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In vivo expression of the whole HOX gene network in human breast cancer

M. Cantile, G. Pettinato, A. Procino, I. Feliciello, L. Cindolo, C. Cillo*

Department of Clinical and Experimental Medicine, Federico II University Medical School, Via S. Pansini 5- 80131, Naples, Italy

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Abstract

The HOX network contains 39 genes that act as transcriptional regulators and control crucial cellular functions during both embryonic development and adult life. Inside the network, this is achieved according to the rules of temporal and spatial co-linearity with 3′ HOX genes acting on the anterior part of the body, central HOX genes on the thoracic part and lumbo-sacral HOX genes on the caudal region. We analysed HOX gene expression in normal breast tissue and in primary breast cancers by reverse-transcriptase-polymerase chain reaction (RT-PCR). 17 out of 39 HOX genes were expressed in the normal breast tissue. The expression of thoracic HOX genes tended to be similar in normal and neoplastic breast tissues suggesting that these genes are involved in breast organogenesis. In contrast, cervical and lumbo-sacral HOX gene expression was altered in the primary breast cancers with respect to normal breast tissue. This supports their involvement in breast cancer evolution and suggests they could be targets for future cancer therapies.

Keywords: HOX genes; Breast cancer

1. Introduction

Breast cancer, a complex disease with regard to its occurrence and treatment, is one of the most frequent human malignancies in the Western world. In spite of recent advances in the assessment of breast cancer risk through the identification of crucial susceptibility genes (*BRCA1/2*), only a small percentage of total breast cancers can be related to these specific genetic lesions [1,2]. In contrast, breast cancer seems to originate from changes in the architectural breast tissue organisation consequent to interactions between the cell genome and the extracellular environment [3]. Thus, basic cellular processes and their molecular regulation are likely to be implicated in the evolution of breast cancer. An increasing number of transcription factor families and the development-specific and tissue-restricted gene programmes regulated by them are involved in crucial cellular mechanisms; any alteration in these mechanisms may give rise to a series of malignancies, including breast cancer [4].

Homeobox genes are transcription factors that act during normal development [5] and contain the homeobox, a 183-bp DNA sequence coding for a 61-amino-acid domain defined as the homeodomain (HD). Different HD types or classes may be identified through sequence similarities within the HDs [6] each characterising a homeobox gene family. Among these, the Drosophila Antennapedia (Antp) HD defines a consensus sequence referred to as class I HD or Hox genes [7]. In mice (Hox genes) and humans (HOX genes) there are at least 39 genes (Fig. 1) organised in four genomic clusters of approximately 100 kb in length, called Hox loci, each localised on a different chromosome (HOX A at 7p15.3, HOX B at 17p21.3, HOX C at 12q13.3 and HOX D at 2q31) [8] and comprising 9–11 genes. On the basis of sequence similarity and position on the locus, corresponding genes in the four clusters can be aligned with each other and with genes of the HOM-C complex of Drosophila in 13 paralogous groups (Fig. 1) [9]. During mammalian development, Hox gene expression controls the identity of various regions along the body axis, from the branchial area through to the tail [10]. This is achieved according to the rules of temporal and spatial co-linearity, with 3′ Hox genes expressed early in development

* Corresponding author.
E-mail address: clecillo@unina.it (C. Cillo).

