ALTERED HOX AND WNT7A EXPRESSION IN HUMAN LUNG CANCER

Alterations in HOX expression have been clearly implicated in leukemia, but their role in most other malignant diseases remains unknown. The present disclosure reports that specific member of the HOX gene family, HOXA9, HOXA10, and HOXB9 are overexpressed in lung cancer cells using are using real-time quantitative assays. In some cases, marked HOX overexpression was associated with elevated FGF10 and 17. During development, the WNT pathway affects cell fate, polarity and proliferation, and WNT7a has been implicated in the maintenance of HOX expression. In contrast to normal lung and mortal short-term bronchial epithelial cultures, WNT7a was frequently reduced or absent in lung cancers. In immortalized bronchial epithelial cells, WNT7a was lost concomitantly with HOX A1, and a statistically significant correlation between the expression of both genes was observed in lung cancer cell lines. Furthermore, we identified a homozzygous deletion of β-catenin in the mesothelioma, NCI-H28, associated with reduced WNT7A and the lowest overall cell line expression of HOX A1, A7, A9 and A10 while HOXB9 levels were unaffected. Of note, both WNT7a and beta-catenin are encoded on 3p which undergo frequent loss in these tumors. Our results indicate that alterations in regulatory circuits involving HOX, WNT, and FGF pathways occur frequently in lung cancer.
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BACKGROUND OF THE INVENTION

Homeodomain containing genes (HOX) encode a set of master transcription factors which function during development to control pattern formation, differentiation and proliferation. A common feature is the presence of a highly conserved 61-amino acid motif, the homeodomain (1). Mammals possess at least 39 class I HOX genes grouped into four major clusters (A-D). During development, HOX expression occurs temporally in accordance with both the position of a gene within its cluster and in a rostral-caudal manner (2). HOX genes in equivalent positions of different clusters (paralogs) are more closely related than adjacent genes in the same cluster.

Recent evidence from chimeric gene experiments suggests that paralogous HOX loci may be functionally equivalent (3) and that it is the expression level of the paralogous group as a whole which is critical for correct development.

Deregulation of HOX genes has been observed in cancer, with the most convincing evidence coming from leukemia. For example, four different translocations in acute myelocytic leukemia (AML) fuse the nucleoporin domain of NUP98 to the homeobox of a major or divergent HOX protein (4-6). Similarly, the T-cell (10;14) chromosomal translocation in Acute Lymphocytic Leukemia (ALL) results in overexpression of HOX11 (7) and the pre-B cell (1;19) translocation fuses the homeodomain of PBX1 with E2A proteins leading to aberrant expression of WNT16 and FGF15 (8, 9). Strikingly, HOXA9 was recently identified as the single gene whose expression was most correlated with treatment failure in AML (10). In solid tumors, rearrangements of HOX genes have not been reported. However, expression surveys have noted differences between normal and tumor samples in kidney and colon (11, 12). In melanomas, Caré et al. (13) reported that HOXB7 was constitutively expressed in melanomas and that antisense HOXB7 inhibited cellular proliferation and expression of basic fibroblast growth factor (bFGF).

Few reports have dealt with HOX expression in lung cancer. Using Northern blots, Tiberio et al. (14) reported that few HOX genes were expressed in normal lung and these patterns were altered in small cell lung carcinoma (SCLC) xenografts. Flagiello et al. (15) noted that
retinoic acid treatment of SCLCs resulted in non-contiguous expression patterns in HOXB and HOXC genes. Lastly, Omatu introduced HOXD3 into A549 cells and reported that this enhanced invasive properties (16). There is a significant need in the art for diagnostic and prognostic assay to detect cancerous tissue and predict the severity and aggressiveness of malignancies in particular to provide information for selection of the most appropriate treatment regime. The present invention provides such diagnostic and prognostic assay methods for lung malignancies based upon measurements of HOX gene expression levels.

SUMMARY OF THE INVENTION

The present invention relates to the identification of specific changes in gene expression that occur during the development of lung cancer. These gene expression changes are markers of lung cancer development and severity. The methods of the present invention can be used for diagnosis of lung cancer and its clinical prognosis, including rate of tumor growth, tendency to metastasize and the like. The methods herein can also be used to assess the efficacy of a method of treatment.

The inventors of the present application found, using quantitative real-time RT-PCR assays, that the severity of lung cancers can be characterized on the basis of their total HOX scores or be evaluation of a set of those HOX genes that most frequently display alteration of expression in lung cancer cells. Lung cancer cell lines (isolated from the most aggressive subset of primary tumors) overexpress these HOX genes more frequently than unselected direct patient tumors that HOX overexpression is associated with a worse prognosis. Specific members of the HOX gene family, in particular HOXA9, HOXA10, and HOXB9, are upregulated during lung cancer development. Therefore, an analysis of HOX gene expression provides an assessment of lung cancer aggressiveness at the molecular level. Based on this discovery, a method is provided for evaluating a suspected lung tumor cell sample from a lung cancer patient comprising a screen for expression of a HOX gene or a WNT gene where the expression of the HOX or WNT gene is compared to that of a non-malignant lung cell sample. The screen of the claimed method can be carried out by any method known in the art suitable for measuring the level of transcription or of translation of a given desired HOX or WNT gene. For example, the level of RNA expressed
(i.e., transcribed) from a given gene can be measured by various art-known methods including, but not limited to Northern analysis, polymerase chain reaction (PCR), and in situ hybridization. Specifically exemplified herein is a real-time RT-PCR. The screen can also be carried out by measuring the level of translation (protein expression) by Western Blot, immunoprecipitation, in situ antibody staining etc. The term, “the level of expression”, is used to describe the amount of expression product measured in the cells of a lung cancer sample compared to that in non-malignant lung cells.

