seen in Figure 3A[f], positive staining in the skin section with dermal nevus was confined to the nuclei of nevus cells and dermal melanocytes. The specificity of cytoplasmic localization of SOX9 in the advanced invasive tumors was also evident from the fact that an ADH exhibited positive immuno-staining mainly in the

Table 1 Immunohistochemical analysis summary of SOX9 expression in human breast carcinomas and normal breast tissue

Sox9 expression	Cytoplasmic	Nuclear	Cytoplasmic + nuclear	Total cases	Percentage showing cytoplasmic accumulation
LN Met	3	0	0	32	3/32 (9%)
IDC	18	3	1	68	18/68 (26%)
ILC	0	1	0	22	0/22 (0%)
SCCA	0	0	0	4	0/4 (0%)
DCIS	4	0	0	22	4/22 (18%)
LCIS	1	0	0	4	1/4 (25%)
NAT	0	0	1*	10	1/10 (10%)
Normal	0	0	0	6	0/6 (0%)

*Indicates adjacent normal tissue with ductal ectasia

LN Met, lymph node metastasis; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; SCCA, squamous cell carcinoma; DCIS, ductal carcinoma *in situ*; LCIS, lobular carcinoma *in situ*; NAT, adjacent-to-tumor normal parenchyma

nuclei of the epithelial cells (Figure 2A[b]). However, the nuclei of surrounding stromal cells or infiltrating inflammatory cells in the invasive carcinoma or the lymph node metastasis specimens failed to show any SOX9 staining (see Figure 3A[b and d]); although a very small percentage of tumors [1/68 ≅1.5%] did exhibit both cytoplasmic and nuclear localization of SOX9.

Cytoplasmic SOX9 staining is significantly associated with proliferation marker Ki-67 staining

Based on the fact that SOX9 is known to be induced in response to growth arresting/differentiating signals such as retinoic acid, we reasoned that its cytoplasmic accumulation may confer proliferative advantage to a tumor cell. To test this hypothesis, a serial section of the TMA was immuno-stained with a proliferation marker Ki-67 and immunoscored to determine if tumors exhibiting cytoplasmic accumulation of SOX9 stained for Ki-67 as well. In our analysis of 204 (4 samples excluded from analysis) paired specimens that were scored for both SOX9 and Ki-67 staining, the probability of SOX9 and Ki-67 staining occurring together was determined by calculating the conditional probability $P(A|B) = P(A \text{ and } B)/P(B)$, where $A =$ "Ki-67 > 0" and $B =$ "SOX9 > 0". Our data indicated that in 54 out of 84 cases, Ki-67 staining was more likely in the event of SOX9 staining. This correlation was determined to be highly significant using the non-parametric Kendall's tau test (Kendall's tau $= 0.337$ with P value < 0.0001). Additionally, the association of cytoplasmic accumulation of SOX9 with Ki-67 expression was more pronounced in the invasive ductal breast tumors (Figures 4a and b). It is worth noting that nuclear staining of SOX9 in a lone ILC was not accompanied by increased Ki-67 staining (Figures 4c and d).

Diversity in the distribution of IHC scores for SOX9 and Ki-67 in DCIS

Intertumoral heterogeneity in the biological responsiveness of different breast tumors in our array was apparent from the SOX9 localization data in different stages of breast cancer, and also from the varied expression of Ki-67 in these specimens. Accordingly, the distribution of SOX9 and Ki-67 IHC score in DCIS and IDC specimens was analyzed to compare the outcome with earlier reports that had highlighted the importance of emergence of diversity during breast cancer evolution.[35] IHC scores of SOX9 and Ki-67 showed wide variability in their distribution among the IDC and DCIS specimens (Figures 5A and B). Specifically, DCIS specimens with a higher IHC score of 2 and 3 for SOX9 and Ki-67 showed a wider spread in the distribution of percent cases (Figure 5A), suggesting tremendous diversity in these specimens. Contrarily, DCIS specimens with an IHC score of 1 for SOX9 and Ki-67 showed much smaller spread. IDC specimens on the other hand showed little variation in percent cases, irrespective of the IHC score across the board (Figure 5B), suggesting less diversity within this subgroup (Figure 5A). Whether this diversity predicts evolution to poorly differentiated breast cancer could not be assessed because our samples were not matched pairs of DCIS and IDC.

