Aberrant Nuclear Localization and Gene Mutation of β-catenin in Low-Grade Adenocarcinoma of Fetal Lung Type: Up-Regulation of the Wnt Signaling Pathway May Be a Common Denominator for the Development of Tumors that Form Morules

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The salient histopathologic features of low-grade adenocarcinoma of the fetal lung type (L-FLAC)/well-differentiated fetal adenocarcinoma (WDFA) include complex glandular structures and morules with biotin-rich optically clear nuclei. Interestingly, these characteristic features are shared by the cribriform-morular variant of papillary thyroid carcinoma, whose morphology is identical to that of familial adenomatous polyposis (FAP)-associated thyroid carcinoma. Furthermore, the single reported case of lung cancer associated with FAP was L-FLAC/WDFA. These observations lead us to hypothesize that up-regulation of the Wnt signaling pathway underlies the development of L-FLAC/WDFA. To verify this hypothesis, 11 cases of L-FLAC/WDFA, including the one FAP-associated case, eight cases of high-grade adenocarcinoma of the fetal lung type (H-FLAC), 24 cases of conventional pulmonary adenocarcinoma (CAC), and 13 fetal lungs were immunostained for β-catenin. All cases of L-FLAC/WDFA showed predominantly aberrant nuclear/cytoplasmic expression, especially in budding glands and morules, whereas six of eight cases (75%) of H-FLAC and all but one case (96%) of CAC showed predominantly membranous expression. Fetal lungs showed nuclear/cytoplasmic expression restricted to the distal branching airway epithelium. Mutational analysis of exon 3 of the *β-catenin* gene in five sporadic cases of L-FLAC/WDFA showed a point mutation at codon 34 and codon 37 in two cases, respectively. The present study indicates that up-regulating disturbances in the Wnt signaling pathway, including mutation of the β-catenin gene, underlie tumorigenesis of L-FLAC/WDFA. The expression pattern of β-catenin in L-FLAC/WDFA resembles that of the developing fetal lung airway. With the expression pattern of β-catenin as a marker, most cases of H-FLAC as well as CAC appear to have different oncogenic pathways from cases of L-FLAC/WDFA. The present study together with other available data also suggests that abnormal up-regulation of the Wnt signaling pathway may be a common denominator for the development of tumors with morular formation from a variety of anatomic sites.

KEY WORDS: Adenocarcinoma of the fetal lung type, APC gene, β-catenin mutation, Biotin-rich optically clear nucleus, Cribriform-morular variant of papillary thyroid carcinoma, Wnt signaling pathway.

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Low-grade adenocarcinoma of the fetal lung type (L-FLAC)/well-differentiated fetal adenocarcinoma (WDFA) was originally reported in 1982 as pulmonary blastoma lacking sarcomatous features (pulmonary endodermal tumor resembling fetal lung) (1). Subsequent studies clarified that L-FLAC/WDFA is a relatively indolent tumor most prevalent in the fourth decade of life with a mild female predominance and a death rate of about 10% (2–4). The salient histopathologic features of L-FLAC/WDFA are complex glandular structures with cells rich in glycogen, resembling the fetal lung airway epithelium or endometrioid carcinoma and morular formation with optically clear nuclei (OCN) that are rich in biotin (4, 5). High-grade adenocarcinoma of the fetal lung type (H-FLAC) was separated from L-FLAC/WDFA because of different clinicopathologic features, including significantly worse prognosis (4), although both tumors have histologic features resembling fetal lung and can be confused with each other (6).

With the progress in our understanding of L-FLAC/WDFA, it has become apparent that tumors with an almost identical histopathologic pattern of complex glandular structures and morular formation with biotin-rich OCN occur in several guises, including endometrioid carcinoma of the ovary (7), thyroid papillary carcinoma (8–10), adenoma of the gallbladder (11), pancreatoblastoma (12), and adenoma of the colon (13). Although female sex hormones were initially implicated as a common denominator for the development of tumors belonging to the "OCN family" (5), this hypothesis has not been verified (5, 13).