Further provided is a discovery that high levels of expression of WNT7a was downregulated or lost as an early change lung cancer development. On the basis of this finding, analysis of WNT7a expression, detected either at the RNA or protein level, or using the technique of methylation-specific PCR at the DNA level, can be used in the evaluation of premalignant epithelial cells. Analysis of the WNT 7a gene can become a routine test in airway lesions that are sampled by bronchoscopy or by sputum analysis.

Alterations in other WNT7a pathway components in lung cancers were also identified. These alterations, exemplified using beta-catenin, involved loss of function as is the case with WNT7a.

The present invention is the first report of specific and quantitative changes in gene expression involving the HOX and WNT pathways which have been identified in lung cancer. The present invention provides specific PCR primers that work under a standardized set of conditions in real time quantitative RT-PCR assays.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B show the results of RT-PCR amplification of HOX and FGF loci. Fig. 1A shows degenerate RT-PCR products from the homeodomain (arrow) amplified from non-malignant ("normal") lung, human bronchial epithelial cells (NHBE), two passages of T-antigen immortalized bronchial epithelial cells (TR5214) and lung cancer cell lines. The leukemia line,
MV4;11 is shown for comparison. Fig. 1B shows the results of RT-PCR for FGF10 and 17 in lung tumor lines, a colorectal carcinoma, NCI-H630, and indicated controls.

Figs. 2A, 2B, and 2C show the results of RT-PCR amplification. Fig. 2A (left) shows quantitative RT-PCR products for G3PDH, HOXA1, A9, A10, B9 and WNT7a from NHBE cells and TR5214, passage-83. (Right) Examples of quantitative RT-PCR products from tumor lines for HOXB9 and A9. NT stands for no template. (Bottom) Similar products shown for WNT7a. Fig. 2B shows Western blot for HOXB9. (Left) The indicated cell lines and direct tumor pair, as described in the text, were analyzed for the 30 kDa HOXB9 protein. Tubulin (55 kDa) served as a loading control. (Right) Identical Western blots contained (lane 1) 3-fold and (lane 2) 1-fold amounts of GST-HOXB9 fusion protein and (lane 3) tumor NCI-H513. No signal was observed for the endogenous HOXB9 (arrow) when the primary antibody was pre-incubated with GST-HOXB9 bound beads (blocked). Fig. 2C shows Southern blot demonstrating specific absence of the beta-catenin gene in EcoRI-digested genomic DNA from NCI-H28. A control probe (D3S30) is shown for comparison. Immunofluorescence confirmed loss of beta-catenin protein in NCI-H28. Nuclei were visualized with DAPI.

Figs. 3A and 3B illustrate the correlation plots for HOX gene expression in cell lines. Fig. 3A shows DCt values for HOXA10 (x-axis) plotted against the sum of DCt values for all HOX loci analyzed (y-axis). The trendline is shown along with the correlation coefficient (Pearson R = 0.77; p=0.0001). Fig. 3B shows similar analysis for HOXA1 (x-axis) vs. WNT7a (y-axis). In this case, the Spearman correlation coefficient is R = 0.56 with p=0.0038. Note that the trendline is skewed by the presence of 1 outlying data point.

DETAILED DESCRIPTION OF THE INVENTION

In general, terms and phrases used herein have their art-recognized meaning, which can be found by reference to standard textbooks, journal references, and contexts known to those skilled in the art. The following definitions are provided to clarify their specific use in the context of the invention.
The term, "quantitative screen", means that the data obtained by measuring the level of expression of a specific HOX or WNT gene in a number of samples can be compared using numerical values. The quantitative screen of the claimed method can be carried out by any method known in the art suitable for quantitatively measuring the level of transcription or of translation of a given desired HOX or WNT gene. For example, the level of RNA expressed (i.e., transcribed) from a given gene can be measured by various art-known methods including, but not limited to Northern analysis, polymerase chain reaction (PCR), and in situ hybridization. Specifically exemplified herein is a real-time RT-PCR by which lung cancers can be characterized on the basis of their total quantitative HOX scores. The quantitative screen can also be carried out by measuring the level of translation (protein expression) by Western Blot, immunoprecipitation, in situ antibody staining etc. The term, "the level of expression", is used to describe the amount of expression product measured in the cells of a lung cell sample compared to that in non-malignant lung cells.

The term "evaluation" is used herein to include obtaining data useful as an aid to diagnosis, to determine clinical prognosis, including rate of growth, tendency to metastasize and the like, to assess the efficacy of a method of treatment and to determine the efficacy of a therapeutic agent. A method of evaluation can include measurement of the expression level of a single gene or it can include measurement of the respective expression levels of plurality of genes. From the data shown herein it can be seen that measurement of the expression level of a single HOX or WNT gene can be increased in the case of certain HOX genes or decreased in the case of WNT genes in most lung cancer cell samples, measurement of two or more such genes can increase the level of confidence of diagnosis, and provide more detail as to the individual pattern of abnormal expression in each individual case. The expression patterns observed in individual cases can be useful in choosing the optimal therapy for each patient and in monitoring the progression of treatment.

