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Genetic Changes in Lung Cancer

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Abstract In an attempt to define the type and temporal sequences of somatic genetic changes that precede the onset of invasive lung cancer, and to search for biological markers useful in screening multiple primary tumors of the upper aerodigestive tract, we have performed a cytogenetic and genetic study using normal bronchial epithelium and primary tumor specimens of 68 patients undergoing pulmonary resection for early stage lung cancer, and normal bronchial epithelium of 5 controls with metastatic sarcomas. Of the 68 lung cancer cases, 31 had a single tumor and 37 displayed multiple synchronous or metachronous tumors. Cytogenetic alterations were observed in 59% (23/39) of the evaluable tumor specimens with complex rearranged karyotypes, particularly involving chromosomes 3 (70%), 17 (39%), 11 (26%), 8, 9, 12 (22%), and 7 (17%). Gene alterations were also detected including overexpression of epidermal growth factor receptor (EGFR) in 63% (36/57), HER2/NEU in 21% (12/56), and p53 mutations in 50% (12/24). The overall frequency of genetic changes (any type) in the tumors was 76% (52/68). In the normal bronchial mucosa, we identified a rearranged karyotype in 20% of the evaluable cases (13/63); particularly simple rearrangements involving chromosomes 3p (6 cases), 7 (6 cases), 17 (3 cases), 9, 11 (2 cases), 8 (1 case); as well as overexpression of EGFR in 39% (20/51) and of HER2/NEU in 14% (7/51). The overall frequency of genetic changes (any type) in the normal epithelium was 46% (30/65). The presence of a rearranged karyotype in the bronchial mucosa was associated with a rearranged karyotype in the tumor sample. Other statistically significant correlations were found between histopathologic and clinical features and the occurrence of the different cytogenetic and genetic changes both in tumors and in the normal bronchial mucosa. No genetic abnormalities were found in the bronchial epithelium of the 5 controls.

Key words: cytogenetics, oncogenes, tumor suppressor genes, growth factor receptors, normal epithelium, lung cancer

Lung cancer arises as the result of multiple genetic hits. Whereas a variety of morphological changes such as squamous metaplasia and bronchial dysplasia have been described in association with lung tumors, little is known about the occurrence and timing of genetic changes during the early, intermediate and late events of lung carcinogenesis. Knowledge of these factors would contribute to the early diagnosis of precancerous lesions of the bronchial mucosa, to the identification of patients at risk for multiple primary tumors of the lung, and to a biological assessment of prognosis in invasive lung carcinoma. Cytogenetic studies have revealed a num-

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ber of chromosomal rearrangements, particularly deletions of the short arms of chromosomes 1, 3, 7, 8, 9, 11, and 17 in non-small cell lung carcinomas. The pattern of cytogenetic abnormalities is consistent among various studies (Table I), although the frequency of these changes may be different for each individual chromosome, depending on the type (established cell lines, short term cultures or direct preparations) and source of biological sample (primary tumor, pleural effusion, distant metastases). Rearrangement of other chromosomes may occur with lesser frequency in the context of complex karyotypes. None of these changes are associated with a specific histotype, with the exception of an i(8q) chromosome which appears to be restricted to adenocarcinomas.

Molecular biology and immunohistochemical studies have demonstrated that dominant oncogenes and tumor suppressor genes are involved in the pathogenesis of lung tumors. A summary of the published data on the frequency and histological correlation of the main oncogenes is shown in Table II. Amplification of the *myc* family is typically observed in small cell lung cancer. Adenocarcinomas are characterized by point mutations of the K-*ras* gene and by overexpression of *erb*B2(NEU), and squamous carcinomas by a high frequency of epidermal growth factor receptor (EGFR) overexpression.

