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The Paralogous Group HOX 13 Discriminates between Normal Colon Tissue and Colon Cancer

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Abstract

Colon diseases are more common in the world with an increasing trend. Despite the different etiology and physiology, the epithelial-mesenchymal transition (EMT), is crucial during transformation, migration and invasive ability of colon cancer cells. Several genes are implicated in the control of EMT (Wnt, Notch, Src, Ras, etc.). The homeobox genes are a transcription factor family, they are arranged in several classes. Class I homeobox genes (Hox in mice and HOX in humans), are 39 transcription factors, mainly involved in the regulation of embryonic development program.

In the present study, we have analyzed the expression of HOX genes, during tumor cell lines differentiation (CaCo2) and compared with the expression of HOX gene network, in the normal intestinal mucosa

Keywords: Colon cancer; HOX genes; CaCo2 cells; Transcriptor factor

Introduction

Colon diseases are more common in the world with an increasing trend [1-3]. Despite the different etiology and physiology, the epithelial-mesenchymal transition (EMT), is crucial during transformation, migration and invasive ability of colon cancer cells [4]. Several genes are implicated in the control of EMT (Wnt, Notch, Src, Ras, etc.). The activation of phosphatidylinositol 3'-kinase (PI3K)/Akt is a key regulator of EMT [5]. PI3K/AKT signal is correlated with the growth and progression of colon cancer. It has been reported mTOR kinases, is a downstream effector of PI3K/Akt and regulates the colon cancer genesis [6,7].

Human colon adenocarcinoma cells (CaCo2), have been used in the approach of mechanisms involved in enterocyte differentiation and in the study of cryptic-like enterocytes.

The cell phenotype is finely controlled by genes the memory cell; epigenetic change in the memory cell program, is critical in the onset and evolution cancer [8]. The fate of cells is determinate by the memory cell (Figure 1), this process control all genetics functions that implies entire genome transfer, from mother cell to daughter cell during replication. The program contains essential biological information as: cell phenotype, number of cell divisions, apoptosis control [8]. Three genes families control this biological processes: Polycomb (H3K27m3), able to block the interaction DNA-chromatin leading to silencing of HOX genes, Trithorax genes (H3K4m3), able to induce the mRNA transcription through an open configuration of DNA-chromatin interaction and leads to activation of HOX genes; finally, the HOX genes involved in the control of the phenotype through a specific gene program, mainly by means the accurate regulation of the mRNA transcription [9]. This biological mechanism also acts in the stem cells that have not yet a specific identity; they are able to generate similar cells to themselves and cells that can differentiate in one of approximately 300 cellular phenotypes present in our body [10].

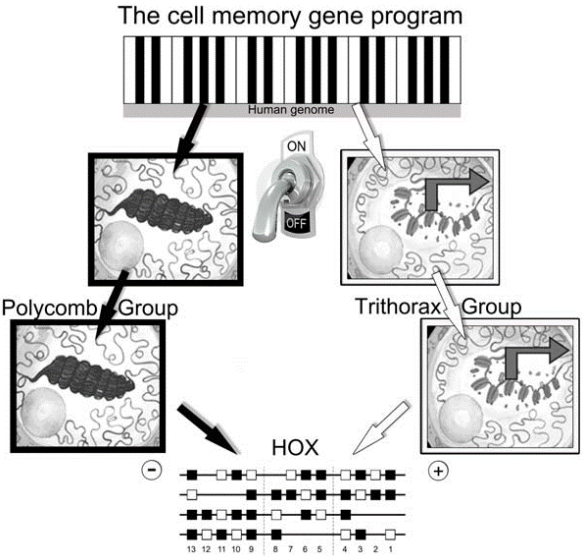

Figure 1: The cell memory gene program (see text for details).

The homeobox genes are a transcription factor family; they are arranged in several classes. Class I homeobox genes (Hox in mice and HOX in humans), are 39 transcription factors, mainly involved in the regulation of embryonic development program; The HOX genes are characterized by a sequence of 183 nucleotides encoding a homeodomain of 61 amino acid that binds to DNA, as a biological gripper, activating or repressing specific genes [11,12].