The term, "housekeeping gene", as used herein, is a gene whose expression is not altered by any physiological changes which affect the level of expression of many genes. Thus, the expression level of a housekeeping gene is often used to control the amount of sample, RNA or
protein, and to normalize the data obtained. Examples of a housekeeping gene include actin, ribosomal subunit gene, and glycerol-3-phosphate dehydrogenase (G-3-PDH).

RT-PCR Survey of HOX Expression Using Degenerate Primers.

We initially surveyed HOX expression utilizing degenerate RT-PCR with primers corresponding to invariant amino acid sequences within the homeodomain (22). PCR products were subcloned and sequenced from 10 cell lines and normal lung. To assess PCR biases, we used genomic DNA as a template since the amplified segment is uninterrupted by an intron. These results, organized into paralogous groups, are shown in Table I as percentages. Only loci that gave products from either cDNA or genomic are listed. This provided an estimate of the most abundantly expressed HOX genes and indicated differences among samples. For instance, while HOX9 paralogs and HOXA10 products were relatively abundant in several lung cancer cell lines, they were not detected in normal lung. This was interesting since HOXA9 is a known target of chromosomal alterations in leukemia (6) and since HOXA9/HOXA10 co-expression has been similarly noted (23). Differences observed are likely due to an overexpression in lung tumors.

We also compared degenerate HOX RT-PCR band intensities as a crude measure of overall expression levels (Fig. 1A). Among lung cancers, there were substantial differences with GLC20 having marked overexpression, followed by NCI-H513. When quantitative real-time RT-PCR assays were applied (see below), these two cell lines had the highest overall levels of HOX expression. Shown for comparison are products from the acute leukemia cell line, MV4;11 (24), which contains a 4;11 chromosomal translocation affecting the MLL (Trithorax) gene. While the degenerate HOX primers produced a band in normal lung, no products were detected in either mortal (NHBE) or immortalized (TR5214) bronchial epithelial cells. As the lung is a highly vascular structure, other cell types such as endothelial or mesenchymal cells could contribute to the bulk of RT-PCR products. From quantitative assays (below), vascular endothelial cells (HUVEC ) express several HOX genes at higher levels than the bronchial epithelium.
In some developmental systems, as well as human cancers, HOX genes have been shown to affect FGF expression (13, 25, 26). Therefore, we screened a subset of lung cancer cell lines for expression of FGF1, 2, 5, 7, 9, 11, 12, 13 and 18. By non-quantitative RT-PCR, these were expressed in all lung cancers tested except for FGF2 which appeared to distinguish SCLCs with a classic phenotype from those with a variant phenotype. However, expression patterns for FGF10 and 17 were different (Fig. 1B). In the lung, FGF10 is produced by mesenchymal cells and induces branching of epithelial lung buds (27). Among 25 lung cancer cell lines, FGF10 was strongly expressed in only GLC20. Expression was also noted in the normal lung and the immortalized bronchial epithelial culture, 5214, but not in the short-term bronchial cultures. During development, FGF17 is preferentially expressed in the brain (28). In the cell lines, FGF17 was greater in SCLCs (lanes I-9) and GLC20, again, had the highest expression. Thus, it is likely that HOX overexpression is associated with upregulation of FGF genes.

Quantitative Real-Time RT-PCR and Western Blot Analysis.

We developed quantitative RT-PCR assays for five HOX loci and WNT7a. In addition to cell lines and control cultures, we analyzed 35 non-SCLC resected tumors of which 25 had adequate amounts of RNA from a matched non-malignant portion of the lung. The results are expressed in terms of ΔCt values, which refer to the cycle number during exponential amplification at which the PCR product (measured in real-time by SYBR green fluorescence) crosses a set threshold. To adjust for variations in the amount of input RNA/cDNA, the average Ct values for each gene were normalized against average Ct values for the housekeeping gene, Glycerol-3-phosphate dehydrogenase G3PDH (i.e., ΔCt = Av. Ct specific gene - Av. Ct G3PDH).

While the resulting ΔCt values are experimentally convenient, they are not readily intuitive (i.e., they reflect exponential amplification and higher ΔCts represent lower expression). These results are shown in Table II and selected examples of the PCR reactions are shown in Fig. 2A.

Neither HOXA9 nor HOXA7 was expressed in control bronchial epithelial cultures, mortal or immortal. While HOXA10 and HOXB9 could be detected, over 30 PCR cycles were required (e.g., Ct G3PDH + Ct HOXB9). In contrast, HOXA1 and WNT7a were relatively abundant (ΔCt range = 4-9). Interestingly, in the T-Ag immortalized culture, expression of both HOXA1 and WNT7a was reduced by 8 to 9 cycles. Control ΔCt values for the non-malignant
lung were obtained from an average of 25 resected samples. However, there was little variation among these suggesting that altered HOX expression is not a common feature of normal appearing airways. Since the lung is an extremely vascular structure, we included human vascular endothelial cells (HUVEC) as a control. Each HOX gene tested was expressed in HUVEC cells, but WNT7a was absent. At least for HOXA1, HOXA9, HOXB9 and HOXA10, the ΔCt values from non-malignant lung were intermediate with those obtained in the mortal bronchial epithelial and HUVEC cultures.