TABLE I. Cytogenetics of Non-small Cell Lung Cancer (NSCLC)

		Chromosome (%)						
	Number	**1p**	**3p**	**7**	**8p***	**9p**	**17p**	**Reference**
	3	66	100	66	66	33	66	[1]
	4	50	100	–	25	–	–	[2]
	20	80	67	67	70	90	50	[3]
	7	–	57	14	14	–	–	[4]
	30**	53	70	43	–	13	17	[5]
	30	53	67	67	20	83	70	[6]
Overall	64	56	70	57	37	70	52	

* i(8q) restricted to adenocarcinomas
** Established cell lines from metastasis or pleural effusion

TABLE II. Genetic Changes in Lung Cancer: Oncogenes

Oncogene	**Histotype**	**Frequency (%)**	**Range**	**Reference**
myc (C-N-L)[1]	SCLC	30	20–50	[7–10]
K-*ras*[2]	ADC	30	25–33	[11–14]
*erb*B1 (EGFR)[1]	SQC	86	74–100	[4,15–18]
*erb*B2 (NEU)[1]	ADC	43	28–80	[19–22]

[1] Overexpression/amplification
[2] Point mutation
SCLC: Small cell lung cancer
ADC: Adenocarcinoma
SQC: Squamous carcinoma

Tumor suppressor genes are listed in Table III. RB1 involvement is observed mainly in small cell lung cancer; p53 mutations and 3p deletions are found in a high proportion of all histotypes. Although the specific genes on 3p remain unidentified, RAR-β and PTPγ have been implicated. Altered expression of RAR-β is observed in 80% of squamous cancer cell lines and is of particular interest for its biological implications, such as modulation of retinoic acid activity through the regulation of gene transcription, in particular those genes related to growth factor receptors. Based on the frequency and consistency of these observations, some studies have searched for a correlation between genetic changes, tumor stage and clinical outcome of lung cancer patients.

While a correlation with tumor stage has been described only for *myc* family amplifications in small cell lung cancer and *erb*B2/NEU overexpression in adenocarcinomas, a prognostic significance has been described for all of these gene abnormalities (Table IV).

Little is known about the presence and type of cytogenetic and genetic changes occurring in preneoplastic lesions of the bronchial epithelium. In a survey of paraffin-embedded lung cancer samples, Vahakangas *et al.* [44] reported immunocytochemical and molecular evidence of p53 mutation in preinvasive, micro-invasive,

TABLE III. Genetic Changes in Lung Cancer: Tumor Suppressor Genes

		SCLC		NSCLC		
Gene	**Mechanism**	**%**	**Range**	**%**	**Range**	**Reference**
RB1	Deletion Mutation	72	18–100	20	–	[23,24]
TP53	Mutation	85	55–100	50	40–55	[25–32]
3p (unident)	Deletion	100	–	53	25–80	[33–39]
RAR-β	Altered expression	–	–	40*	30–80	[37,40]
PTPγ	Altered expression Deletion	15	–	50	–	[41,42]

* 80% in squamous carcinoma
SCLC: Small cell lung cancer
NSCLC: Non-small cell lung cancer

TABLE IV. Genetic Changes in Lung Cancer: Clinical Correlations

Oncogene	**Histotype**	**Stage**	**Survival**	**Reference**
myc (C-N-L)	SCLC	+	+	[8]
K-*ras*	ADC	–	+	[12–14]
*erb*B1 (EGFR)	SQC	–	+	[18]
*erb*B2 (NEU)	ADC	+	+	[19,21]
p53	NSCLC	–	+	[43]

SCLC: Small cell lung cancer
ADC: Adenocarcinoma
SQC: Squamous carcinoma
NSCLC: Non-small cell lung cancer

and invasive lesions of one patient. Six cases of bronchial dysplasia adjacent to lung cancer have been reported by Sundaresan [45], showing 3p deletions in 100% and p53 mutations in 83% of the samples, respectively. In 3 additional samples obtained from patients without lung tumors, allelic loss of loci at 3p were found in all cases, whereas p53 mutations were present in 2 of 3 samples.

