Intracellular Location of the SOX9 Protein in Breast Disease

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Abstract: SOX9, an important factor for testis determination and chondrogenesis, was previously detected in the nucleus of testicular sertoli cells and chondrocytes but was found in only a subset of ovarian sertoli cell tumors, carcinoid tumors, and endometrioid carcinomas, and occasionally only in the cytoplasm of those cells. SOX9 has also been detected in metaplastic carcinomas of the breast exhibiting chondroid differentiation so it became of interest to examine SOX9 in other breast carcinomas and normal breast. Normal breast lobules were studied immunohistochemically for SOX9 along with examples of metaplastic breast carcinoma exhibiting chondroid differentiation, lobular neoplasia (ALH/LCIS), invasive lobular carcinomas, and ductal carcinomas (invasive and *in situ)*. SOX9 was identified solely in the nucleus in over 90% of the normal breast epithelial cells examined. In 10 of 12 breast carcinomas exhibiting chondroid metaplasia, and in 15 of 16 invasive lobular carcinomas, SOX9 was present primarily in the nucleus much like that seen in normal breast epithelium. In 14 of 20 invasive ductal carcinomas, however, it was missing or identified mainly in the cytoplasm of the malignant cells. The functional importance of SOX9 in the breast is unknown although transcription factors involved with the development of certain tissues may have similar capabilities in other organs. The retention of SOX9 in the nucleus of metaplastic or lobular carcinomas could indicate some functional expression of this protein in these tumors with an even more compelling reason why it is often restricted to the cytoplasm, or missing, in ductal carcinomas of the breast.

INTRODUCTION

SOX9, the SRY (sex-determining region on the Y chromosome)-type high-mobility-group box transcription factor 9, is known to be involved in testis formation and chondrogenesis. SOX9 was first identified as a testis-determining factor although many downstream target genes have yet to be identified [1]. SOX9 was found to have a male specific role in sex determination and was shown to be specific to the sertoli cell lineage [2, 3]. SOX9 expression also correlates with chondrocyte gene expression involving the production of collagen type II while having a major role in chondrogenesis and skeletal development [4-6]. Using an antibody recognizing SOX9 we detected the protein in the nucleus of testicular sertoli cells and chondrocytes. These cells and the nuclear location are in keeping with the described function of this transcription factor.

Mutations in the SOX9 gene can lead to human disease. Changes in SOX9 resulting in decreased DNA binding ability were shown to be present in patients with the cartilage disease campomelic dysplasia [7]. Also, SOX9 was shown to be present in over 95% of the nuclei of chondrosarcomas [8]. In our previous study, SOX9 was identified, inconsistently, in a subset of sertoli cell tumors of the ovary [9]. However, a subset of endometrioid carcinomas and carcinoid tumors of the ovary were also found to contain this protein. Moreover, other carcinomas were additionally found to be reactive with the SOX9 antibody but only in the cytoplasm or on the cytoplasmic membrane. Since SOX9 functions as a transcription factor, the nuclear location of the protein in certain cells was an expected finding, but the solely cytoplasmic or membranous location seen in some malignant cells was unexpected.

Because of SOX9's presence in the nucleus or the cytoplasm of some ovarian carcinomas it became of interest to analyze breast carcinomas as well; a gland also under hormonal influence in the female. Recently, a report by Kusafuka *et al*. showed SOX9 to be present in 4 out of 4 cases of a subset of breast carcinomas that are metaplastic with cartilaginous features [10]. Metaplastic breast carcinoma is a rare tumor with a worse prognosis than typical breast cancer [11]. Since some metaplastic breast carcinomas exhibit chondroid differentiation it is reasonable to think that SOX9 might be present. We wanted to expand on this finding by examining more metaplastic breast carcinomas along with ductal and lobular carcinomas of the breast for the presence of SOX9 in order to determine if a possible role for SOX9 exists in the formation of these and other lesions.

