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Epigenetic alteration of Wnt pathway antagonists in progressive glandular neoplasia of the lung

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Background: Atypical adenomatous hyperplasia (AAH) is now recognized as a precursor lesion from which lung adenocarcinomas arise and thus represents an ideal target for studying the early genetic and epigenetic alterations associated with lung tumorigenesis such as alterations of the Wnt pathway.

Methods: We assessed the level of Wnt signaling activity in lung cancer cell lines by determining the level of active β-catenin and determined the level of expression of Wnt antagonists APC, DKK1, DKK3, LKB1, SFRP1, 2, 4, 5, WIF1 and RUNX3 using reverse transcription–polymerase chain reaction. Using multiplex nested methylation-specific polymerase chain reaction, we analyzed promoter region methylation of these genes in resected lung tissue in the histopathologic sequence of glandular neoplasia (normal lung parenchyma, low-grade and high-grade AAH, adenocarcinoma).

Results: The majority of non-small cell lung cancer cell lines (11 of 16, 69%) have evidence of active Wnt signaling and silencing of Wnt antagonists correlated with promoter hypermethylation. Promoter region methylation of Wnt antagonists was common in primary lung adenocarcinoma and there was a significant increase in the frequency of methylation for Wnt antagonist genes and the number of genes methylated with each stage of tumorigenesis (test for trend P ≤ 0.01). Additionally, odds ratios for promoter hypermethylation of individual or multiple Wnt antagonist genes and adenocarcinomas were statistically significantly elevated and ranged between 3.64 and 48.17.

Conclusion: These results show that gene silencing of Wnt antagonists by promoter hypermethylation occurs during the early stages of glandular neoplasia of the lung and accumulates with progression toward malignancy.

Introduction

Over the last decade, Wnt signaling has been described as a critical pathway involved in the maintenance of the stem-cell populations in the gut, skin and bone marrow (1). Among the Wnt signal transduction pathways that can be triggered upon binding of Wnt ligands to the frizzled receptors, canonical Wnt signaling, also referred to as the β-catenin/TCF-signaling pathway, remains the best described for its role in cancer. In colon cancer, constitutive activation of the β-catenin/TCF-signaling pathway occurs through APC, β-catenin or axin mutations (2–4). Other pathways can likewise interfere with the β-catenin/TCF-signaling pathway. KRAS mutation at codon 12 can lead to Wnt pathway upregulation via the phosphorylation of GSK3β at serine 9 and its inactivation (5). RUNX3, a downstream target of the TGFβ-signaling pathway, has been shown to sequester β-catenin in the nucleus, preventing it from acting as a transcriptional activator (6). Additionally, LKB1, a serine threonine kinase whose mutation has been linked with the Peutz-Jeghers syndrome, has been shown to activate GSK3β and downregulate the pathway (7,8). Importantly, KRAS mutation and epigenetic silencing of Wnt antagonists, such as those of the SFRPs family, were found in colonic atypical crypt foci, in the absence of APC or β-catenin mutation (9,10).

There is increasing evidence, including overexpression of cyclin D1 and COX2, to suggest that the β-catenin/TCF-signaling pathway may also be constitutively active in lung adenocarcinomas (11–14). Lemjabbar-Alaoui et al. (15) recently showed that smoke-induced tumorigenesis in the lung was mediated through embryonic signaling pathways, including activity of the Wnt and sonic hedgehog pathways. This latest report is particularly interesting given that smoking might contribute to the development of multiple primary lung adenocarcinomas in particular in patients with atypical adenomatous hyperplasia (AAH) (16).

Unlike colorectal adenocarcinomas, lung adenocarcinomas rarely harbor mutations that target APC or β-catenin (17–19). Instead, disruption of the Wnt signaling pathway in lung adenocarcinoma mainly occurs via promoter hypermethylation of genes antagonizing the β-catenin/TCF-signaling pathway including RUNX3, SFRP1, WIF1 and APC (20–23). Although epigenetic silencing of these genes individually has been identified as a common event in lung adenocarcinomas, little is known about the timing of these alterations. Specifically, it is not known whether disruption of Wnt signaling by promoter hypermethylation is an important mechanism during the early stages of lung tumorigenesis.