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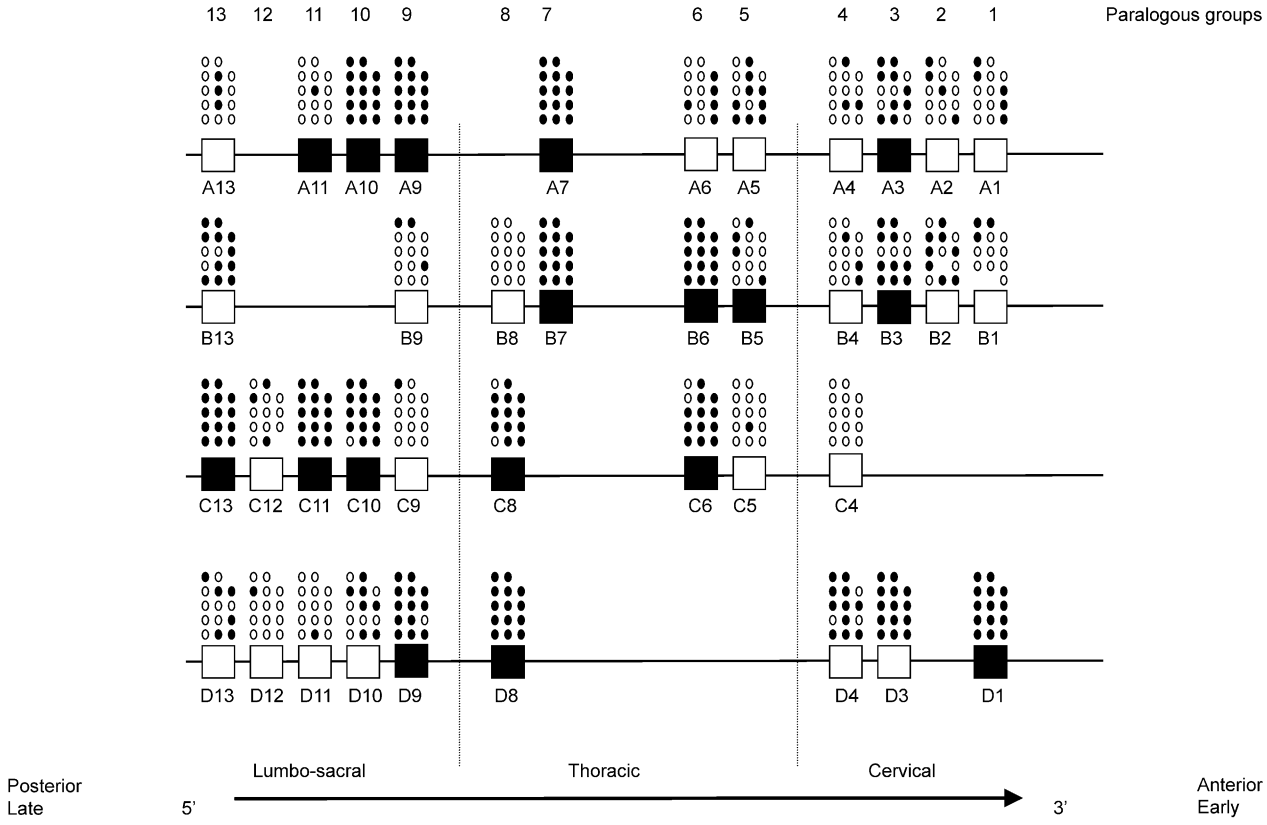


Fig. 1. Diagram of HOX gene expression in normal and neoplastic breast. Closed or open symbols indicate active or silent HOX genes, respectively. Large squares represent normal breast; small ovals represent each individual cancer biopsy tested. Small ovals in the same position in the figure refer to the same breast cancer biopsy.

and controlling anterior regions, followed by progressively more 5′ genes expressed later and controlling more posterior regions [11]. In particular, 3′ Hox genes in groups 1–4 (cervical) primarily control the development of the branchial area and the rhombencephalon, the embryonic region corresponding to the hindbrain [12]. Central Hox genes in groups 5–8 control the thoracic portion of the body, whereas 5′ Hox genes in groups 9–13 control the lumbo-sacral region. The HOX gene network, the most repeat-poor regions of the human genome [13], is also expressed in normal adult human organs. Each adult organ (kidney, colon, liver, lung) displays a peculiar combination of HOX gene expression representing the molecular portrait of the organ [14]. Hox and homeobox genes appear to regulate normal development, normal and abnormal cell proliferation in addition to crucial cellular physiological processes, as proven by the recent description of congenital [15], somatic [16] and metabolic [17] defects involving mutations in these genes.

Recent studies have demonstrated hox gene involvement in leukaemias [18] and solid cancers, such as those involving the kidney [19], colon [20], lung [21] and breast [22]. In the case of breast cancer, specific hox genes may either be involved in mammary gland organogenesis [23] or interact with crucial cancer genes, such as *TP53* [24].

In the present paper, we describe the molecular portrait of normal human breast according to the expression of the HOX network and report a survey of the whole HOX gene network expression in human breast cancer tissue.

2. Materials and methods

Normal and malignant breast tissue was obtained from non-selected patients operated upon at the Surgery Department of our Medical School. During mastectomy, samples from non-necrotic breast cancer tissue were taken, as well as intact normal breast tissue from an area within a few centimetres of the tumour. Each sample was split into two parts and analysed by the pathologist for diagnosis (cancer biopsies), and to exclude the presence of infiltrated tumour cells (normal breast). The other part of the sample was snap-frozen in liquid nitrogen and stored at −80 °C until RNA was extracted.