Sporadic cases of thyroid papillary carcinomas with morules containing OCN have been termed to be cribriform-morular variant (14), and it has been recognized that an identical morphology also occurs in familial adenomatous polyposis (FAP)-associated thyroid carcinoma (15, 16). The single lung cancer so far reported in association with FAP has been L-FLAC/WDFA (17). Based on these observations, we hypothesized that the development of L-FLAC/WDFA is closely related to abnormal up-regulation of the Wnt signaling pathway (18).

The Wnt signaling pathway participates in embryonic development and leads to tumor formation when deranged (18). The Wnt signal is transmitted to the nucleus by cytoplasmic β-catenin, which activates target genes by forming complexes with lymphoid enhancer factor/T-cell factor (LEF/TCF) within the nucleus. In the normal state, cytoplasmic β-catenin is maintained at a low level because of its degradation by a multiprotein complex, including the adenomatous polyposis coli (APC) tumor suppressor protein. In FAP, mutational inactivation of the APC results in reduced degradation of β-catenin and nuclear accumulation of the protein, leading to activation of oncogenic target genes such as *c-myc* and *cyclin D1*. Furthermore, β-catenin degradation also may be blocked by mutation of β-catenin, which is present in approximately half of the colorectal cancers that lack APC mutations, and thus may play a role in tumorigenesis (19). Because aberrant nuclear/cytoplasmic localization of β-catenin as a final common event of either APC or β-catenin mutations can be detected immunohistochemically (20), we investigated the localization of β-catenin in L-FLAC/WDFA as well as in related lung tumors and developing fetal lungs. Furthermore, mutational analysis of the *β-catenin* gene was performed in five cases of L-FLAC/WDFA.

MATERIALS AND METHODS

Seven surgically resected L-FLAC/WDFAs, eight H-FLACs, 24 conventional adenocarcinomas, and 13 fetal lungs from cases of spontaneous abortion and stillbirth were collected from the files of our departments and from the consultation files of one of the authors (E.J.M.). In addition, four cases of L-FLAC/WDFA were kindly provided by Drs. S. Kuwao, K. Kashima, and S. Hamazaki of Japan, as well as Drs. W.S. Hwang and S.K. Field of Canada. The clinical and pathologic features of most of the L-FLAC/WDFA and H-FLAC cases in the present series have been described elsewhere (4, 17). Of the 24 conventional adenocarcinomas, seven were well-differentiated, 13 moderately differentiated, and four poorly differentiated. The gestational age for the 13 fetal lungs ranged from 9 to 26 weeks.

Immunostaining for β-catenin was performed on formalin-fixed paraffin-embedded tissue sections by the Envision+ technique (DAKO). Antigen retrieval was conducted by heating in an autoclave for 10 minutes. The primary mouse monoclonal anti-β-catenin antibody (1:200, clone 14; Transduction Labs, Lexington, KY) was applied to the sections at 4°C overnight. Final visualization was carried out by diaminobenzidine.

Mutational analysis of the *β-catenin* gene was performed in five sporadic cases of L-FLAC/WDFA, using DNA extracted from three to five 4-mm-thick paraffin sections of a representative tissue block in each case. For the extraction of DNA, the tumor sections were placed in a microtube containing 0.5 mL of a DNA extraction solution (TaKaRa DEXPAT™, Takara, Otsu, Japan). The mixture was boiled for 10 minutes, centrifuged at 12,000 rpm for 10 minutes, and the supernatant was used as a DNA extract according to the manufacturer's instructions. Next, 5 μL of a DNA extract was amplified by polymerase chain reaction (PCR) in a total reaction volume of 50 μL, containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 50 μM of each dNTP,