In agreement with our initial degenerate RT-PCR survey, HOXA9 and HOXB9 were overexpressed in many lung cancer cell lines compared to non-malignant lung. Using at least two PCR cycle differences, HOXB9 overexpression occurred in 18/25 (72%) of the cell lines and HOXA9 overexpression was identified in 10/25 (40%). Nine cell lines (GLC20, NCI-H187, H1048, H1341, H433, H1264, H460, H290, H292) overexpressed both genes. Similarly, HOXA10 was overexpressed in 18/25. Of the cell lines that overexpressed both HOXA9 and HOXB9, all but one (H1341) had elevated HOXA10. In the direct tumors, overexpression of these same genes was observed, but less frequently. For instance, HOXB9 was overexpressed in 9/35 (26%), HOXA9 in 11/35 (31%) and HOXA10 in 20/35 (57%). Thus, overexpression of HOX9 paralogous genes and HOXA10 occurs frequently in lung cancer.

These data were analyzed using both parametric (Pearson) and non-parametric (Spearman) correlation coefficients in conjunction with the UC Cancer Center Biostatistics Core. In the lung cancer cell lines, which are free of contaminating non-malignant components, HOXA9 expression was significantly correlated with both HOXA10 (Pearson R = 0.559, p = 0.0036) and HOXA7 (Spearman R = 0.404, p = .0453). Likewise, HOXA7 expression was correlated with HOXA10 (Pearson R = 0.472, p = 0.0166). In contrast, neither HOXA1 nor HOXB9 expression was correlated with any other HOX locus (see below). However, there was a significant correlation (Spearman R = 0.43, p = 0.0319) when HOXB9 was compared to overall HOX expression measured as the sum of the individual ΔCt values for HOXA1, A7, A9, A10 and B9. Nevertheless, this relationship was weaker than the observed correlations between HOXA7, HOXA9 or HOXA10 (Fig. 3A) with the total HOX score (Spearman R = 0.768, p = 0.0001; R = 0.676, p = 0.0002; and R = 0.684, p = 0.0002, respectively).
For HOXB9, we confirmed that elevated RNA levels were associated with elevated protein expression (Fig. 2B, left). Two cell lines (NCI-H513 and H460) predicted to express HOXB9 protein on the basis of their low ΔCt values were compared to cell lines (N177 and NCI-H196) with much higher ΔCts. As anticipated, HOXB9 protein was identifiable by Western blot in extracts from NCI-H513 and H460, but not from H196 and N177. Anti-tubulin antibody was used to assess protein loading. We also confirmed that protein overexpression occurs in direct tumors, as can be seen in the matched pair, 6802. To demonstrate that the antibody was specific, we produced GST-HOXB9 protein. The antibody detected recombinant protein (Fig. 2B, right) and, in addition, excess GST-HOXB9 attached to glutathione agarose beads was capable of blocking the endogenous HOXB9 band in H513 extracts (arrow). In summary, elevated HOXB9 RNA correlates with elevated HOXB9 protein in cell lines and direct tumors. However, the degree of protein overexpression was less than what would have been predicted from the RNA measurements alone. How factors such as alternative splicing, translational regulation or protein stability, either alone or in combination, affect detectable HOXB9 levels using this antibody is unknown.

A Relationship Between HOXA1 and WNT7a Expression in Lung Tumors.

In contrast to the above results, there were no cell lines with elevated HOXA1 levels that differed from mortal bronchial epithelial cells by more than two PCR cycles and only one, H513, had expression that was greater by one PCR cycle. If the non-malignant lung value is taken as the reference, then three cells lines (NCI-H513, H1648 and H290) had elevated HOXA1 expression by two PCR cycles. Interestingly, NCI-H513 and H290 represent two of the three mesotheliomas. This relationship becomes more obvious when WNT7a expression is considered (see below). Among direct tumors, there were no examples of significantly elevated HOXA1 expression. There was only one mesothelioma (7362) in the set of direct tumors and it was among the highest 10% in terms of HOXA1 expression.

We tested whether other pathways known to affect HOX expression during normal development might be operative in lung tumors. The WNT pathway (29-31) was an obvious candidate since there are several well described interactions between these pathways in model
organisms plus other reports of interactions involving human cancers. We carried out a survey of WNT pathway components in this same collection of lung cancer cell lines. Interestingly, compared to either NHBE cells or non-malignant lung, WNT7a expression was almost uniformly reduced in the tumors. In the cell lines, WNT7a was undetectable in 10 of 26 (38%) (Fig. 2A, bottom). Moreover, WNT7a was lost concomitantly with HOXA1 in SV40 immortalized bronchial epithelial cells (Fig. 2A, left).

Chromosome 3p21.3 is recognized as a somatic deletion target in lung cancer (32) and includes the gene for β-catenin. In a PCR survey for homozygous deletions affecting 3p, we discovered that β-catenin was missing in the mesothelioma, NCI-H28. This was confirmed by Southern blot analysis and at the protein level by immunofluorescence (Fig. 2C). The absence of β-catenin should result in downregulation or loss of genes dependent upon an activated WNT pathway. In Drosophila, a positive autoregulatory WNT feedback loop has been postulated (17) and, in the mouse uterus, WNT7a has been associated with maintenance of some HOXA loci (33). Strikingly, loss of β-catenin in NCI-H28 was associated with loss of WNT7a and the lowest overall expression of HOX loci among all cell lines despite the fact that HOXB9 appeared relatively unaffected. While, as mentioned above, HOXA1 expression did not correlate with any other HOX genes, nor with the total HOX score, it was strongly associated in the cell lines with WNT7a expression using either Pearson (R=0.506, p=0.0099) or Spearman (R=0.558, p=0.0038) correlation coefficients (Fig. 3B).

