Expression of lumbosacral HOX genes, crucial in kidney organogenesis, is systematically deregulated in clear cell kidney cancers

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Homeobox-containing genes are involved in different stages of kidney organogenesis, from the early events in intermediate mesoderm to terminal differentiation of glomerular and tubular epithelia. The HOX genes show a unique genomic network organization and regulate normal development. The targeted disruption of paralogous group 11 HOX genes (HOX A11, HOX C11 and HOX D11) results in a complete loss of metanephric kidney induction. Despite a large amount of data are related to the early events in the kidney development, not much is known about HOX genes in advanced kidney organogenesis and carcinogenesis. Here, we compare the expression of the whole HOX gene network in late-stage human foetal kidney development with the same patterns detected in 25 pairs of normal clear cell renal carcinomas (RCCs) and 15 isolated RCC biopsy samples. In the majority of RCCs tested, HOX C11 is upregulated, whereas HOX D11, after an early involvement becomes active again at the 23rd week of the foetal kidney development, is always expressed in normal adult kidneys and is deregulated, together with HOX A11 and lumbosacral locus D HOX genes. Thus, through its function of regulating phenotype cell identity, the HOX network plays an important role in kidney carcinogenesis. Lumbosacral HOX genes are involved in the molecular alterations associated with clear cell kidney cancers and represent, through their deregulation, a molecular mark of tubular epithelial dedifferentiation occurring along tumour evolution, with the restoration of genetic programs associated with kidney organogenesis. The deregulation of lumbosacral HOX genes in RCCs supports (i) the consideration of the HOX gene transcriptome as the potential prognostic tool in kidney carcinogenesis and (ii) the possibility to foresee clinical trials with the purpose of targeting these genes to achieve a therapeutic effect in RCC patients. *Anti-Cancer Drugs* 22:392–401 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The molecular mechanisms involved in early kidney organogenesis require the expression, in the matanephric blastema, of the homeobox genes HOX A11 and HOX D11 [1], to induce the outgrowth of the ureteric bud from the Wolffian duct through the expression of several transcription factors (Wt 1, Pax 2, SALL 1, Fox C1 and Eya 1) [2–6], of the nuclear protein formin [7], growth factors GDNF [8], its tyrosine kinase receptor (Ret) [9] and the coreceptor (GFRa1) [10]. Wnt signals are crucial inductors of kidney tubologenesis, as deduced by the Wnt4-mutant mice lacking epithelial–mesenchymal transition and tubulogenesis [11].

Homeobox genes are transcription factors that function during normal development [12], and contain the homeobox, a 183-bp DNA sequence coding for a 61-amino acid homeodomain. Different homeodomain types or classes may be identified [13], each characterizing a homeobox gene family. Among these, the homeodomain of the homeotic gene of *Drosophila Antennapedia* defines a consensus sequence referred to as class I homeodomain or HOX genes [14]. In mice (HOX genes) and humans (HOX genes) there are 39 genes organized into four genomic clusters of approximately 100 kb in length, defined as HOX loci, each localized on a different chromosome (HOX A at 7p15.3, HOX B at 17p21.3, HOX C at 12q13.3 and HOX D at 2q31) [15] and comprising 9–11 genes (Fig. 1 right). On the basis of sequence similarity and position on the locus, the corresponding genes in the four clusters can be aligned with each other and with genes of the homeotic-C complex of drosophila in 13 paralogous groups [16]. During mammalian development, HOX gene expression controls the identity of various regions along

