

Expression pattern of a homeotic gene, HOXA5, in normal breast and in breast tumors

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Abstract. *Introduction:* Homeotic (HOX) gene products are now known to be functionally associated with breast cancer biogenesis. Recent evidence has indicated that HOXA5 regulates both p53 and progesterone receptor expression levels in breast cancer cells. In addition, HOXA5 has been shown to interact and regulate the activity of another protein referred to as Twist. As homeotic genes play a pivotal role in development, we sought to decipher the expression pattern in both normal breast tissues and in breast carcinomas. *Methods:* RT-PCR and immunohistochemistry were performed, to assay the levels of HOXA5 expression, on a panel of normal breast tissue and its corresponding primary breast tumors. *Results and Conclusions:* We show that HOXA5 expression was maintained at stable levels at different reproductive stages of a woman's life, except during lactation. This evidence indicates that HOXA5 may play a role in maintaining the differentiated state within the breast epithelium. However, nearly 70% of all breast carcinomas had decreased HOXA5 protein levels as compared to normal breast tissues. In addition, we demonstrate that HOXA5 protein expression levels in breast carcinomas inversely co-relates with Epidermal Growth Factor Receptor (EGFR) expression. Furthermore, we found that the survival rate amongst the different low levels of HOXA5 expressing breast tumors was not significant, indicative of an early tumorigenesis process in the absence of innate levels of HOXA5 in normal breast cells.

Keywords: Breast, homeotic gene, epidermal growth factor receptor

1. Introduction

The *HOX* family of homeobox-containing genes encodes transcription factors that are highly conserved from *Drosophila* to *Homo sapiens* [16,41]. In humans, there are 39 homeobox genes organized in four clusters (A, B, C, and D) that are localized on chromosomes 7, 17, 2, and 12, respectively [16,41]. During development, the homeotic transcription factors control the segmentation identity of the embryo by regulating a number of target genes that have a direct role in cellular morphogenesis and its associated functions [27,32].

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Thus, the misappropriate expression of Hox genes during development can give rise to developmental anomalies [19].

A series of studies that include both misexpression and targeted disruption of *HOX* genes in mice showed that some *HOX* genes function in both the growth of limbs and its patterning [14,25]. Interesting parallels are being noted in human syndromes [14,25]. For instance, *HoxA13* mutations in mice cause hypodactyly [29], while in humans they give rise to hand-foot-genital (HFG) syndrome [28] characterized by limb anomalies, partially divided uterus, and urinary tract malformations. In addition to skeletal abnormalities, targeted disruption of Hox genes like that of the HoxD cluster can alter the peripheral nervous system development [13,42]. Also, loss of HoxB6 in mice results in increased numbers of early erythrocyte progenitors [22] while that of HoxB8 exhibits behavioral defect such as excessive grooming that leads to hair loss and

skin ruptures at these sites [20,34]. Thus, there is growing evidence to indicate that Hox genes not only play an important role in embryogenesis but also are crucial for hematopoiesis [2,18] and neuronal differentiation [3]. Such studies indicate that dysregulation of these Hox expressions can perturb normal development and alter cellular differentiation leading to a variety of disease state [1,4]. Furthermore, several studies have linked HOX function to neoplastic growth, where translocations involving HOX genes were shown to be an underlying cause of leukemias and lymphomas [1, 4,21,36]. Also, selected HOX genes have been shown to be differentially expressed in neoplasms of the colon [17], lung [39], kidney [10], and breast [7,38], but their functional relationship to the neoplastic phenotype remains to be elucidated.

In a previous study, we showed that *HOXA5* expression is higher in immortalized normal breast cell lines than in breast cancer cell lines [33]. Seeking a functional significance to these findings, we searched the promoter sequences of tumor suppressor genes for consensus HOX binding sites and identified candidate sequences in the p53 promoter [33]. Functional studies involving co-transfection revealed that *HOXA5* specifically transactivates the p53 promoter, an effect that is also observed using mouse *HoxA5* and the mouse p53 promoter [33]. Recent evidence has also shown that *HOXA5* can induce apoptosis independent of p53 [9]. In addition, *HOXA5* has been demonstrated to interact with an anti-apoptotic factor Twist and abrogate its functions [37]. Another study showed that *HOXA5* and *HOXA7* were down-regulated in cultured keratinocytes induced to differentiate, while *HOXA7* expression was induced by EGF receptor activation [23], the latter being a frequent phenomenon in breast cancer [15].