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Methodology

The purpose of this study was to define a possible sequence of genetic changes occurring in the multi-step process of lung carcinogenesis. Cytogenetic and genetic studies were conducted in normal bronchial epithelium and primary tumor specimens of patients undergoing pulmonary resection for non-small cell lung cancer. A schematic representation of the methodology adopted for tissue sampling of normal bronchial epithelium and the lung cancer is shown in Figure 1. A frozen section of the bronchial margin between the two samples was always analyzed to rule out any contamination of the normal bronchial epithelium by the adjacent primary tumor. The results obtained in 68 cases, collected from August 1988 to May 1992, are reported here. Normal bronchial epithelium of 5 patients with metastatic sarcomas was evaluated as a control group.

Clinical features of the series were as follows: mean age 64 years (46–80); 64 males and 4 females; 35 squamous, 25 adeno, 3 mucoepidermoid, 2 small cell, 2 bronchioloalveolar, and 1 atypical carcinoid; 45 stage 1, 10 stage 2, and 13 stage 3; 20 pT1, 42 pT2, and 6 pT3; 48 N0, 12 N1, and 8 N2. Multiple primary tumors occurred in 37 patients, either synchronous (4) or metachronous (33). Thirty-one tobacco-related tumors were defined as "infield" (15 lung, 10

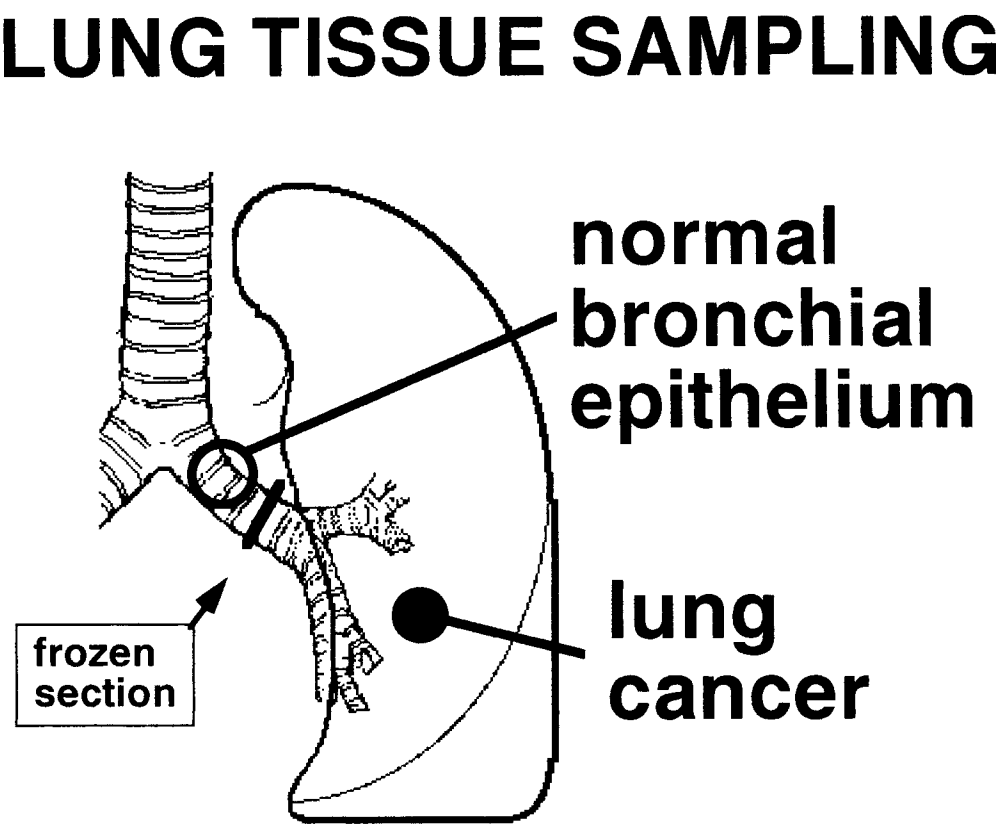

Fig. 1. Schematic representation of tissue sampling for normal bronchial epithelium and lung cancer.

larynx, 4 bladder, 2 oral cavity) and 6 as "outfield" (3 skin, 1 breast, 1 colorectal, 1 sarcoma). The average interval between lung cancer and the other primary tumor was 47 months (range 0–209). Updated follow-up information was available for 60 patients.