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Fig. 1

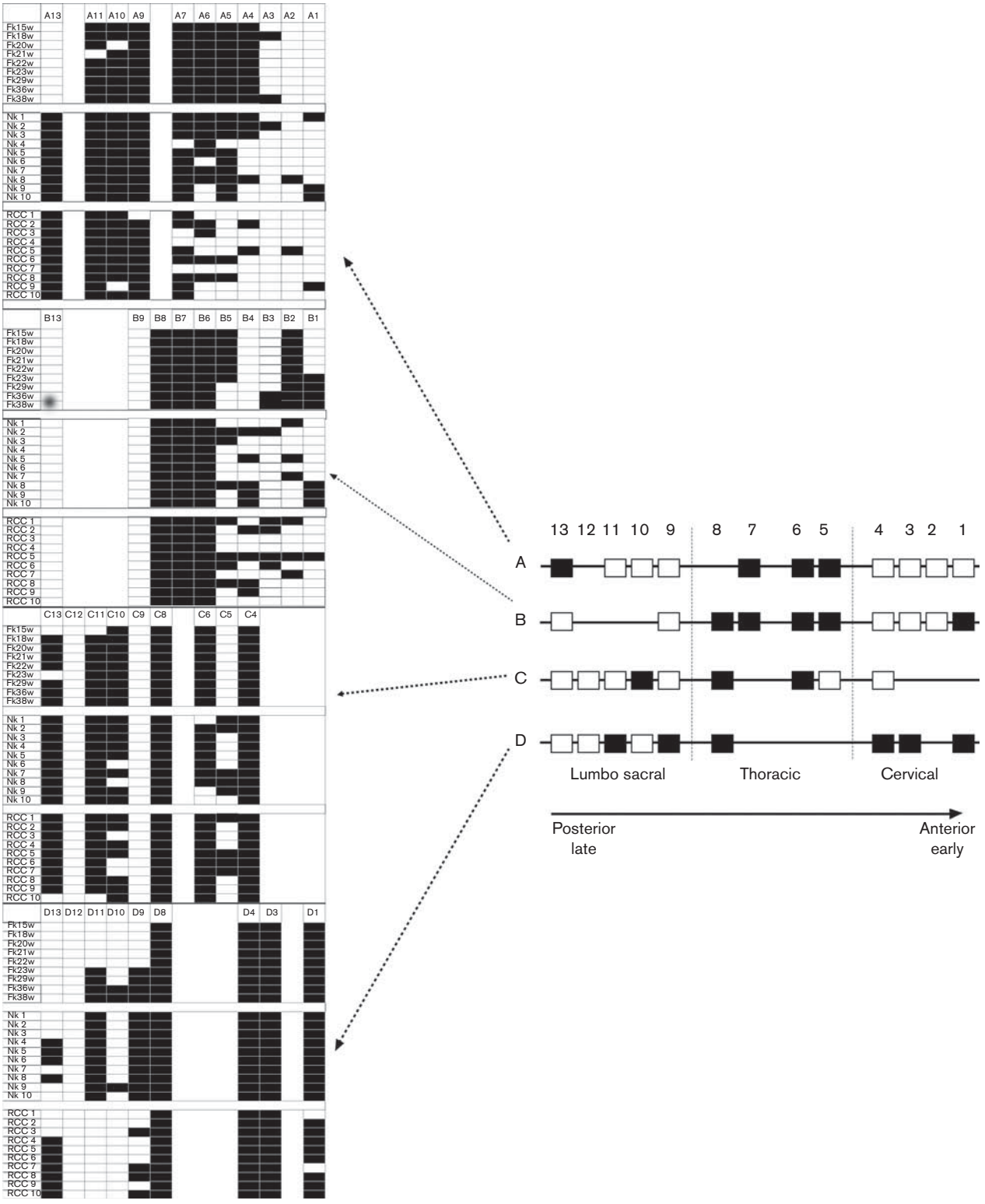


Diagram of whole HOX gene network expression detected by reverse transcription-PCR in foetal kidney between 15 and 38 weeks of development (Fkw15–Fkw38), in ten pairs of human samples from normal kidneys (Nk1–Nk10) and kidney cancer tissues [renal cell carcinoma (RCC1–RCC10)] taken from the same patients (left). Small black and white rectangles indicate active or silent HOX genes, respectively. Schematic representation of the HOX gene network (see the text for details) and expression of the whole HOX gene network in human primary epithelial tubular cells (right). Black and white squares indicate active or silent HOX genes, respectively.

the body axis, from the branchial area to the tail [17]. This is achieved according to the rules of temporal and spatial colinearity, with 3′ HOX genes expressed early in development and controlling anterior regions, followed by progressively more 5′ genes expressed later, and controlling more posterior regions [18]. In particular, 3′ HOX genes in groups 1–4 (cervical), primarily control the development of the branchial area and the rhomboencephalon, the embryonic region corresponding to the hindbrain [19]. Central HOX genes in groups 5–8, control the thoracic portion of the body, whereas 5′ HOX genes in groups 9–13, control the lumbosacral region. In the HOX gene network, the most repeated poor regions of the human genome [20], is also expressed in normal adult human organs [21]. HOX and homeobox genes seem to regulate normal development, cell differentiation [22,23] and control other cellular processes, as proven by the description of congenital [24], somatic [25], metabolic [26] and neoplastic alterations [27,28] involving these genes.

Homeobox and HOX genes are directly involved in kidney organogenesis [29,30]. In mice that are triple mutant for paralogous group 11 HOX genes (HOX A11, HOX C11 and HOX D11), the induction of metanephric development is arrested with 100% penetrance [31]. Furthermore, HOX genes regulate the expression of renal morphogens [32,33]. Despite a large amount of data are related to the early events in the kidney development, not much is reported on the role of *HOX* genes in advanced kidney organogenesis.

Here, we have compared the role of HOX genes in kidney organogenesis and carcinogenesis through the expression of the whole gene network in late kidney development (from the 15th to the 38th week) with the same expression patterns detected in 25 pairs of normal clear cell renal carcinomas (RCCs) and 15 isolated RCC biopsy samples. RCC is generated by the neoplastic transformation of the epithelial compartment of the kidneys' proximal tubules. RCC represents the most widespread and lethal adult kidney cancer of the urinary tract. It includes several types: clear cells (70–80%), papillary (15–20%), chromophobe (4–5%) and collecting ducts (< 1%) [34]. The main genetic alterations associated with RCCs are located on chromosome 3 and on the von Hippen–Lindau gene mutation [35].

Lumbosacral HOX genes play a crucial role in kidney organogenesis. These data that we present here, support the view of an involvement of the same genes in kidney carcinogenesis: HOX C11, already active in the kidney development and in normal adult kidney, becomes upregulated in the majority of RCCs tested. HOX D9, silent between the 15th and 22nd week of kidney development, becomes active from the 23rd week to the 38th week, is always active in the normal kidney, whereas it tends to be inactive, together with the other paralogous 9 HOX genes, in the RCCs tested. Furthermore, HOX D11, one of the paralogous group 11 HOX genes that generate bilateral kidney agenesis in triple-mutant mice, becomes active again after early uretheric bud induction in the 23rd week of the development, is always active in the normal kidney and becomes inactive in the majority of the tested clear cell kidney cancers. This gene, already involved with paralogous group 11 HOX genes during early metanephric induction through the interaction with the Pax/Eya/Six regulatory cascade [31], seems to be heavily involved in the molecular alterations associated with clear cell carcinomas. The involvement of lumbosacral HOX D in kidney carcinogenesis further confirms a nonsecondary role played by the chromosomal region 2q31-33, in which the HOX D locus is located, in the epithelial–mesenchymal interaction.