In order to obtain insights into the subcellular localization of *HOXA5* protein, in this study, we examined the expression of *HOXA5* in both normal and neoplastic tissues of the breast in relation to expression of other breast cancer biomarkers.

2. Methods

2.1. Immunohistochemistry

Samples for the assessment of *HOXA5* expression at different stages of normal breast development (one sample each from proliferative parous, secretory nulliparous, post-menopausal parous and two sam-

ples each from proliferative nulliparous and lactating parous) were obtained from Fox Chase Cancer Centre, Philadelphia, USA. For the paraffin-embedded tissues (normal breast sections and breast carcinomas) and tissue micro arrays (TMA) were retrieved from Department of Pathology of the Johns Hopkins Oncology Center, Baltimore, USA) and from Gerhard-Domagk-Institute of Pathology, University of Munster, Germany respectively. The normal tissues were obtained from reduction mammoplasties. Three micron thick sections were cut from blocks for immunohistochemistry with the custom made rabbit polyclonal antibody raised against *HOXA5*. After rehydration, endogenous peroxidase activity was blocked for 30 minutes in a methanol solution containing 0.3% hydrogen peroxide. After antigen retrieval in citrate buffer, a cooling off period of 30 minutes preceded the incubation (overnight at 4°C) with the primary antibody (1:100 in PBS/1%BSA). The primary antibody was detected using a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA). The signal was amplified by avidin-biotin complex formation and developed with either diaminobenzidine or chloronaphthol followed by counterstaining with haematoxylin, dehydrated in alcohol and mounted. Percentage of nuclei staining and staining intensity in each core was estimated. In addition, immunohistochemistry was performed on breast cancer tissues that were previously described [6] for which follow-up data are available.

2.2. RT-PCR for *HOXA5* mRNA

Total cellular RNA was extracted from subconfluent cultures of normal breast cell lines (H16N and HBL100), finite lifespan Human Mammary Epithelial Cells (HMECs-184AD, 184 mm, HMEC-4372Q and HMEC-66372), immortalized Human Mammary Epithelial Cells (184A1 and 184B5) and primary breast carcinomas (approximately 80% of the cells in the section were that of tumor origin following microdissection) using TRIzol, a modification of the guanidine isothiocyanate method (Invitrogen, Carlsbad, CA).

RNA was treated with RNase free DNase I (Ambion, Austin, TX) prior to RT-PCR. RT reactions were performed using SuperScript as described by the manufacture (Invitrogen, Carlsbad, CA). The PCR were carried out using *HOXA5* and 36B4 specific primer sets: *HOXA5*-5'-ACCCACATCAGCAGCAGAG-3' (sense) and 5'-TCGGAGAGGCAAAGAGCAT-3' (antisense) and 36B4-5'-GAAGGCTGTGGTGCTGATGG-3' (sense) and 5'-CCCCTGGAGATTTT-

AGTTGGT-3' (antisense). PCR amplification of a ribosomal protein gene, 36B4, served as an internal loading control.

2.3. *In situ hybridization*

Samples for RNA *in situ* hybridization (three normal breast and six invasive breast cancer samples) were retrieved from the archives of the Department of Pathology of the Johns Hopkins Oncology Center, Baltimore, USA. ³⁵S-antisense riboprobes were synthesized from a mouse *HoxA5* cDNA (first 400 bp of the open reading frame) template cloned in the bi-directional plasmid, pCRTm II (Invitrogen), in antisense orientation relative to the T7 promoter. In the 400 bp, there are only four nucleotides that are different between the mouse and human *HOXA5* cDNA sequence [40]. Tissue specimens were fixed in 4% paraformaldehyde, embedded in OCT (Miles, Elkhart) and processed further as described in [5]. The autoradiographs were photographed using bright and dark field illumination on a Zeiss Axioptan microscope.

