Involvement of multiple developmental genes on chromosome 1p in lung tumorigenesis

Cathie Garnis*, Jennifer Campbell‡, Jonathan J. Davies‡, Calum MacAulay, Stephen Lam and Wan L. Lam

British Columbia Cancer Research Centre, Vancouver, BC V5Z 1L3, Canada

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Lung cancer is the leading cause of cancer death in North America. Despite advances in lung cancer treatment, the overall 5 year survival rate for those diagnosed with the disease is bleak presumably due to the late stage of diagnosis. Owing to the difficulty of early detection, preneoplastic specimens are rare. However, studying both preinvasive and invasive stages of disease is necessary to fully understand lung cancer progression. Aberration of chromosome arm 1p is common in lung and other cancers. In this study, we used a genomic array with complete tiling coverage of 1p to profile preinvasive and invasive squamous non-small cell lung carcinoma samples. With this technology, multiple novel submegabase alterations were identified. Three of the 1p alterations harbored genes belonging to gene families known to be involved in cancer development through either the Wnt or the Notch developmental pathways. Our finding of a 0.4 Mb amplified region at 1p36.12 containing WNT4 in preinvasive lung cancer, coupled with the identification of three additional alterations in invasive tumors that also contain genes related to the Notch and Wnt pathways, strongly suggests an intricate role of these pathways in early and late stages of lung cancer development. Furthermore, ectopic expression of DVL1, LRP8 and Notch2 in malignant lung tissue validates the biological impact of these genetic alterations. Importantly, this implication of pathways known only to be activated in fetal lung development lends support to the proposed model of lung cancer ontology whereby tumors arise from dysregulated pleuripotent stem cells.

INTRODUCTION

Aberration of the short arm of chromosome 1 (1p) is a common event in lung and many types of cancer (e.g. colorectal, gastric, neuroblastoma, liver, pancreatic and breast cancer) (1). Cytogenetic studies have shown that 1p harbors structural rearrangements in non-small cell lung cancer (NSCLC) (2), whereas microsatellite analysis demonstrates frequent loss of heterozygosity (LOH) at 1p36 in all major lung cancer subtypes (3). Moreover, the alignment of LOH data revealed multiple regions of alteration on 1p (3–6). Although there appears to be a high frequency of LOH across the entire chromosome arm, three large but distinct regions (1p36, 1p32 and 1p12) have been reported, suggesting the presence of more than one oncogene/tumor suppressor gene on the short arm of chromosome 1. Notably, there are several genes associated with the Wnt and Notch developmental pathways mapping to chromosome 1p.

Pathways involved in development may play a role in tumorigenesis. Recent focus on the Wnt pathway in colorectal cancer has prompted gene expression analysis of pathway components in other cancer types including breast, prostate and lung (7–9). Wnt ligands bind to their target membrane receptors, frizzled (FZD) and low-density lipoprotein receptor-related proteins (LRPs), and interfere with the multi-protein APC/β-catenin destruction complex, resulting in the downstream activation of gene transcription by β-catenin. While the complex role of β-catenin in cell proliferation and cell adhesion has been the main focus of many mechanistic studies, it is becoming increasingly evident that upstream components such as WNT, FZD, SFRP, LRP and DVL genes are also involved in cancer (9–19). Recent reports of DVL3 and WNT2 overexpression in NSCLC suggest a potential role of this pathway in cell proliferation (17,20). However, WNT7a silencing, which leads to β-catenin-mediated loss of E-Cadherin expression and subsequently impacts cell...
migration, suggests that the Wnt pathway plays a role in the later stages of lung cancer (including metastasis) (21). In addition, the Notch developmental pathway, which interacts with Wnt components, has also been implicated in cancer. For example, NOTCH3 translocation and overexpression has been observed in lung cancer (22). Interestingly, aberrant Wnt pathway signaling is an early event found in ~90% of colorectal cancers (23). This possibility has not been explored in lung cancer.

In this study, we investigated 1p alterations in early stage development of lung cancer. Hitherto, genomic analysis of early stage lesions has been limited presumably owing to the difficulty in obtaining preinvasive lesions and the minute size of such material, which yield limited DNA sufficient only to support locus-specific analysis but far too impractical for large-scale gene discovery. In this work, we combine the use of fluorescent bronchoscopy to capture preinvasive lesions and a bacterial artificial chromosome (BAC) genomic array to comprehensively analyze the 1p arm in microdissected preinvasive bronchial carcinoma in situ (CIS) and invasive squamous cell carcinoma (SqCC) specimens. The BAC clones of the 1p specific array span the chromosome arm in a tiling fashion, allowing focused characterization of genetic alterations in order to identify candidate genes that may be causal to the development of lung cancer.