The HOX genes are organized into four chromosomal clusters or loci (HOXA Chr 7p15.3, HOXB Chr 17q21.3, HOXC Chr 12q13.3 and HOXD Chr 2q31), each having 9-11 genes. On the basis of position into the locus and similarity of homeobox sequence, corresponding genes, of the four clusters, can be aligned with each other in 13 paralogous groups (Figure 2) [13]. The HOX network is active in adult human tissues and organs, controls the spatial-temporal generation of biological structure expected during embryonic development and regulates the cell memory program (Figure 2).

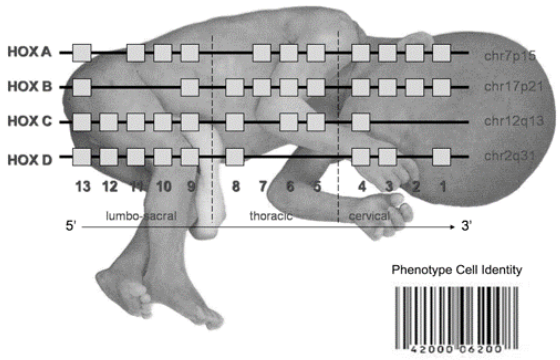

Figure 2: Schematic representation of the HOX gene network (see text for details).

It has recently been demonstrated the involvement of homeobox genes in evolution of colon cancer [14]. Cdx1 and Cdx2 genes are involved in differentiation of intestinal epithelium and regulate the early stages of colon embryonic development [15]. Isolated HOX genes expression (HOX B7 and HOX C6), it was studied, in vitro, during the CaCo2 cell differentiation [16,17].

In the present study, we have analyzed the expression of HOX genes, during tumor cell lines differentiation (CaCo2) and compared with the expression of HOX gene network, in the normal intestinal mucosa. We have also studied the quantitative change in gene expression of HOX 13 paralogous group, in normal colon vs. colon cancer.

Materials and Methods

Cell cultures

The human colon cancer cell line, $CaCO_2$, was grown in Petri dishes in a humidified incubator equilibrated with 5% CO2 at 37°C, using DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml sreptomycin. The cells were detached for RNA extraction at 2 days (50% confluent), 7 days (post-confluent), 15 days and 21 days (differentiated).

RNA extraction and RT-PCR analysis

The samples will be first lysed and then homogenized in the presence of a highly denaturing guanidine-thiocyante-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the samples will be then applied to a specific column, where the total RNA will bind to the membrane and contaminants will be efficiently washed away. High-quality RNA is then eluted in 30-100 microL water (Qiagen Kit cat. No. 74004), and in order to obtain cDNA molecules one microg of total RNA will be subjected to cDNA synthesis for 1 h at 37°C (Amersharm Biosciences cod. 27-9264-01, in a reaction mixture containing 0.5 µg oligo-dT, nucleotide and Reverse Transcriptase (Amersharm Biosciences cod. 27-7610-01).

Polymerase chain reaction (PCR) amplification of cDNA will be performed in a reaction mixture containing 4 µl of cDNA sample and different primer sets (20 p/mol each) (Pure Taq Ready to go PCR-beads Amersharm Biosciences cod. 27-9558-01). The sense/anti-sense HOX primers for PCR were designed as previously reported [Cantile et al.] to prevent genomic DNA contamination, the sense and anti-sense primers are designed to frame a sequence that crossed at least one intron on the genes. The co-amplification of the specific gene and human beta-actin gene, as an internal control, will be achieved using two primer sets, in a single reaction mixture. We'll select two pairs of β-actin primers to obtain amplified fragments with a different molecular weight, to be used alternatively in the co-amplification reaction. PCR products will be separated by 1.2% agarose gel electrophoresis.

QRT-PCR analysis

QPCR will be performed using Taq-Man or technology (QPCR 7500 Applye Biosystem). This assay uses a specific oligonucleotide probe, annealing between the two primer sites, which is labelled with a reporter fluorophore and a quencher. Cleavage of the probe by exonuclease activity of Taq polymerase during strand elongation releases the reporter from the probe resulting in an increase in reporter emission intensity owing to its separation from the quencher. This increment in net fluorescence is monitored in real-time during each PCR amplification.