2.4. *Sample collection and characterization*

To evaluate the expression levels of *HOXA5* and assess correlations with *EGFR* expression, 222 breast cancer samples were retrieved from the archives of the Gerhard-Domagk-Institute of Pathology, University of Munster, Germany, to produce a tissue microarray. The distribution of the T-category was T1: 63, T2: 77, T3: 59, T4: 23. Of all the cases, 56% were lymph node negative. Fifty-nine cases were grade 1, 99 grade 2 and 64 grade 3. Two punches per tumor were taken on a tissue microarray according to standard procedures [30,31]. For the co-localization studies, only 144 samples out of 222 could be evaluated. In order to study the prognostic value of *HOXA5* expression a total of 104 samples were analyzed. The patient age was in the range between 22–84 (median 55). Patient follow-up started after the end of the initial therapy. The median follow-up was 59 months. Also, the sample used for RT-PCR, *in situ* hybridization and immunohistochemistry did not overlap in their respective analyses. The samples collected and used for this study were approved by the local ethic committee.

2.5. *Statistical analysis*

Statistical analyses were carried out using the Wilcoxon test. For survival analysis, Kaplan–Meijer curves were plotted and the differences between the curves were analyzed by LogRank statistics. *p* values less than 0.05 were considered significant.

3. Results

3.1. *HOXA5 expression at different stages of normal breast development*

In order to establish the pattern of expression of *HOXA5* in normal breast tissue during sexual maturation, we compared the immunostaining pattern from normal breast tissues at different developmental stages such as post-menarchal (parous and nulliparous), postmenopausal and lactating women. Expression of *HOXA5* protein was observed in ductal epithelial cells, myoepithelial cells, stromal cells, and lymphocytes in parous breast tissue (Fig. 1). Subcellular distribution of the *HOXA5* protein was predominantly nuclear, with some diffuse cytoplasmic staining (Fig. 1). Darkly stained cells were detected through all the four stages of breast development examined. In addition, lactating breast cells showed little *HOXA5* expression (Fig. 1). These results show that the proliferative influences of puberty or parity, or lack thereof in the postmenopausal stage, do not have a profound effect on *HOXA5* expression, which is maintained at a high level throughout life. The results are summarized in Table 1.

3.2. *Comparison of HOXA5 expression levels between HMECs and primary breast carcinomas*

In order to evaluate the expression levels of *HOXA5* in breast cancers, we tested the levels of *HOXA5* mRNA in a panel of normal breast cell lines, HMECs, immortalized HMECs and primary breast carcinomas by RT-PCR. We observed a significant decrease in *HOXA5* mRNA in primary breast carcinomas as compared to the immortalized HMECs, HMECs and normal breast cell lines (Fig. 2). In more than 80% of the primary breast carcinomas little or no *HOXA5* mRNA could be detected.

Table 1

HOXA5 expression levels at various stages of normal breast development

Age	Menstrual status	Parity	<i>HOXA5</i> staining
17	Proliferative	Parous	+++
18	Proliferative	Nulliparous	+++
28	Proliferative	Nulliparous	+++
40	Secretory	Nulliparous	+++
60	Post-Menopausal	Parous	+++
29	Lactating	Parous	---
35	Lactating	Parous	---