As mentioned earlier, the role of developmental pathways has become increasingly appreciated in cancer etiology. Remarkably, candidate genes within three of the five novel regions of genetic alteration defined in this study are associated with the Notch and Wnt developmental pathways.

RESULTS AND DISCUSSION

Detecting genetic alterations by 1p array CGH

Tumor DNA samples isolated from microdissected lung cancer cells were analyzed for regional copy number changes using a BAC array spanning the chromosome 1p arm at tiling resolution. In order to define gains and losses, we first established the baseline of technical variation by performing normal versus normal reference DNA comparisons. Clones with aberrant signal intensity were removed from the analysis. To ensure the detection of single copy alterations, normal male and female DNA samples were differentially labeled and co-hybridized to the 1p array, which contains control spots of X chromosome loci as a measure of detection sensitivity. Figure 1 shows the 1p profiles from two SqCC samples. Multiple segmental copy number changes were evident across the chromosome arm. Owing to the redundancy and the high degree of overlap of clones, small regions of genetic alterations were readily detected. While such small alterations may have gone undetected by marker-based techniques and conventional chromosomal CGH, the tiling nature of the array enables the definition of breakpoints flanking an altered region. In our example, we magnified three regions to illustrate simultaneous localization of multiple breakpoints (Fig. 1). One of the regions located at 1p12 contains Notch2.

Recurrent amplification in preinvasive stage

Genetic alterations critical to lung cancer are likely to be present in multiple patients. Twenty lung SqCCs (Eight CIS and 12 tumors) were microdissected and the DNA analyzed. Although there are sporadic alterations on 1p in the CIS samples, only one region was deemed to be recurrent under our stringent criteria (half of the CIS samples had to contain the amplification). This recurrent alteration was identified by the alignment of 1p profiles, generated from a two clone moving average across the entire chromosome arm (Fig. 2). Amplification at 1p36.12 was observed in six of the eight CIS samples defining the ~0.2 Mb minimal region of alteration between BAC clones RP11-489K12 and RP11-415K20. This region contains two known genes, interestingly, one of which is WNT4, a member of the wingless-type MMTV integration site family (region A1, Table 1). WNT4 has been shown to be abnormally expressed in human breast cancer cell lines (24). The involvement of the Wnt pathway in cancer is best known through adenomatous polyposis coli (APC) mutations in colorectal cancer (23). In the context of lung cancer, this pathway has been implicated in NSCLC, owing to the overexpression of dishevelled homolog 3 (DVL3) (17). Although the amplification was observed in six of the eight samples, the small sample size precludes the generalization of frequency in squamous cell lung cancer.
Wnt-signaling activation and cell growth in NSCLC (17,27).

identity with results, raising the possibility of more than one candidate region on the basis of LOH studies have yielded conflicting frequently altered region. However, definitions of the minimal chromosome 1p in NSCLC have largely focused on this frequent alteration region, which is consistent with our observation that the \(L\text{-MYC}\) region is amplified in only one of the eight CIS samples. The 0.13 Mb region C at 1p32.3 is present in seven of the 12 tumors (Table 1; Fig. 3). Region D is at 1p32.1. Amplification is observed in eight of the 12 tumor samples but contains no known genes. Interestingly, region D is only 90 kb centromeric to the \(JUN\) oncogene, but RT–PCR assay of paired normal and tumor samples did not detect elevated \(JUN\) expression levels (discussed later). However, five ORFs are located in the amplified region according to the Genescan track at UCSC (http://genome.ucsc.edu/), and its recurrence suggests an importance in lung cancer development. Region E is the smallest of the recurrent alterations, spanning only 0.05 Mb between BACs RP11-641F2 and RP11-754O9 at 1p11 (Table 1; Fig. 3). This region near the centromere is amplified in nine of the 12 tumors and contains a single Refseq gene \(NOTCH2\) (\(Drosophila\) Notch homologue 2). Amplification of this region has been recently reported in small cell lung cancer cell lines (33).

Interestingly, nearly all of the altered regions identified on chromosome 1p harbored genes belonging to gene families known to be involved in cancer development through either the Wnt or the Notch developmental pathway. Coupling our early observation of an amplified region containing \(WNT4\) in CIS and the identification of amplified regions containing additional genes related to these developmental pathways in the tumors prompted further evaluation of these genes at the expression level.