AAH is a localized clonal proliferation of cytologically atypical cells lining alveoli (24), resulting in focal lesions no larger than 5 mm (Figure 1). The importance of AAH lays in the recent recognition that it probably represents a precursor lesion from which lung adenocarcinomas arise and therefore represents a target for studying the sequence and timing of genetic and epigenetic events involved in glandular neoplasia of the lung (25,26). Additionally, mouse models for lung adenocarcinoma either induced by carcinogen or by genetic manipulation further support AAHs as precursor lesions (27,28).

In an effort to separate early from late mutational events, AAH has been evaluated for key genetic alterations that are commonly present in lung adenocarcinomas including activation of important oncogenes such as KRAS, inactivation of the p53 tumor suppressor gene, loss of heterozygosity at selected chromosomal arms and activation of telomerase (25). Several of these studies have indicated that the accumulation of key genetic alterations appears to drive histologic progression of glandular neoplasia. For example, when AAH is further subclassified by the degree of cytarchitectural atypia, loss of p53 expression was detected in 5% of low-grade atypical adenomatous hyperplasias (LG-AAHs), 9% of high-grade atypical adenomatous hyperplasias (HG-AAHs) and 50% of lesions showing transition between HG-AAH and adenocarcinoma (29).

The purpose of the present study was to determine the prevalence and timing of silencing of Wnt antagonists by promoter hypermethylation in lung adenocarcinomas. We first set out to examine the level of activity of the β-catenin/TCF pathway in non-small cell lung cancer (NSCLC) cell lines by using unphosphorylated β-catenin as a readout for Wnt pathway activation. We then assessed the relationship between gene silencing of Wnt antagonists and the activity status of the Wnt signaling pathway in NSCLC cell lines. As such, we examined the effect of 5-aza-2’-deoxycytidine on the expression and the methylation status of Wnt antagonists affecting Wnt ligands or the frizzled receptors (e.g. SFRPs, WIF1), the LRPS/6 receptors (DKKs), GSK3β (LKB1) or β-catenin itself (APC, RUNX3) in lung cancer cell

Abbreviations: AAH, atypical adenomatous hyperplasia; HG-AAH, high-grade atypical adenomatous hyperplasia; LG-AAH, low-grade atypical adenomatous hyperplasia; MSP, methylation-specific polymerase chain reaction; NSCLC, non-small cell lung cancer; RT–PCR, reverse transcription–polymerase chain reaction; TCF, T cell factor.
lines. Finally, we assessed the frequency of promoter hypermethylation of these genes in AAH, adjacent normal lung parenchyma and synchronous adenocarcinomas from 16 patients using multiplex nested methylation-specific polymerase chain reaction (PCR).

Material and methods

Cell culture and 5-aza-2′-deoxycitidine treatment

NSCLC cell lines (H1993, A549, H2, H1666, H920, H1155, H1435, H460, H1395, H1209, H838, H358, H125, H157, U1752 and H1703) and colon cell lines (H1395, H1299, H838, H358, H125, H157, U1752 and H1703) and colon cell lines (HCT116 and RKO) were purchased from American Type Culture Collection and grown in recommended media (ATCC, Manassas, VA). The DKO lines (HCT116 and RKO) were purchased from American Type Culture Collection. H1395, H1299, H838, H358, H125, H157, U1752 and H1703) and colon cell lines. Finally, we assessed the frequency of promoter hypermethylation of these genes in AAH, adjacent normal lung parenchyma and synchronous adenocarcinomas from 16 patients using multiplex nested methylation-specific polymerase chain reaction (PCR).