2.1. RNA extraction and analysis

Frozen tissues were pulverised in a blender. Total RNA was extracted by the guanidinium thiocyanate

technique [25]. Four μg of total RNA were subjected to cDNA synthesis for 1 h at 37 °C using the 'Ready to go You-Primer First-Strand Beads' kit (Amersham Pharmacia Biotech cod. 27-9264-01) in a reaction mixture containing 0.5 μg oligo-dT (Amersham Pharmacia Biotech cod. 27-7610-01).

Polymerase chain reaction (PCR) amplification of cDNA was performed in a reaction mixture containing 4 μl of cDNA sample and different primer sets (20 p/mol each). To prevent genomic DNA contamination, the sense and anti-sense primers were designed to frame a sequence that crossed at least one intron on the genes. The sense/anti-sense HOX primers for PCR were designed as reported in Table 1. The co-amplification of the specific HOX gene and human *β-actin* gene, as an internal control, was achieved using two primer sets in a single reaction mixture. We selected two pairs of *β-actin* primers to obtain amplified fragments with a different molecular weight (149 and 433 bp—see Table 1), to be used alternatively in the co-amplification reaction.

PCR products were separated by 1.2% agarose gel electrophoresis.

Table 1
Sequence of PCR primers

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	Gene Bank Accession no.
HOX A1	ATG AAC TCC TTC CTG GAA TA	CGT ACT CTC CAA CTT TCC	AC004079
HOX A2	GAC GCT TTC ACA CTC GAC	TGG TGT AAG CAG TTC TCA G	AC004079
HOX A3	ATG CCA ATC AGC AGC CGT A	TGT ACT TCA TGC GGC GAT	AC004079
HOX A4	TGC ATG CGA GCC ACG TCC T	TTG ACC TGG CGC TCA GAC AA	NM002141
HOX A5	ATG GCA TGG ATC TCA GCG T	GTA ACG GTT GAA GTG GAA CT	AC004080
HOX A6	CTG ATA AGG ACC TCA GTG	TCA GGT AGC GGT TGA AGT G	AC004080
HOX A7	AAT GCC GAG CCG ACT TCT T	AGA TCT TAA TCT GGC GCT CG	AC004080
HOX A9	ACG GCA GGT ACA TGC GCT	GAA CCA GAT CTT GAC CTG C	AC004080
HOX A10	CTA CTG CCT CTA CGA CTC	AAG TTG GCT GTG AGC TCC	AC004080
HOX A11	ACG TGC TGG CCA AGA GCT	TGA CTT GAC GAT CAG TGA GG	AC004080
HOX A13	CGG ACA AGT ACA TGG ATA C	TAT AGG AGC TGG CAT CCG A	AC004080
HOX B1	CCT TCT TAG AGT ACC CAC TCT G	GCA TCT CCA GCT GCC TCC TT	X16666
HOX B2	TCC TCC TTT CGA GCA AAC CTT CC	AGT GGA ATT CCT TCT CCA GTT CC	X16665
HOX B3	TAC CAG TGC CAC TAG CAA CA	GAA CCA GAT CTT GAT CTG C	X16667
HOX B4	CGA GGA ATA TTC ACA GAG CGA T	CCA GAT CTT GAT CTG GCG CT	AF287967
HOX B5	GCT CTT ACG GCT ACA ATT ACA ATG	GCT GTA GCC AGG CTC ATA CT	AF287967
HOX B6	AAG AGC AGA AGT GCT CCA CT	TGA TCT GCC TCT CCG TCA	g184302
HOX B7	AGC CGA GTT CCT TCA ACA TG	CGC GTC AGG TAG CGA TTG TA	XM008559
HOX B8	TTC TAC GGC TAC GAC CCG CT	CGT GCG ATA CCT CGA TTC GC	AY014293
HOX B9	CGA TCA TAA GTC ACG AGA GTG	TCC TTC TCT AGC TCC AGC GT	AY014296
HOX B13	CTG GAA CAG CCA GAT GTG TT	TTG GCG AGA ACC TTC TTC TC	NM006361
HOX C4	CTG AAC ACA GTC CGG AAT A	TTG ATC TGC CTC TCA GAG AG	NM014620
HOX C5	TGG ATG ACC AAA CTG CAC ATG AGC	CAA GTT GTT GGC GAT CTC TAT GCG	X61755
HOX C6	ACC TTA GGA CAT AAC ACA CAG A	ACT TCA TCC GGC GGT TCT GGA A	NM004503
HOX C8	CCA CGT CCA AGA CTT CTT CCA CGG C	CAC TTC ATC CTT CGA TTC TGA AAC C	NM022658
HOX C9	TCA GTC GTC CGT GGT ATA TCA C	AGT TCC AGC GTC TGG TAC TTG	AY014301
HOX C10	TGT TGG CAG GCC GCT GTC CT	CTC CAA TTC CAG CGT CTG GTG T	AF255675
HOX C11	CTT CGA CAA CGC CTA CTG CG	GTC CGT CAG GTT CAG CAT CC	AJ000041
HOX C12	TGC GCT CGG CTT CAA GTA CG	TGG CGT GTG ATG AAC TCG TTG AC	AF328963
HOX C13	TGT CGC ACA ACG TGA ACC TG	CTT CAG CTG CAC CTT AGT GTA G	NM017410
HOX D1	TTC AGC ACG TTC GAG TGG AT	TGC GTG TCA TTC AGG TGC AA	AF202118
HOX D3	CAT CAG CAA GCA GAT CTT C	AGC GGT TGA AGT GGA ATT C	NM002146
HOX D4	TGG ATG AAG AAG GTG CAC GT	AGA TGA GGA CGA TGA CCT G	X17360
HOX D8	GGA TAC GAT AAC TTA CAG AGA C	TAG AGT TTG GAA GCG ACT GT	X15507
HOX D9	GAG TTC TCG TGC AAC TCG T	CAG CTC AAG CGT CTG GTA T	g32390
HOX D10	TAG ACT GAG TCA GAC CTA CG	TCC AAT CCT GGC CTC TGA T	X59373
HOX D11	CTT CGA CCA GTT CTA CGA G	CAG ACG GTC TCT GTT CAG T	AF154915
HOX D12	AGC AGG CTA AGT TCT ATG CG	CAA TCT GCT GCT TCG TGT AG	AF154915
HOX D13	AGA AGT ACA TGG ACG TGT CA	GTC ACT TGT CTC TCA GAT AG	AB032481
β-actin 149	TCT ACA ATG AGC TGC TGG T	TGG ATA GCA ACG TAC ATG G	M10277
β-actin 433	CAC CAT GGA TGA TGA TAT CG	TGG ATA GCA ACG TAC ATG G	M10277