Expression of candidate genes in the Wnt and Notch pathways

We investigated the expression pattern of \(WNT4\), DVLI and \(EGFL3\) (region A), \(LRP8\) (region C), \(JUN\) (region D) and \(NOTCH2\) (region E). As a control, \(NOTCH2\) expression levels were compared with those of the neighboring \(ADAM30\) and \(REGIV\). We examined the expression levels of these genes in 13 microdissected matched normal and tumor SqCC cases by RT–PCR (Fig. 4). The expression analysis revealed significant overexpression in tumors of Notch and Wnt pathway gene family members \(DVLI\) (\(P = 0.003\)), \(LRP8\) (\(P = 0.008\)) and \(NOTCH2\) (\(P = 0.029\)). \(EGFL3\), \(JUN\), \(ADAM30\) and \(REGIV\) showed no significant expression changes. \(WNT4\) levels were too low to allow for the detection of differential expression. However, though expression levels of \(WNT4\) are not high in tumors, this does not preclude the importance of \(WNT4\) in early development as the genetic evidence suggested. Further investigation of the role of \(WNT4\) at
the expression level in invasive lesions—albeit challenging to obtain—will be necessary for validation.

Pair-wise comparison of DVL1 expression data showed a large difference between five of the 11 (Tumor 1, 3, 5, 10, 11) matched tumors and normal samples (Fig. 4A). This is in contrast to Uematsu et al. (17), who report only the overexpression of DVL3 and not DVL1 and DVL2 in NSCLC; however, their sample set was small, comprising only four squamous and four adenocarcinomas. The Drosophila dishevelled gene (Dsh) encodes a cytoplasmic phosphoprotein that regulates cell proliferation, acting as a transducer protein for developmental processes that include segmentation and neuroblast specification (34). It has been reported that Dsh is required for the function of the Wingless gene product, Wg, a segment polarity gene homologous to the mammalian proto-oncogene WNT1 (35–37) (Fig. 5). Human DVL1 shares ~64% amino acid identity with DVL3 and therefore may serve similar functions (27).

Expression analysis of LRP8 was striking, with overexpression observed in 11 of the 13 samples (Fig. 4B). The LRP family encodes membrane proteins involved in receptor-mediated endocytosis of many ligands and signal transduction pathways (32). Although LRPs appear to have multiple functions (38), LRP5, 6 and 12 have been linked to carcinogenesis (12,39–41). LRP5 and LRP6 are known to be co-receptors for Wnt ligands in Drosophila, Xenopus, and mice (32). The role of LRP5 and 6 in Wnt signaling in humans is observed, but the mechanism remains unclear (42). It has been suggested that LRP5 is a possible marker for progression in high-grade osteosarcoma (39). In addition, LRP12 has recently been shown to be amplified and overexpressed in oral SqCC (12). LRP8, or ApoER, is a lipophilic protein that mediates high-affinity binding of APOE-containing lipoproteins, one of which is reelin, to the very low-density lipoprotein (VLDL) receptor. Binding of reelin to the receptor results in the phosphorylation of Dab1; this activates an unknown chain of events that ultimately suppresses the activity of glycogen synthase kinase (GSK) 3β. Interestingly, the Wnt signaling pathway also functions through the regulation of GSK3β (43,44). The amplification and overexpression of LRP8 in squamous cell lung carcinoma reported here combined with previous functional reports of other LRP family members suggests that LRP8 may play an oncogenic role in lung cancer.

In addition to alterations affecting the Wnt developmental pathway, we also observe alterations in the Notch developmental pathway. Not only is NOTCH2 frequently amplified in lung tumors (discussed earlier) but expression is also higher in the tumor samples assayed (Fig. 4C). The Notch signaling pathway affects a wide variety of cellular processes, including the maintenance of stem cells, specification of cell fate, differentiation, proliferation and apoptosis (45). Although little is known about the role of NOTCH2 in cancer, its involvement is not surprising as NOTCH1 has been repeatedly implicated in disease (45). Furthermore, a NOTCH3 translocation has been previously observed in NSCLC (22). The Notch and Wnt developmental pathways interact through a number of mechanisms. Depending on the mechanism of action, these pathways can act either synergistically or antagonistically (46–52).

Of the 13 tumor normal pairs, we were able to obtain DNA for five of the samples (case number 3, 5, 8, 10, 13 in Fig. 4) for the purpose of genomic profiling. Notably, there was concordance between amplification and overexpression for these three genes. All amplifications correspond to overexpression.