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Patients and lesion classification

All clinical samples were obtained as part of protocol approved by the institutional review board at the Johns Hopkins University. We evaluated 121 formalin-fixed and paraffin-embedded tissues from 16 patients. The 121 samples were taken from 16 lung resections identified through a histologic review of all lung resections performed at the Johns Hopkins Hospital between 1995 and 2002. These samples included 56 AAHs, 19 synchronous primary lung carcinomas and 46 histologically normal lung samples (also referred to as normal-adjacent) taken from the lung parenchyma adjacent to the AAHs. For patients 1, 3, 8 and 12, multiple parts of the same tumor were analyzed. The AAHs were identified using the criteria of Nakanishi (31). Specifically, AAHs classified as low grade were characterized by round to cuboidal epithelial cells lining delicate septa. AAHs classified as high grade had increased cellular density, larger cell size, greater variation in cell size and shape and mild fibrosis of the alveolar septa. AAHs classified as high grade had increased cellular density, larger cell size, greater variation in cell size and shape and mild fibrosis of the alveolar septa. AAHs tend to be microscopic lesions (<5 mm), and only those cases in which sufficient cells remained for DNA isolation were included in this study. Slides of the corresponding synchronous primary lung neoplasms were reviewed, and when available, an appropriate tissue block of the primary lung neoplasm was microdissected for evaluation. Smoking histories were obtained from a review of the medical records of the patient (Supplementary Table 2 is available at Carcinogenesis Online). Additionally, four normal lung tissues were obtained postmortem from individuals without cancer.

DNA/RNA/protein extraction

DNA extraction. Following deparraffinization of the paraffin-embedded slide in xylene and rehydration in 95, 90, 75, 50% ethanol and water, areas of interest (as defined on the corresponding hematoxylin–eosin slides) were manually microdissected from each paraffin-embedded slide. To prevent sample contamination, normal-adjacent areas were sampled first followed by AAH and tumor areas. Tissue specimens and cell pellets were resuspended in lysis buffer (50 mM Tris, 50 mM ethylenediaminetetraacetic acid, 2% sodium dodecyl sulfate, 10 mg/ml protein kinase) (Sigma-Aldrich) and incubated overnight at 60°C. The next day DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), ethanol precipitated and resuspended in 50–200 μl of Tris–HCl buffer (pH 8.0). DNA was quantified using the NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE).

RNA extraction. Cell pellets were extracted using the RNeasy mini kit (QIAGEN Inc., Valencia, CA) and quantified using the NanoDrop® ND-1000 Spectrophotometer. Two micrograms of RNA was then used for complementary DNA synthesis using the SuperScript™ II Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA).

Whole-cell lysate preparation. Each cell pellet was resuspended in 1× radioimmunoprecipitation assay buffer (1× phosphate-buffered saline, 10% sodium dodecyl sulfate, Nonidet P-40 and sodium deoxycholate) completed with 1× complete (Roche Diagnostics Corporation, Indianapolis, IN) and 1 mM 4-(2aminoethyl)benzene sulphonyl fluoride (EMD Chemicals, Inc., San Diego, CA). Samples were incubated on ice for 30 min, passed through a 20 gauge
Western blot
Ten micrograms of protein was mixed with 4× loading dye, 10× reducing reagent (Invitrogen Corporation) boiled for 10 min and loaded onto a 4–12% gradient bisacylamide gels (Invitrogen Corporation). The gel was transferred onto a nitrocellulose membrane that was then incubated overnight in blocking solution (5% non-fat dry milk in phosphate-buffered saline/0.1% Tween 20). Unphosphorylated S33/S37 β-catenin antibody (1/500 dilution; catalogue number 05-665; Upstate USA Inc., Charlotteville, VA) was used to probe for active β-catenin and β-actin antibody (1/10 000; catalogue number: A5441, Sigma-Aldrich) was used as a loading control. Enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL) was used as a detection reagent.