0.5 μM primers, and 2.5 U *Taq* DNA polymerase (Promega, Madison, WI). Two sets of primers were used for nested PCR with final amplification of a 200-bp fragment of exon 3 of the *β-catenin* gene encompassing the region of the GSK-3β phosphorylation site that contains activating mutations. The primer set for the first round of PCR included the forward primer: 5'-CCAATCTACTAA-TGCTAATA-CTG-3' and the reverse primer: 5'-CTGCATTCTGA-CTTTCAGTAAGG-3'. The second primer set used was the forward primer: 5'-ATGGAACCAGACAG-AAAAGC-3' and the reverse primer: 5'-GCTACTTG-TTCTTGAGTGAAG-3'. Following an initial denaturation step at 95°C for 5 minutes, 20 cycles of amplification were performed (denaturation at 95°C for 30 sec/annealing at 55°C for 30 sec/elongation at 72°C for 90 sec), followed by a final elongation step of 5 minutes at 72°C. Diluted external PCR products (2 μL; 1:100) were submitted to the second round PCR for 30 cycles using the same temperature profile. The PCR products were sequenced directly by the same primers as those used for the second round of PCR amplification with the dye terminator cycle sequencing method and the CEQ2000 multi-capillary DNA sequencing system (Beckman Coulter, Fullerton, CA). Because the direct sequence analysis showed heterozygous substitution mutations, those fragments demonstrating heterozygous sequence profiles were subcloned into pGem-T-easy plasmid vector (Promega, Madison, WI), and more than 10 clones were sequenced with both M13 forward and reverse universal sequencing primers to confirm the sequence.

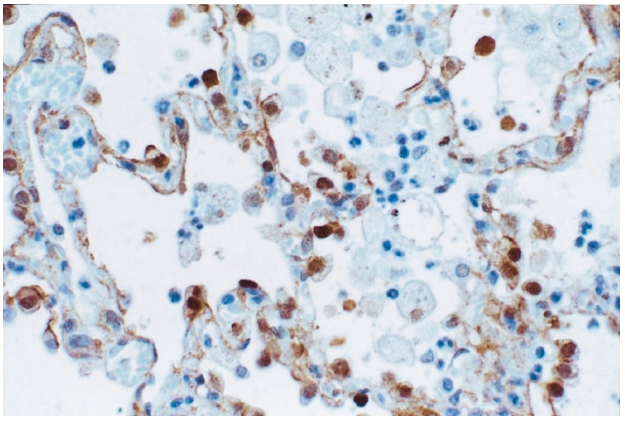

FIGURE 1. Nuclear/cytoplasmic expression pattern of β-catenin can be seen in reactive type II pneumocytes.

RESULTS

Intense membranous expression pattern (MP) of β-catenin was observed in the normal bronchial epithelium present in the non-neoplastic portion of the tumor sections. It was weakly observed in the normal alveolar lining cells as well, whereas reactive type II pneumocytes in the vicinity of the tumor growth commonly showed the aberrant nuclear/cytoplasmic expression pattern (NCP) (Fig. 1). All 11 cases of L-FLAC/WDFA, including the one FAP-associated case (17), predominantly exhibited NCP. NCP was especially prominent in cells comprising the peripherally budding glands and morules, including cells with optically clear nuclei (Fig. 2). MP was markedly reduced or absent in the neoplastic cells of all L-FLAC/WDFA cases. Of eight cases of H-FLAC, six showed predominantly MP and two showed NCP (Fig. 3). One of the tumors with NCP exhibited histologic features intermediate between L-FLAC/WDFA and H-FLAC. In 24 cases of conventional adenocarcinoma, MP was predominant in all but one case (96%). However, poorly differentiated adenocarcinomas showed reduced expression of β-catenin. Only one case of moderately differentiated adenocarcinoma showed NCP predominantly. In eight cases (33%), NCP was focally seen as well, often in the peripheral portion of the tumors abutting the surrounding stroma. Stromal cells within the surrounding young fibrous tissue often showed NCP. In the 13 fetal lungs, the peripheral branching airway epithelium predominantly showed NCP from 9 to 22 weeks of gestation, while all airway and respiratory epithelia, at later stages of gestation, exhibited only MP (Fig. 4). The proximal airway epithelium constantly showed MP throughout the gestational periods examined. In the lung at 9 weeks' gestation, many of the primitive stromal cells surrounding the peripheral branching airway also showed NCP (Fig. 4A).

Direct sequence analysis of the PCR products of *β-catenin* gene exon 3 in Case 9 and Case 6 demonstrated a mixed pattern of the wild-type and mutant peaks. Specifically, Case 9 exhibited a T*C*T (Ser) to T*G*T (Cys) transversion at codon 37 (Fig. 5A) and Case 6 a G*GA* (Gly) to G*TA* (Val) transversion at codon 34. The remaining three cases showed no abnormality. Further sequence analysis of the subcloned PCR fragments confirmed that both wild and mutated alleles actually existed. The confirmed sequences of the mutated alleles are shown in Fig. 5B.