Materials and methods

Specimens

Human foetal kidneys were obtained from legally approved therapeutic abortions carried out at the Department of Pathology, Federico II University of Naples. The sampling was performed under the control of the University's guidelines for human experimentation (autopsy protocol). Informed consent was obtained from all the patients involved in the experiments, and the study protocols were reviewed and approved by the University Ethics Committee. The age of the foetuses was calculated from anamnestic and ultrasonographic data, which ranged from 15 to 38 gestational weeks. Kidneys were dissected, typically within 2 h after death. Each tissue sample was split into two upright parts, one to be fixed and the other part to be snap frozen in liquid nitrogen and stored at −80°C until used for RNA extraction. After fixation and dehydration, tissue samples were embedded in wax at 54°C, and section of 3–5 mm thickness were cut. Paraffin sections were placed on gelatin-coated glass slides and dried overnight at 37°C. Sections were counterstained with haematoxylin–eosin for morphological examination.

Normal and malignant kidney tissue was obtained from nonselected patients operated at the Urology Department of our medical school. During surgery, samples from non-necrotic clear cell kidney cancer tissues and from intact normal kidneys from an area within a few centimetres of the tumour were taken. The tumour sample was split into two parts and analysed by the pathologist for diagnosis, tumour staging and grading according to the Tumor Node Metastasis system [34] (Table 1). Normal kidney was also split into two parts and analysed by a pathologist to exclude the presence of dysplastic changes. The presence of a minimal amount of dysplasia in normal kidney justified the exclusion of both normal and corresponding cancer biopsy samples from RNA extraction. All samples of normal kidney and clear cell RCCs were

Table 1 Clinicopathological features and HOX gene expression in clear-cell kidney cancers

	Age (years)/sex	Histology	Grading	Staging	HOX A4	HOX A5	HOX A6	HOX B2	HOX C12	HOX D9	HOX D11
1	8/F	RCC	G2	T2NxM0	−	−	−	+	−	−	−
2	45/M	RCC	G2	T2 N0M0	+	−	+	−	−	−	−
3	70/F	RCC	G3	T1NxM0	−	−	+	−	−	+	−
4	67/F	RCC	G2	T4 N0M0	−	−	−	−	−	−	−
5	61/M	RCC	G2	T2NxM0	+	−	−	+	−	−	−
6	69/F	RCC	G2	T2 N0M0	−	+	+	−	−	−	−
7	55/M	RCC	G2	T1 NxM0	−	−	−	+	−	+	−
8	69/F	RCC	G3	T1 NxM0	−	+	+	−	−	+	−
9	57/F	RCC	G3	T3aN0M1	−	−	−	−	−	−	−
10	49/F	RCC	G2	T2N1M0	−	−	−	−	−	+	−
11	63/F	RCC	G2	T1NxM0	−	−	+	+	−	−	−
12	62/M	RCC	G2	T1 NxM0	−	−	−	−	−	+	−
13	51/F	RCC	G2	T1 NxM0	−	−	+	−	−	+	−
14	46/F	RCC	G2	T2 NxM0	−	−	−	−	−	−	−
15	43/F	RCC	G2	T2N0M0	−	−	+	−	−	−	−
16	57/M	RCC	G3	T4 N0M0	+	+	+	−	−	−	−
17	60/M	RCC	G3	T3aN0M0	−	−	+	+	−	−	−
18	58/M	RCC	G2	T2NxM0	−	−	−	−	−	−	−
19	49/F	RCC	G2	T1 NxM0	+	−	+	−	−	−	−
20	52/F	RCC	G2	T1 N0M0	−	−	+	−	−	+	−
21	67/F	RCC	G2	T2 N0M0	−	−	−	−	−	−	−
22	70/M	RCC	G2	T2 N0M0	−	+	+	−	−	−	−
23	62/M	RCC	G3	T4 N0M0	−	−	−	−	−	−	−
24	51/M	RCC	G2	T2NxM0	−	−	+	+	−	−	−
25	49/F	RCC	G2	T2 NxM0	−	−	+	−	−	−	−

Classification of the 25 kidney cancer biopsies (1–10 from normal–tumour pairs; 11–25 from isolated tumours) according to grades (G2 and G3), stage [34]. Reverse transcription (RT)-PCR expression (positive or negative) of HOX A4, HOX A5 and HOX A6, HOX B2, HOX C12, HOX D9 and HOX D11 as detected by RT-PCR. F, female; M, male; RCCs = clear-cell renal carcinoma.

reviewed by a single pathologist. The other part of the samples was snap frozen in liquid nitrogen and stored at −80°C until used for RNA extraction. Isolated kidney cancer biopsy samples were obtained through surgery and followed the same treatment as that for cancer biopsy samples belonging to normal–tumour pairs. An overview of the histological and clinical data is given in Table 1.

RNA extraction and analysis

Total RNA was isolated from frozen liver biopsy samples using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Samples were treated with RNase-free DNase (Qiagen GmbH, Hilden, Germany) to prevent amplification of genomic DNA. The concentration of isolated RNA and the ratio of absorbance at 260–280 nm were measured by the average of three readings using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, Delaware, USA). RNA (1 μg) was subjected to cDNA synthesis for 1 h at 37°C using the Ready-To-Go You-Primer First-Strand Beads (GE Healthcare, Buckinghamshire, UK) in a reaction mixture containing 0.5 μg of random hexamers (Applied Biosystems, Foster City, California, USA).