The cDNA, synthesized as previously described, will be used for real-time PCR performed in 96-well optical reaction plates with cDNA equivalent to 100ng RNA in a volume of 25 ml reaction containing Taqman Universal Master Mix (Applied Biosystem 4304437), optimized concentrations of FAM-labelled probe and specific forward and reverse primer for the genes of paralogous group 13 HOX (Applied Biosystem) from Assay on Demand.

The results will be analyzed using a comparative method, and the values will be normalized to the β-actin expression as an endogenous control.

Results

Expression of HOX genes in normal colon

We observed the expression of circuit HOX genes (by RT-PCR) in CaCo2 cells during growth phases: 2 days, corresponding to reach 50% confluence (Phase 0 or proliferating); to 7 days or phase post confluence (Phase 1 or not differentiated), 15 days (Phase 2) and 21 days (Phase 3) corresponding to achievement complete cell differentiation stage (Figure 3); on the left, is reported the expression HOX gene network in normal mucosa colon, 29/39 HOX genes are expressed. All genes of HOX A locus are expressed; HOX B locus shows 8/10 genes are actived, HOX B1 and HOX B13 are always (respectively the most cephalic and most caudal of circuit) silenced. The genes of the HOX C locus are actived in cephalic and thoracic region (from HOX C4 to C8), while caudal five genes are silent (from HOX C9 to HOX C13). Locus HOX D showed three consecutive active

genes (HOX D4, HOX D8 and HOX D9) and there are active some genes in the cephalic area (HOX D1) and caudal area (HOX D11 and HOX D13). All thoracic genes (11/11) of the network are actived in the normal mucosa. The genes appear to be less active in lumbo-sacral (8/16) than cephalic (10/12).

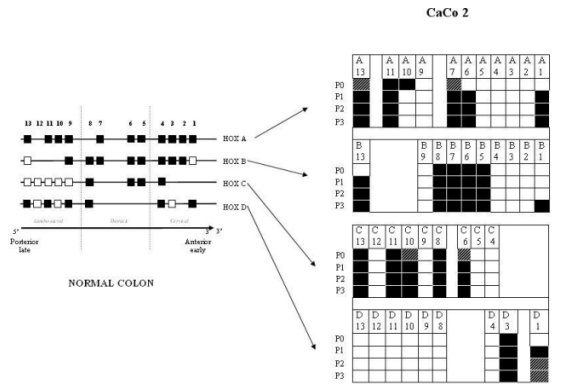

Figure 3: Schematic representation of the HOX gene network (see the text for details). Expression of the whole HOX gene network, detected by RT-PCR, in human normal colon mucosa (left) and in CaCo2 cells (right) harvested after 2days=P0 (50% confluent, proliferating, phase 0); 7 days = P1 (post-confluent; post-proliferative, non-differentiated, phase 1); 15 days = P2 (differentiated, phase 2) and 21 days = P3 (post-differentiated, phase 3). Black and white squares indicate active or silent HOX genes, respectively. Grey squares indicate intermediate active HOX genes

Expression of HOX genes CaCo2 cells

In the Figure 3, right, are shown the results from HOX genes expression related to the locus HOX A, HOX B, HOX C and HOX D, during CaCo2 cell differentiation. (The result was obtained at least three times). The analysis of gene expression at different phases of differentiation (Phase 0, Phase 1, Phase 2, Phase 3), showed the HOX gene network is always activated in all analyzed stages. Locus HOX A and HOX B are the most active (as in normal colon), while the HOX C and HOX D locus are the least active during cells proliferative phases (Phase 0). During phase 0, 14/39 HOX genes are activated, while 17/39 HOX genes are expressed in the differentiating stages 1 and 2; 18/39 HOX genes are activated in the phases 3. In all stages, 20 HOX genes are always silent; HOX A2, HOX A3, HOX A4, HOX A5 and HOX A9 on HOX A locus, HOX B2, HOX B3, HOX B4 and HOX B9 on HOX B locus, HOX C4, HOX C5, HOX C9 and C12 on HOX C locus and the entire thoracic and lumbo-sacral HOX D locus (from HOX D4 to HOX D13). Only 13/39 HOX genes are always expressed in the early stages of CaCo2 cells differentiating: HOX A7, HOX A11 and HOX A13 on HOX A locus; HOX B5, HOXB6, HOXB7 and HOX B8 on HOXB locus; HOX C6, HOX C8, HOX C10, HOX C11 and HOX C13 on HOX C locus; finally HOX D3 on HOX D locus.