MATERIALS AND METHODOLOGY

Formalin-fixed, paraffin-embedded, samples of breast carcinoma were obtained from the files of the Armed Forces Institute of Pathology (AFIP) after Institution Review Board (IRB) approval and studied immunohistochemically using the SOX9 antibody. The lesions examined, in addition to normal terminal duct lobular units, included 20 invasive ductal carcinomas, 18 examples of ductal carcinoma *in situ* (DCIS), 16 invasive lobular carcinomas, 23 examples of lobular neoplasia (ALH/LCIS) and 12 specimens of metaplastic breast carcinoma exhibiting chondroid

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differentiation only. Lobular lesions were defined as having the morphologic appearance of lobular breast disease and being E-cadherin negative.

Immunohistochemical stains were performed as previously described [9, 12]. Essentially, a polyclonal antiserum reactive against SOX9 (Chemicon Inc., Temecula, CA, USA), was detected on tissue using an ABC technique (Vector Laboratories, Burlingame, CA, USA) following pretreatment in a pressure cooker with Reveal (Biocare Medical, Concord, CA, USA). Normal rabbit immunoglobulins assayed on duplicate sections served as negative controls. A known SOX9 positive section of normal testis demonstrating sertoli cells and a section of cartilage were used as positive controls on the assay. All sections were reviewed for intensity and distribution of reaction product. Results were tabulated for each final diagnosis by intracellular location and recorded qualitatively. A positive result was determined to represent at least 30% of the tumor cells being reactive.

RESULTS

The results are listed in Table **1**. SOX9 was strongly identified solely in the nucleus in over 90% of the normal breast epithelial cells examined in all of the samples where normal foci existed whether close to, or away from, the lesion studied (Fig. **1a**, **b**). All negative controls were non-reactive. Positive controls demonstrated a nuclear reaction product in both sertoli cells of the testis and chondrocytes. In

10 of the 12 breast carcinomas exhibiting chondroid metaplasia SOX9 was found only in the nucleus much like that seen in normal breast epithelium, testicular sertoli cells, and chondrocytes (Fig. **1c**, **d**). The protein was identified in most of the tumor cells with an increase in intensity associated with those cells fully exhibiting chondroid differentiation. In the remaining two cases the majority of the tumor cells were non-reactive with anti-SOX9.

Substantial nuclear reactivity was also seen in most of the invasive lobular carcinomas tested (Fig. **2a**, **b**). SOX9 was expressed in the nucleus in 15 out of the 16 (> 90%) invasive lobular carcinoma samples. Interestingly, in 14 of the 20 invasive ductal carcinomas examined, SOX9 was either missing or identified mainly in the cytoplasm of the malignant cells (Fig. **2c**, **d**). When present, SOX9 was found in the nucleus in only 30% of these lesions, whereas 65% contained SOX9 predominantly in the cytoplasm.