Results
To determine whether Wnt-signaling activity was altered in human lung cancer, we first determined the level of unphosphorylated β-catenin (also known as active β-catenin) in NSCLC cell lines by western blot (38). As seen in Figure 2A, H358, H1435 and H1666 had high levels of active β-catenin. Unphosphorylated β-catenin was also detected in H23, H1395, H1993, A549, H460, H1299, H157 and H175 (Figure 2A and data not shown). In contrast, H920, H38, H1155, H125 and U1752 had little or no detectable active β-catenin, suggesting that the pathway is not active in these cell lines (Figure 2A and data not shown). Among the cell lines with evidence of active β-catenin, only H358, A549 and H157 had KRAS mutation at codon 12, as detected by restriction fragment length polymorphism (Figure 2A and data not shown). However, cell lines without KRAS mutation also exhibited Wnt-signaling activation, suggesting that KRAS mutation was not required or solely responsible for Wnt activation.

A recent study in colon cancer suggested that methylation of Wnt antagonists such as SFRP1 could result in high pathway activity (10). Since there are a number of other Wnt antagonists that have been reported to be epigenetically silenced in human cancers, we systematically examined the effect of 5-aza-2′-deoxycitidine on the expression level and methylation status of 10 Wnt antagonists (APC, DKK1, LKB1, SFRP2, SFRP1, SFRP5, DKK3, WIF1, RUNX3, and both MSP and nested primer sets were synthesized (Integrated DNA Technologies, Coralville, IA) (Supplementary Table 1 is available at Carcinogenesis Online). All analyses were accomplished using the STATA statistical software (College Station, TX).
of methylation in the silencing of these genes. For 31 genes/cell line pairs with absent expression at baseline, 26 had restored expression to levels seen in unmethylated cell lines by RT–PCR, whereas 4 increased to only low-level expression and one did not increase at all (Figure 2D). Thus, all silenced genes were reactivated by demethylating agents. For cell lines with minimal but not absent expression, expression was increased to high level in eight cases. All examples of high-level expression remained unchanged with 5-aza-2’-deoxycitidine treatment. Thus, loss of expression was highly correlated to gene silencing and could be reversed by treatment with a demethylating agent.

While cell lines which had active β-catenin were found to have silencing of multiple Wnt antagonists (H358, H1666, H1435, H460 and H1703), we observed that not all cell lines with inactivation of Wnt antagonists had evidence of Wnt activity. Indeed six Wnt antagonists were silenced in H38, but the pathway was not active in this cell line (Figure 2A and B). This lack of correlation between pathway activation and Wnt antagonists gene silencing was explained by the...
Epigenetic alteration of Wnt pathway antagonists

fact that β-catenin protein was not detected by western blot in this cell line (data not shown). Similarly, the colon cancer cell line RKO that has wild-type APC and β-catenin does not show Wnt pathway activation (data not shown) despite having 8 out of 10 Wnt antagonists silenced by promoter hypermethylation. A previous study has shown that many of the Wnt ligands are repressed in RKO, including Wnt-1, 2, 3, 5a, 5b, 6, 7a and 7b (10). These observations highlight the complexity of the Wnt pathway regulation and suggest that active Wnt signaling in cell lines lacking constitutive mutation at APC or β-catenin is achieved in the context where Wnt ligands are expressed and Wnt antagonists silenced.

Overall, Figure 2 shows that the Wnt pathway is active in many NSCLC cell lines and that the absence of expression of Wnt antagonists in various NSCLC cell lines is due to gene promoter hypermethylation. Although most genes were demethylated and reexpressed after treatment with the demethylating agent, LKBI was expressed and unmethylated at baseline in all the cell lines tested. Published previous data have shown that LKB1 inactivation through DNA methylation is an uncommon event in lung cancers (39). Nevertheless, given that LKB1 downregulation has been shown to occur in 10 of 96 (10%) of AAH lesions, we retained examination of this gene in our panel (40).