On the first stage (7 days), four HOX genes are expressed, HOX A1, HOX A6, HOX B13 and HOX D1 (Figure 3). HOX A10 is not expressed at stages 1, 2 and 3. HOX D1 is constantly silent in step 0, is activated in phase 1 and then regresses in steps 2 and 3. HOX B1 is exclusively expressed in phase 3 (21 days) and HOX D1 is activated in step 1 to regress in steps 2 and 3. The expression of HOX A7, HOX A13, HOX C6 and HOX C10 genes increases in phase 1.

The paralogous group 1: (HOX A1, HOX B1, HOX D1) showed the most significant changes of gene expression during the CaCo2 cells differentiation (Figure 3). Indeed HOX A1 is active only in phase 1.

The paralogous group HOX 6: HOX A6 is silenced in phase 0, but activated in 0, 1, 2 and 3 phases (Figure 3); HOX B6 is always expressed in all phases of cell differentiation; HOX C6 is less active in phases 0 than at stages 1, 2 and 3.

Paralogous groups HOX 4 and 9: This HOX genes are always silenced in all phases of differentiation.

Paralogous HOX 10: HOX A10 is expressed in phase 0 but is reduced at 1, 2 and 3 phases. Conversely, HOX C10 is poorly expressed in phase 0 but increased in the phase 3, HOX D10, instead, is always silenced in all phases (Figure 3).

Paralogous group HOX 13: HOX A13 is weakly active in phase 0 and actively expressed in step 1, 2 and 3; HOX B13 silent in phase 0 but expressed in step 1,2 and 3; HOX C13 is always active during the four stages of differentiation unlike HOX D13 is not expressed in the four stages of differentiation

Interestingly the expression of HOX 13 paralogous group, in normal colon vs. CaCo2 cells, showed HOX A13 gene is always expressed in normal mucosa while in CaCo2 cells is activated during the transition from phase 0 to phase 1 and remained active even in steps 2 and 3. The HOX genes HOX B13 and HOX C13 are always silent in normal mucosa, conversely in CaCo2 cells, HOX B13 is always silent in the phase 0 but is activated in 1, 2 and 3 phases, while HOX C13 is consistently expressed in all stages; finally HOX D13 has always been silent in all different phases **(Figure 3)**.

Quantitative analysis of gene expression of HOX paralogous group 13, in the normal human colon mucosa and colon cancer

The results obtained from quantitative analysis of gene expression (QRT-PCR), showed that HOX 13 paralogous group (Figure 4).

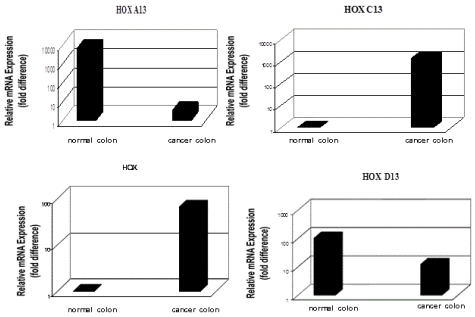

Figure 4: QRT-PCR of HOX13 paralogous group in normal colon vs. colon cancer, HOXA13 and HOXD13 are hyper expressed in normal colon compared with colon cancer tissue; instead HOXC13 and HOXB13 are activated in colon cancer compared with normal colon.

In the normal colon tissue and colon cancer, is involved in colon cancer evolution. HOX A13 is strongly expressed in normal colon, conversely, is down regulated in neoplastic tissue. Instead, the HOX B13 and HOX C13 genes are always silenced in normal mucosa, but are activated in the tumor tissue, showing a high degree of gene expression. Finally HOX D13, is always expressed in normal colon and is down regulated in colon cancer.