3. Results

HOX gene expression was detected using total RNA extracted from normal and neoplastic tissues, through duplex Reverse Transcriptase (RT)-PCR using specific oligonucleotides designed based on the sequence of each of the 39 genes of the HOX network (Table 1).

The pattern of HOX gene expression, as observed in seven biopsies from normal breast, is illustrated in Fig. 1. No qualitative differences in HOX gene expression were detectable between normal epithelial mammary tissues. Of the 39 genes tested, 17 HOX genes were expressed in normal adult breast. Overall, normal breast showed a prevalent expression of loci A and C (five HOX genes expressed per locus) compared with loci B and D (only three HOX genes expressed on locus D). In locus A, the expression involved *HOX A3* and a combination of four adjacent genes from *HOX A7* to *HOX A11*. On locus B, *HOX B3* and three adjacent genes from *HOX B5* to *HOX B7* were expressed. A pair of thoracic HOX C genes (*HOX C6* and *HOX C8*) were expressed in locus C, together with dispersed lumbo-sacral *HOX C10*, *HOX C11* and *HOX C13* genes. The three HOX D genes that were expressed included *HOX D1* and the adjacent genes *HOX D8* and *HOX D9*.

According to the expression patterns in the paralogous groups, HOX gene expression in the normal breast mostly concerned thoracic (7/11 active genes) and lumbo-sacral (7/16) HOX genes, whereas only three out of 12 cervical HOX genes were expressed. Furthermore, in the cervical region of the network, there was no expression pattern in adjacent HOX genes. In contrast, adjacent 3′ or 5′ end HOX genes were expressed both in the thoracic and lumbo-sacral regions of the network.

The complex expression pattern illustrated in Fig. 1 (large squares) represents the molecular portrait of normal human breast according to the expression of the whole HOX gene network. The same analysis performed in other organs (kidney, colon, liver, lung) shows different patterns of expression with different HOX genes being switched on or off [26].