In summary, this study provided evidence for the genetic basis of Wnt and Notch involvement in both the early and the late stages of tumorigenesis. Furthermore, previous description of Wnt7a involvement in cell adhesion suggests its role in lung cancer metastasis (21). Through the comparison of preinvasive and invasive lung carcinomas, we have identified novel genetic alterations which contain overexpressed genes related to both the developmental pathways (Fig. 5). These pathways are important for normal lung development (53–55); however, the ectopic expression of genes within these pathways reported here may lead to abnormal cellular activity. Importantly, our implication of developmental pathways in lung cancer supports the proposed model of lung cancer ontology whereby tumors arise from dysregulated pleuripotent stem cells (56). Also, by identifying five novel regions on chromosome 1p in tumors as well as a recurrent alteration in CIS samples, this study demonstrates the value of genetically profiling early preinvasive lesions and the use of a comprehensive tiling set approach to array CGH. Extension of this approach to the analysis of entire tumor genomes, for example, using the newly developed whole genome sub-megabase resolution tiling set array (57), will provide more complete understanding of lung cancer progression.

**MATERIALS AND METHODS**

**Lung tissue samples**

The detection and capture of minute precancerous and cancerous bronchial lesions has been facilitated by the development

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**Table 1. Summary of regions of recurrent amplification on 1p**

<table>
<thead>
<tr>
<th>Region</th>
<th>Cytoband</th>
<th>Size</th>
<th>Genes</th>
<th>Boundary clones</th>
<th>Occurrence in tumors</th>
<th>Occurrence in CIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1p36.12–p36.33</td>
<td>21 Mb</td>
<td>184</td>
<td>158F2–277F18</td>
<td>5/12</td>
<td></td>
</tr>
<tr>
<td>A*</td>
<td>1p36.12 (CIS only)</td>
<td>0.2 Mb</td>
<td>2*</td>
<td>489K12–415K20</td>
<td>6/8</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1p32.3</td>
<td>0.13 Mb</td>
<td>1*</td>
<td>351E14–809G7</td>
<td>1/8</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1p32.1</td>
<td>0.32 Mb</td>
<td>0</td>
<td>206P1–592P11</td>
<td>1/8</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>1p11.2</td>
<td>0.05 Mb</td>
<td>1*</td>
<td>641F2–754O9</td>
<td>2/8</td>
<td></td>
</tr>
</tbody>
</table>

*AI is within region A and contains the WNT4 gene.

* A 0.79 Mb alteration containing L-MYC was observed in one additional tumor.

*LRP8 is the only gene in region C, and NOTCH2 is the only gene in region E.
of fluorescence bronchoscopy technology (58). The isolation of CIS specimens was guided by the use of the LIFE-Lung device (59). Formalin-fixed, paraffin-embedded CIS tissue blocks were retrieved from the cancer imaging collection at the British Columbia Cancer Agency and diagnoses were confirmed by a lung pathologist. In addition, formalin-fixed and frozen lung tumor specimens (for DNA profiling and gene expression analysis, respectively) were provided by Dr James Hogg, St Paul’s Hospital, Vancouver, BC. Tumor cells were microdissected from tissue sections and DNA was isolated as previously described (60).

**BAC array synthesis**

Construction of a 1p chromosome arm specific BAC array for CGH analysis has been described by Henderson et al. (33). Clone lists with map position of individual BACs are available publicly at WWW.bccrc.ca/cg/ArrayCGH_Group.html. The procedures used for BAC DNA isolation, HindIII fingerprint clone verification and linker-mediated PCR amplification have been described previously (61). All clones were spotted in triplicate using a VersArray ChipWriter Pro (BioRad).
with Stealth Micro Spotting Pins (Telechem/ArrayIT SMP2.5) as previously described. Quality control was applied to each batch of arrays synthesized to ensure reproducibility and robust detection of single copy gains and losses (33,62). While this 1p array containing 642 unique BACs was constructed to accommodate the profiling of minute CIS samples, in order to identify progressive changes on 1p during tumorigenesis, CIS profiles were compared with tumor profiles created from the 1280 clones that mapped to 1p on the SMRT array (57).

