

# Relationship between DNA methylation and gene expression of the *HOXB* gene cluster in small cell lung cancers

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**Abstract** The expression pattern of the *HOXB* gene cluster in four xenografted small-cell lung cancers was compared to the methylation of the DNA in the corresponding genomic regions. In 90% (17/19) of the studied cases, the expressed genes were in methylated regions whereas 70% (12/17) of the unexpressed genes were in unmethylated regions. This specific behavior could correspond to a particular gene expression regulation mechanism of the *HOX* gene network. Since some genes (*HOXB2*, *HOXB4*, *HOXB7*) were always inactive when unmethylated, this unexpected relationship might indicate their key function(s) in the *HOX* gene network.

**Key words:** Methylation; Expression; Homeogene; Cancer; Lung

## 1. Introduction

Homeobox-containing genes are a family of evolutionarily highly conserved transcription factors (reviewed in [1–3]). The homeobox encodes a 61 amino acid domain, the homeodomain, which includes an helix-turn-helix motif responsible for the DNA-binding ability of homeobox-containing proteins.

The class I homeogenes encode proteins containing a homeo-domain closely related to that of the archetypal *Drosophila antennapedia* domain. In human (*HOX*) and mouse (*Hox*), the 38 members of the family are tandemly arranged in 4 clusters of 90–120 kilobases (kb). The *HOX* gene clusters are borne by chromosomes 7 (*HOXA*), 17 (*HOXB*), 12 (*HOXC*) and 2 (*HOXD*). Within the clusters, each gene can be assigned to one of 13 paralogous groups, with successively higher numbered paralogous groups being located more 5' within each cluster.

The expression of *HOX* genes has been extensively studied in human embryonal carcinoma cells (reviewed in [4]) and, in some instances, the promoter regions were studied in detail [5–8]. These regions are characterized by the presence of multiple positive and negative regulatory elements including retinoic acid-responsive sites. It has been shown that the retinoic acid-induced expression of silent *HOX* genes proceeded in a sequential order: the 3'-end *HOX* genes respond earlier than the 5'-end *HOX* genes [9,10]. Similar sequential responses were observed after inhibition of the *HOX* gene expression by antisense oligodeoxynucleotides [11]. Taken together, these data suggest a cascade mechanism involved in the regulation of *HOX* gene

expression, operating on extended regions, with a 3' to 5' polarity.

*HOX* genes are expressed during embryogenesis in a tissue-specific and often stage-related fashion. After embryogenesis, *HOX* genes may continue to be transcribed according to a tissue-specific pattern of expression. In cancer, alterations of this pattern, as compared to the corresponding normal mature tissues, have been described [12–21]. However, few data are presently available on *HOX* gene regulation in adult tissues or on the mechanisms responsible for the changes observed in cancer cells.

Little is known about the possible involvement of DNA methylation in the regulation of *HOX* gene expression, although available sequences indicate that the *HOX* loci are CpG-rich regions. In mouse, the homeobox genes are unmethylated in the gametes and throughout embryonic life. In certain tissues, some *Hox* genes become methylated after birth by a de novo mechanism acting on extended regions reaching up the 20–30 kb [22]. The lack of data on the nucleotidic sequences of most *HOX* genes, in particular in the 5' regulatory regions, prevents the detailed study of the regulation of each gene's expression by DNA methylation.

In this study, we attempted to estimate the global methylation of the DNA along a *HOX* locus to search for a possible relationship between DNA methylation and *HOX* gene-expression patterns [15]. We took advantage of a panel of xenografted small-cell lung cancers (SCLC) to select cases with different expression patterns, thereby allowing expression-methylation cross-correlation studies. The *HOXB* locus was selected for these studies because it contains a row of 9 *HOX* genes, with no missing paralogous genes, which, at least in embryonic cells, seems to be coordinately regulated.

## 2. Materials and methods

### 2.1. Tumors

The main characteristics of the xenografted SCLC used in this study have been reported elsewhere [15]. Briefly, human SCLC tissues, obtained through fibroscopy or surgical excision of tumors from patients with histologically confirmed diagnoses of SCLC, were xenografted into 6–8-week-old Swiss/athymic mice (IFFA-Credo, Lyon, France) by s.c. implantation. The resultant tumors were serially transplanted in nude mice and the retention of their human karyotypes and histology was controlled. In this study, we used 2 xenografts (SCLC-10 and SCLC-82), each from primary tumors of 'oat-cell' histology, and 2 xenografts (SCLC-75 and SCLC-6), each from tumors of 'intermediate-grade' histology; the latter originated from a lymph node metastasis. DNA were extracted according to standard methods [23] and RNA were prepared using a Trizol kit according to the manufacturer's instructions (Gibco BRL, Gaithersburg, USA).