Fig. 1 also compares the patterns of HOX gene expression, for the whole HOX network (39 genes), in normal breast (large squares) and in fourteen breast cancer biopsies (small circles). Due to the technical approach (duplex RT-PCR) we used to analyse small size samples of breast cancer tissues, only qualitative differences in HOX gene expression pattern can be determined.

Several differences in the expression of individual HOX genes were detected when comparing normal breast and primary breast cancer tissues. Eleven of the 12 cervical HOX genes became alternatively expressed in the breast cancer biopsies tested, whereas in normal breast, the cervical region of the HOX network showed only *HOX A3*, *HOX B3* and *HOX D1* expressed. A single gene, *HOX C4*, appeared to be constitutively silent in normal breast as well as in all of the breast cancers tested (Fig. 2). In contrast, the *HOX D1* gene was active in all normal tissue and breast cancers, whereas *HOX D3* appeared to be silent in normal breast and expressed in all of the breast cancer tissues tested (Fig. 2). The cervical part of the HOX gene network displayed the largest difference when comparing the expression of each HOX gene in the breast cancer tissues with the expression of the same gene in normal breast tissue.

Thoracic genes of the HOX network were actively expressed in normal breast and manifest only minor differences when their expression was compared with the breast cancer biopsies. The unique block of four adjacent HOX genes expressed in normal breast and located on locus HOX A, starts with the thoracic gene *HOX A7* (Figs. 1 and 2), which, together with *HOX B7*, belongs to the paralogous group 7, and is invariantly expressed in all normal and neoplastic tissues tested. Anterior thoracic HOX A genes, *HOX A5* and *HOX A6*, were silent in the normal breast tissue tested, but expressed in the breast cancer biopsies. In the same biopsies, we have tested the expression of *TP53*, which paralleled the expression of *HOX A5* in only 50% of the samples tested (data not shown). Three out of 4 thoracic HOX B genes were expressed in normal breast and, apart from *HOX B5* which was switched off in most of the breast cancer tissues (Fig. 2), did not display qualitative differences in their expression between normal and neoplastic breast tissues. Minor differences between normal and neoplastic breast tissues emerged from the analysis of the expression of the thoracic HOX C genes. Finally, *HOX D8* appeared to be invariantly expressed in both normal and neoplastic breast tissues.

Lumbo-sacral HOX genes displayed expression of seven out of 16 HOX genes in normal breast tissue. The adjacent genes *HOX A9*, *HOX A10* and *HOX A11*, were all expressed in normal breast, but behaved differently in breast cancer biopsies: *HOX A9* and *HOX A10* were invariantly expressed in the cancer biopsies whereas *HOX A11* appeared to be silent in all, but one, of the breast cancer tissues tested. Finally, *HOX A13* was switched off in the normal breast tissue and in the majority of breast cancer samples tested, being active in only three breast cancer tissues. Locus B HOX genes were silent in normal breast. *HOX B9* was also silent in all, but three, breast cancer samples. In contrast, *HOX B13* was actively expressed in all, but three, of the breast cancer tissues tested (Fig. 2). The HOX genes on locus C were alternatively switched on and off, without any major differences in their expression between normal breast and breast cancer tissues. The lumbo-sacral part of locus D appeared to be silent in four of its five HOX genes and major differences between normal and neoplastic breast tissue were only detectable for *HOX D10* (Fig. 2) and *HOX D13*.