Polymerase chain reaction amplification

The primers are systematically tested against new primer design programs to increase their score and are validated before being introduced into experimental use. To prevent genomic DNA contamination, sense and antisense primers were designed to frame a sequence that crossed at least one intron on the genes. The coamplification of each specific gene and of the 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 different molecular weights (149 and 433 bp), to be used alternatively in the coamplification reaction. Duplex-PCR products were separated by ethidium (1.2%) agarose gel electrophoresis.

Real-time polymerase chain reaction

TaqMan analysis was carried out on a 7900HT Sequence Detection System (Applied Biosystems). Single-plex PCR reactions were performed in fast-gene quantification in 96-well plates. The thermal cycling conditions included a step of 20 s at 95°C, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s with TaqMan Fast Universal PCR Master Mix (10 μl) in a volume of 20 μl containing 2 μl of cDNA and 1 μl of specific TaqMan Gene Expression Assays for human HOX and other genes, according to the manufacturer's directions. All reactions were performed in triplicate. All reagents were from Applied Biosystems. The comparative Ct method was used to determine the human HOX and other gene variations in kidney pair samples, using as reference gene TaqMan Endogenous Controls Human β-actin Endogenous Control (Applied Biosystems). For HOX fold in isolated kidney samples, we identified a calibrator sample (a nontumour sample pair under study) that represents the unitary amount of the target of interest; the other samples express *n*-fold mRNA relative to the calibrator. Final amounts of target were

determined as follows: target amount = 2^{-C_t}, where $C_t = [C_t\ (\text{HOX}) - C_t\ (\text{ACTB})]_{\text{sample}} - [C_t\ (\text{HOX}) - C_t\ (\text{CTB})]_{\text{calibrator}}$.

Immunohistochemistry

Immunostainings were performed on paraffin-embedded sections, using a previous step of the step-induced antigen-retrieval technique. Specimens were selected from our Pathology Unit (Federico II University Medical School) including embryonal, non-neoplastic and neoplastic samples. Before incubation with the primary antibody, the slides were heated in a pressure cooker for 3 min in a solution of sodium citrate (0.01 mol/l). After incubation with the primary antibody (HOX D9 sc-8320, Santa-Cruz Biotechnology Inc., California, USA), at a dilution of 1/100 for 1 h, immunodetection was performed with biotinylated antimouse immunoglobulins, followed by peroxidase-labelled streptavidine (LSAB-DAKO, Glostrup, Denmark) with diaminobenzidine chromogen as the substrate. Sections were counterstained with haematoxylin.

Results

HOX gene expression in foetal, normal adult, neoplastic kidney and in primary tubular kidney cells

We have analysed the expression of the entire HOX gene network in late-stage human kidney organogenesis (from the 15th to the 38th week of development) in 10 pairs of kidney tissue biopsy samples, each pair from the same patient, and comprising, according to the pathologist, clear cell RCC and adjacent normal kidney (Fig. 1, left; Table 1, samples 1–10), in 15 isolated biopsy samples of clear cell RCCs (Table 1, samples 11–25) and in primary cultures of epithelial tubular cells obtained from normal adult kidneys (Fig. 1, right). All tumour samples are clear cell RCCs with different stages and grades [34] (Table 1).

The analysis of HOX loci gene expression through the real-time reverse transcription (RT)-PCR duplex (Fig. 1, left), starting from locus A, indicates that in the foetal kidneys a block of genes (from HOX A4 to HOX A11) are almost constitutively active from the 15th to the 38th week of kidney development. In contrast, the most cervical HOX genes, HOX A1 and HOX A2, and the most lumbosacral HOX gene, HOX A13, are constitutively silent and HOX A3 is rarely detectable. In normal kidneys, the most cervical HOX A genes, HOX A1, HOX A2 and HOX A3, are only episodically active; HOX A4 is expressed in four of 10 kidneys, whereas all the remaining genes of the HOX A locus, from HOX A5 to HOX A13, are substantially active, with a sporadic lack of expression in HOX A5, HOX A6 and HOX A7. In clear cell RCCs, anterior HOX A genes, from HOX A1 to HOX A6, tend to be less active compared with normal kidneys (Fig. 1, left); in particular, thoracic HOX genes tend to increase their expression anteroposteriorly from HOX A5, which is expressed in four of 25 RCCs (Table 1), to HOX A6 and HOX A7, which are active in 14 of 25 (Table 1) and 17 of 25 (data not shown) clear cell kidney cancers, respectively. Lumbosacral HOX genes (from HOX A9 to HOX A13) are almost always active in the totality of kidney cancer biopsy samples that were tested (Fig. 1, left).

In the HOX B locus, HOX B1 is expressed in foetal kidney starting from the 23rd week of human foetal kidney development. HOX B2 is constitutively active, together with HOX B6, HOX B7 and HOX B8. HOX B3 is active at 36 and 38 weeks of development; HOX B4, as well as HOX B9 and HOX B13, is constitutively silent, whereas HOX B5, which is an active from the 15th to the 23rd week, becomes silent in the final stages of foetal kidney development. In normal adult kidney, cervical HOX genes (from HOX B1 to HOX B5) tend to be occasionally or more frequently (HOX B4 = 5/10) expressed. In contrast, thoracic HOX genes (from HOX B6 to HOX B8) are constitutively active, whereas lumbosacral HOX genes (from HOX B9 to HOX B13) are constitutively silent. As far as the HOX B locus is concerned, no substantial alterations on gene expression are detected through RT-PCR on comparing normal kidney and clear cell kidney cancers. Out of the cervical HOX genes, HOX B1 is only sporadically active in three of 25 RCCs; HOX B2 is expressed in six of 25 neoplastic kidneys (Table 1); and HOX B3 and HOX B4 are both active in 10 of 25 kidney tumour samples (data not shown). In contrast, thoracic HOX B genes are constitutively active, whereas both the lumbosacral genes are systematically inactive in RCCs and in normal kidneys.