Tumor and bronchial mucosal samples were mechanically minced and enzymatically digested with 0.8% collagenase type II (Sigma Chemical Co., St. Louis, MO) in culture medium for 18 hours. The culture medium was RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 5% fetal calf serum (Flow Laboratories, Irvine, Scotland), penicillin (200 U/ml), streptomycin (200 μg/ml), transferrin (10 μg/ml), hydrocortisone (0.5 μg/ml), epidermal growth factor (5 ng/ml) (Sigma Chemical Co., St. Louis, MO), and insulin (5 μg/mg) (Eli Lilly, Indianapolis, IN). Bronchial epithelial cells were cultured in plastic dishes, whereas tumor cells were seeded in dishes coated with collagen (25 μg/ml, Sigma) alone or in combination with laminin (20 μg/ml, Flow Laboratories). Direct preparations or short term cell cultures of 8–10 days were used to harvest tumor cell chromosomes following the method of Gibas *et al.* [46]. Chromosome preparations from the normal epithelium were performed within one week of culture without passages. G-banding was achieved by Wright's stain or trypsin-Giemsa. PHA-stimulated lymphocytes were analyzed in all patients in order to exclude constitutional abnormalities. Karyotype was determined by analyzing at least 10 metaphase cells from the bronchial epithelium, 2 to 10 metaphase tumor cells, and 10 metaphase cells from lymphocyte cultures for each individual. Chromosome abnormalities were described according to the International System for Human Cytogenetic Nomenclature (ISCN 1985) [47].

Immunohistochemical analysis was performed on frozen sections and cell suspensions from both malignant and nonmalignant bronchial cells. The immunoperoxidase technique was performed on frozen sections with avidin–biotin peroxidase complex (ABC) kits (Vector, Burlingame, CA). Indirect immunofluorescence of cell suspensions was performed using fluorescein isothiocyanate conjugated to goat anti-mouse Ig (Meloy Laboratories Inc., Springfield, VA) [48]. The monoclonal antibodies (MAbs) used were MGR1 and MGR2 directed against the EGF receptor [49] and p185 HER-2/NEU [50], respectively. Immunocytochemical staining of the p53 protein on frozen sections was carried out using the ABC technique with S-DAB enhancement according to Frigo *et al.* [51]. The monoclonal antibody PAb240, raised against mutant forms of p53 [52], was used at dilution of 1:1000. Immunocytochemistry was performed on paraffin-embedded sections using the polyclonal antiserum CM-1 against wild-type human p53 (Novocastra Lab., Newcastle, England) by a modification [53] of the method described by Shi *et al.* [54]. Tumor DNA was extracted from frozen surgical specimens using conventional methods [55]. Previously identified dysplastic areas from stained sections were micro-dissected from parallel 5 μm frozen sections and collected in microfuge tubes followed by conventional DNA extraction. Oligonucleotide sequencing and single strand conformational polymorphism (SSCP) procedures are described by Gaidano *et al.* [56]. Gel-purified PCR products were sequenced directly using 5′-^{32}P-labelled primers and a heat-stable DNA polymerase as described by Murray *et al.* [57].

Genetic Changes in Lung Tumors

Table V describes the genetic changes observed in the tumor samples. Cytogenetic alterations were observed in 59% (23/39) of evaluable tumor specimens including complex, rearranged karyotypes, particularly involving chromosomes 3 (70%), 17 (39%), 11 (26%), 8, 9, 12 (22%), and 7 (17%); overexpression of EGFR and HER2/NEU were detected in 63% (36/57) and in 21% (12/56) of the cases, respectively. p53 mutations were found in 50% (12/24). The overall frequency of genetic changes (any type) in tumors was 76% (52/68).