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## 2.2. Expression analysis

The expression of the *HOXB* gene cluster, previously studied by Northern blot, was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Reverse transcription was performed using the Gibco BRL kit with 2 µg of total RNA, 200 ng of oligo d(T) and 0.5 mM of each dXTP. One-tenth of the preparation was used for PCR amplification. The amplification procedure involved denaturation at 94°C for 1 min, annealing for 1 min at the appropriate temperature (see below) and extension at 72°C for 1 min during 40 PCR cycles. The buffer conditions were those recommended by the Taq-polymerase supplier (Appligene-Oncor, Illkirch, France). The following primers were used: (1) *HOXB1*: 5'CCTTCTTAGAGTACCCACTCTG3' and 5'GCATCTCCAGCTGCCTCTT3', which gave a 826-bp fragment [24], annealing temperature 55°C; (2) *HOXB2*: 5'AGAAATCCG-CCAAGAAACCCAGCC3' and 5'GAGAAGACGTCTTCTGGC-AATGGC3', which yielded a 587 bp fragment [24], annealing temperature 64°C; (3) *HOXB5*: 5'CGTACTTTGTAACTCCTTCTCGGG3' and 5'TCTGGAACCAGATCTTGATCTGGCG3' which generated a 723 bp fragment [25], annealing temperature 55°C; (4) *HOXB6*: 5'CCTATTTCTGTAAGTCCACC3' and 5'TTCATGCGTGGTTC-TGGAA3', which gave a 589-bp fragment [26], annealing temperature 55°C. The primers used for *HOXB4* [16] *HOXB7* [27], *HOXB3*, *HOXB8* and *HOXB9* [28] were as previously reported. For *HOXB3*, *HOXB4*, *HOXB8* and *HOXB9*, the amplified fragment did not include an intron. Control experiments were performed in which the reverse transcription step was omitted to assure the absence of contaminating DNA.

## 2.3. DNA methylation investigation

The methylation status of *HOXB* locus segments was studied using methylation sensitive-insensitive enzymes *HpaII/MspI*. DNA (5 µg) were digested overnight with 50 units of enzyme and run on a 1% agarose gel. A second round of digestion did not affect the results. Southern blots were hybridized with [ $\alpha$ -<sup>32</sup>P]dCTP nick-translated DNA probes in previously described conditions [29]. The hybridization patterns were recorded with a PhosphorImager (Molecular Dynamics, Sunnyvale, USA) and the images were processed using Persuasion software. Methylation was also studied in the same experimental conditions with methylation-sensitive restriction enzymes *HhaI* and *BstUI*. Eight probes of the 3' regions of the *HOXB* genes were previously used for gene-expression studies [15]. One probe of the 5' region of the *HOXB4* gene was obtained from the American Type Culture Collection (ATCC 65146) [30]. The *HOXB8* probe is a 1.5 kb-cDNA fragment whose sequence is not available. However, its digestion by *MspI* gave a band of about 0.6 kb and several bands below 0.2 kb, indicating the presence of numerous *MspI/HpaII* sites. No informative probe was available for Southern blot analysis of the *HOXB2* region. In some cases, when the nucleotide sequence was available, restriction-enzyme digestions were studied by PCR and the products of the reaction analyzed on 1% agarose gel. DNA were digested in the same conditions as those for Southern blotting. The following primers were used: (1) *HOXB1*, first exon [24], 5'CCTTCTTAGAGTACCCACTCTG3' and 5'AACCTTCATCCAGTCCGAAGGC3', which yielded a 531-bp fragment; (2) *HOXB1*, second exon [24], 5'AAGGTGTCAGAC-CCAGGCT3' and 5'GCATCTCCAGCTGCCTCTT3', which gen-

erated a 269-bp fragment; *HOXB2* promoter [6], 5'CAGACCA-TACAGCGAGGG3' and 5'TACAGGGAATTCGTGGTCA C3', which gave a 609- bp fragment. The amplification procedure, performed on 200 ng of digested DNA, was the same as for RT-PCR with an annealing temperature of 55°C during 26 PCR cycles. The MgCl<sub>2</sub> concentration was 2.5 mM for the *HOXB2* primers. One-tenth of each sample was separated on 1% agarose gel and stained with ethidium bromide. The images were captured by a CCD camera and processed using Image software.