Locus C HOX genes apparently show minor variations in their RT-PCR expression when comparing foetal, adult normal and cancerous kidneys (Fig. 1, left). HOX C4 is constitutively active and HOX C5 is undetectable in foetal kidney and active in half of the normal and neoplastic kidneys. HOX C6 and HOX C8 are almost always active; HOX C9 is constitutively inactive. HOX C10 is active in foetal kidneys and in the majority of normal and cancerous kidneys. HOX C11 and HOX C13 are almost always active in foetal, adult normal and cancerous kidneys. HOX C12 is constitutively silent.

Finally, locus D HOX genes show a constitutive expression of cervical and thoracic genes (from HOX D1 to HOX D8) in foetal, adult normal and neoplastic kidneys. Lumbosacral HOX D genes represent the part of the HOX network that, together with the cervical side of locus A genes, manifests the largest variations in gene expression when comparing foetal, adult normal and cancerous kidneys. HOX D9, silent from the 15th to the 22nd week of development, becomes active from the 23rd week to the 38th week and is always expressed in normal adult kidneys; in contrast, HOXD9 is active only in four of 10 (Fig. 1, left) and seven of 25 RCCs (Table 1). HOX D10 is active throughout the end of kidney development, and is only occasionally expressed in

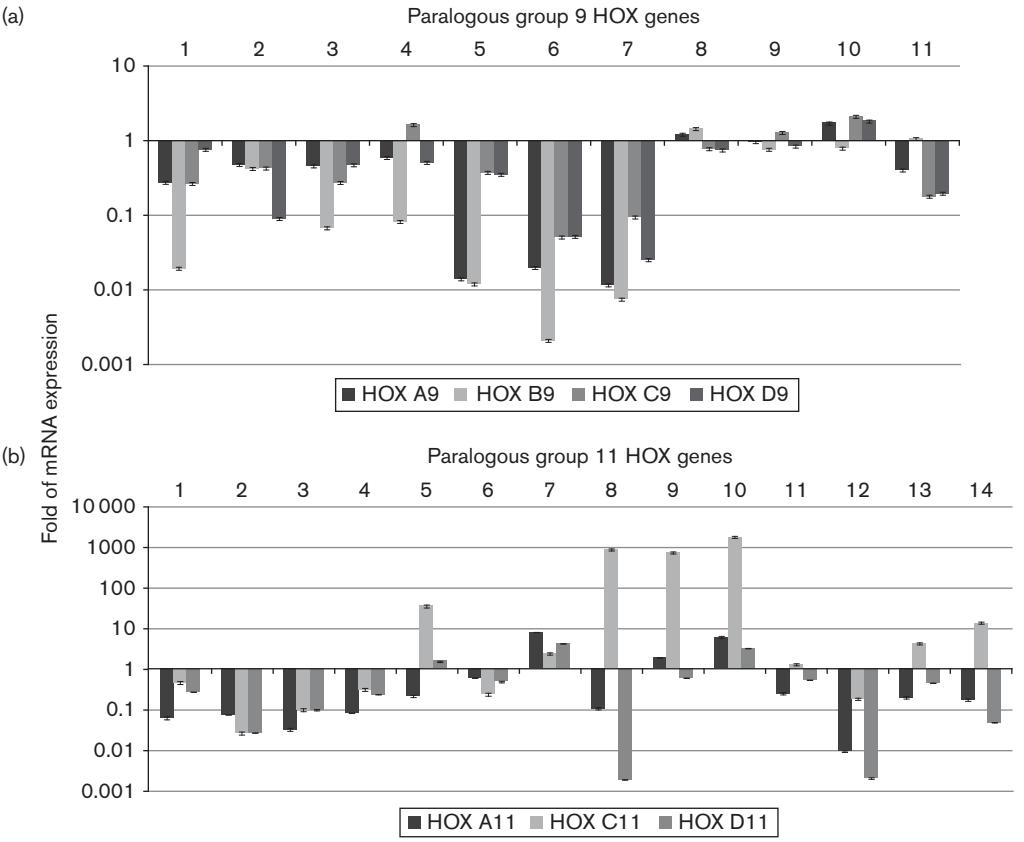
normal adult kidneys and is always silent in RCCs. HOX D11 is silent from the 15th to the 22nd week, becomes active from the 23rd to the 38th week of kidney development and is always active in adult normal kidneys, whereas it is systematically silent in clear cell kidney cancers (Table 1). HOX D12 is constitutively silent. Finally, HOX D13, always inactive in foetal kidneys, is expressed in four of 10 normal kidneys and in seven of 10 RCCs.

Figure 1 (right) shows the HOX gene network expression in primary tubular epithelial cells obtained from normal adult human kidneys. This is the cell phenotype of which the neoplastic transformation generates clear cell RCCs. Both cervical and lumbosacral regions of the HOX gene network are scarcely active, whereas the thoracic HOX region is almost completely active with its component genes, except HOX C5, which is silent. In the cervical HOX region, only four genes are active, HOX B1 and three HOX D genes, HOX D1, HOX D3 and HOX D4. Four genes are also active in the lumbosacral HOX region, HOX A13, HOX C10 and two locus D genes, HOX D9 and HOX D11. In primary epithelial kidney cells, HOX D is the most active locus, whereas HOX C is the least active.