The results disclosed above indicate that lung cancers are associated with substantial changes in HOX gene expression. Moreover, this expression is not haphazard but rather regulated and appears integrated with the WNT and FGF pathways. The degenerate PCR approach provided an initial estimate of the most abundantly expressed HOX genes in lung cancer cell lines and normal lung. However, not all HOX loci could be amplified and, clearly, there are more genes of interest not yet investigated by quantitative analysis. For example, HOXA5 was recently shown to positively regulate p53 expression (34). From the degenerate analysis (Table I), HOXA5 levels varied from 0-8%. Considerably more variation is suggested for other genes such as the HOX3, HOX4, HOX6 and HOX7 paralogues (Table I). We were initially surprised that the degenerate PCR results did not detect more differences among cell types, especially SCLC.
vs. non-SCLC. However, Anbazhagan et al. (35) recently described similar findings using cDNA arrays in which the patterns of gene expression for SCLC were more similar to epithelial cells than neuroendocrine tumors.

Interestingly, as in leukemia (23, 36), genes of the HOX9 paralogous group as well as HOXA10 were frequently overexpressed in lung cancers. Chen and Capocci (37) demonstrated that HOX9 paralogous genes influenced mammary gland proliferation and suggested they should be examined in mammary carcinomas. Increased expression of HOX9 paralogous genes was not simply related to proliferation as they were transcriptionally silent in the SV40 immortalized epithelial culture, TR5214. In leukemia, HOXA9 expression has been associated with a poor outcome (10). The finding that HOX9 genes were more frequently overexpressed in lung cancer cell lines than in resectable primary tumors indicates that a similar relationship can exist. In several tumors, such as neuroblastoma and melanoma, cell lines are more readily established from biologically aggressive tumors and metastatic sites. From our quantitative results, there are subsets of cell lines and direct tumors that overexpress most of the genes tested and vice versa. Thus, HOX genes can be important biomarkers and possible therapeutic targets.

To search for consequences of altered HOX expression, we looked at FGFs which are known target genes in some instances (38, 39). In general, most FGFs tested were widely expressed although FGF10 and 17 were more restricted. Interestingly, GLC20, which had the highest overall HOX expression by both degenerate RT-PCR and quantitative analyses, also had the highest expression for both FGF10 and 17. In the breast cancer cell line, SkBr3, HOXB7 was shown to induce bFGF (39). While we have not yet examined HOXB7, the paralogous HOXA7 was most highly expressed in GLC20 and H661 (ΔCt = 6.8 and 5.6, respectively), both of which expressed FGF10. During lung development, FGF10 is produced by mesenchymal cells and influences epithelial branching (40) and FGFs can influence proliferation, transformation and angiogenesis (36, 41, 42). Thus, in lung cancer, HOX overexpression could function in part by upregulating FGFs.

The apparent alteration of HOX genes in lung cancers indicted that pathways affecting these loci might be altered. In C. elegans, the WNT pathway controls expression of HOX genes
including mab-5 during development of the migratory QL neuroblast (43) and lin-39 during vulval development (30). HOX loci can also regulate Wnt expression in both Drosophila development and in human leukemias (31, 38, 44). We found that WNT7a was significantly correlated with HOXA1 in several instances. In lung cancer cell lines, the only significant HOXA1 correlation was with WNT7a. In the bronchial epithelial cultures, WNT7a and HOXA1 expression were lost concomitantly following immortalization with T-Ag. Finally, a homozygous deletion of β-catenin in the mesothelioma, H28, was associated with loss of both genes which were otherwise strongly expressed in the two other mesotheliomas, H513 and H290. In the mouse, a knockout of WNT7a was associated with uterine alterations and loss of HOXA10 and HOXA11 (45). HOXA1 expression was not mentioned.

Similar relationships were observed in the direct tumors. However, the expression of all the HOX genes examined by HUVEC cells suggests that contamination by non-malignant stroma may complicate the analysis. WNT7a was not expressed by HUVEC cells and, interestingly, was substantially downregulated in most tumors. WNT7a is located at 3p25, a common site for genetic loss in lung cancer (46). Thus, it is possible that 3p alterations, or epigenetic changes, could lead to reduced WNT7a expression and loss of target genes including HOX loci. Likewise, imbalances have been noted for the chromosome arms containing the HOXA and HOXB clusters (7p and 17q, respectively), but these changes have not been sufficiently delineated to suggest the HOX clusters as targets. The WNT pathway has been implicated in differentiation processes in various settings including avian mesoderm (47), neural crest (48), hair follicle development (49) and axonal remodeling (50). However, WNT7a is also weakly transforming when expressed in C57MG cells (51). However, based on its frequent loss, we hypothesize that WNT7a signaling in lung epithelial cells affects differentiation, possibly through corresponding changes in HOX expression. HOX genes can also be influenced by other signaling pathways, including TGF-b (52). Interestingly, TGF-b signaling can directly interact with WNT signaling by a complex between β-catenin/TCF/LEF and Smad4 on the Xtwn promoter (53). Since TGF-b signaling is commonly disrupted in epithelial tumors (54), it is plausible that both the WNT and TGF-b pathways may synergistically interact to affect specific HOX gene expression.
Table 1. HOX expression survey by degenerate RT-PCR. The percentage of subclones corresponding to each identified HOX locus is listed. The number of clones sequenced is given at the top along with the source. The sequence data did not discriminate between HOXC6 and C8 which are listed as C6/C8.