Statistically significant correlations were found between the histopathologic and clinical features of the patients and the occurrence of different cytogenetic and genetic changes in the tumor samples (Table VI). In fact, overexpression of EGFR was higher in squamous histotypes than in adenocarcinomas (79% versus 50%, $p = 0.03$), as was true for p53 mutations (75% versus 37%) as well. In addition, a tendency, although not statistically significant, was observed for a higher occurrence of an isochromosome (8q) in the tumor karyotype of adeno-

TABLE V. Genetic Changes in Lung Tumor Samples

Abnormality	Number Evaluable	Number Positive	% of Total
Cytogenetic (any)	39	23	59
3		16	70*
7		4	17*
8		5	22*
9		5	22*
11		6	26*
12		5	22*
17		9	39*
EGFR	57	36	63
HER2/NEU	56	12	21
p53	24	12	50
Any Change	68	52	76

* Percent of the rearranged cases

TABLE VI. Genetic Changes in Lung Tumors: Clinical Correlations (Proportion of Positive)

	Histotype		Stage		Nodal Status	
Abnormality	SQC	ADC	I	II–III	Neg	Pos
Cytogenetic (any)	67	43	52	75	52	75
3p	24	16	12	36*	11	40**
i(8q)	3	16	7	9	7	10
11	12	4	5	18	4	20*
HSR	9	4	2	14	2	15*
EGFR	79	50*	54	78	54	81
HER2/NEU	14	32	16	33	15	38
p53	75	37*	31	73*	40	67
Any Change	83	72	67	91*	67	95*

* $p < 0.05$
** $p < 0.01$
SQC: Squamous carcinoma
ADC: Adenocarcinoma
HSR: Homologous staining region

carcinomas ($p = 0.078$), and for increased HER2/NEU overexpression in adenocarcinomas.

The analysis of correlations with the clinical features revealed that whereas no specific (cyto)genetic abnormalities were more frequent in the tumor samples of patients with multiple versus single tumors, a significant association was found for cytogenetic changes such as 3p rearrangements with advanced tumor stage (II + III, $p = 0.01$) and nodal positivity ($p = 0.007$), whereas chromosome 11 abnormalities and the presence of homologous staining regions and/or double minutes correlated with the presence of nodal positivity (both $p = 0.04$). p53

mutations were significantly more frequent in advanced stages (II + III) than in stage I ($p = 0.04$), whereas p53 mutations showed a tendency, although not significant, to increase in relation to the tumor size (pT). The overexpression of at least one of the growth factor receptors was significantly increased in node-positive over node-negative patients ($p = 0.02$). This was specifically true for EGFR ($p = 0.05$). Finally, we observed that the presence of at least one cytogenetic or genetic lesion was significantly increased in stages II + III with respect to stage I tumors ($p = 0.03$) and in node-positive versus node-negative patients ($p = 0.01$).

Genetic Changes in Bronchial Epithelium

In normal bronchial mucosa (Table VII), we identified a rearranged karyotype in 20% (13/63) of the evaluable cases, with simple rearrangements particularly involving chromosomes 3p (6 cases), 7 (6 cases), 17 (3 cases), 9, 11 (2 cases), and 8 (1 case). In addition, overexpression of EGFR was detected in 39% (20/51) and HER2/NEU in 14% (7/51) of the cases. The overall frequency of genetic changes (any type) in the normal epithelium was 46% (30/65). The samples of bronchial mucosa obtained from the 5 control patients with metastatic sarcomas did not show cytogenetic or genetic abnormalities.

Interestingly, the presence of a rearranged karyotype in the bronchial mucosa was significantly associated with a rearranged karyotype in the tumor sample. In fact, of the 37 cases with evaluable tumor and epithelial tissue, all 8 cases of cytogenetic abnormality in the bronchial mucosa occurred in patients whose tumors also displayed a rearranged karyotype.