## 3. Results

Expression of the *HOXB* gene cluster was analyzed by RT-PCR in 4 xenografted SCLC tumors. The results are summarized in Table 1. SCLC-10 and SCLC-82 tumors had the same expression pattern with 5 out of 9 genes being actively expressed (*HOXB2*, *B3*, *B4*, *B6* and *B7*). In contrast, SCLC-75 and SCLC-6 displayed dramatically different *HOX* gene patterns of expression: in SCLC-75, all but *HOXB7* genes were expressed, whereas SCLC-6 expressed only *HOXB3*. Although the general trends remained the same as those published previously [15], slight differences in the expression patterns were observed: SCLC-6 now expressed *HOXB3* but not *HOXB4*, SCLC-10 and SCLC-82 expressed *HOXB6*, and SCLC-75 no longer expressed *HOXB7*. These differences might be explained by different sensitivities between RT-PCR and Northern blotting and/or by slight modification of the *HOX* pattern as a consequence of additional xenograft passages in nude mice.

The methylation status of segments localized in the 9 *HOX* genes of the cluster were studied by Southern blotting or by PCR using methylation-sensitive/insensitive restriction enzymes. The patterns of hybridization of the 9 *HOX* gene probes (8 localized in the 3'-end and 1 in the 5'-end) obtained with the DNA extracted from the 4 SCLC xenografts, digested by restriction enzymes *MspI* and *HpaII* and subjected to Southern blotting are shown in Figs. 1 and 2. For *HOXB1*, for example, digested with the methylation-insensitive enzyme *MspI*, a single band of about 1 kb was detected in all 4 tumors. However, with *HpaII* (methylation-sensitive), several band patterns were observed: a band of about 1 kb was seen in SCLC-6, SCLC-10 and SCLC-82, but not in SCLC-75 which exhibited bands at 4 and 5.5 kb; SCLC-10, and to a much lesser extent SCLC-82, also contained longer fragments indicating the presence of methylated restriction sites. The small size difference between the bands recurrently observed near 1 kb after *HpaII* or *MspI* digestion could be explained by the presence in all the tumors of a methylated restriction site giving rise to a small undetected

Table 1

Summary of *HOXB* gene expression and methylation status of the corresponding genomic region in SCLC-10, SCLC-82, SCLC-75 and SCLC-6

Tumor	Parameter	<i>HOXB1</i>	<i>HOXB2</i>	<i>HOXB3</i>	<i>HOXB4</i>	<i>HOXB4</i> 5'	<i>HOXB5</i>	<i>HOXB6</i>	<i>HOXB7</i>	<i>HOXB8</i>	<i>HOXB9</i>
SCLC-10	Expression	-	+	+	+	+	-	+	+	-	-
	Methylation	+	+++ <sup>a</sup>	+	+	+	+	++	+	-	+
SCLC-82	Expression	-	+	+	+	+	+	+	+	-	-
	Methylation	-	+ <sup>a</sup>	+	+	+	+	+	+	-	+
SCLC-75	Expression	+	+	+	+	+	+	+	-	+	+
	Methylation	++ + <sup>a</sup>	+ <sup>a</sup>	+	++	+	+	-	-	-	+
SCLC-6	Expression	-	-	+	-	-	-	-	-	-	-
	Methylation	- - <sup>a</sup>	- <sup>a</sup>	++	-	-	-	-	-	-	-

Probes were cDNA from the 3' end (B1, B3, B4, B5, B6, B7, B8, B9) or the 5' end (B4 5') of the corresponding *HOX* gene. <sup>a</sup>Data from PCR experiments. Expression: +, expressed gene; -, unexpressed gene. Methylation: ++, heavily methylated; +, methylated; -, unmethylated.