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The percentage of subclones corresponding to each identified HOX locus is listed. The sequence data did not discriminate between HOXC6 and C8, which are listed as C6/C8. The number of clones analyzed from each sample is as follows: DNA control (153), normal lung (62), CLC20-SCLC (48), H187-SCLC (51), H460-large cell (49), H661-large cell (42), H1648-adenocarcinoma (59), H322-bronchioalveolar (35), H226-squamous (36), H290-mesothelioma (61), H513-mesothelioma (44), H28-mesothelioma (36).
Table 2. Quantitative RT-PCR for HOX expression. Values represent ΔCtS obtained from 6 controls, 25 lung tumor cell lines and 35 resected tumors, as indicated. Non-malignant lung values represent the average of 25 independent resections which were tumor free. The All HOX column contains the sum of ΔCt values for the 5 HOX loci analyzed. The average standard deviation was 0.12 cycles.

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Cell lines and Tumors: Twenty-five lung cancer cell lines (available from American Type Culture Collection, Manassas, VA) and 25 matched tumor/non-malignant lung pairs were obtained through the Colorado Lung Cancer SPORE. The non-malignant samples were obtained from a site equivalent, but removed from the tumor. Ten additional tumors were from pathology archives (E.B.) at CHU, Grenoble. The lung cancer cell lines have been previously described (18). GLC20 was provided by Dr. C. Buys, Univ. of Groningen, The Netherlands. Normal human bronchial epithelial (NHBE) cells were obtained from Clonetics (San Diego, CA). In addition, three short-term bronchial epithelial cultures, 302-1, 604-2 and 2523 were derived from non-malignant biopsies of heavy smokers. A fourth bronchial culture, TR5214-2, was immortalized with large-T antigen and examined at passages 50, 78 and 83. HUVEC cells were obtained from BioWhittaker (Belgium).

RNA isolation, cDNA preparation: Total RNA was isolated using Trizol (Life Technologies, Rockville, MD) and further purified on RNeasy columns (Qiagen, Valencia, CA) with RNase-free DNase treatment. Absence of contaminating genomic DNA was verified by PCR using multiple primer pairs which amplify genomic DNA segments. Reverse-transcription reactions used ~2 μg of total RNA in 20 μl with 200 units of SuperScript II (Life Technologies, Rockville, MD) and random hexamers.

Homeobox Amplification using Degenerate Primers: Two blocks of completely conserved amino acids, ELEKEF (SEQ ID NO:1) and KIWFQN (SEQ ID NO:2), within the homeodomain were chosen for degenerate primers. These were; ELEKEF: 5'-gct ga(a/g) (c/t)(a/c/g/t) ga(a/g) aa(a/g) ga(a/g) tt (SEQ ID NO:3) and KIWFQN: 5'-gga att c(a/g)t (c/t)t g(a/g)a acc a(a/g/t)a t(c/t)t t (SEQ ID NO:4). PCR conditions consisted of 32-35 cycles of amplification (94° C, 1 min; 40° C, 1 min; 72° C, 1 min) with 4.5 mM MgCl2 and 2 μM primers. A band of ~120 bp was gel isolated and cloned into a T-vector. Individual clones were sequenced and analyzed using BLAST.
Real-Time RT-PCR: An ABI5700 (PE Biosystems) with SYBR green fluorescence was used. Primers were designed to amplify segments of < 200 bp to maximize efficiency. Primer sequences (5' to 3') were: HOXA1(for) acc cct egg acc ata gga tta c (SEQ ID NO:5), (rev) aag gcg ccc tga agt tct gtt (SEQ ID NO:6); HOXA7(for) act cta cct cgt aaa acc gac ac (SEQ ID NO:7), (rev) aca taa tac gaa gaa ctc ata att tgt acc (SEQ ID NO:8); HOXA9(for) cag aac tgg tgg att tag gta g (SEQ ID NO:9), (rev) cca ctg aag taa tga agg gca gtt (SEQ ID NO:10); HOXA10(for) gag agc agc aaa gcc tgc c (SEQ ID NO:11), (rev) cca gtt tct ggt gct tgc tgc (SEQ ID NO:12); HOXB9(for) aaa aag cgc tgt ccc tac acc (SEQ ID NO:13), (rev) agg agt ctt gcc act tgc tgc (SEQ ID NO:14); WNT7a(for) tgc ccc gac tct cat gaa c (SEQ ID NO:15), (rev) gtt tgg tcc aag gct tct (SEQ ID NO:16); G3PDH(for) tgc acc acc aac tgc tta gc (SEQ ID NO:17), (rev) ggc atg gac tgt ggt cat gag (SEQ ID NO:18). Twenty 41 reactions were used under conditions suggested by ABI. Data were analyzed using GeneAmp 5700 SDS software (version 1.1) and converted into Ct values (see Results). Non-quantitative primers included FGF10 (for) cat tgt gcc tca gcc ttt c (SEQ ID NO:19); (rev) tcc att ttc ctc tat cct ctc c (SEQ ID NO:20); FGF17 (for) tgc tgc cca acc tca ctc (SEQ ID NO:21), (rev) tct tgt ctt ttc ccc ctg (SEQ ID NO:22).