The *in situ* lesions were similar to their invasive counterparts in intracellular location of SOX9 with almost 80% nuclear expression seen in cases of lobular neoplasia (ALH/LCIS) and 55% cytoplasmic expression seen in the DCIS samples (Fig. **3**). In DCIS, predominant nuclear expression was seen in less than 40% of the samples, and in lobular neoplasia (ALH/LCIS) SOX9 was either missing or present in the cytoplasm in only a little over 20% of the samples.
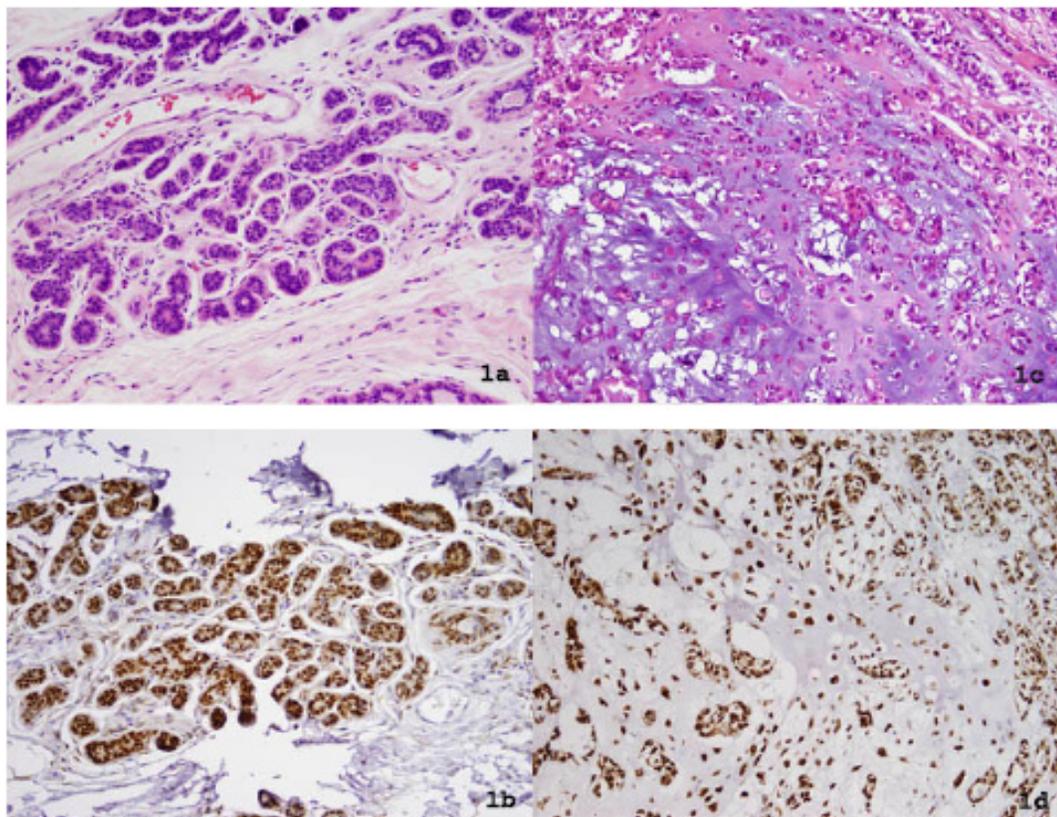
Fig. (1). (**a**) Hematoxylin and Eosin stain of normal breast. (**b**) Immunohistochemical demonstration of SOX9 in normal breast. Note location (brown) in the nuclei of the luminal breast epithelial cells. (**c**) Hematoxylin and Eosin stain of a metaplastic breast carcinoma exhibiting chondroid differentiation. (**d**) Immunohistochemical demonstration of SOX9 in the nuclei of the tumor cells showing chondroid differentiation in this breast carcinoma. X200.
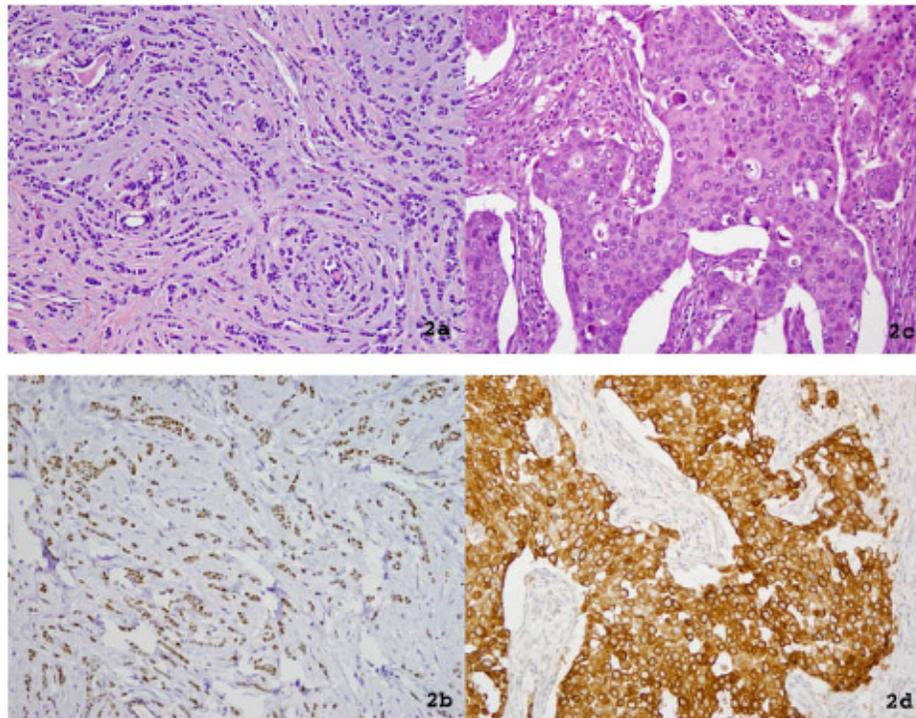
Fig. (2). (**a**) Hematoxylin and Eosin stain of an invasive lobular carcinoma of the breast. (**b**) Immunohistochemical demonstration of SOX9 in the nuclei of the tumor cells in this lobular breast carcinoma. (**c**) Hematoxylin and Eosin stain of an invasive ductal carcinoma of the breast. (**d**) Immunohistochemical demonstration of SOX9 in the cytoplasm of the cells in this ductal breast carcinoma.