Significant correlations were found between the histopathologic and clinical features of the patients and occurrence of different cytogenetic and genetic changes in the normal bronchial mucosa (Table VIII). Overexpression of HER2/NEU was significantly increased in patients with adenocarcinoma-positive histology of their tumor samples ($p = 0.0048$). HER2/NEU overexpression was found to be significantly higher in patients with multiple versus single tumors of the respiratory tract, or multiple tumors in other sites ($p = 0.0048$). A similar tendency, although not significant ($p = 0.054$), was noted for abnormalities of chromosome 3p.

Whereas no significant differences were found in the frequency of the various (cyto)genetic changes in the bronchial mucosa of patients with tumors of different T or N stage, we observed a higher overall frequency of genetic changes in the normal epithelium of patients with multiple tumors in the upper aerodigestive tract (60%) as compared to those with single or multiple tumors in other sites (32%, $p = 0.019$). The follow-up, although still brief, has shown an increased presence of rearranged karyotypes in the bronchial epithelium in patients present-

TABLE VII. Genetic Changes in Normal Bronchial Samples

Abnormality	Number Evaluable	Number Positive	% of Total
Cytogenetic (any)	63	13	20
3		6	46*
7		6	46*
8		1	8*
9		2	15*
11		2	15*
17		3	23*
EGFR	51	20	39
HER2/NEU	51	7	14
p53	19	1	5
Any Change	65	30	46

* Percent of the rearranged cases

TABLE VIII. Genetic Changes in Normal Bronchial Mucosa: Clinical Correlations (Proportion of Positive)

	Histotype		Multiple Infield		Follow-up	
Abnormality	**SQC**	**ADC**	**Yes**	**No**	**Ned**	**Rec**
Cytogenetic (any)	26	17	28	15	13	40*
	9	13	18	3	10	10
EGFR	43	40	56	33	37	67
HER2/NEU	0	30*	28	5*	20	0
Any Change	34	56	60	32*	38	67

* $p < 0.05$
SQC: Squamous carcinoma
ADC: Adenocarcinoma
Ned: No evidence of disease
Rec: Recurrence (any site)

TABLE IX. Genetic Changes in Bronchial Dysplasia

	Histology	p53 Mutation	Cytogenetics
Case 1	Severe Metaplasia	+	3p deletion
Case 2	Moderate Dysplasia	++	17p deletion
Case 3	Severe Dysplasia	+++	17p deletion + marker
Case 4	Carcinoma *in situ*	+++	not evaluable

ing relapse or second primary tumor in the respiratory tract (40%) as opposed to those patients with no evidence of disease (13%, $p = 0.047$).

Genetic Changes in Bronchial Dysplasia

Using genetic alterations previously reported in invasive lung tumors, we performed a cytogenetic and genetic study in 4 patients with various degrees of morphological abnormalities of the bronchial mucosa, either distant or adjacent to lung cancer. Our intent was to identify the earliest morphological changes in which genetic lesions are detectable.

Table IX shows the genetic abnormalities observed in relation to histopathological features. p53 immunostaining of sections from these lesions displayed nuclear positivity using the antibody PAb240, which specifically detects the conformationally altered mutant p53 protein, as did the polyclonal antiserum CM-1, specific for wild-type p53. Immunoreactive nuclei were more numerous in the areas of carcinoma *in situ* and severe dysplasia than in those with moderate dysplasia, whereas severe metaplasia showed positive dispersed nuclei. Normal bronchial mucosa samples from these patients did not stain. Cytogenetic analysis revealed the presence of a 3p deletion in Case 1, a 17p deletion in Case 2, and a 17p deletion plus a marker chromosome in Case 3. Case 4 was not evaluable. The cytogenetic deletion of the short arm of chromosome 3 in Case 1 was confirmed at the molecular level using a PCR-based detection of the loss of heterozygosity (LOH) at the THRB locus on 3p24. In Case 3, the p53 mutation observed by immunohistochemistry was examined at the molecular level using SSCP and DNA direct sequencing. These techniques identified a point mutation in codon 161 in exon 5.