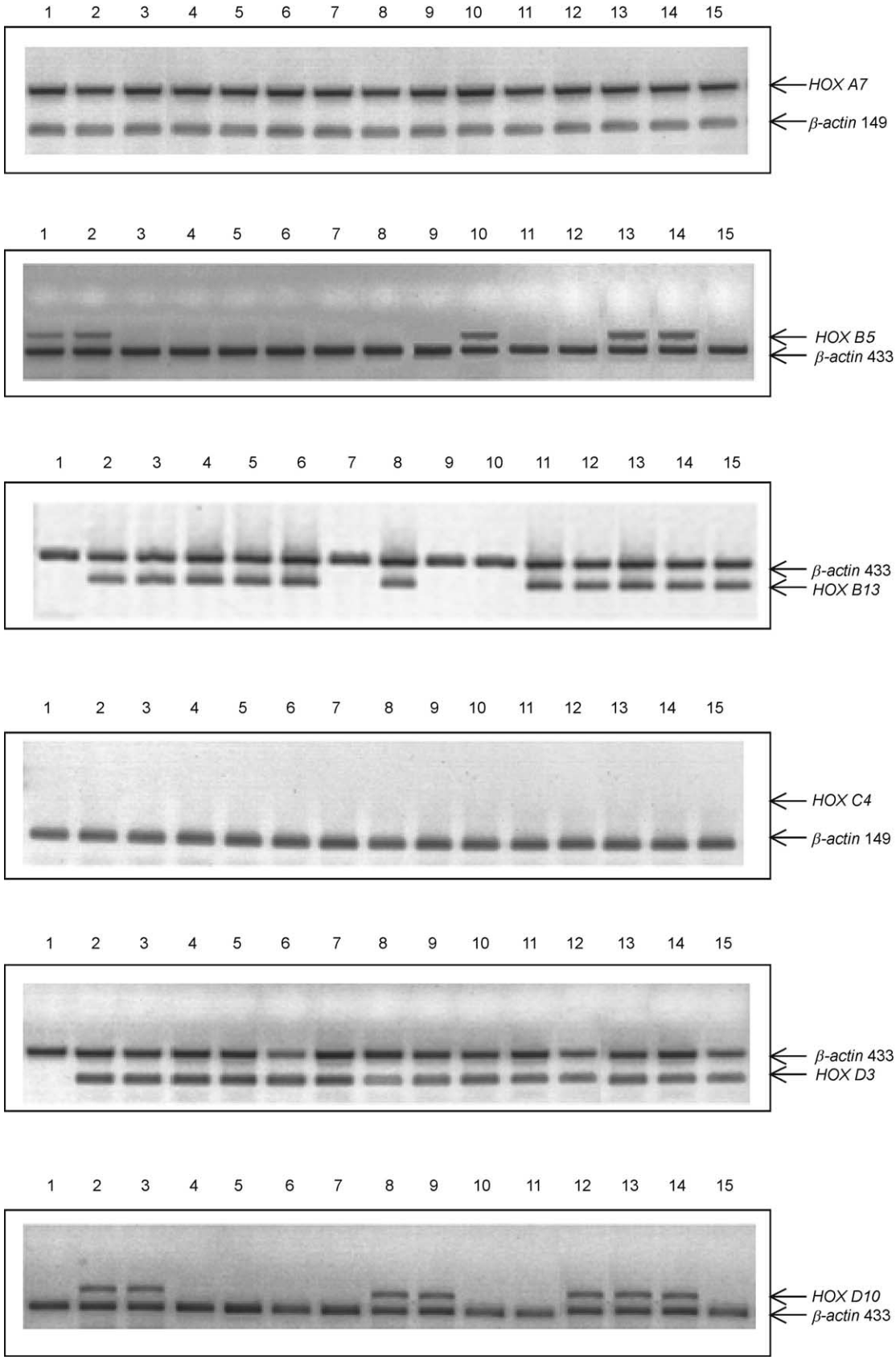
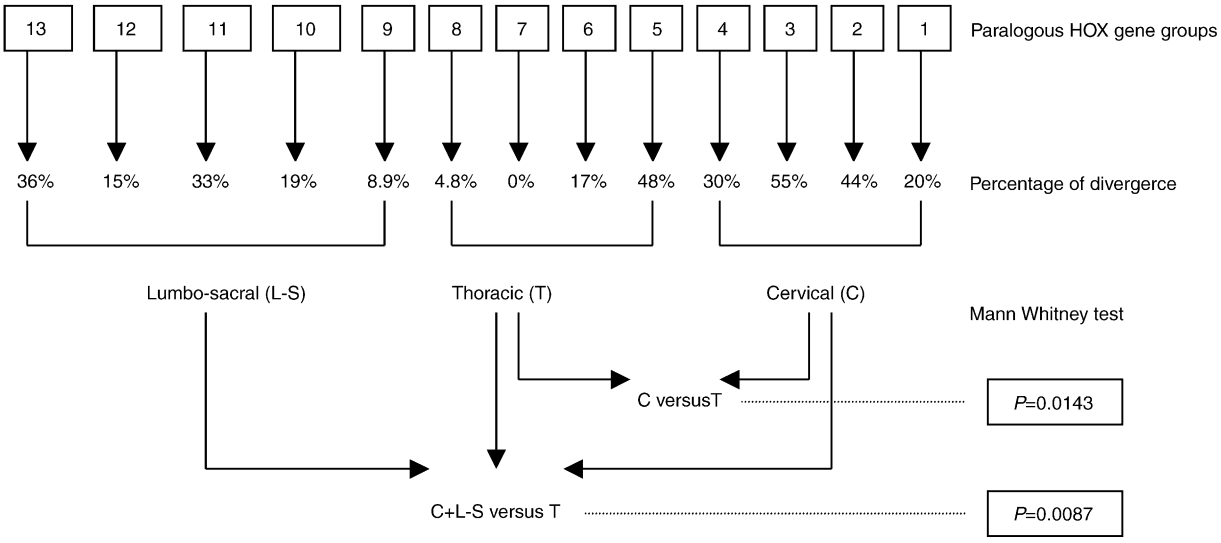