There was a little expression overlap seen with 31% of all of the lobular lesions and 54% of all of the ductal lesions with SOX9 predominantly expressed in the nucleus also demonstrating some cytoplasmic expression while none of the ductal or lobular lesions expressing SOX9 predominantly in the cytoplasm demonstrated any nuclear expression.

Whether SOX9 was missing or found only in the cytoplasm of invasive ductal carcinomas, the adjacent normal cells were shown to express the SOX9 protein in the nucleus (Fig. **4a**, **b**). In some cases, where available, it appeared that the level of SOX9 cytoplasmic reactivity seen in DCIS increased in the adjacent invasive carcinoma component (Fig. **4c**). Also, benign proliferative glands like Flat Epithelial Atypia were often observed to have lost luminal cell SOX9 expression while maintaining SOX9 in the myoepithelial cell layer (Fig. **4d**).

DISCUSSION

Although the expression of SOX9 has been shown to be important for a number of organ systems its aberrant expression can lead to indicators of disease [13]. Since sex hormones have been shown to play a role in SOX9 expression it is not surprising that its expression has been noted in some ovarian tumors [9, 14]. SOX9 was also observed to be activated in basal cell carcinomas of the skin, gastric as well as colorectal cancers, and in prostate cancer cells refractory to hormone therapy it was shown to enhance tumor cell proliferation [15-18]. Therefore, despite the necessity of SOX9 involvement in the development of normal tissues it seems that it might also play a role in disease under certain conditions. The ultimate effects of SOX9 depend upon the genes on which it acts and the accessibility of those genes in various cells.