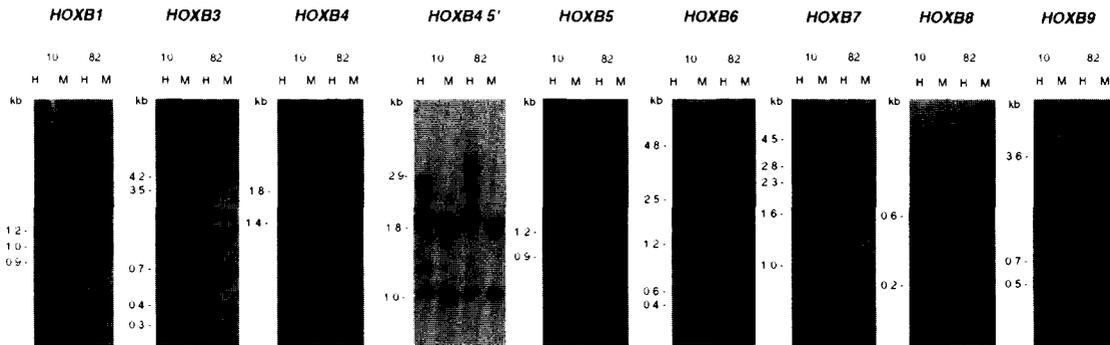


Fig. 1. Southern blot analysis of the methylation status of the *HOXB* locus genes in SCLC-10 and SCLC-82. Each tumor's DNA was digested by *HpaII* (H, methylation-sensitive) or *MspI* (M, methylation-insensitive) restriction enzyme and hybridized. Probes were cDNA from the 3' end (B1, B3, B4, B5, B6, B7, B8, B9) or the 5' end (B4 5') of the corresponding *HOXB* gene. The presence of higher molecular weight bands in the *HpaII* digestions as compared with the *MspI* digestions indicates the methylation status of the studied region.

fragment in the *MspI* digestion. Because a complete restriction map is not available, it was not possible to precisely localize the methylated/unmethylated restriction sites. However, it was possible to establish the relative methylation status of the tested segments by mean of the molecular weight distribution of the bands. According to this criterion (Table 1), the *HOXB1* locus was not methylated in SCLC-6 and SCLC-82 (-), methylated in SCLC-10 (+) and heavily methylated in SCLC-75 (++). The lower methylation of *HOXB1* in SCLC-6 as compared to SCLC-75 was confirmed by the PCR analysis of the methylation of exons 1 and 2 for which none of the 9 tested *MspI/HpaII* sites were unmethylated in SCLC-75 whereas, in SCLC-6, at least one was not methylated in each exon (not shown).

Analysis of the Southern blots (Figs. 1 and 2), according to the interpretation delineated above, allowed us to establish the relative methylation status of the genes of the cluster in the 4 tumors (Table 1): *HOXB3* was methylated in all 4 tumors (hypermethylated in SCLC-6); *HOXB5* and *HOXB9* were methylated in SCLC-10, SCLC-82 and SCLC-75 but not in SCLC-6; *HOXB6* and *HOXB7* were methylated in SCLC-10 and SCLC-82 but not in SCLC-75 and SCLC-6. In the case of *HOXB4* for which probes were available for the 3' and 5' regions of the gene, methylation was analyzed in the two parts of the gene with similar results: SCLC-10, SCLC-82 and SCLC-75, but not SCLC-6, were methylated in both 3' and 5' regions. As regards *HOXB8*, the same hybridization pattern was observed for the 4 tumors: the 0.6 kb band, also obtained after *MspI* digestion of the probe, was observed when the DNA were digested by *MspI* or *HpaII*. One band, about 0.2 kb, was also

hardly visible and the lower molecular weight bands, present in the digested probe, could not be detected under the present hybridization conditions. It can be concluded that this region of the cluster was not methylated in the 4 tumors studied.

Similar Southern blotting investigations of the methylation of the *HOXB* locus using methylation-sensitive restriction enzymes *HhaI* or *BstUI* generated the same relative methylation status for the 4 tumors (not shown).

Since the sequence of the promoter region of *HOXB2* gene is known, it was possible to evaluate the methylation of this region by PCR after digestion by methylation-sensitive enzymes (Fig. 3). A 609-bp fragment containing a single site for each enzyme *HhaI*, *BstUI*, *BstBI* and *HpaII* was obtained with the selected primers. For SCLC-10, all the restriction sites were methylated, whereas for SCLC-6, only the more 5' *HhaI* site was methylated. For SCLC-82 and SCLC-75, the 2 more 5' restriction sites (*HhaI* and *BstUI*) were methylated.