Fig. 2. Expression of *HOX A7*, *HOX B5*, *HOX B13*, *HOX C4*, *HOX D3* and *HOX D10* in normal breast (lane 1) and in breast cancer biopsies (lanes 2–15). Control co-amplification of *HOX A7* and *HOX C4* with a 149bp *β- actin* primer is reported. Control co-amplification of *HOX B5*, *HOX B13*, *HOX D3* and *HOX D10* with a 433 bp *β-actin* primer is reported.

Table 2
Percentage of divergence in HOX gene expression between normal breast and breast cancer tissues, relative to the number of tissues tested for each HOX paralogous gene group (1–13)[a]



[a] Statistical comparison (Mann–Whitney test) of cervical versus thoracic HOX paralogous gene groups and of cervical plus lumbo-sacral versus thoracic HOX paralogous gene groups.

For each paralogous gene group (1–13—top of Fig. 1) the HOX network can be subdivided into, we have determined the percentage of breast cancer tissues whose HOX gene expression differs from normal breast, relative to the total number of breast cancer tissues tested; for example, in parologous group 1 (three HOX genes: HOX A1 = 14 biopsies tested, HOX B1 = 12 biopsies tested and HOX D1 = 14 biopsies tested—giving a total of 40 determinations) in eight out of 40 determinations (20%), HOX gene expression was different in the cancer biopsies compared with the expression of the same genes in normal breast tissue (Table 2). The highest peak of divergence between breast cancer and normal breast tissue occurred in the paralogous group 3 (55%). On the basis of this analysis, it is possible to identify three parts of the network: one corresponding to cervical paralogous groups HOX genes where the divergence in HOX gene expression between normal and malignant breast tissue was high (paralogous groups 1–4); one for thoracic paralogous HOX gene groups (paralogous groups 5–8) which displayed the lowest divergence; and, finally, the lumbo-sacral part of the HOX network where the divergence increased without reaching the values of cervical paralogous HOX genes. The statistical comparison of the percentages of divergence in HOX gene expression between cervical versus thoracic paralogous HOX groups, according to the Mann–Whitney test, indicated a P value of 0.0143. When we compared the percentages of divergence in HOX gene expression between cervical + lumbo-sacral paralogous HOX groups versus thoracic paralogous HOX groups, the P value reached 0.0087 (Table 2). The boundary between cervical and thoracic HOX gene paralogous groups where divergence abruptly decreases (no divergence is observed in paralogous group 7) corresponds to the antero-posterior physical location of the human breast.

4. Discussion

We have analysed the expression of the complete HOX gene network (39 genes) in normal human breast and primary breast carcinomas to determine whether the HOX network is implicated in breast cancer evolution and to identify, inside the network, specific HOX genes primarily involved with breast tumorigenesis. Our results show that the HOX gene network displays an overall expression pattern characteristic of normal breast tissue. Furthermore, the expression of specific HOX genes is similar in normal and neoplastic breast tissue indicating that these genes may be involved in breast organogenesis. In contrast, other HOX genes manifest an altered expression in primary breast cancers with respect to normal breast, which supports their involvement in breast cancer tumorigenesis.

In normal breast, seventeen out of the 39 HOX genes of the network were actively expressed. Their overall expression represents the molecular portrait of human breast. Different normal adult human organs such as the liver, colon, kidney and lung display an overall pattern of HOX gene expression that is characteristic of

each organ. Thus, the expression of the HOX gene network makes it possible to define the molecular portrait of normal adult human organs.