Real-time expression of HOX genes in pairs of RCCs/normal kidneys

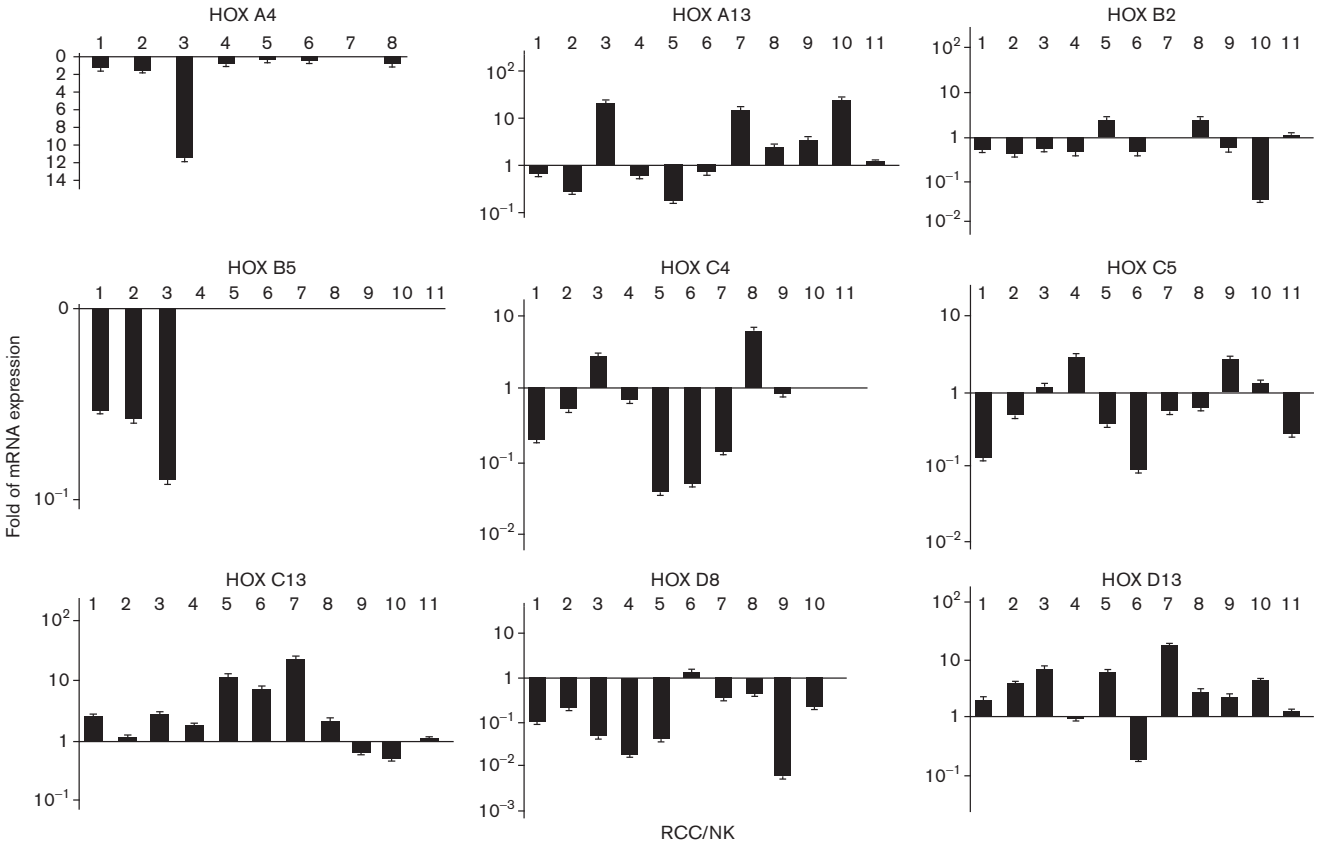
To quantify the variation of expression in pairs of RCCs/normal kidneys for specific HOX genes in the network, we have detected the real-time mRNA expression of paralogous group 9 (four HOX genes), paralogous group 11 (three HOX genes) and of selected genes of the HOX network (HOX A4, HOX A13, HOX B2, HOX C4, HOX C5, HOX C13, HOX D8 and HOX D13). These data shown in Fig. 2 indicate a substantial decrease of HOX A9, HOX B9, HOX C9 and HOX D9 gene expression in RCCs versus normal kidneys in eight of 11 tested pairs, with HOX D9 decreasing in all but one pair. Paralogous group 11 HOX genes (HOX A11, HOX C11 and HOX D11), the ablation of which determine kidney agenesis [31], seem to be also involved in kidney carcinogenesis: HOX A11 and HOX D11 decrease their expression in 10 of 14 pairs tested, whereas HOX C11 is increasingly active in eight of 14 pairs tested, at low level (between one and three-fold increase in three pairs), an intermediate level (between 10 and 100-fold increase in two pairs) and at high levels (between 100 and 1000-fold increase in three pairs; Fig. 2). With regard to the other selected genes of the

Fig. 2



Fold increase or decrease of the real-time mRNA expression of paralogous group 9 HOX genes (HOX A9, HOX B9, HOX C9 and HOX D9; a), and paralogous group 11 HOX genes (HOX A11, HOX C11 and HOX D11; b) in 11 (HOX9) and 15 (HOX11) pairs of renal cell carcinomas/normal kidneys. All reactions were performed in triplicate and data are expressed as the mean of the relative amount of mRNA levels.