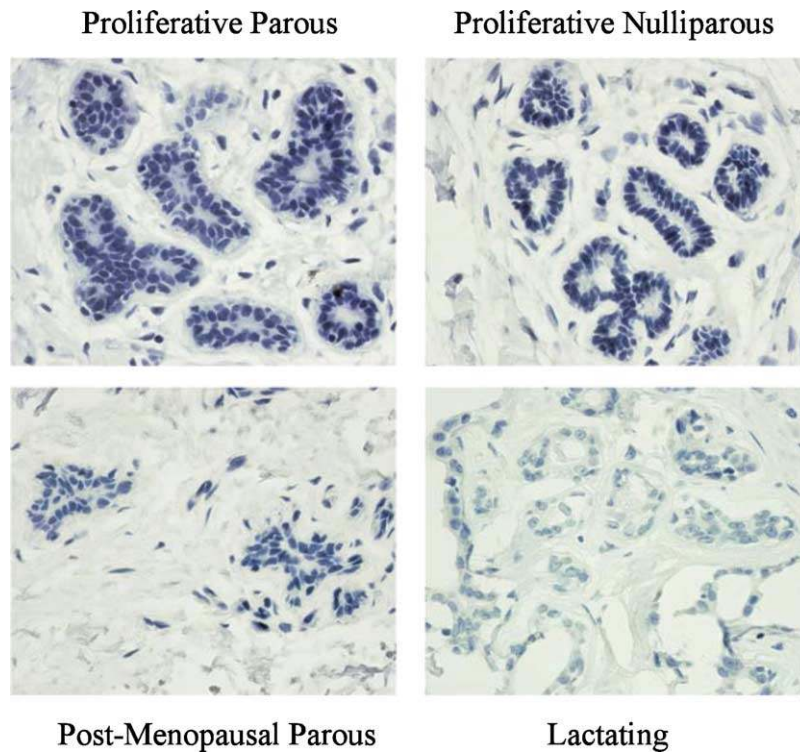


Fig. 1. Immunohistochemical detection of *HOXA5* during the different phases of the reproductive history of the women. Representative photomicrographs of sections of breast tissue stained with a 1:250 dilution of polyclonal anti-*HOXA5* antibody. Paraffin-embedded breast tissues from proliferative parous, proliferative nulliparous, post-menopausal parous and lactating stages are shown. As evident from the photo-micrograph, lactating breast cells have very light *HOXA5* staining compared to the other stages.

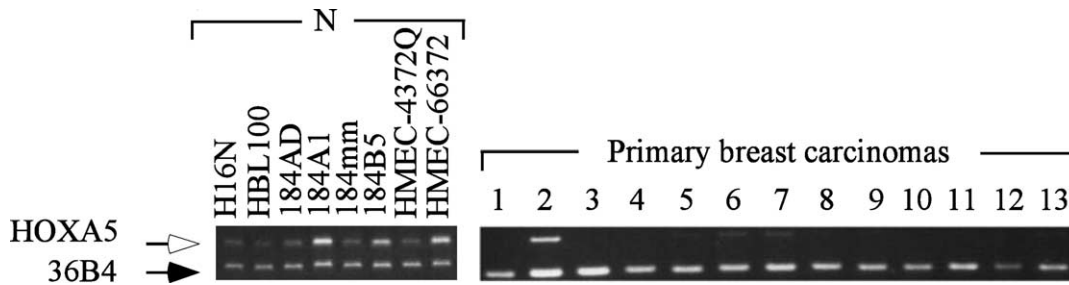


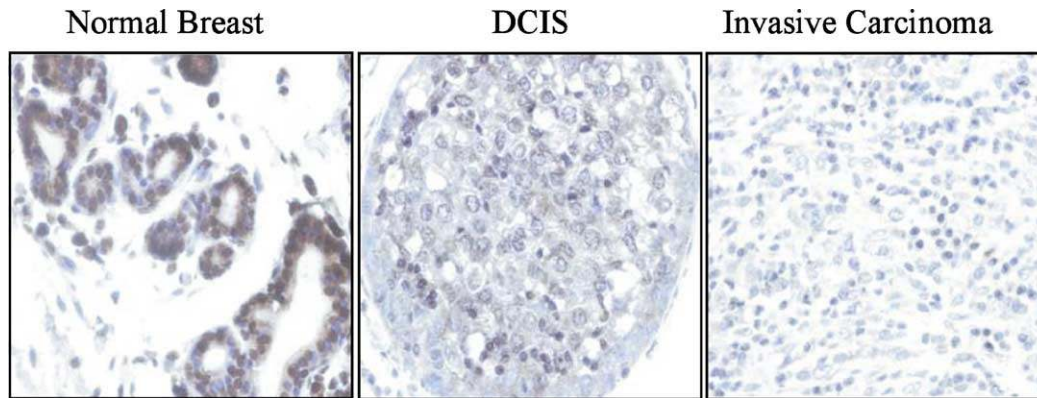
Fig. 2. *HOXA5* expression in normal mammary epithelial cultures and primary breast carcinomas. RT-PCR analysis for the expression of *HOXA5* was performed on a series of normal breast cultures and primary carcinomas (1–13). Amplification of the mRNA coding for the human ribosomal protein 36B4 was used as an internal loading control. As observed, normal breast cells expressed *HOXA5* (open arrow). However, in primary breast carcinomas *HOXA5* expression was decreased. *HOXA5* (open arrow) and 36B4 (closed arrow) cDNAs were amplified using the following primer sets: *HOXA5*-5'-ACCCACATCAGCAGCAGAG-3' (sense) and 5'-TCGGAGAGGCAAAGAGCAT-3' (antisense); 36B4-5'-GAAGGCTGTGGTCTGATGG-3' (sense) and 5'-CCCTGGAGATTTAGTTGGT-3' (antisense).