Immunofluorescence and Western Blots: For immunofluorescence, cells were fixed as described (19). The anti-β-catenin mAb was C19220 (Transduction Labs, Lexington, KY) diluted 1:100. The secondary antibody was FITC conjugated goat anti-mouse IgG (Jackson Immuno Research) diluted 1:100. Images were captured with a Vysis Smart Capture System on an Olympus BX60 microscope equipped with a Photometrix SenSys CCD camera. For HOXB9 Westerns, filters were incubated with anti-HOXB9 antibody (Berklee Antibody Company, Richmond, CA) at 1:1000 dilution and processed as suggested by the manufacturer. To control for protein loading, parallel gels were incubated with anti-tubulin antibody (Ab-4, clone DM1A + DM1B, NeoMarkers, Inc., Freemont, CA) at 1:500 dilution. For detection, an enhanced chemiluminescence kit was utilized (Amersham). To demonstrate specificity of the HOXB9 antibody, we prepared a GST-HOXB9 fusion protein using a bacterial expression construct (20) kindly provided by Dr. Laura Corbo (Lyon, France). GST-HOXB9 protein was affinity purified from 500-mls of BL21 cells as described by Harper and Speicher (21). For blocking, primary antibody was absorbed for 2 hr. with 50 ml of HOXB9 beads or, as a control, glutathione agarose beads.
In addition to the primer sets for a specific HOX gene or a specific WNT gene which are disclosed in the present application, one of ordinary skill in the art can readily identify other primer sets specific for a given HOX gene or a WNT gene for use in a quantitative screen disclosed herein based particularly on sequence information that is known and available to those in the art. The nucleotide sequences of various human HOX and WNT genes are available in the GenBank database. Those of ordinary skill in the art can apply the method of the invention to currently known HOX genes, WNT genes and to those, for example, HOX or WNT whose sequence becomes available.

While the foregoing Specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention encompasses all of the usual variations, adaptations, or modifications, that come within the scope of the following claims and their equivalents.


The foregoing exemplary descriptions and the illustrative preferred embodiments of the present invention have been explained in the drawings and described in detail, with varying modifications and alternative embodiments being taught. While the invention has been so shown, described and illustrated, it should be understood by those skilled in the art that equivalent changes in form and detail may be made therein without departing from the true spirit and scope of the invention, and that the scope of the present invention is to be limited only to the claims except as precluded by the prior art. Moreover, the invention as disclosed herein, may be suitably practiced in the absence of the specific elements which are disclosed herein.

All references cited in the present application are incorporated in their entirety herein by reference to the extent not inconsistent herewith.

References:

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    P. R., Linnoila, R. I., Matthews, M. J., Bunn, P. A. Jr, Carney, D., Minna, J. D., and
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    Oncogene 16, 3285-9.
    124, 4867-78.
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We claim:

1. A method for evaluating a lung cell sample of a lung cancer patient which comprises a screen for expression of a HOX gene or a WNT gene wherein the level of expression of the HOX gene or WNT gene is compared to that in a sample of non-malignant lung cell.

2. The method of claim 1 wherein the HOX gene is a HOXA or HOXB gene.

3. The method of claim 2 wherein the HOX gene is a HOXA gene.

4. The method of claim 3 wherein the HOXA gene includes one or more genes selected from the group consisting of HOX A1, HOX A7, HOX A9 and HOX A10.

5. The method of claim 2 wherein the HOX gene is a HOXB gene.

6. The method of claim 5 wherein the HOXB gene is HOXB9.

7. The method of claim 1 wherein the WNT gene is WNT7a.

8. The method of claim 1 wherein the screen is carried out by RT-PCR.

9. The method of claim 8 wherein the RT-PCR is quantitative real-time RT-PCR.

10. The method of claim 1 wherein the screen is carried out by Northern analysis or Western analysis.

11. A kit useful for evaluating a lung cell sample of a lung cancer patient which comprises a set of primers, probes or antibodies specific for one or more HOX genes.

12. A method for evaluating a lung cell sample of a lung cancer patient comprising the steps of:
   a) preparing RNA or protein samples from a lung cancer tissue and non-malignant lung tissue
   b) measuring the level of a HOX or WNT gene expression in these samples
   c) comparing the relative levels of the HOX or WNT gene expression between the samples

   whereby the level of the HOX or WNT gene in the lung cancer tissue is altered compared to that of the non-malignant lung tissue.

13. The method of claim 12 wherein the HOX gene is a HOXA or HOXB gene.
14. The method of claim 12 wherein the HOX gene is a HOXA gene.

15. The method of claim 14 wherein the HOXA gene includes one or more genes selected from the group consisting of HOXA1, HOXA7, HOXA9 and HOXA10.

16. The method of claim 12 wherein the desired gene is a HOXB gene.

17. The method of claim 16 wherein the HOXB gene is HOXB9.

18. The method of claim 12 wherein the WNT gene is WNT7a.

19. A method for detecting a gene expression pattern associated with lung cancer comprising the steps of:

   measuring expression levels of genes selected from the group consisting of HOX and WNT genes in a test cell sample and in a control cell sample of non-malignant lung cells,

   identifying any of said genes having increased expression or decreased expression in the test cell sample compared to the control cell sample,

   Whereby decreased expression of a WNT gene or HOXA1 gene, or increased expression of a HOX gene other than HOXA1, or both, is associated with lung cancer.

20. The method of claim 19 wherein the HOX gene is a HOXA or HOXB gene.

21. The method of claim 20 wherein the HOX gene is HOXA.

22. The method of claim 21 wherein HOXA includes one or more genes selected from the group consisting of HOXA1, HOXA7, HOXA9, and HOXA10.