With this high frequency of methylation-associated gene silencing of many Wnt antagonists in NSCLC cell lines, we examined the frequency and timing of hypermethylation of Wnt antagonists in early and advanced glandular neoplasia of the lung by means of multiplex nested MSP. Multiplex nested MSP is a two-stage reaction that allows better amplification of archived tissues (41,42) and achieves greater sensitivity (typically at a detection level of 0.1% of alleles) while maintaining specificity (43). The first-stage reaction (multiplex nested) uses primers that are not biased toward the methylation status of promoters and allow for enough templates to be generated for subsequent analysis. In the second-stage reaction (MSP), unmethylated and methylated MSP primers discriminate between unmethylated and methylated template. Multiplex nested MSP was first used to analyze the methylation pattern of Wnt antagonists in four postmortem lung samples obtained from individuals without cancer. None of these genes were methylated in these postmortem specimens (data not shown), while control lung cancer cell lines remained positive for methylation, suggesting that these genes are normally unmethylated in lung tissue from individuals without cancer and demonstrating the specificity of this approach.

We then determined the methylation status of Wnt antagonists in AAH, adjacent lung parenchyma and synchronous lung carcinomas from 16 patients. Multiplex nested MSP successfully determined the methylation of these 10 genes in 1174 (97%) of the 1210 assays from these 121 microscopic samples. Methylation of each of these 10 genes was detected in adenocarcinomas and in many pre-invasive tissues (Figure 3). Figure 3 demonstrates intra- and interpatient heterogeneity, particularly for AAH lesions, in the methylation status of Wnt antagonists affecting the binding of Wnt to frizzled receptors (e.g. SFRP4, SFRP5 and WIF1), the LRPS6 receptors (e.g. DKK1 and DKK3) or β-catenin itself (e.g. RUNX3) for patients 12 and 13. (Supplementary Table 2 for complete information is available at Carcinogenesis Online). The analysis of methylation for these genes clearly show a steady increase in the frequency of methylation in normal adjacent, LG-AAH, HG-AAH and adenocarcinoma samples (test for trend P ≤ 0.01) for all genes except WIF1 and LKB1 (Figure 4A and Supplementary Table 3 is available at Carcinogenesis Online). For WIF1, however, the frequency of methylation was higher in adenocarcinomas than in HG-AAHs but surprisingly higher in LG-AAHs than in the HG-AAHs. LKB1 methylation was found only infrequently in adenocarcinomas as has been previously suggested and was rarely observed in early lesions (39). Thus, although there was not a linear increase in methylation through all histologic grades for LKB1 or WIF1, there was an increase with progression. APC, DKK1, RUNX3, WIF1, SFRP2 and SFRP5 were nearly as frequently methylated in the normal adjacent as in the LG-AAH. These genes appear to be epigenetically silenced during the earliest stages of glandular neoplasia, even preceding the onset of microscopic alterations. DKK3, SFRP1 and SFRP4 were more frequently methylated in the LG-AAH than in the normal adjacent. Additionally, DKK1 and RUNX3 were the only two genes in our panel showing a statistical increase in methylation (P value ≤ 0.05) in HG-AAH compared with LG-AAH (Supplementary Table 3 is available at Carcinogenesis Online), although the trend was clearly observed for the other Wnt antagonists.

In addition to gene-specific increases in methylation, we also observed an increase when looking at the number of genes methylated (test for trend P ≤ 0.01) (Figure 4B and Supplementary Table 4 is available at Carcinogenesis Online). Indeed, adenocarcinoma samples had more genes methylated (mean = 6.42) than the HG-AAH (mean = 3.29) or the LG-AAH (mean = 1.72) (Figure 4B).