SOX9 has been implicated in breast disease with activity in breast cancer cell lines suggesting it is stimulated by agonists for the retinoic acid receptor [19]. These receptor ligands inhibit the growth of normal and carcinoma cell lines. This growth inhibition is concomitant with a stimulation of SOX9 expression and the introduction of a dominant negative SOX9 was recently shown to inhibit the effect of retinoic acid induced expression [20]. While SOX9 has been shown as a collagen type II stimulator in chondrocytes and has a role in male differentiation in the testis, its role is largely unknown in mediating growth inhibition in breast tumors. Its up-regulation by retinoic acid receptor agonists suggests it may play a role in normal breast physiology [19]. A recent report identified SOX9 in the nucleus of 4 out of 4 metaplastic breast carcinomas exhibiting chondroid differentiation [10]. There was little mentioned about the presence of SOX9 or the intracellular location of the protein in normal breast epithelial cells. In this present study we found nuclear expression of SOX9 in the majority of the studied metaplastic breast carcinomas exhibiting chondroid differentiation. We also found, however, consistent expression of SOX9 in over 90% of normal breast epithelial cell nuclei and in the nuclei of a vast majority of lobular carcinomas as well. Kusafuka *et al*. found less nuclear expression in the areas of typical carcinoma in their 4 metaplastic breast carcinoma cases [10]. Likewise, in our ductal breast carcinomas that did not exhibit any metaplastic features, SOX9 was occasionally absent. However, in almost two thirds of these ductal breast lesions, SOX9 was located in the cytoplasm, not the nucleus, of the malignant cells. It is indeed interesting whenever the intracellular expression of regulatory proteins is altered in abnormal cells.
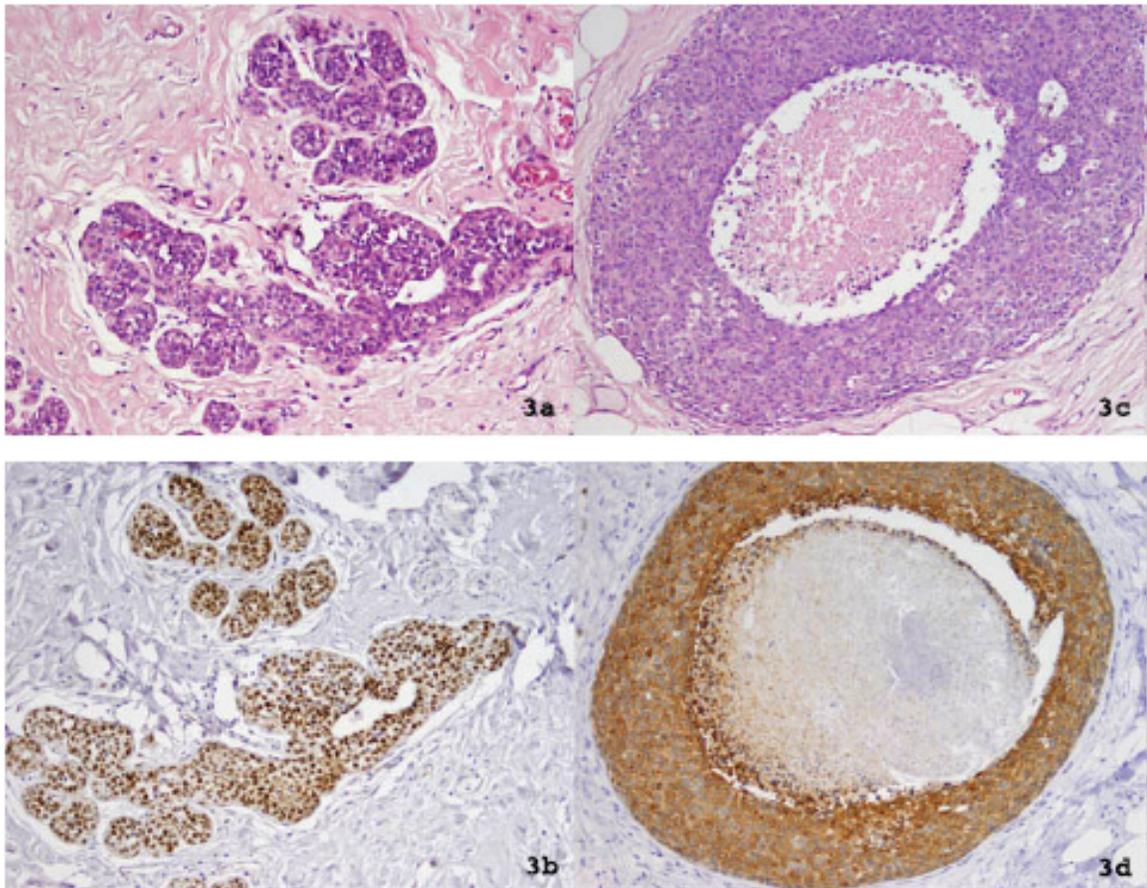
Fig. (3). (**a**) Hematoxylin and Eosin stain of lobular neoplasia (ALH/LCIS) of the breast. (**b**) Immunohistochemical demonstration of SOX9 in the nuclei of the cells in this breast lesion. X200. (**c**) Hematoxylin and Eosin stain of ductal carcinoma *in situ* (DCIS) of the breast. (**d**) Immunohistochemical demonstration of SOX9 in the cytoplasm of the cells in this *in situ* carcinoma. X200.

Table 1. Expression and Intracellular Distribution of SOX9 in Breast Lesions

Diagnosis	SOX9 Predominant Location			
	Total Samples	Nuclear	Cytoplasmic	Negative
LN (ALH/LCIS)	23	18 (78%)	3 (13%)	2 (9%)
Invasive Lobular CA	16	15 (94%)	1 (6%)	0
DCIS	18	7 (39%)	10 (56%)	1 (5%)
Invasive Ductal CA	20	6 (30%)	13 (65%)	1 (5%)
Metaplastic Carcinoma	12	10 (83%)	0	2 (17%)
Normal Breast Epithelium	80	80 (100%)	0	0

LN: lobular neoplasia; ALH: atypical lobular hyperplasia; LCIS: lobular carcinoma. *In situ*; DCIS: ductal carcinoma *in situ*; CA: carcinoma.