DISCUSSION

To the best of our knowledge, no abnormality of the Wnt signaling pathway comparable with that detected in L-FLAC/WDFA in this study has been reported previously in human lung cancers. Retera and associates investigated 101 cases of non-small cell lung cancers and noted an association of reduced expression of β-catenin with an unfavorable prognosis, but they did not refer to aberrant nuclear localization of β-catenin (21). Pirinen and associ-

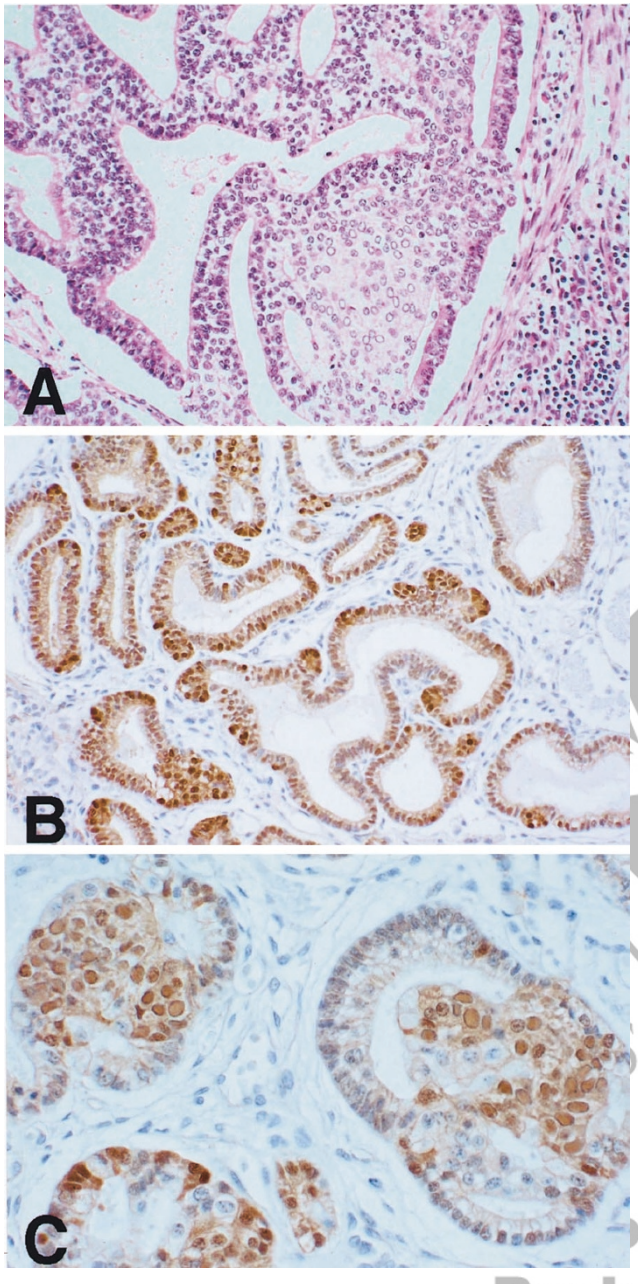

FIGURE 2. L-FLAC/WDFA. (**A**) Complex glands resembling fetal lung airway epithelium with morular formation. (**B**) Note predominant localization of the nuclear/cytoplasmic expression pattern of β-catenin in budding glands and morules. (**C**) Optically clear nuclei in morules are immunoreactive for β-catenin.