Normal-adjacent samples had the least number of genes methylated (mean = 1.63), which was similar to what we observed for LG-AAH. We also observed an increase in frequency (individual genes) and prevalence (number of genes) of methylation with histologic progression when looking at tissues from individual patients. Examples of the methylation profile for each gene in the normal adjacent, LG-AAH, HG-AAH and adenocarcinoma from patients 1, 3 and 12 are shown (Table I and Supplementary Table 2 for complete information is available at Carcinogenesis Online). Multiple AAH lesions were present in these patients, allowing the differences according to lesion type to be seen. Overall, we observed intra- and interpatient heterogeneity in the methylation signature of Wnt antagonists. Nevertheless, the mean number of genes methylated increased with histological progression for each patient, and Wnt antagonists found to be methylated in normal tissues were consistently methylated in the AAH and adenocarcinoma lesions with hypermethylation of additional genes with progression.

The clear increase in methylation events according to the histological categories suggests that methylation at individual or multiple loci may be useful to predict the malignant evolution of AAH lesions in patients. Thus, we determined the odds ratios for the presence of methylation as determinants of histological categories (Table II). The presence of methylation was associated with statistically significant odds ratios for most genes. For example, the presence of promoter hypermethylation of RUNX3 and SFRP1 had an odds ratios of 10.43; 95% confidence interval (4.17; 26.07) and 8.94; 95% confidence interval (2.70; 29.61), respectively, for the sample being an invasive tumor or a HG-AAH, with hypermethylation of four cancer genes having an odds ratio approaching 18-fold. However, while the odds ratios of hypermethylation were strongly able to predict that a sample was an invasive tumor or a HG-AAH, there was an even stronger association of hypermethylation in predicting tumor versus HG-AAH, LG-AAH or normal lung (Table II, right panel). Increasing numbers of genes hypermethylated were associated with increasing odds ratios for the sample being an invasive tumor/HG-AAH, with hypermethylation of four cancer genes having an odds ratio of 26.59 and 17.28 times more probably to be adenocarcinomas than HG-AAH, LG-AAH or normal lung specimens (P value <0.01). All other genes showed statistically significant odds ratios ranging from 3.64 for LKB1 to 26.59 for APC and RUNX3 (P value <0.01), and once again, the increasing number of methylation events also showed large and statistically significant odds ratios. For example, promoter hypermethylation of at least four genes was 48 times more probably to be found in adenocarcinomas than HG-AAH, LG-AAH or normal lung specimens.

Discussion

Dysregulation of embryonic pathways including Wnt is increasingly implicated in cancer initiation and is thought to provide malignant cells with stem-cell properties (1). Recent studies also suggest that the chromatin surrounding genes that regulate embryonic pathways are maintained in a bivalent state allowing change in expression during
development (44). However, during malignant transformation, such genes appear to be susceptible to a deeper level of silencing, and the expression may become much more completely silenced by gene promoter hypermethylation, allowing constitutive pathway activity (45). In this study, we examined whether the silencing of Wnt antagonists by promoter hypermethylation was involved in the development of lung adenocarcinoma. We showed that Wnt-signaling pathway is active in NSCLC cell lines and find that gene silencing of 10 Wnt antagonists in NSCLC cell lines directly results from promoter hypermethylation. We demonstrate for the first time that a number of Wnt antagonists are methylated in lung adenocarcinoma precursor lesions and that these epigenetic events accumulate in the multistep progression of lung adenocarcinoma. Indeed, there was an increasing trend in the frequency of gene-specific methylation (test for trend \( P < 0.01 \)) and the number of genes methylated as a function of histologic progression from normal lung to LG-AAH to HG-AAH to adenocarcinoma. Lung specimens obtained at autopsy from patients without cancer were unmethylated for all the genes in our panel suggesting that promoter hypermethylation of these genes occurs during cancer progression. Together, this suggests that the silencing of Wnt antagonists plays a role in the earliest stages of lung cancer development, consistent with the role that this pathway plays in allowing the malignant proliferation of cancer cells (1).