Discussion

We report here three observations that indicate a clear biological rationale to use SOX9 as a biomarker for identifying poor prognostic invasive breast cancers. The first observation is based on gene expression analysis of publicly available breast cancer databases. This analysis revealed that SOX9 expression is significantly associated with ER− phenotype, higher tumor grade and poor overall survival. The second observation indicates that SOX9 protein is undetectable using IHC in normal breast tissue, but it is significantly over-expressed in some IDCs and lymph node metastasis specimens. Finally, unlike ADH, where SOX9 expression is nuclear, DCIS and IDCs show cytoplasmic expression of SOX9 that significantly correlates with Ki-67 expression in the IDC specimens. These observations suggest a hitherto unknown but important functional role for SOX9 in a subset of invasive breast cancers and justify the use of SOX9 localization as a biomarker to mirror its functional status in human tumors.

Invasive breast cancer evolves through alterations in many regulatory pathways over time. Thus, the key to finding effective measures of intervention would be to identify biomarkers that not only change with the earliest changes in breast epithelium, but also continue to reflect the tumor's transition to invasive phenotype. Our data show that SOX9 possesses several characteristics of such

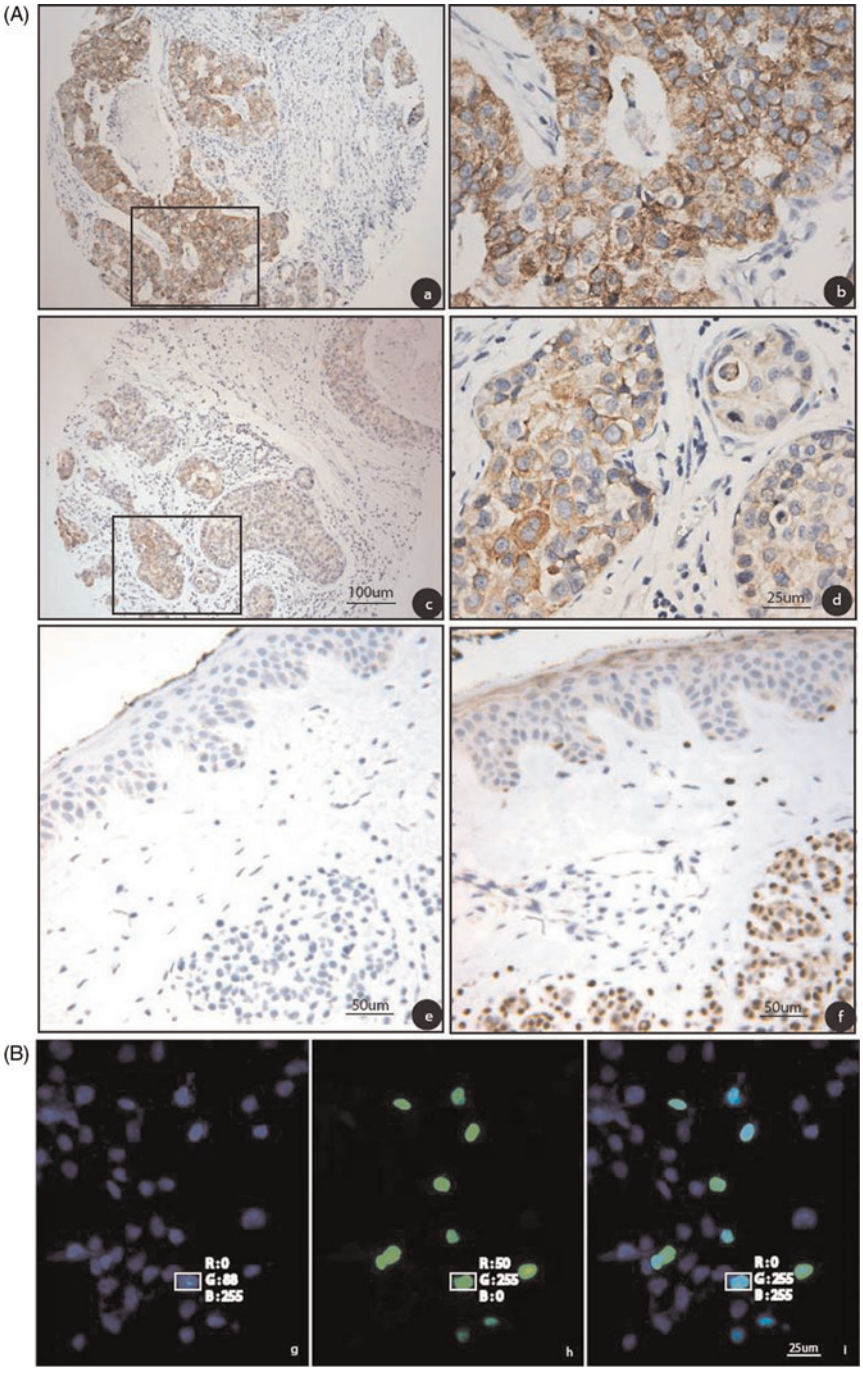

Figure 3 Localization of SOX9 protein in IDC, DCIS and in cells transfected with full-length SOX9 cDNA. (A) Cytoplasmic localization of SOX9 by IHC in IDC (a–b) and DCIS (Figs c–d). The area enclosed by the rectangles in (a) and (c) are shown at higher magnification in (b) and (d). (e) shows human adult