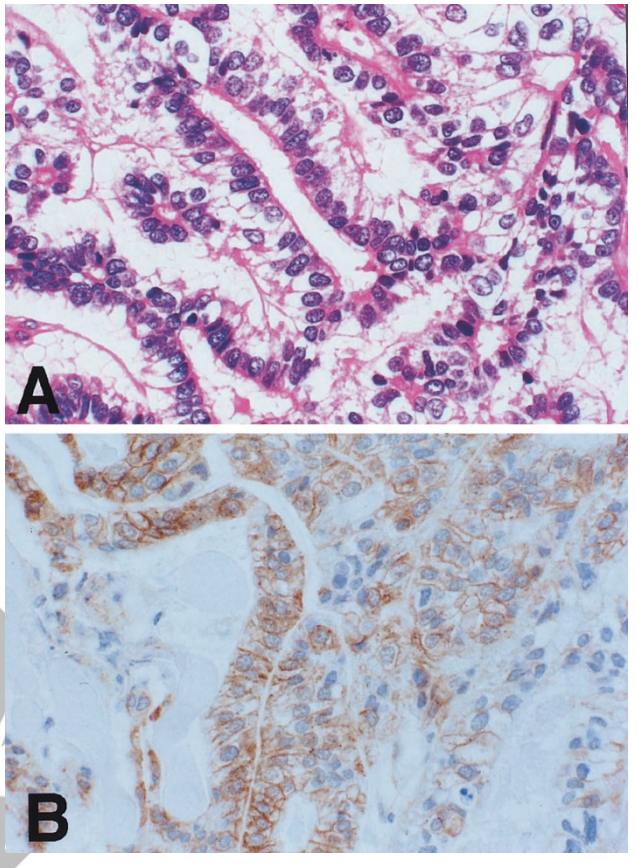

FIGURE 3. H-FLAC. (**A**) Complex glycogen-rich glands resemble those of L-FLAC/WDFA, but show more significant nuclear atypia. (**B**) Predominantly membranous expression pattern of β-catenin can be seen.

ates recently reported nuclear/cytoplasmic expression for β-catenin in only 16 (7%) of 261 non-small cell lung cancers (22). Sunaga and associates studied 46 cultured cell lines of lung cancer as well as 47 resected lung cancer specimens and found mutations in exon 3 of the *β-catenin* gene in only 1 (2%) of the 46 cell lines and 2 (4%) of the 47 lung tumors (23). Moreover, in the present study, nuclear/cytoplasmic expression of β-catenin was seen only rarely as the predominant pattern in conventional adenocarcinomas of the lung, which was consistent with the results of the previous studies, indicating that abnormality of the Wnt signaling pathway may play a relatively minor role or none at all in the tumorigenesis of conventional lung cancers. In contrast, all 11 cases of L-FLAC/WDFA in the present study showed predominantly nuclear/cytoplasmic expression of β-catenin, suggesting that the development of this unique lung tumor may be closely associated with abnormality of the Wnt signaling pathway. In support of this is our finding of mutations in the phosphorylation sequence for GSK-3β in exon 3 of the *β-catenin* gene in two cases of L-FLAC/WDFA. Mutations in these phosphorylation sites lead to failure of β-catenin degradation by GSK-3β, resulting in β-catenin accumulation and the subsequent activation of oncogenic target genes (18). Mutational inactivation of the *APC* gene, another critical event leading to up-regulation of the Wnt signal transduction, may be partly responsible for the development of those sporadic cases of L-FLAC/WDFA that lack β-catenin mutations, as has been demonstrated recently in a sporadic case of the cribriform-morular variant of papillary thyroid carcinoma (24). Mutations of a tumor suppressor gene *PTEN* are also possible because inactivation of PTEN leads to nuclear accumulation of β-catenin