Morandi et al. (46) recently combined sequencing of mitochondrial DNA with loss of heterozygosity analysis and found that in 9 of 13 (69%) of informative cases, AAH and the adenocarcinoma within each patients were not clonally related, suggesting that individual AAH lesions may represent independent neoplastic foci. Such multiclonality of cancer was first suggested to result from a ‘field defect’ at a number of sites within the bladder, allowing for the independent transformation of epithelial cells sharing genetic alterations (47). It is now believed that such a field defect may also exist in lung cancer, often as a result of smoking exposure, leading to mosaic clones with different genetic and epigenetic alterations (48). We find that not all AAH lesions had the same epigenetic pattern for these genes, suggesting that AAH lesions are indeed individual clones with distinct epigenetic signatures. However, whereas there were differences in some of the genes methylated in each patient, many of the same genes were...
consistently methylated through progression in each patient. This suggests that distinct ‘epigenetic fields’ exist within the lung tissues of patients who ultimately develop lung cancer. Given the clear association of the number of genes methylated with histological grade, we suggest that clones with the highest number of genes methylated are the most likely to progress to adenocarcinoma. This cannot be directly tested on these specimens since they were all removed at the time of resection of the primary lung cancer. However, the abnormalities present in normal appearing lung may provide an explanation for the risk of development of second primary tumors in patients.

Fig. 4. Increases in the frequency and prevalence of methylation of Wnt antagonist genes parallels histological progression. (A) The increase in the frequency of methylation across tissue types was statistically significant for all the genes but WIF1 (test for trend $P \leq 0.01$). For example, SFRP1 was methylated in 2% of normal lung specimens from cancer patients (normal adjacent), 11% of LG-AAH, 14% of HG-AAH and 58% of adenocarcinoma samples. The normal lung specimens obtained postmortem from disease-free patients were all unmethylated for each of the Wnt antagonists analyzed. The difference in frequency of methylation between HG-AAH and LG-AAH was statistically significant for DKK1 and RUNX3 ($P$ value $\leq 0.05$) (Supplementary Table 3 is available at Carcinogenesis Online). (B) The percentage of samples according to number of genes methylated presented for normal lung (normal-adjacent lung), LG-AAH, HG-AAH and adenocarcinoma samples. The mean number of genes methylated was 1.63 for normal adjacent, 1.72 for LG-AAH, 3.29 for HG-AAH and 6.42 for adenocarcinoma, with this trend of increasing number of genes methylated with grade reaching statistical significance (test for trend $P \leq 0.01$). Complete statistical analysis is presented in Supplementary Table 4 is available at Carcinogenesis Online).
following surgical resection of the primary tumor. The frequency of methylation for many genes in the earliest lesions implicates the epigenetic silencing of Wnt antagonists in the initiation of lung adenocarcinoma and suggests that the therapeutic targeting of the Wnt pathway by epigenetic therapy or non-steroidal anti-inflammatory drugs (e.g. COX2 inhibitors) be investigated further. Finally, we found that promoter hypermethylation of Wnt antagonists showed higher odds ratio for predicting adenocarcinomas than HG-AAH or LG-AAH. This may suggest that adenocarcinomas of the lung may require the silencing of a full spectrum of direct and indirect regulators of the Wnt pathway in order to acquire full malignant capabilities. Promoter hypermethylation of genes (i.e. Wnt antagonists) may, in addition to conventional histology analysis, help to refine the diagnosis and possibly predict the development of lung adenocarcinoma.

### Table 1. Heterogeneity of methylation patterns and increase in the number of methylation events in individual patients

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<tr>
<th>Patient</th>
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<th>AAH grade</th>
<th>APC</th>
<th>DKK1</th>
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<th>RUNX3</th>
<th>WIF-1</th>
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### Supplementary material

Supplementary material can be found at [http://carcin.oxfordjournals.org/](http://carcin.oxfordjournals.org/)

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References