According to our data, the expression of specific HOX genes appeared altered in breast cancer tissue compared with the expression observed in the normal breast. This modification mostly concerned the cervical HOX genes belonging to paralogous groups 1–4 and the lumbo-sacral HOX genes of paralogous groups 9–13, whereas HOX genes located in the thoracic group of the HOX network appeared to be invariantly expressed in normal and neoplastic breast tissues.

Specific HOX genes, such as *HOX A1*, *HOX B2*, *HOX A3*, *HOX D3* and *HOX D4*, in the cervical part of the network and *HOX D10*, *HOX A11*, *HOX B13* and *HOX D13* in the lumbo-sacral part of the network, are good candidate genes to be tested further in order to confirm their role in breast cancer evolution.

Initial observations on HOX gene involvement in breast cancer have already suggested a role for genes from loci A and C of the HOX A1 network [23]. *Hox A1* has been implicated in mouse and human breast epithelial cell differentiation, mostly through its regulation by retinoic acid [27], steroids [22] and its link to the expression of laminin [28]. *HOX C6* has been detected in breast carcinomas, where it is capable of contributing to the breast cell phenotype and through its interaction with other HOX genes (*HOX B7*) of repressing their target genes [29]. Alterations in *Hox A1* and *Hox B7* expression are detectable in mammary epithelial cells following their contact with the extracellular matrix (ECM) and changes in cellular structure [30]. Increased *HOX D3* expression has been linked with angiogenesis through the regulation of αv β3 integrin and the urokinase plasminogen activator (u-PA) [31]. Transduction of breast carcinoma cells with *HOX B7* induces basic fibroblast growth factor (bFGF) expression and increases the malignancy of breast cells *in vitro* [32,33]. Paralogous group 9 Hox genes (*Hox a9*, *Hox b9* and *Hox d9*) work together in mutant mice controlling the differentiation of their mammary epithelial ductal system during pregnancy [34]. Recently, it has been shown that in breast carcinomas, *HOX A5* behaves as a transcriptional regulator of multiple target genes, two of which are *TP53* [24] and the progesterone receptor [35]. In contrast to a previous report [24], which describes a close correlation between *TP53* and *HOX A5* mRNA levels in human breast cancer cells, the interaction between *HOX A5* and *TP53* diverged in half of the biopsies we tested. However, the small number of human samples tested, in our study as well as in Ref. [24], hampers any comparisons.

Apart from the first-identified function of controlling antero-posterior patterning during embryonic development [10], the HOX gene network regulates multiple cellular processes such as (a) cell growth and proliferation through interactions with the cell-cycle [36] and the apoptotic pathways [24]; (b) the acquisition and maintenance of spatial and temporal cell allocation [11]; (c) the establishment of cell identity, achieving specific cell phenotypes through the decoding of micro-environmental signals [29]; (d) the process of cell–cell communication through cross-talk with morphogenetic molecules, growth factors and cytokines and signal transduction pathways [37]; (e) cell compartmentalisation and architectural organisation during the formation and maintenance of tissues and organs [38]. However, cancer is a multistage process that manifests itself through the uncoordinated alteration of most of these functions [39]. Thus, it is conceivable that changes in the expression of networks of genes acting in a complex coordinated manner (such as the HOX network), might be involved in cancer evolution [26], despite modifications concerning specific genes.

The HOX gene network manifests expression patterns corresponding to specific cell phenotypes [40]. The combination of the seventeen HOX genes we have reported, is the result of the HOX network expression in the different cell phenotypes involved in the generation of normal breast. Alterations of the breast phenotype consequent to cancer evolution paralleled changes in HOX gene expression concerning HOX genes that are not crucial for breast organogenesis (paralogous cervical and lumbo-sacral HOX groups). In contrast, breast-specific HOX genes (thoracic paralogous HOX groups) remained invariantly expressed, in spite of cancer progression. Thus, thoracic paralogous HOX gene groups may be able to control the antero-posterior patterning of the HOX network during thoracic embryonic development and its homeostatic regulation during adult life. In contrast, cervical and lumbo-sacral paralogous HOX groups may be involved in signalling pathways related to the determination of the breast cell phenotype and therefore their expression is altered during breast cancer progression. These observations emphasise the importance of the HOX network in the evolution of cancer and highlight the possibility of using the HOX genes as potential targets for future cancer therapies.

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