skin stained with rabbit IgG alone as the negative control for the assay and (f) shows human adult skin section with nevus stained with SOX9 antibody as the positive control. Note, in skin section, SOX9 expression is nuclear in nevus cells and dermal melanocytes as opposed to its cytoplasmic localization in tumors. (B) HEK 293T cells transfected with a full length cDNA of SOX9 and stained with SOX9 antibody show that the antibody specifically recognizes nuclear staining in these cells. (g) shows DAPI staining in the field (blue nuclei), (h) shows SOX9 expression as detected with an Alexa488 secondary in the same field (green nuclei) and (i) shows the merged image of DAPI and Alexa 488 staining. A representative nucleus is boxed in the blue, green and merged fields, and the average RGB values of the brightest region within the nucleus is shown to indicate complete merger of the blue and the green fields. Please see supplementary data (Supplementary Figure 2S and Table 4S), for the RGB values of the remaining nuclei expressing SOX9. IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; IHC, immunohistochemical (A color version of this figure is available in the online journal)

an early biomarker. For instance, its expression is evident in ADH, the earliest lesion of breast cancer. Previous studies have shown that some ADH give rise to low-grade/non-comedo DCIS, while the poorly differentiated DCIS are thought to evolve from occult precursors.[36] These observations, together with our result that SOX9 was nuclear in the ADH sample, suggest that SOX9 expression may be associated with those ADH that progress to well-differentiated DCIS, and upon accumulation of additional genetic hits give rise to invasive breast cancer. Humanized