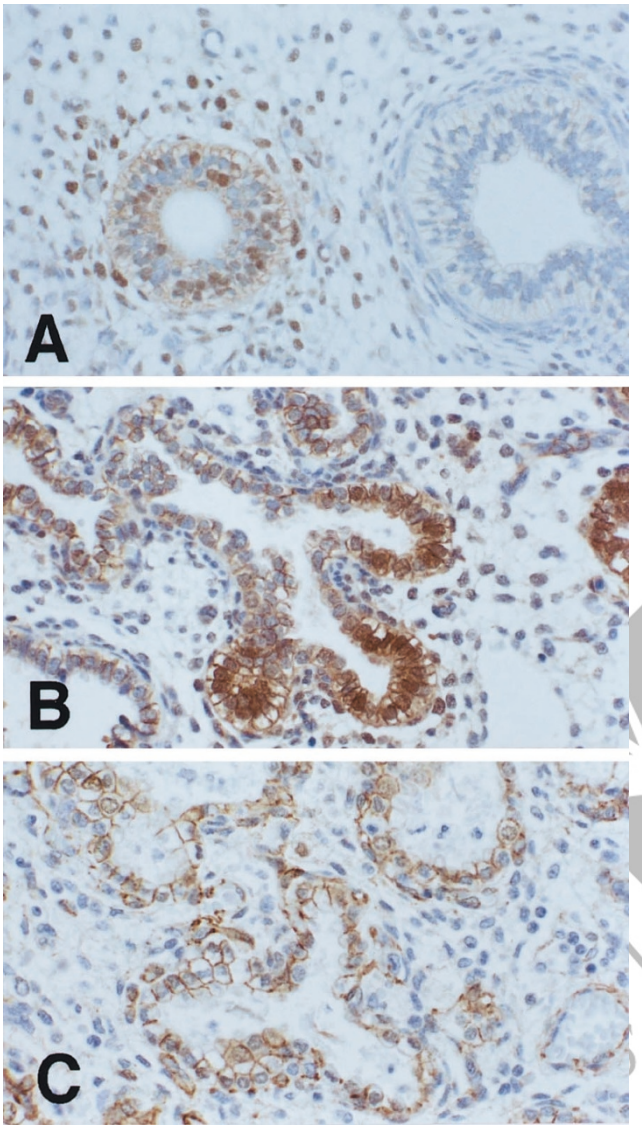

FIGURE 4. Fetal lungs at various gestational periods. (**A**) Nine weeks. Note nuclear/cytoplasmic expression pattern of β-catenin both in the branching airway epithelium and primitive stromal cells surrounding it. (**B**) Seventeen weeks. Note β-catenin immunostaining with a predominant membranous expression pattern in the proximal airway and nuclear/cytoplasmic expression pattern in the distal branching epithelium. (**C**) Twenty-three weeks. Only the membranous expression pattern of β-catenin can be seen.

