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Microsatellite Alteration at Chromosome 3p Loci in Neuroendocrine and Non-Neuroendocrine Lung Tumors

Histogenetic and Clinical Relevance

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Although chromosome 3p regions are the most frequent site for genetic alterations in small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC), the extent of such abnormality in carcinoid tumors remained to be investigated. Moreover, the histogenetic and biological implications of these findings in non-carcinoid lung tumors remain unclear. We studied eight microsatellite loci on chromosome 3p regions by multiplex polymerase chain reaction in paired normal and tumor DNA from 17 carcinoid tumors, 5 SCLCs, and 38 NSCLCs to determine the histogenetic and the clinical significance of their alterations in these neoplasms. Our results revealed a lack of microsatellite abnormalities at all loci tested in both typical and atypical carcinoid tumors. SCLCs and NSCLCs showed loss of heterozygosity in 100% (5/5) and 58.0% (22/38), respectively. Loss of heterozygosity at more than two loci correlated significantly with poor histological differentiation and were preponderantly found in high proliferative index and DNA aneuploid NSCLCs. Microsatellite instability was noted in only one (1.7%) of the lesions. Our study suggests that 1) the difference in chromosome 3p alterations between carcinoid tumors and SCLCs favors a stochastic rather than linear evolution of these tumors, 2) 3p alterations may constitute an initial event in the development of small cell carcinomas, and 3) loss of heterozygosity at 3p loci is associated with aggressive tumor characteristics in non-small-cell carcinomas. (Am J Pathol 1996, 149:613–620)

The histogenetic and the biological relationships between certain major subtypes of pulmonary neoplasms remain a subject of controversy. This is clearly notable in studies of carcinoid tumors and small-cell carcinomas at this location. Although these tumors share common cytomorphological and immunophenotypic features,[1–4] they widely differ in their cytogenetic, epidemiological, and biological characteristics.[1,5–7] Furthermore, the presence of neuroendocrine features in non-small-cell lung carcinoma (NSCLC) and hybrid small-cell lung carcinoma (SCLC) and NSCLC are not uncommonly encountered.[8,9]

Several studies of chromosome 3 in diverse human solid tumors,[10–12] including SCLC and NSCLC, have shown frequent alteration at certain regions on the short arm of this chromosome.[6,13–16] These findings indicate that these regions may harbor a putative tumor suppressor gene associated with their oncogenesis.[7,17–19] The lack of similar analysis in carcinoid tumors and the insufficient clinicopathological correlation in these studies, however, hamper efforts to determine the pathobiological significance of these findings.

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The objectives of this study were 1) to analyze the molecular alterations at 3p loci in the histopathological entities of neuroendocrine and non-small-cell lung neoplasms and to elucidate their histogenetic relationship and 2) to correlate the findings with the clinicopathological features of these neoplasms.

Materials and Methods

Seventy paired normal lung or lymph node and tumor tissue specimens from twenty carcinoid tumors, six small-cell carcinomas and forty-four non-small-cell carcinomas obtained fresh shortly after surgery from July 1991 to February 1995 at the frozen section of the Department of Pathology, The University of Texas, M. D. Anderson Cancer Center, formed the initial materials for this study. Specimens were carefully dissected from grossly viable tumor and distant normal lung tissue or uninvolved lymph nodes. Tumor specimens contained less than 20% normal host elements by cytospin cytological and/or frozen section evaluation. Tissues were immediately snap-frozen and stored at −80°C until used.

DNA Extraction

Snap-frozen tissues were homogenized and treated with a lysis buffer solution containing 0.2 mol/L Tris-Cl, 1% sodium dodecyl sulfate, 0.25% NaCl, and 25 mmol/L EDTA (pH 8.5). Proteinase K was added to a final concentration of 200 μg/μl. DNA was then purified by using the standard phenol/chloroform method and precipitated with 3 mol/L sodium acetate/ethanol.

Microsatellite Analysis

Eight polymorphic microsatellite markers covering three putative tumor suppressor regions on the short arm of chromosome 3 were analyzed from 60 lung tumors and their corresponding normal tissue. Microsatellite markers (Research Genetics, Huntsville, AL) consisted of D3S1307 (3p26), D3S966 (3p21.3), D3S1766 (3p21–14), D3S1312 (3p14), D3S1285 (3p14), D3S1217 (3p14), D3S1261 (3p13), and D3S1284 (3p12).

We used mutiplex polymerase chain reaction (PCR), in which two loci were amplified simultaneously in one reaction tube. In this procedure, pairings of microsatellite markers were based on 1) the type of the markers used (dinucleotide, tetranucleotide, etc.), 2) at least a 30-bp difference in the size of the PCR product, and 3) the microsatellite loci being at least 1 to 2 cM apart on the same chromosomal band. Loci that did not meet all of the criteria were amplified individually.