Discussion

We studied the expression of HOX gene network in normal colon and in the CaCo2 cells differentation; moreover, we have evaluated changes in the gene expression of the HOX 13 paralogous group. We have observed an active involvement of HOX genes in the development of the bowel. Paralogous groups HOX 1, 6, 10 and 13 appear to be particularly altered in their expression, during the differentiation of CaCo2 cells, suggesting that these genes may play a crucial role in the enterocytes differentiation process. When cells begin to develop the epithelial absorbent phenotype, 5 genes of locus A appear to be up or down regulated. The HOX genes HOX A1 and HOX A6 are silent in phase corresponding to 50% confluence, they become constitutively expressed in the proliferation and differentiation phases, conversely HOX A10 active in pahse 0 became silent in 1, 2 and 3 phases. HOX 13 genes, HOX A7 and HOX A13 weakly expressed in phase 0 are up-regulated in stages 1 and 2. In HOX B locus, only HOX B1 and HOX B13 modify their expression, HOX B1 is active in the phase 3 (21 days) while HOX B13 active in the proliferative and differentiation phases. In the HOX C locus, HOX C6 and HOX C10 are weakly expressed in phase 0 and then become actively expressed at later stages. Finally, the HOX D locus, essentially silent, shows only the HOX D1 gene silent in phase 0, conversely, it is hyper expressed in proliferative phase and weakly expressed in cell differentiation phase. Pralogous group 1 seems to be more involved in maturation of CaCo2 cells. Other paralogous groups HOX, are altered during different stages of CaC02 cells differentiation. HOX6 Paralogous group defines the development of thoracic area during embryogenesis, including the gastrointestinal tract. It has demonstrated the crucial role of these genes, in regulation of cellular differentiation processes that may be realized both in vivo and in vitro. HOX B6 gene expression is down regulated during cell differentiation, induced by erythropoietin in the erythroid cell line MB02 and it's over expression results in loss of erythroid phenotype in K562 and HEL cells [18,19]. The HOX gene has become involved in the development of bladder cancer tumor [10]. In addition, the HOX A6 and HOX C6 genes appear linked to the differentiation of pancreatic islands a and b [20]. Paralogous group 13 HOX genes, defines the posterior region of the circuit that controls embryonic development of lumbo-sacral body, including urogenital system, terminal region of the bowel and limb. The HOX13 paralogous group, are implicated in many diseases and malformations. Mutations in HOX A13 and HOX D13 determine alterations affecting the limbs (synpolydactly) and the urogenital tract (hand-foot-genital syndrome) [21-24]. The genes HOXA13, HOXB13, HOXC13 and HOXD13, control urogenital embryogenesis and are deregulated in normal urothelium, compared with neoplastic tissue [10,25]. Expression of HOXB13 gene discriminate to bladder cancer invasive and non-invasive and is an important determinant of prostate cancer to androgen response [26,27]. Paralogous group 13 and the HOX D locus genes are discriminating in kidney cancer than normal kidney. The HOX13 paralogous group is hyperexpressed in kidney cancer [27]. Recently has been shown HOXA13 hyperexpression in human HCC and seems to be a specific marker that characterize this neoplasia [28]. Paralogous HOX13 genes expression, in normal and cancer colon confirm the role of specific markers of the neoplastic phenotype (Figure 4). The HOX genes, HOX A13 and HOX D13 are always expressed in normal and cancer mucosa, but decreases in colon cancer, conversely HOX genes, HOX B13 and HOX C13 are always silent in normal colon and constitutively expressed in neoplastic tissue. These results show, the involvment of HOX gene network in the control of enterocytes cell phenotype; these events are finely controlled by epigenetic mechanisms that controls the memory cell program. Alteration of these mechanisms leading to epigenetic reprogramming of the cellular genome, with increased risk of cancer [29]. The model of colon tumorogenesis is due to several genetic changes that induces colon cancer genesis [30].

HOX network acts as a complex genetic system where, behind the properties of the single gene, complex biological functions are realized by the network as a whole. [29]. Recent studies have identified small RNAs (single stranded, 21-23 nucleotides in length) inside the genome displaying a regulative role (miRNAs) as well as long non coding RNA (ncRNAs) ranging from 300 nucleotides to over 10kb that are spliced, polyadenylated, and as diverse as protein-coding RNAs [31]. miR-34b/c, miR-9-1, miR-129-2 and R-137 genes was observed in colon cancer cell lines and in primary tumor compared with normal mucosa [32]. Epigentic control of chromatin may be regulated by the ncRNA [33].