3.3. *HOXA5* expression is decreased in breast cancers

In order to determine the cell type in which *HOXA5* is expressed as well as whether expression is lost in breast cancer tissues, we performed *in situ* mRNA hybridization assays on three normal breast and six invasive breast cancer samples. A high level of *HOXA5*

expression, mainly confined to the epithelial cells lining the ducts, was observed in normal breast sections, while only non-specific background levels of signal were detectable in all six samples of invasive poorly differentiated breast carcinomas (data not shown). The use of the sense strand as a control produced little or no signals.

A



B

Tissue	n	Nuclear*			Cytoplasmic		
		Low	Med	High	Low	Med	High
Normal breast	6	0	0	6	0	1	5
Fibroadenoma	6	0	1	5	1	0	5
Ductal carcinoma <i>in situ</i>	6	4	2	0	3	2	1
Carcinoma total	24	22	2	0	7	14	3
Ductal	15	14	1	0	4	11	0
Lobular	7	7	0	0	2	2	3
Mucinous	1	0	1	0	0	1	0
Metaplastic	1	1	0	0	1	0	0

Fig. 3. Immunohistochemical detection of *HOXA5* in breast tissues. Representative photomicrographs of sections of breast tissue stained with a 1:250 dilution of polyclonal anti-*HOXA5* antibody. **A.** Paraffin-embedded tissues from normal breast (note intense nuclear, and diffuse cytoplasmic staining of epithelial and myoepithelial cells, stromal cells, and lymphocytes), ductal carcinoma *in situ* and invasive poorly differentiated ductal carcinoma (remarkably light staining in carcinoma cells compared to dark staining in nucleus of normal breast epithelial cells). **B.** Analysis of the *HOXA5* expression levels in the normal breast tissues and the different breast carcinomas. Immunoperoxidase staining was assessed in more than 300 cells and the numerical values are as follows: low-0 to <20% of cell showed staining; med - >20% to <50% of cell showed staining; high - >50% of cell showed staining.

To further investigate the possibility that *HOXA5* expression is specifically lost in breast cancer, we performed immunohistochemical analyses on the following paraffin embedded sections: 6 samples of normal breast tissue, 6 fibroadenomas, 6 ductal carcinomas *in situ*, and 24 unpaired invasive breast cancer specimens. The tumor samples comprised of ductal carcinoma *in situ* samples (DCIS grade I–III), invasive ductal carcinomas (IDC Elston grade I–III), invasive lobular carcinomas (ILC Elston grade II), mucinous carcinoma (Elston grade I) and metaplastic carcinoma (Elston grade III). Representative photomicrographs of one case of normal breast tissue and two breast cancers are shown in Fig. 3A. Normal breast tissues retained a high level of expression of *HOXA5* (Fig. 3A). In pre-malignant and malignant breast tissue samples (Fig. 3A), the level of *HOXA5* was significantly lower

than in normal breast tissue. The stromal cells and infiltrating lymphocytes in the same tumor sections retained dark, nuclear staining with the *HOXA5* antibody, lending support to the idea that *HOXA5* expression is downregulated specifically in the epithelial cells (data not shown). Also, the *HOXA5* positive tumors did have lower signal intensity than the normal breast tissue. The results of immunostaining and the total numbers of samples analyzed for *HOXA5* expression using different techniques are summarized in Fig. 3B and Table 2 respectively.