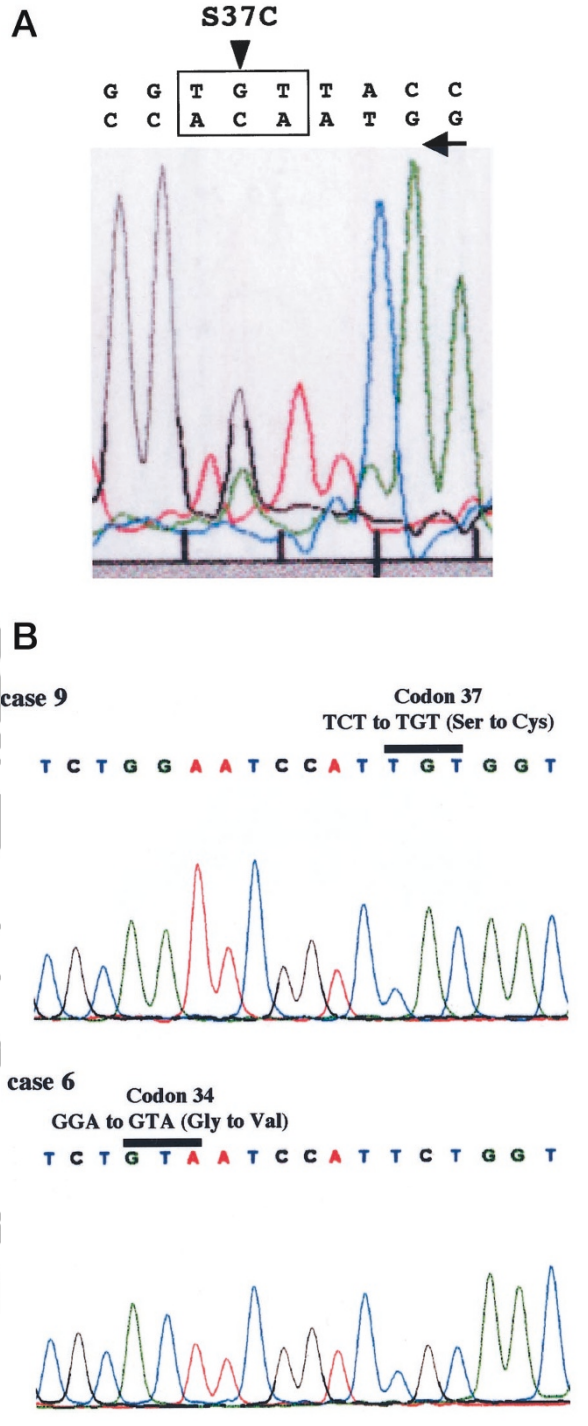

FIGURE 5. Sequence analysis for exon 3 of the β-catenin gene in L-FLAC/WDFA. (**A**) Sequencing chromatogram in Case 9 demonstrating a mixed pattern of the wild-type (T*C*T) and mutant (T*G*T) peaks at codon 37. The mutation results in an amino acid change of serine to cysteine (S37C). The nucleotide sequence is shown above the chromatogram. Codon 37 is boxed, and the mutated guanine nucleotide is indicated by an arrow head. The horizontal arrow demonstrates the direction of sequencing. (**B**) Sequence analysis of the subcloned PCR fragments confirming the presence of the mutated alleles in Case 9 and Case 6.

and TCF transcriptional activation (25). Further study is required to determine precisely which gene mutations are involved in what proportions in the abnormal up-regulation of the Wnt signaling pathway in L-FLAC/WDFA.

With the expression pattern of β-catenin as a marker, H-FLAC appears to be closer to conventional adenocarcinoma than to L-FLAC/WDFA despite its morphologic similarity to L-FLAC. This observation lends support to the concept of discrimination of H-FLAC from L-FLAC/WDFA (4), suggesting that most if not all H-FLAC cases may arise *de novo* rather than as a progression from L-FLAC. This is in keeping with the significant difference in gender and age distribution and natural history of the two subtypes (4). We have previously proposed the hypothesis that what have been reported as biphasic pulmonary blastoma consist of

those derived from dedifferentiation of L-FLAC/WDFA and those from dedifferentiation of H-FLAC (4). Investigation of abnormality of the Wnt signaling pathway in biphasic pulmonary blastomas certainly will contribute to understanding their relationship to L-FLAC/WDFA and H-FLAC.

Because most cases of L-FLAC/WDFA so far reported are likely to have been sporadic, except for the one FAP-associated case (17), some postnatal mutagenic factor(s) are likely to play an important role in tumorigenesis. Thus, it is noteworthy that 76% of patients with L-FLAC/WDFA have had a history of smoking (4). Tsujiuchi and associates recently reported frequent mutations of the *APC* and*β-catenin* genes in pulmonary adenocarcinomas induced by *N*-nitrosobis(2-hydroxypropyl)amine (BHP) in rats (26). BHP is a carcinogenic compound postulated to be an intermediate metabolite of one of the closely related *N*-nitrosoamines that are contained in cigarette smoke (27).

Another interesting aspect of the aberrant nuclear/cytoplasmic expression of β-catenin in L-FLAC/WDFA is its predominant localization in branching glands and morules. An identical pattern of aberrant β-catenin expression in branching glands has been noted in colorectal adenoma/carcinoma (28), a neoplasm that is well known for its frequent β-catenin mutations (19). This pattern of β-catenin expression is analogous to that seen in invagination of endoderm during embryogenesis (29). Everhart and Argani recently reported nuclear localization of β-catenin in pulmonary acinar buds and mesenchymal cells surrounding the acini in the fetus (30), which concurs with our finding in the present study. Branching of the distal airways during fetal lung development may be regarded as an extension of endodermal invagination, which is consistent with the finding of nuclear/cytoplasmic expression of β-catenin in branching airway epithelium. *β-catenin* gene mutations and extracellular matrix molecules in the microenvironment around the tumor may be affecting expression of β-catenin and its distribution in nucleus and cytoplasm in these tumors (28). The signaling pathway involving growth factors and integrin-linked kinase may be a link between the extracellular matrix and up-regulation of the Wnt signaling pathway (25, 31). The nuclear/cytoplasmic expression of β-catenin in reactive type II pneumocytes suggests that the Wnt signaling pathway also may play a role in cell proliferation in response to alveolar injury. Thus both tumorigenesis of L-FLAC/WDFA and regenerative change of the adult respiratory epithelium appear to resemble embryogenesis of the lung with respect to the pattern of β-catenin expression. Furthermore, the NCP in stromal cells of fibrous tissue around invasive tumors and in neoplastic cells in the invasive front of conventional adenocarcinomas may be a recapitulation of early pulmonary embryogenesis.