In Case 1, the spectrum of cytogenetic and genetic lesions observed at the level of dysplastic bronchial mucosa (3p del/LOH, p53+) was different from that observed in the concurrent adenocarcinoma tumor specimen (complex karyotype, no 3p del/LOH, p53–). This patient also had a history of prior squamous lung cancer.

DISCUSSION

The type and frequency of cytogenetic abnormalities observed in our tumor samples are in accordance with published literature data, and strengthen the importance of specific chromosomal and genetic changes in the histological characterization and pathogenesis of lung tumors. A typical example of the genetic changes observed is the overexpression of EGFR, and to a lesser extent p53 mutations occurring in a higher proportion of squamous carcinomas. On the other hand, overexpression of HER2/NEU tends to be higher in adenocarcinomas. Some of these genetic changes are significantly correlated with tumor stage and nodal involvement. In particular, 3p rearrangement, EGFR overexpression, and p53 mutations are more frequent in advanced tumors. Although these findings need to be substantiated by a long term follow-up, they indicate the relevance of biological markers as independent prognostic factors in lung cancer [18,43,58].

A major point of interest in our study was the attempt to analyze normal bronchial mucosa and the tumor simultaneously, particularly in patients with multiple primary cancers of the upper aerodigestive tract. We confirmed our previous report of a high frequency (46%) of genetic abnormalities in normal-appearing epithelium with a larger number of cases [4]. Overexpression of HER2/NEU was significantly increased in patients whose tumor sample showed adenocarcinoma histology ($p = 0.0048$), indicating that HER2/NEU overexpression is an early specific marker in these patients. Also noteworthy was the fact that patients with multiple tumors of the upper aerodigestive tract showed a significantly higher frequency of genetic changes (60% versus 32%), HER2/NEU overexpression in particular.

The analysis of data derived from a few patients with differing degrees of morphological change in their bronchial mucosa offers a further contribution to the definition of the sequential events in the lung carcinogenesis process [59]. p53 mutations were not only observed in cases of altered bronchial morphology, but a clear quantitative trend in p53 immunostaining

TABLE X. Putative Sequence of Genetic Events in Lung Carcinogenesis

	Chromosomal Changes	%	p53 Immunostaining	p53 Molecular
Normal mucosa	simple rearrangements (3p, 7, 11, 17)	20	negative	negative
Bronchial metaplasia			spots only	undetectable
Bronchial dysplasia	same + 17p mutations		positive	mutations
Carcinoma *in situ*			positive	mutations
Early invasive cancer	complex karyotypes (3p, 7, 8, 9, 11, 17, HSR/DMs)	60	extensive	mutations
Metastatic cancer	more complex karyotypes	100	extensive	mutations

HSR/DMs: Homologous staining regions/double minutes

from severe metaplasia to *in situ* carcinoma was established.

In Case 1 (Table IX), the difference in the pattern of cytogenetic and genetic lesions observed at the level of bronchial metaplasia and in the lung tumor specimen suggests that these lesions arise independently and are genetically distinguishable. This finding indicates a "field cancerization" effect resulting in the appearance of detectable genetic lesions at sites distant from the tumor. Moreover, in another patient whose two synchronous lung tumors were resected and analyzed, a different pattern of p53 mutations was found in each of the two lesions, again suggesting an independent pathway of carcinogenesis. Altogether, these data provide evidence that specific genetic alterations may be detectable in the various stages of lung tumorigenesis, including severe metaplasia, bronchial dysplasia, carcinoma *in situ*, early stage invasive cancer, as well as locally advanced and metastatic lung cancer. A tentative sequence of these events is depicted in Table X. In the near future, cytogenetic and molecular analyses may become useful clinical tools for the identification of high-risk individuals, the early diagnosis of precancerous lesions of the bronchial mucosa, and the screening of lung cancer patients.

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