Taken together, the results of *in situ* hybridization and immunostaining show decreased levels of *HOXA5* in breast cancer as compared to normal breast tissues. Moreover, this loss of expression appears to occur during cancer progression and is detectable in carcinoma *in situ* regardless of their nuclear grade.

Table 2
Techniques used to analyze HOXA5 expression

Technique	Cell lines/Tissues	Sample numbers
RT-PCR	Normal breast cell lines	2
	Finite lifespan human mammary epithelial cells (HMECs)	4
	Immortalized human mammary epithelial cells	2
	Primary breast carcinomas	13
RNA in situ hybridization	Normal breast	2
	Tumor Grade 3	6
Immunohistochemistry	Normal breast	13
	Fibroadenoma	6
	Tumor Grade 1	59
	Tumor Grade 2	99
	Tumor Grade 3	94

Table 3

Expression of HOXA5 inversely correlates with tumor grade ($p < 0.05$) and EGFR levels ($p < 0.05$)

A		
Grade	HOXA5	
	Negative	Positive
1	4%	11%
2	28%	25%
3	20%	12%
B		
EGFR	HOXA5	
	Negative	Positive
Negative	37%	45%
<50% of cells staining	8%	3%
>50% of cells staining	2%	2%
Staining in all cells	3%	0%

3.4. Inverse co-relation between HOXA5 and epidermal growth factor receptor (EGFR) expression in breast cancer patient samples

In order to systematically correlate the expression of HOXA5 in breast carcinomas, we performed immunohistochemical analysis on a TMA. Of the 222 patient samples included in the array, only 144 samples could be evaluated for both HOXA5 and EGFR expression. Similar to our earlier findings in breast cancer lines [2], we show that HOXA5 expression inversely correlates with tumor grade with a p value < 0.05 (Table 3A). Also, we found that HOXA5 expression within this sample set did exhibit inverse correlation with EGFR expression with a p value < 0.05 (Table 3B). In addition, there were no significant correlations between HOXA5 and other cancer biomarkers such as p53 ($p = 0.64$) and PR ($p = 0.41$) within the samples analyzed.

3.5. Overall outcome is not altered between different levels of HOXA5 expression in patient samples

In order to determine if the amount of HOXA5 within the tumor contributed to the overall survival, we plotted a survival probability curve using data from 104 breast cancer cases. As depicted in Fig. 4, there was no significant difference in the survival rate between the various low levels of HOXA5 expression in breast carcinomas (p -value = 0.50 and the LogRank statistic is 0.45).

4. Discussion

There is increasing evidence indicating that the expression of HOX genes has an important function in breast cancer biogenesis. We have previously shown that of the HOX family of genes, HOXA5 is one of the trans-activating components of p53 gene [33]. Given the knowledge that p53 mutations occur in a minority of breast cancers, it was tempting to speculate that alternate mechanisms exist that decrease p53 expression. In an effort to decipher the functional decrease of p53 functions in breast cancer, we have previously demonstrated that HOXA5 is a potent activator of the p53 gene [33]. In addition, the p53 promoter contains HOXA5 binding sites and is able to bind to HOXA5 protein. Given these findings, we were interested to determine the expression levels of HOXA5 both during normal breast development and in tumor progression.

In our present study, we initially evaluated the expression pattern of HOXA5 during the different reproductive phases of human breast, we carried out immunohistochemistry for HOXA5 in a series of samples from normal breast at different developmen-