Metaplastic carcinomas of the breast are rare tumors often with P53 mutations [21]. SOX9 normally regulates the transformation of mesenchymal cells to chondrocytes in the formation of bone and cartilage. Since some metaplastic carcinomas demonstrate chondrocytic differentiation it is reasonable to assume that SOX9 may be active in these cells. Interactions with beta-catenin are involved in the process of chondrogenesis and beta-catenin has been shown to be present in different locations in normal and abnormal breast epithelial cells [22, 23]. SOX9 has been shown to be expressed in colon carcinoma cell lines where a Wnt pathway beta-catenin-TGF4 complex is required possibly helping to keep a Wnt dependent progenitor phenotype [24]. It is already known that genetic changes in the Wnt pathway genes are common events in metaplastic carcinomas of the breast [25]. SOX9 is also down-regulated when there is increased collagenase activity [26]. During fracture healing, the rise and fall of SOX9 expression correlates with specific expression of the cartilage proteins, collagen type II, and retinoic acid sensitive protein [27]. It stands to reason therefore that an active SOX9 can have the ability to stimulate the formation of cartilage under the right conditions. The cartilage formation may occur through a retained, active, SOX9 coupled with other conditions allowing for the transcription of genes perhaps identical to the ones seen in mesenchymal cells undergoing chondrogenesis. In this case, SOX9 may be functioning in a normal way for mesenchymal cell differentiation. In the mouse, prostate tumor endothelial cells can demonstrate chondrogenic differentiation when accompanied by the up-regulation of SOX9 [28]. Hypoxia up-regulates the expression of SOX9 through HIF-1alpha in mesenchymal cells that differentiate to chondrocytes [29]. Perhaps an increased state of hypoxia present within certain metaplastic breast cancers allow for this up-regulation as well. Also,
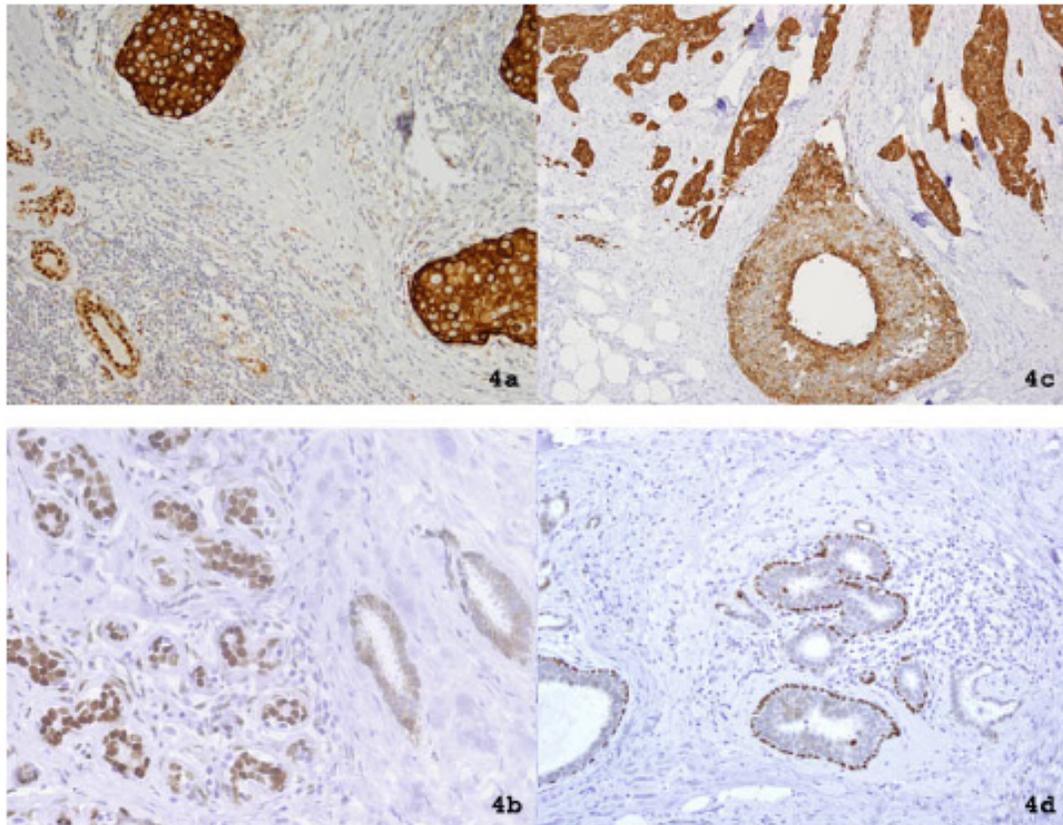
Fig. (4). (**a**) SOX9 expression in the cytoplasm of invasive ductal carcinoma of the breast adjacent to normal glands. X200. (**b**) Lack of SOX9 expression in tubular carcinoma of the breast adjacent to normal glands. X400. (**c**) Increased intensity of SOX9 cytoplasmic expression in breast invasive ductal carcinoma over that expressed in the adjacent DCIS. X100. (**d**) SOX9 expression in the myoepithelial but not the luminal cell layer of Flat Epithelial Atypia adjacent to tubular carcinoma of the breast. X200.