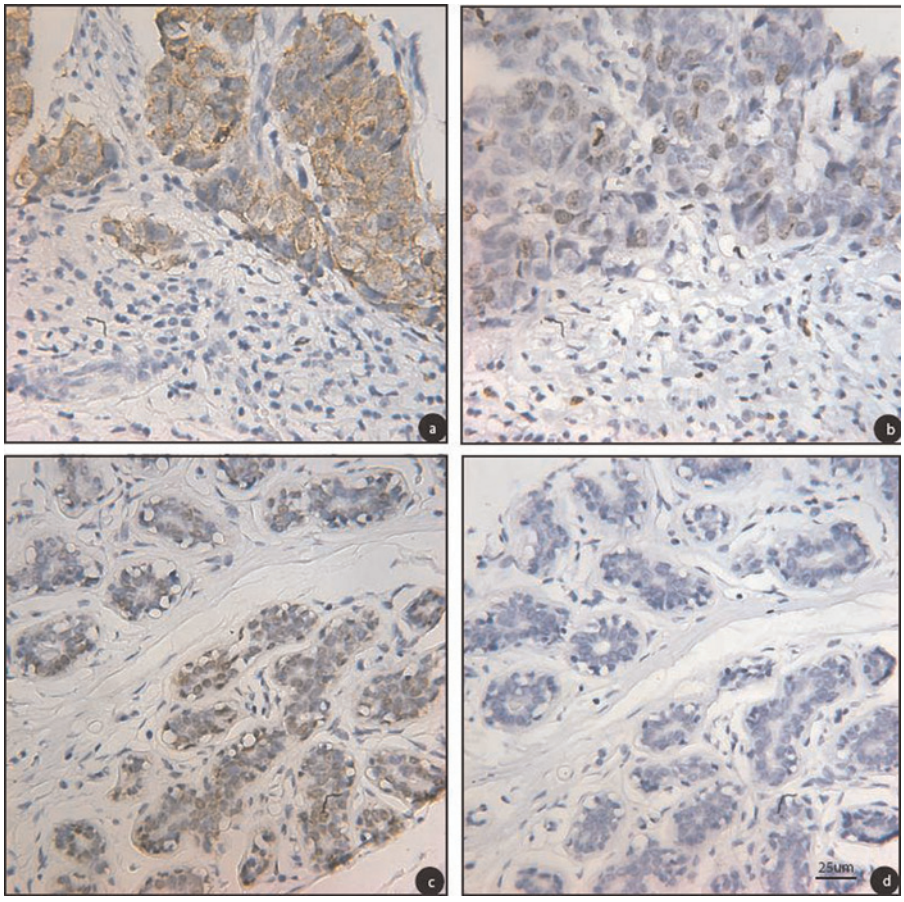

Figure 4 Cytoplasmic SOX9 accumulation is associated with increased cell proliferation as indicated by Ki-67 staining in the serial sections of the TMA. (a) Invasive ductal carcinoma stained with anti-SOX9 antibody. (b) Serial section of the invasive ductal carcinoma in (a) stained with Ki-67. (c) Invasive lobular carcinoma stained with anti-SOX9 antibody. (d) Serial section of the invasive lobular carcinoma in (c) stained with Ki-67. Note, only the specimen with cytoplasmic SOX9 shows Ki-67 staining in the corresponding serial section. TMA, tissue microarray (A color version of this figure is available in the online journal)

models of breast tumor progression[37–39] may help elucidate SOX9's role in the transition of ADH to DCIS, and then to invasive carcinoma.

Our studies show that SOX9 displays yet another characteristic of a good prognostic biomarker – its ability to reflect the inherent aggressiveness of a tumor. This is best revealed by SOX9's widely different expression levels that range from undetectable in normal tissues to nuclear in benign and cytoplasmic overexpression in DCIS, IDC and metastatic breast cancers. Such variable expression levels of SOX9 in normal and cancer, preinvasive and invasive carcinomas provide an excellent rationale to use SOX9 as a surrogate to identify potentially aggressive and metastatic breast cancers. However, cytoplasmic expression of SOX9 in almost a similar percentage of DCIS specimens as IDCs (Table 1) may raise the concern that SOX9 expression levels alone may be insufficient to unambiguously predict progression to invasive disease. This may be reconciled, considering previous studies have shown that DCIS are propagated to IDCs in a manner independent of progression to invasion,[35] and that DCIS are obligate precursors for invasive breast cancers.[36,40,41] All of these studies support our premise that changes in expression patterns of SOX9 alone should be sufficient to predict a tumor's propensity to evolve into invasive breast cancer.

Our study of random assorted breast tumors, representing various stages of breast cancer, highlights other important facets of SOX9 such as its preferential expression in ductal carcinoma specimens. Similarly, the SOX9 expression pattern changes as the disease evolves (Figure 2A). Thus, SOX9's ability to demarcate earliest changes in the breast epithelium along with its ability to distinguish the pathobiology of tumors originating from distinct types of epithelial cells, such as ductal and lobular epithelial cells, may lead to new approaches for better management of these cancers. Furthermore, strong association between higher expression of SOX9 and ER− phenotype in our study, and the prior knowledge that ER has often been clinically used as a predictive marker for hormonal therapy,[42] strongly suggests that combining the analysis of SOX9 and ER would substantially enhance the prognostic power of these markers, as together they would more accurately reflect the natural history of the disease.

One of the most intriguing results of our study that highlights the importance of assessing SOX9 expression in human IDCs is our observation that mostly tumors with cytoplasmic accumulation of SOX9 showed Ki-67 expression (Figures 4 and 5), indicating that this phenotype may represent a non-mutational mechanism for abrogation of SOX9's growth arrest function. While we find this