23. The method of claim 19 wherein the HOX gene is HOXB.

24. The method of claim 23 wherein the HOXB gene is HOXB9.

25. The method of claim 19 wherein the WNT gene is WNT7a.

26. A method for measuring gene expression in a cell sample comprising:

   preparing RNA from the cell sample,

   amplifying a selected gene and a housekeeping gene from the RNA sample by reverse transcriptase polymerase chain reaction (RT-PCR) thereby providing DNA corresponding to the selected gene and the housekeeping gene,

   measuring the number of rounds of amplification needed to obtain a preset threshold amount of DNA corresponding to the selected gene, and of DNA corresponding to the housekeeping gene,
normalizing any sample-to-sample variation in the number of rounds of amplification of
the selected gene to a predetermined number of rounds of amplification of the
housekeeping gene needed to obtain the threshold amount of DNA of said housekeeping
gene.

27. The method of claim 26 wherein the housekeeping gene is glycerol-3-phosphate
dehydrogenase (G-3-PDH).

28. A method for determining the level of expression of one or more HOX genes in a lung
cell sample taken from a patient suspected to have a malignancy compared to the level
of expression of said one or more HOX genes in a control sample of non-malignant lung
tissue, whereby elevated levels of expression of one or more HOX genes in the sample
over the control indicates the presence of malignant cells in the sample.

29. A method for determining the prognosis of a lung cancer by detecting a gene expression
pattern associated with lung cancer comprising the steps of

measuring expression levels of genes selected from the group consisting of HOX and
WNT genes in a test cell sample and in a control cell sample of non-malignant lung cells,

identifying any of said genes having increased expression or decreased expression in the
test cell sample compared to the control cell sample,

whereby decreased expression of a WNT gene or HOXA1 gene, or increased expression
of a HOX gene other than HOXA1, or both, indicates poor prognosis, increased rate of
tumor growth, increased tendency to metastasize.

30. A method for assessing a lung cancer for having a tendency to metastasize by detecting
a gene expression pattern associated with lung cancer comprising the steps of

measuring expression levels of genes selected from the group consisting of HOX and
WNT genes in a test cell sample and in a control cell sample of non-malignant lung cells,

identifying any of said genes having increased expression or decreased expression in the
test cell sample compared to the control cell sample,

whereby decreased expression of a WNT gene or HOXA1 gene, or increased expression
of a HOX gene other than HOXA1, or both, indicates increased tendency to metastasize.
FIGURE 2
<110> Drabkin, Harry A.
    Gemmill, Robert M.

<120> ALTERED HOX AND WNT7a EXPRESSION IN HUMAN LUNG CANCER

<130> 118-01WO

<140> Not assigned
<141> 2001-10-11

<150> 60/239,596
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Description of Artificial Sequence: Forward primer for HOXA7

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Description of Artificial Sequence: Reverse primer for HOXA7

acataactcg aagaactcat aatgtgacc 30

Description of Artificial Sequence: Forward primer for HOXA9

cagaacctgt cggtgatatta ggtag 25
Artificial Sequence

Description of Artificial Sequence: Reverse primer for HOXA9

caactgaag taatgaagggc agtg

Description of Artificial Sequence: Forward primer for HOXA10

gagagcagca aagcctgc

Description of Artificial Sequence: Reverse primer for HOXA10

ccaagtgtctg gtgcttcctg

Description of Artificial Sequence: Forward primer for HOXB9

aaaaagcgcgt gccctacac c

DNA
Artificial Sequence

Description of Artificial Sequence: Reverse primer for HOXB9

agagtcctgg ccacttcgtg

Description of Artificial Sequence: Forward primer for WNT7a

tgcccgagct ctcatgaac

Description of Artificial Sequence: Reverse primer for WNT7a

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Description of Artificial Sequence: Forward primer for G3PDH

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Description of Artificial Sequence: Forward primer for FGF10

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Description of Artificial Sequence: Reverse primer for FGF10

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Description of Artificial Sequence: Forward primer for FGF17

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Artificial Sequence

Description of Artificial Sequence: Reverse primer for FGF17

tctttgctct tcogctg
INTERNATIONAL SEARCH REPORT

A: CLASSIFICATION OF SUBJECT MATTER

IPC(3) : C10Q 1/06, G01N 31/20, C07H 21/04, C10P 10/24
US CL : 466/5, 91.1, 91.2; 493/01; 596/24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B: FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 465/6, 91.1, 91.2; 493/01; 596/24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

DIALOG, EAST

C: DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>US 5,866,404 A (BRADSHAW et al) 02 February 1999 (02.02.1999), Example 4, column 19.</td>
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<td>X</td>
<td>HEID et al Real Time Quantitative PCR, Genome Research, 1996, Vol. 6, pages 986-994.</td>
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<td>Y</td>
<td>WO 00/38709 A1 (CHIRON CORPORATION) 06 July 2000 (06.07.2000), pages 7, 27-29, 49 and 50.</td>
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<td>THORSTEINSDOTTIR et al. Overexpression of HOXA10 in Murine Hematopoietic Cells Perturbs both Myeloid and Lymphoid Differentiation and Leads to Acute Myeloid Leukemia. Molecular and Cellular Biology, January 1997, Vol. 17, No. 1, pages 495-505.</td>
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[ ] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

Date of the actual completion of the international search: 27 DECEMBER 2001

Date of mailing of the international search report: 17 JAN 2002

Authorized officer: [Signature]

Form PCT/ISA/210 (second sheet) (July 1999)*
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