Our results as well as published data suggest that up-regulating disturbances in the Wnt signaling pathway is a common denominator for the development of tumors with morular formation from a variety of anatomic sites. Besides the cribriform-morular variant of papillary thyroid carcinoma/FAP-associated thyroid carcinoma and colonic adenoma/adenocarcinoma, which show mutations of the *APC* or *β-catenin* genes, recent studies have demonstrated aberrant nuclear/cytoplasmic expression of β-catenin in association with *β-catenin* gene mutations in tumors with morular formation, such as endometrioid carcinoma of the ovary (32) and adenoma of the gallbladder (33). Although morules within adenoacanthoma of the uterus usually do not show OCN, endometrioid carcinoma (34, 35), especially adenoacanthoma (36), frequently show *β-catenin* gene mutations. Based on this hypothesis, we investigated pancreatoblastoma, which shows morules with biotin-rich OCN (12). We found that this tumor constantly shows aberrant nuclear/cytoplasmic expression of β-catenin with two of the five cases examined showing missense mutations of the *β-catenin* gene (44). We also found aberrant nuclear/cytoplasmic expression of *β-catenin* in all 13 sporadic cases of cribriform-morular variant of papillary thyroid carcinoma examined, three of which showed *β-catenin* gene mutations as well (37). Apparently not all types of glandular tumors with β-catenin or APC mutations show morular formation, as pituitary adenoma (38) and hyperplastic fundic gland polyp (39), which may harbor β-catenin mutations, do not do so. Colonic adenoma/adenocarcinoma with common β-catenin mutations (19) only rarely exhibit morular formation (13). Accordingly, an up-regulating disturbance in the Wnt signaling pathway is probably a prerequisite condition, and some other factor(s) must also underlie the morular formation.

The clinically significant feature common to most of these tumors with morular formation is that they are either benign (11, 13) or of low-grade malignancy with a relatively favorable prognosis (3, 4, 8, 32, 35). This is in sharp contrast to another group of tumors with frequent β-catenin or APC mutations and high-grade malignancy with a poor prognosis, such as hepatoblastoma (40, 41) and anaplastic carcinoma of the thyroid (42). Further study is necessary to clarify the mechanism by which mutations of the *β-catenin* and *APC* genes are related to the grade of malignancy.

ADDENDUM

After submission of the manuscript, Abraham and associates reported frequent *β-catenin* gene mutations in sporadic pancreatoblastomas (43).

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Book Review

Nadler S: The Language of Cells: Life as Seen Under the Microscope, 197 pp, New York, Random House, 2001 ($24.95).

Within the genre of "books by physicians" one encounters everything from biography, through medicine for the layman, to simply a second interest in writing. The present title is not about signaling among cells (which is in vogue in cell biology) but rather observations on people and life by someone who happened to devote his professional activity to the cellular level. The author is a widely published surgical pathologist who had his formative years in Canada, engaged in specialty training in New York and Los Angeles, and then, for the bulk of his career, was employed by a large community hospital in Southern California.

The book consists of eight essays, all written in a sensitive and insightful manner. The subjects will conjure up further contemplations in the broad audience for which it seems intended. However, this reviewer had difficulty in finding profound and novel concepts. In the introduction, Spencer Nadler contrasts his "cellular" days behind the microscope with his "whole patient" encounters in the past decade. "The Old Soldier" exemplifies this theme, the author's seeking out patient bonding after so many years peering at slides, and being once removed. This approach has obviously fulfilled the writer, but the average reader may wish for a tad less morbidity.

The format and size of the text make for pleasant reading. The selection of color illustrations, reproductions of microscopic slides according to the essay, makes sense but is largely lost by having them ganged together in the front of the book, after the table of contents, rather than inserted as chapter introductions. This was surely due to an unhappy cost reduction, imposed by the production department.

In Australia in the 1940s and 1950s I recall that it was not uncommon for mothers to consult family doctors on career counseling for their sons and daughters. It was intended to be based on cumulative observations during the pediatric years, on the perceived fitness of their minds and bodies for future activities. At second thought it was not all that quaint, but I doubt that it happens anymore. Perhaps this circumstance pertains because today's medical professionals do not enjoy the community respect of their antecedents, and they are normally not as broadly educated, especially within the humanities. His essays indicate that Dr. Nadler is an exception on both counts.

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