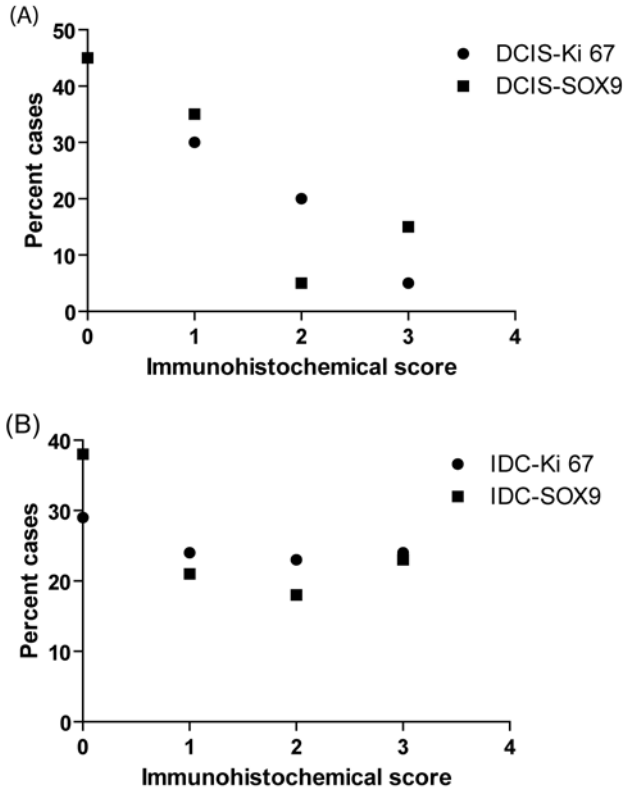

Figure 5 SOX9 and Ki-67 immunohistochemical score distribution in DCIS and IDC specimens of the TMA. The *X*-axis shows the IHC scores of the specimens based on percentage of cells immunopositive for SOX9 or Ki-67. An IHC score of 0 implies no staining, score of 1 implies staining of 0–10% cells; score of 2 indicates 10–50% cells are immunopositive and score of 3 indicates >50% cells are immunopositive. The *Y*-axis shows the percentage of cases with different IHC scores for Ki-67 (•) or SOX9 (■) in (A) DCIS ($n = 20$) and (B) IDC ($n = 66$) specimens. IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; IHC, immunohistochemical

association to be significant, intertumoral diversity was equally apparent and most pronounced in DCIS, consistent with those reported earlier.[35] These results demonstrate the relevance of our findings for human breast tumor progression and raise the possibility that cytoplasmic SOX9 may represent a gain of function SOX9 allele. Therefore, unlike Ki-67 that detects only proliferating cells, serial staining for SOX9 may help identify tumors that would have a higher propensity to pursue an aggressive course.

Our finding that SOX9 over-expression correlates with reduced overall survival is consistent with those of Lu Bingjian *et al.*[43] for colorectal cancer patients and substantiate our claim that SOX9 may directly contribute to breast cancer progression. However, we do recognize that by comparing only the top and bottom 10% SOX9 expressors and ignoring the remaining 80% of the samples, our analysis might suffer some minor bias. Such inherent biases may be minimized using larger data-sets that compare the groups as tertiles of expression with sufficient number of samples per group, and per tertile. A larger sample size study would also help determine how ER status may be influencing the survival outcome in these patients. This is important because we find that SOX9 is over-expressed in ER− breast cancers that are known to have poor overall survival. Nonetheless, if cytoplasmic expression of SOX9 is an indicator of possible progression to invasive disease, relative assessments of nuclear versus cytoplasmic expression of SOX9 in breast cancer patients prior to and after therapies, and stratification of the data based on hormone receptor status would be helpful in determining whether cytoplasmic up-regulation of SOX9 results in a more malignant phenotype of mammary tumors with reduced overall survival.

The fact that SOX9 protein is unstable with a $t_{1/2}$ of 3.6 ± 0.22 h, yet a sizable proportion of DCIS, IDCs and lymph node metastasis samples show strong cytoplasmic localization of the protein is reminiscent of cytoplasmic sequestration of p53 in human tumors that also results in worse prognosis. However, unlike p53, there are no data available to suggest that SOX9 locus is amplified or mutated in breast cancer or other cancers. This poses the question whether SOX9 locus/gene undergoes mutation, and whether the loss of its nuclear functions up-regulates the expression of invasion and metastasis genes.

Although the present studies suggest that other SOX family members may not compensate for SOX9 function, conservation of the HMG domain and high homology with the E-group members indicates possible functional overlap with other SOX family of genes. Our reverse transcriptase-polymerase chain reaction data from human mammary epithelial cells and breast cancer cell lines confirm previously reported expression of SOX2 and SOX4 in breast cancer cells. However, none of the cell lines under investigation express the other two E group gene (SOX8 or SOX10) mRNAs (see Supplementary Figure 1S and Table 3S), suggesting only SOX2 or SOX4 may partner with SOX9, or, share common functional targets. However, both SOX2 and SOX4 are single exon genes, while SOX9 encodes a triple exon gene, and, apart from sharing the conserved HMG domain with these two members, it has additional flanking sequences that may allow it to interact with many additional proteins to form diverse transcriptional complexes. More importantly, in the present study, only SOX9 mRNA is up-regulated in a manner that was representative of our observation in human tumors, suggesting that although these genes may be co-expressed, they may have little to no functional overlap during cancer progression, especially in influencing the metastatic phenotype of breast cancer cells.

In conclusion, our data indicate that SOX9 may contribute directly to the poor clinical outcomes associated with invasive breast cancer. Thus, monitoring its expression over the course of the disease and modulating its signaling, particularly in cases where it is preferentially localized in the cytoplasm, may be a novel way to curb the growth of a subset of metastatic breast cancers.

Author contributions: GC conceptualized, executed, wrote and communicated the manuscript, GC, SAL, SEG, PRC conducted the experiments, KM provided pathological evaluations, NMM interpreted the single nucleotide polymorphism data, MRL provided statistical inputs, KA and DM provided critical feedback.

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