Fig. 3



Fold increase or decrease of real-time mRNA expression of HOX A4, HOXA 13, HOX B2, HOX B5, HOX C4, HOX C5, HOX C13, HOX D8 and HOX D13 in pairs of renal cell carcinomas (RCCs)/normal kidneys (NKs). All reactions were performed in triplicate and data are expressed as the mean of the relative amount of mRNA levels.

network (Fig. 3), HOXA4 and HOXB2 only occasionally manifest variations on comparing neoplastic with normal kidneys; paralogous group 13 HOX genes (HOXA13, HOXC13 and HOXD13) tend to increase their expression in RCCs versus normal kidneys (Fig. 4). HOXC4, HOXC5 and HOXD8 tend to be downregulated in RCCs. Finally, HOXB5 shows a marked decrease of expression in three pairs of RCCs/normal kidneys and no variations in the other eight pairs tested.

Histopathological features of kidney biopsies

We have compared the expression of HOX genes mostly involved in kidney cancer evolution with the histopathological features of the tumour biopsy samples in our analysis. The data shown in Table 1 indicate the lack of correspondence for the histological grade and stage [34] with respect to the altered expression of the HOX genes observed in our molecular analysis.

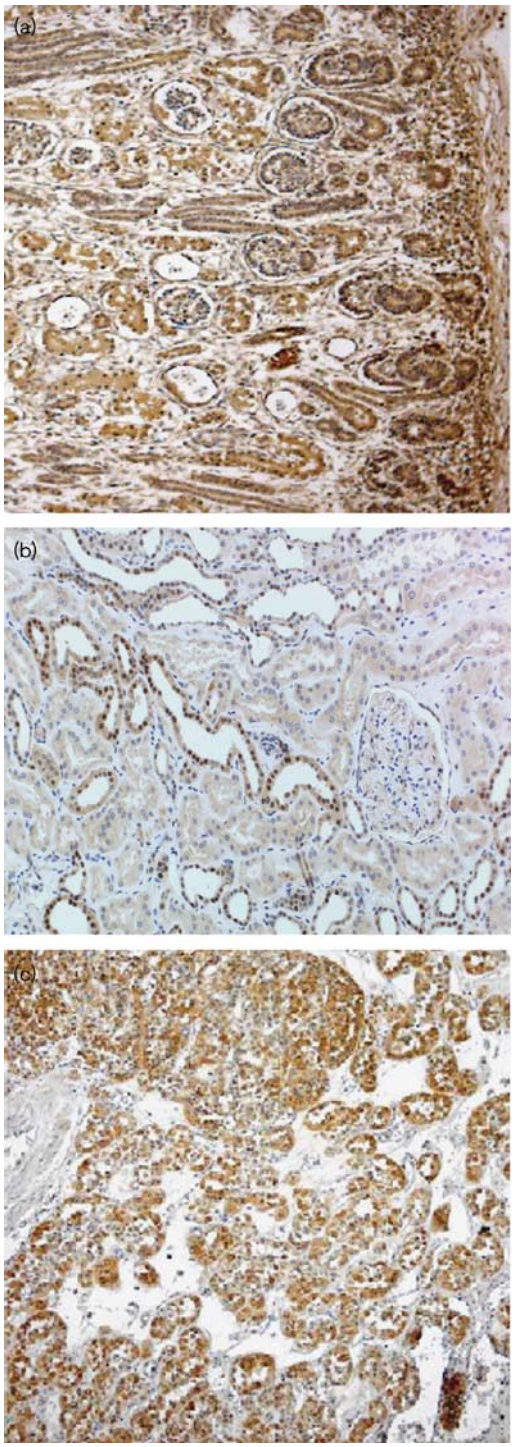
Immunohistochemical staining of the HOX D9 homeoprotein

Immunohistochemical staining of HOX D9, one of the locus D lumbosacral homeoproteins involved in RCCs, on tissue sections from foetal kidneys, normal adult kidneys and clear cell kidney cancers shows an abundant localization in the epithelial compartment of a 23-week foetal kidney (Fig. 3a). Anti-HOX D9 antibody homogeneously stains kidney epithelial tubules of normal adult human kidney (Fig. 3b) and less well the epithelial compartments of the RCC sample (Fig. 3c).

Discussion

To detect the HOX gene involvement in late kidney organogenesis and carcinogenesis, we have compared the expression of the whole HOX gene network in foetal kidneys, from the 15th to the 38th week of human foetal kidney development, in normal adult kidneys and in clear cell kidney cancers (RCCs). We have also determined the expression of the HOX gene network in primary tubular epithelial cells *in vitro* derived from normal adult human kidneys, as these cells are the kidney cell phenotype of which the neoplastic transformation generates clear cell kidney cancers. These results highlight the involvement, in kidney carcinogenesis, of lumbosacral HOX genes, already described as being crucial during early kidney organogenesis and confirmed by our data as being deeply

Fig. 4



(a) Immunohistochemical localization of the HOX D9 homeoprotein in a foetal kidney (aged 23 weeks). Brown staining identifies the HOX D9 protein expression in epithelial kidney cells. S-shaped bodies, characteristic of foetal kidney, are located on the right-hand side of the figure (× 20 magnification). (b) Anti-HOX D9 antibody homogeneously stains the epithelial tubules of normal adult human kidney (× 40 magnification). (c) HOX D9 homeoprotein localization in an adult kidney tumour sample. The brown nuclear staining identifies the HOX D9 protein expression in epithelial neoplastic kidney cells (× 20 magnification).

involved in late kidney organogenesis. More specifically, paralogous groups 9, 11 and 13 and locus D HOX genes manifest a substantial deregulation in RCCs compared with normal kidneys. HOXC11 and paralogous group 13 tend to increase their expression in RCCs, whereas paralogous group 9, HOXA11 and HOXD11 manifest a decreased expression in RCCs compared with normal kidneys.