with metaplastic carcinomas being generally of the basal phenotype, and with most of the myoepithelial cells demonstrating expression of SOX9, it is possible that other types of metaplastic breast carcinomas may express nuclear SOX9 regardless of cartilage formation.

SOX9 shifts from cytoplasmic to nuclear location at the time of testis differentiation and anti-mullerian hormone expression [30]. Since an active SOX9 has been functionally linked to testis differentiation, and a nuclear location has been seen for this protein in the testis, the nuclear location observed for SOX9 in normal breast epithelial cells could be indicative of an active SOX9. While SOX9 is capable of stimulating different genes in various cell types the genes involved in SOX9 mediated activity in the breast have yet to be determined. The presence of SOX9 in the nucleus of malignant lobular epithelial cells may indicate normal SOX9 functions in these cells, or may merely indicate the presence of a structural antigenicity having lost some function through malignancy. Since lobular lesions are also defined as luminal, the presence of SOX9 in the nucleus in these lesions demonstrates a luminal as well as a basal identity for this protein.

Of additional interest is the qualitative reduction of SOX9 expressed in the nucleus in most ductal carcinomas of the breast. While SOX9 was mostly detected in the nucleus of normal cells it was either missing or found only in the cytoplasm of most *in situ* or invasive ductal breast cancers. In bladder cancer the SOX9 DNA was shown to be

hypermethylated, and thus down-regulated, and the hypermethylated state of SOX9 was shown to correlate with tumor grade progression and overall survival [31]. This evidence leads to speculation that perhaps the absence of active SOX9 in ductal breast carcinomas could be a factor in increasing epithelial cell growth. An examination of the mutational or methylation status of SOX9 in ductal breast carcinomas warrants further study along with a comparison to prognostic markers seen in other histologic and molecular grades of breast cancer. Much larger sample sizes and concentration on specific phenotypes of breast disease would allow for valid statistical correlations between these markers.

CONCLUSION

The nuclear location of SOX9 in normal cells of the breast suggests that it may play a role in normal breast epithelial cell maturation by targeting breast specific metabolic genes and the fact that it is missing or restricted to the cytoplasm in the majority of ductal breast carcinomas may indicate an alteration that prevents normal cell metabolism leading to disease. The retention of SOX9 in the nucleus of most lobular breast lesions perhaps is indicative of an active SOX9 either with the same functionality seen in normal cells or an aberrant one involving a lobular carcinoma specific pathway. In the rare case of metaplastic breast cancer cells that continue to express nuclear SOX9 leading to cartilage formation the switch to a more mesenchymal chondroid phenotype in these cells may occur

through a novel availability of matrix forming SOX9 target genes.

ACKNOWLEDGEMENT

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