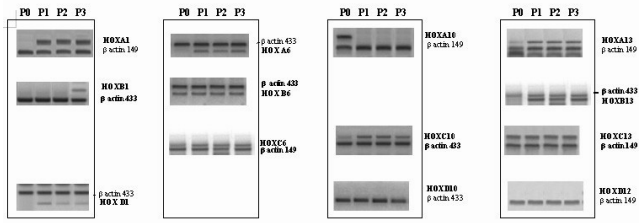

Figure 5: RT-PCR expression of paralogous group 1 (HOX A1, HOX B1 and HOX D1), paralogous group 6 (HOX A6, HOX B6 and HOX C6), paralogous group 10 (HOX A10, HOX C10 and HOX D10) and paralogous group 13 (HOX A13, HOX B13, HOX C13 and HOX D13) HOX genes during the different (P0, P1, P2 and P3) phases of CaCo2 differentiation in vitro. Control co-amplifications of HOX A1, HOX C6, HOX A10, HOX A13, HOX C13 and HOX D13 with a 149bp β actin primer and of HOX B1, HOX D1, HOX A6, HOX B6, HOX C10, HOX D10 and HOX B13 with a 433bp β actin primer is reported. Duplex PCR products were separated by bromide 1.2% agarose gel electrophoresis

Inside network HOX genes have been identified 231 ncRNA [34-36]. In locus HOXC has been identified a ncRNA termed HOTAIR, which acts as a transcriptional repressor in trans to the locus HOX D by interaction with Polycomb Responsive Element (PRE) [34]. In region of the 3' end locus HOX A between HOX A1 and HOXA2, has been identified another ncRNA called HOTAIRM1; HOTAIRM1 modulates the gene expression of HOXA locus, during myelopoiesis [27]; The ncRNA can suppress distant domains interacting with specific chromosomes areas.

Recently has been identified a lncRNA, HOTTIP, transcribed from the 5' end of the HOXA locus that coordinates the activation of several 5'HOX A genes in vivo [35,36]. HOTTIP is expressed by the

development to adulthood in lumbo-sacral anatomical locations. Depletion of HOTTIP in mice induces defects resembling HoxA11 and HoxA13 inactvation, suggesting the in vivo control of lumbo-sacral Hox genes by HOTTIP [35,37]. Finally, the molecular interactions described between HOX genes inside the network, support the concept of molecular software able to regulate cell identity and cell-cell communication.

Our results confirm that the HOX gene network is actived in the bowel mucosa, while is altered during in vitro differentiation of CaCo2 cells. HOX 1, 6, 10 pasalogous, are the most involved genes (Figure 5) and in details the HOX 13 paralogous group, show a specific pattern of expression in normal mucosa compared with neoplastic tissue; this may be suggest the involvement of HOX gene network, during enterocytes differentiation. [38-45] In conclusion, we have demonstrated the involvement of paralogous group HOX 13, in epigenetic alterations of molecular mechanisms that control neoplastic transformation in normal colon [46-51].

Conclusion

In this study our data show the alteration of HOX genes, can induce alteration of cell phenotype and the onset of diseases such as cancer. Quagliata et al. 2014 has already demonstrated the role HOX A13 gene and lncRNA HOTTIP are positive markers of the hepatocarcinoma.Moreover, the detection of HOXD13 homeoprotein in pancreas-tissue microarrays shows that its negative expression has a significant and adverse effect on the prognosis of patients with pancreatic cancer independent of the T or N stage at the time of diagnosis [2]. In this study I identify the HOX 13 paralogous group as discriminating between normal tissue versus tumor tissue. The detailed study of the interaction between HOX genes, miRNAs and lncRNA, will help to understand the nature of a complex disease such as colon cancer and to identify specific therapies able to correct errors in the HOX network program.

Considering the role of Class I Homeobox genes in the epigenetic control of human cell memory program, cell phenotypes, HOX cluster could be used as "*the Rosetta stone*" of human cell biology.

Acknowledgement

To my wife Donatella and my daughter Andreasophje.

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