#### 4. Discussion

The relationship between DNA methylation and gene expression does not appear to be an all-or-none situation (reviewed in [31,32]). Four situations were described, with unequal occurrence. First, in a majority of cases, the hypomethylation of the promoter regions coincided with the expression of the gene. However, in several cases, genes were fully methylated in both expressing and non-expressing cells whereas, in many other cases, some sites were specifically hypomethylated in both expressed or non expressed genes. A second group of genes, for

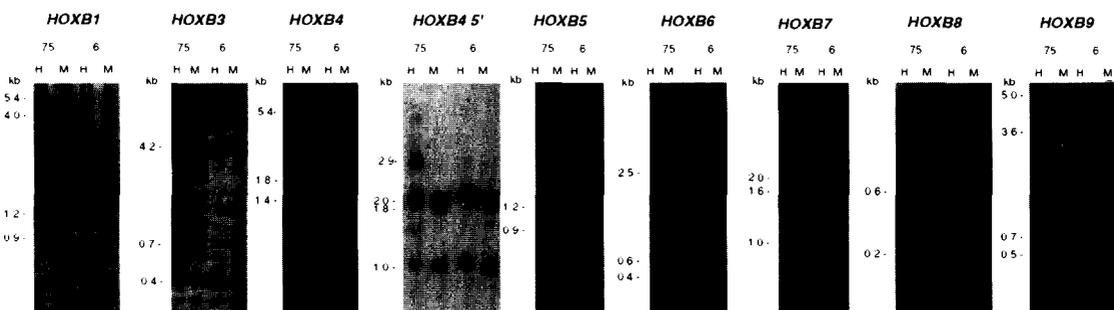


Fig. 2. Southern blot analysis of the methylation status of the *HOXB* locus genes in SCLC-75 and SCLC-6. Experimental conditions were described in the legend to Fig. 1.

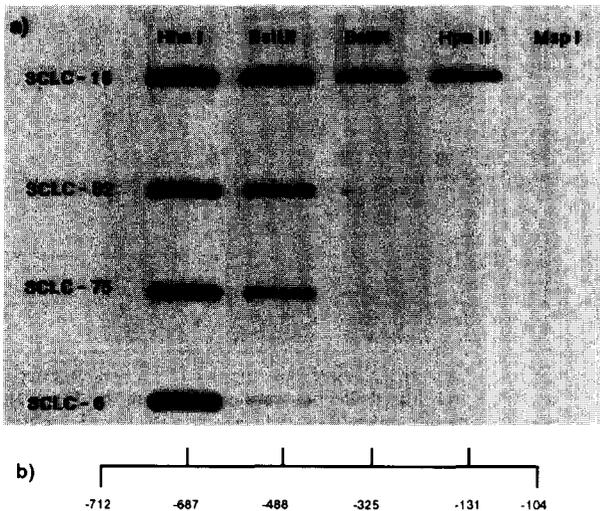


Fig. 3. (a) PCR analysis of the methylation of the *HOXB2* promoter region in SCLC-10, SCLC-82, SCLC-75 and SCLC-6. The 609-bp fragment obtained with the selected primers was digested by 1 of the 5 methylation sensitive restriction enzymes *HhaI*, *BstU1*, *BstBI*, *MspI* or *HpaII* and the digestion products were analyzed by gel electrophoresis. When the site is methylated, the enzyme does not cut and the fragment can be amplified by PCR. When the site is not methylated, the enzyme cuts and the PCR fragment cannot be obtained. With the methylation-insensitive restriction enzyme *MspI*, the fragment is cut in all cases. (b) Position of the restriction enzyme sites. Numbering starts from the initiation site. The primers used were in positions -104 and -712.

the most part the housekeeping genes, were generally unmethylated in their CpG island, regardless of their expression. The third group contained genes which were fully methylated even when expressed. Finally, for a few genes, no apparent relationship existed between their methylation status and their expression.