**Probe labeling and hybridization**

Probe labeling was previously described (61). Briefly, Cyamine3 and Cyamine5 dCTPs were used to label the reference and test DNA, respectively (100 ng each for CIS samples and 200 ng each for tumors). The DNA probes were pooled, denatured and annealed in a solution containing 100 μg of human Cot-1 DNA (Invitrogen, Mississauga, Ontario) in 25 μl of DIG Easy hybridization solution (Roche, Quebec), 50 μg of sheared herring sperm DNA (Sigma-Aldrich, Oakville, Ontario) and 250 μg of yeast tRNA (Calbiochem, CA, USA). The probe mixture was applied to the slide surface and hybridized for 36 h at 42°C. Arrays were washed five times with 0.1 × SSC, 0.1% SDS for 5 min each at room temperature, rinsed five times with 0.1 × SSC and then dried by centrifugation.

**Array CGH imaging and analysis**

Hybridized arrays were imaged using a charge-coupled device-based imaging system and analyzed using the SoftWorx array analysis program (ArrayWorx eAuto, API, Issaquah, WA, USA). Data for each dye channel were normalized by applying a scale factor to each channel, which placed the median signal ratio to 1. The average signal ratios and standard deviations for each triplicate spot set were calculated as previously described (62) and displayed as a plot of the normalized Cyanine5/Cyamine3 log 2 signal ratio versus relative tiling path position using Excel for the 1p array and versus basepair position using SeeGH software for the SMRT array (63). A log 2 signal ratio of 0 at a spot represents equivalent copy number between the sample and the reference DNA. Normal versus normal hybridizations on the 1p array and SMRT array were performed to establish the technical variation throughout the arrays. We found that the standard deviations for all clones were 0.074 and 0.086 for the 1p array and SMRT array, respectively. As previously described by Veltman et al. (64), we selected thresholds of 0.15 for the 1p array and 0.18 for the SMRT array, which is slightly more conservative than two times the standard deviation for a normal versus normal hybridization. In addition, alterations had to have more than one consecutive BAC with copy number change to be scored as a region of alteration. For a region to be considered as altered, the local average had to be greater than or equal to 0.15 on the 1p array and 0.18 on the SMRT array. To identify regions of recurrent alteration, the data from each sample were aligned and visualized as heat maps of the copy number profiles using Java TreeView (http://jtreeview.sourceforge.net).

**Gene expression analysis**

Semi-quantitative analysis of gene expression was performed by RT–PCR. RNA was extracted from frozen tissue sections using Trizol reagent (Invitrogen, Burlington, Ontario). cDNA was synthesized using the Superscript II RNase H reverse transcriptase system (Invitrogen). Gene expression levels were determined by gene-specific PCR, using β-actin levels for normalization between samples. Gene-specific primers were used to assay the quantity of DVL1 (5'-CCCCACCCCCCTTTGTTGCTGTT-3', 5'-GGTGGGACA GATGACAGGG-3'), EGFL3 (5'-AGTTTGACTGAGGA CAGTTG-3', 5'-AGCGTGCAGACACGTAACTC-3'), JUN (5'-AACCACGTCGTCGTCCTCAA-3', 5'-CACAGTCTGCG GTAGATGAC-3'), NOTCH2 (5'-GCCAATCGAAGATCAGT CCAGA-3', 5'-CCCAAGATGACATGAGTGA-3'), WNT4 (5'-ACATGCAACAGACTGCTCAA-3', 5'-CATCGCAGA CGTGTCGACT-3'), LRPs (5'-ATGTTGCCCAAGGT AACCA-3', 5'-TGAGTCAAGTGATCTGCACTGAT-3'), REG4 (5'-GAAACAGCAGGCTGAGA-3', 5'-ATGACGCA CTAGGCCAGGC-3') and ADAM30 (5'-GGTGGTTTGGACA CAAAAGAA-3', 5'-TGGACACACAAAATGGAC-3'). PCR cycle conditions were as follows: one cycle of 95°C, 1 min; 30–35 cycles of 95°C, 30 s; annealing at temperature optimal for primers used, 30 s; 72°C, 1 min; and a 10 min extension at 72°C. Annealing temperatures were as follows: 55°C for β-actin, ADAM30 and NOTCH2; 56°C for EGFL3; 59°C for DVL1; 53°C for WNT4; 50°C for JUN and LRPs and 55°C for REG4. PCR products were resolved by polyacrylamide gel electrophoresis, imaged by SYBR green staining (Roche, Laval, Quebec) on a Storm phosphoimager and quantified using ImageQuant software (Molecular Dynamics, Piscataway, NJ). Because these genes were hypothesized to be overexpressed owing to DNA amplification, a one-tailed Wilcoxon sign-rank test was used to determine whether
overexpression of these genes was significant in a set of matched tumor and normal samples.

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REFERENCES