Lumbosacral HOX D genes are all silent in foetal kidneys from the 15th to the 22nd week of development. HOX D9 and HOX D11 start to be expressed from the 23rd week, and remain active until the 38th week of development. The expression of these genes can be temporally connected to kidney functions as the kidney starts functioning from the 23rd week [36]. HOX D9 and HOX D11 are constitutively expressed in normal adult human kidneys and are the only lumbosacral HOX D genes active in primary epithelial tubular kidney cells to prove that these homeoproteins function in tubular epithelial kidney cells [37]. Finally, HOX D9 and HOX D11 are inactive in the majority of clear cell RCCs tested. It has been suggested that, during tumour evolution, the gene profiles responsible for identifying specific cell phenotypes undergo a dedifferentiation program towards early developmental stages [38]. Comparing the patterns of lumbosacral HOX D gene expression in foetal kidneys (from the 15th to the 22nd week of development) with the same patterns in clear cell RCCs, we note that they overlap. We can thus conclude that, in clear cell carcinomas of the kidney, the expression of lumbosacral HOX D genes marks a molecular dedifferentiation process towards embryonic life.

Paralogous group 11 HOX genes in triple-mutant mice show bilateral kidney agenesis because of alterations in the epithelial–mesenchymal interaction, consequently preventing ureteric bud outgrowth [31]. According to our data, HOX A11, the effector of which is α8 integrin [39], is again active at the 15th week of development; HOX C11 is expressed from the 18th week and HOX D11 resumes being active from the 23rd week until birth and is always active in normal adult human kidneys. Thus, in timing, the expression of HOXD11 and HOXD9 parallels the acquisition of kidney functions. The lack of the expression of these same HOX genes, which we have detected in clear cell kidney carcinomas, suggests the alteration of a kidney function as related to HOX D homeoproteins. This function is probably associated with the role played by lumbosacral HOX D genes in controlling epithelial cell differentiation and epithelial–mesenchymal transition through the interaction of a putative mesenchymal enhancer (acting from HOX D12 to HOX D9) and a ureteric bud enhancer (acting from HOX D9 to HOX D1). HOX D9 is the only HOX D gene responsive to both enhancers [37].

Recently, 231 noncoding RNAs (ncRNAs) have been identified inside four human HOX loci [40]. Between them,

a 2.2 kb ncRNA residing in the HOX C locus on chromosome 12q13-15 between HOX C11 and HOX C12, termed HOTAIR, is able to control in-cis transcription of adjacent genes and repress in-trans transcription across 40 kb of the 5′ lumbosacral HOX D locus (from HOX D9 to HOX D13). Thus, transcription of ncRNAs may act on chromosomal domains of gene silencing at a distance (from chromosome 12 to chromosome 2 where HOX C and HOX D are, respectively, located). The upregulation of HOX C11 that we detected in RCCs may be connected with (i) the involvement of HOTAIR in kidney cancers and (ii) the downregulation of posterior HOX D genes in RCCs. Interestingly, the same chromosomal area 12q13-15 in which the HOX C locus is located harbours the gene AQP2 coding for the water channel aquaporin-2 involved in water excretion, as part of the vasopressin hormone system, in renal collecting duct cells. The proteomic profiling of renal inner medullary collecting duct cells has recently identified HOX genes among the transcription factors expressed by duct cells [41]. Future experiments will be aimed at dissecting a potential interaction between HOTAIR, HOX C11 and AQP2.

With regard to the diagnostic, prognostic and therapeutic role of HOX genes despite the limited understanding of the mechanisms involved, it has already been possible to identify specific HOX genes deregulated in certain types of human cancer with considerable benefit for cancer patients: the expression ratio of HOXB13:IL17BR is a strong independent predictor of treatment outcome in deciding adjuvant tamoxifen therapy in breast cancers [42]. Detection of HOX D13 homeoprotein expression across 79 tumour tissue types has allowed the identification of the lack of HOX D13 expression as having a significant and adverse effect on the prognosis of patients with pancreatic cancer [43]. The possibility of targeting the posttranslational interaction between HOX proteins and their PBX cofactors, through the short peptide HXR9 that is able to bind a conserved six-amino acid sequence in the HOX proteins, to block *in vivo* and *in vitro* the HOX/PBX-regulated gene expression of melanoma cells has been shown recently [44]. With less sensitivity to HXR9 with respect to B16 melanoma cells, however, the interaction between HOX and PBX proteins is also blocked in kidney cancer cells and HXR9 spares normal adult kidney cells [45]. Thus, the HOX/PBX interaction may be considered a potential therapeutic target in kidney cancer.

In conclusion, by comparing the HOX gene network expression in foetal, adult normal and neoplastic kidneys, we have identified the lumbosacral region of HOX D locus on chromosome 2q31-33 as being potentially involved in the epithelial–mesenchymal interactions characteristic of kidney organogenesis and deregulated in kidney carcinogenesis. This chromosomal area houses the HOX D9 and HOX D11 genes, which are known to play a crucial role in kidney organogenesis and, according to our data, are systematically altered in their expression in clear cell kidney cancers. The key involvement of HOX D genes in kidney cancers suggests the possibility of targeting these homeoproteins for diagnostic and therapeutic purposes in the clinical management of clear cell kidney cancers.

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