To investigate the role of DNA methylation in *HOX* gene network expression, the expression patterns of the *HOXB* gene cluster in xenografted SCLC tumors were compared to the methylation of the DNA in the corresponding genomic regions. Due to the lack of complete sequence data for each gene, DNA methylation was assessed by methylation-sensitive/insensitive restriction enzymes only at a few sites generally localized in the 3' region of the gene, with only one promoter region being presently analyzable (*HOXB2*). When it was also possible to investigate the 5' region (*HOXB1* and *HOXB4*), concordant data were obtained for the two regions of the genes.

For the two tumors with similar expression patterns, SCLC-10 and SCLC-82, similar but not strictly identical methylation patterns were observed (Fig. 1). These differences indicate that, despite the global methylated or unmethylated status of the DNA region, at least some CpG sites were differently involved in each case. These sites could correspond to fine regulatory points of the gene expression or to tumor-specific methylation sites.

Taking each gene individually, a direct relationship was found between gene expression and methylation of the corresponding genomic region in 90% of the cases (19 expressed genes and 17 methylated regions). The situation associating an unexpressed gene and an unmethylated region was found in 70% of the cases (17 unexpressed genes and 12 unmethylated

regions). This relationship between unmethylation and gene expression was particularly evidenced in the study of the *HOXB2* promoter region (Fig. 3), where the progression of the demethylation from 3' to 5' led to gene expression extinction. For each tumor, when a series of neighboring genes was all turned on or off, the relationship between DNA methylation and gene expression or DNA unmethylation and no gene expression was generally observed for all the series (*HOXB2* to *HOXB4* in SCLC-10 and SCLC-82; *HOXB4* to *HOXB9* in SCLC-6 or *HOXB1* to *HOXB5* in SCLC-75; Table 1). However, this relationship was not absolute: unexpressed genes could be embedded in methylated regions (*HOXB5* in the *HOXB2* to *HOXB7* methylated region of SCLC-10 or SCLC-82, Table 1) and an isolated unexpressed gene could be associated with an unmethylated island (*HOXB7* in SCLC-75, Table 1). In contrast, in SCLC-6, the completely unmethylated locus harbored a methylated island corresponding to the expressed *HOXB3* gene. Four of the 9 genes studied followed the rule methylation/expression – unmethylation/unexpression very strictly in all the studied cases: *HOXB2*, *HOXB3*, *HOXB4* and *HOXB7*. For the 5 others, exceptions were observed. It can be noted that this general rule was established by determining the methylation in the 3' region of the gene (*HOXB7*), in 3' and 5' ends (*HOXB4*) or in the promoter region (*HOXB2*) indicating that a large DNA region containing the gene was methylated.

It has been previously reported that some methylated genes are expressed but, that the same genes (*HOXB2*, *HOXB4* and *HOXB7*) could be inactive when not methylated and active when methylated had not yet been demonstrated. However, this behavior can be compared to the one of the imprinted *Igf2r* mouse gene [31]. For expression to occur, both hypomethylation of the 5' flanking region and methylation of a site in an intron is required. Hypomethylation at this later site may silence the gene even if the 5' flanking region remains hypomethylated. In contrast with *HOX* genes, *Igf2r* is not expressed when the 5' region is methylated. Despite this main difference, the presence of 'gene silencer' regions in the *HOX* cluster can be postulated. These regions, acting when unmethylated, could correspond to repressor factor recognition regions.

At the present time, the biological significance of these observations is necessarily speculative. It may reflect the particular regulatory functions of the *HOX* gene network whose expression pattern can be considered to be tissue-specific in adults [12–21]. Like imprinted genes, homeobox-containing genes behave unusually as regards DNA methylation during development: sites in non-island regions escape global de novo methylation during the pregastrula stage and became methylated late in development [32–34]. In addition, the CpG islands of these two types of genes also become methylated at a later stage. It may be postulated that the methylation patterns of *HOX* genes reflect signals established earlier in development possibly modified during the tumoral progression which is often associated with striking DNA methylation modifications (reviewed in [35]). The specific methylation of the *HOX* genes could be required to distinguish key genes with respect to their function(s) in the network. In this hypothesis, if the rule 'unmethylated DNA/unexpressed gene' characterizes the *HOX* gene function, the degree of fidelity to this rule could indicate the hierarchy of these genes in the network: the functions dependent on *HOXB2*, *HOXB3*, *HOXB4* and *HOXB7* could be more important than those of other genes. Further studies on other normal

and neoplastic tissues, are needed before the significance of the methylation pattern of *HOX* genes can be fully understood.

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