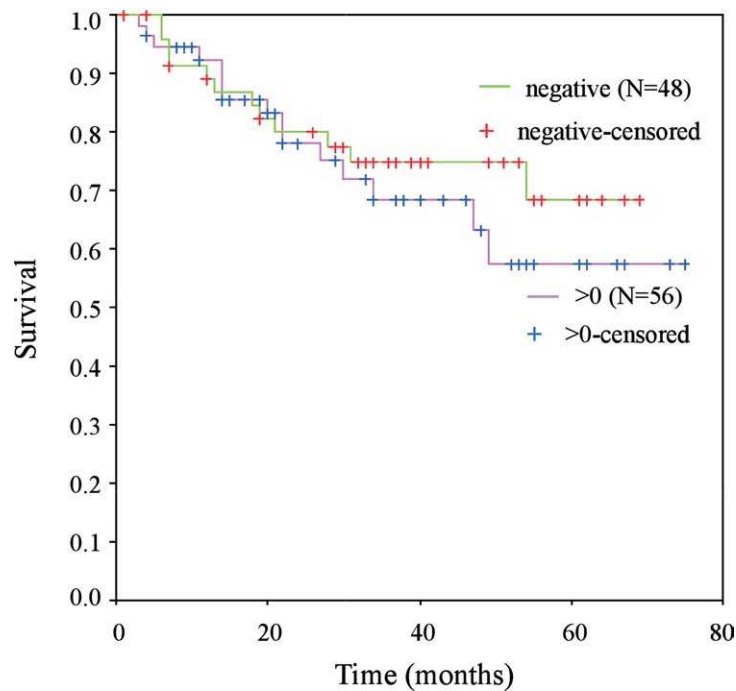


Fig. 4. Correlating HOXA5 expression with survival probability.

tal stages. Pubertal, nulliparous, parous and post-menopausal breast tissue expressed HOXA5, indicating that HOXA5 may not be an anti-proliferative gene. In general, post-menopausal breast epithelium is not undergoing proliferation [35]. Furthermore, lack of HOXA5 expression in lactating breast epithelium indicates that the functional activity of HOXA5 is diminished following terminal differentiation of the epithelial cells [26]. As these cells are programmed for cell death, they do not require the finite controls for cell division. On the other hand, it is possible that down-regulation of HOXA5 is required for proper lactational differentiation of normal breast epithelium. However, much more research is required to substantiate the findings regarding the decreased expression of HOXA5 in lactating breast cells. To our knowledge, this is the first evidence of differential expression of HOXA5 in human adult mammary glands at different reproductive stages of the women's life.

Subsequently, expression levels of HOXA5 were determined by RT-PCR in a panel of HMECs, normal breast cells and primary breast tumors. The majority of the breast tumors had no detectable HOXA5 expression, indicating a possible tumor suppressor role for HOXA5. To further examine this question, we carried out RNA *in situ* hybridization and immunohistochemistry in a panel of normal breast sections and primary

breast tumors. HOXA5 was found to be decreased in the epithelium of invasive breast cancer indicating tissue specific expression. We found a higher incidence of HOXA5 expression in DCIS than in invasive cancers, indicating that the down-regulation may be occurring at the pre-malignant stage of cancer progression. In addition, loss of HOXA5 expression inversely correlated to EGFR expression. EGFR is a transmembrane receptor tyrosine kinase known to be involved in numerous cellular processes such as growth, cellular proliferation and motility [8,11]. The expression levels of EGFR have been correlated with the pathogenesis of a broad range of human cancers [12,24]. As EGFR over-expression has been demonstrated to be a marker of poor prognosis [24], it is possible that loss of HOXA5 expression, in some way, alleviates the restriction on EGFR expression. The mechanism by which EGFR is over-expressed includes an autocrine growth factor loop that constitutively keeps the receptor active. However, further work is required to elucidate the association between HOXA5 and EGFR expression. Taken together, our data supports the conclusion that loss of HOXA5 expression correlates with progression of breast cancer to higher-grade lesions. In addition, the different low levels of HOXA5 expression in these tumors may affect the overall survival probability. It appears that the effect of decreased HOXA5 levels in

the tumor ontogeny may have preceded the observable phenotype. However, a much larger cohort of patients must be studied prior to making any definitive conclusion on the levels of HOXA5 expression to survival rate.

These findings provide further evidence for the putative role of HOXA5 in normal breast development and that the loss of HOXA5 can perturb the balance towards increased growth. Also, it raises the possibility of using HOXA5 as a biomarker for breast cancer progression by helping to predict breast cancer formation at earlier stages, thus providing a platform for more effective treatment.

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