The forward primer from each pair was end-labeled using T4 polynucleotide kinase (U.S. Biochemicals, Cleveland, OH) and [γ-^{32}P]ATP (10 mCi/ml) (DuPont New England Nuclear, Boston, MA). PCR was performed in a final volume of 25 μl, containing 200 ng of genomic DNA, 0.0125 μmol/L labeled primer, 0.5 μmol/L each unlabeled primer, 250 μmol/L deoxynucleotide triphosphate, 6.25% dimethylsulfoxide, 0.25 mmol/L spermidine (Sigma Chemical Co., St. Louis, MO), 10 mmol/L Tris-Cl (pH 8.4), 40 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, and 0.5 U of *Taq* DNA polymerase (Perkin Elmer, Norwalk, CT). Twenty-five cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute were performed using an initial denaturation step and final elongation step of 94°C for 5 minutes and 72°C for 3 minutes, respectively. After addition of 10 μl of loading buffer, the PCR products were heat denatured and electrophoresed on 7% urea-formamide-polyacrylamide gels at constant 80°C for 3 to 5 hours depending on the fragment size. The gels were dried and exposed to Kodak X-O-Mat-Ar film (Eastman Kodak Co., Rochester, NY) at −80°C with intensifying screens. Loss of heterozygosity (LOH) was determined by visual comparison of the band densities between normal and corresponding tumor samples. A reduction in band density of >50% was considered as LOH. Microsatellite instability was scored 1) if there were additional band(s) in tumor tissue that were not observed in the corresponding normal pattern or 2) if there were a band shift in tumor samples that contrasted to those of corresponding normal bands.

To exclude the possibility of technical artifacts or contamination, all of the differences described were reproduced by independent PCR reactions and separate gel loadings.

Acridine Orange Flow Cytometry

Single-cell suspensions from solid tissues were prepared by mechanically mincing fresh tissue in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA). Acridine orange staining was performed using a two-step method previously described.[20]

Results

Of the initial seventy specimens, PCR amplification of the extracted DNA was successful in sixty samples. Ten specimens (three carcinoid tumors, one

Table 1. *Clinicopathological Features, Tumor Characteristics, and LOH of Carcinoid Tumors*

Case	Age (years)	Sex	Size (cm)	Type	Location	DI	PI	LOH
5	20	F	2.0	Typical	RL	1.00	3	No
6	48	F	NA	Typical	RL	1.00	3	No
7	68	F	2.7	Atypical	LL	1.00	7	No
8	71	M	1.6	Typical	RL	NA	NA	No
9	65	M	3.1	Typical	LL	1.00	4	No
10	69	F	1.6	Typical	LL	1.20	7	No
11	52	F	NA	Atypical	RL	1.00	3	No
12	45	M	4.7	Atypical	RL	0.91	1	No
13	28	F	2.5	Typical	RL	1.14	10	No
14	59	F	NA	Atypical	RL	1.89	11	No
15	40	F	1.5	Typical	RL	1.13	2	No
16	78	F	1.7	Typical	LL	1.00	4	No
46	42	F	2.5	Typical	RL	NA	NA	No
47	39	M	2.4	Typical	RL	NA	NA	No
48	74	F	4.2	Atypical	LL	NA	NA	No
49	58	F	1.8	Atypical	RL	1.00	6	No
50	54	M	2.8	Atypical	RL	1.00	3	No

F, female; M, male; DI, DNA index; PI, proliferative index; RL, right lung; LL, left lung; NA, not available.

SCLC, and six NSCLCs) failed DNA amplification and were excluded from the analysis. Tables 1 and 2 represent the clinicopathological, the flow cytometric, and the LOH results of the carcinoid and lung carcinomas in this cohort. None of the seventeen carcinoid tumors showed alterations at the microsatellite markers used, whereas 100% of the SCLCs (five of five) and 58.0% of NSCLCs (twenty-two of thirty-eight) showed LOH. Of the NSCLCs, large-cell undifferentiated carcinoma showed the highest LOH with 75.0% (three of four), followed by squamous carcinoma with 68.4% (thirteen of nineteen) and adenocarcinoma with 46.6% (seven of fifteen).

The highest incidence of LOH was at markers D3S1307 (3p26) and D3S1217 (3p14) with 38%. This was followed by markers D3S1284 (3p12) and D3S1261 (3p13), which showed 33 and 29% LOH, respectively. The remaining markers, D3S1766 (3p21–14), D3S1285 (3p14), D3S966 (3p21.3), and D3S1312 (3p14) showed 26, 24, 24, and 21% LOH, respectively. Four tumors showed microsatellite instability; three showed instability at one locus and were considered a background, and only one tumor showed alteration at more than two loci (1.7%).

Table 3 presents the correlation between LOH and clinicopathological and tumor characteristics in lung carcinomas. Significant statistical correlation between poor histological differentiation and LOH at >2 loci was found (P = 0.001). Although a higher number of tumors with DNA aneuploidy and high proliferative activity displayed LOH, this did not reach a statistical significance (P = 0.44). No correlation between LOH and the histological subtypes of non-small-cell carcinoma and tumor stage was found (Figures 1 to 3).

Discussion

Pulmonary neoplasms represent widely diverse clinicopathological entities that not uncommonly manifest hybrid microscopic and submicroscopic features. Of these, neuroendocrine tumors (carcinoid and SCLC) are the most controversial. These tumors, although they share common neuroendocrine fea-

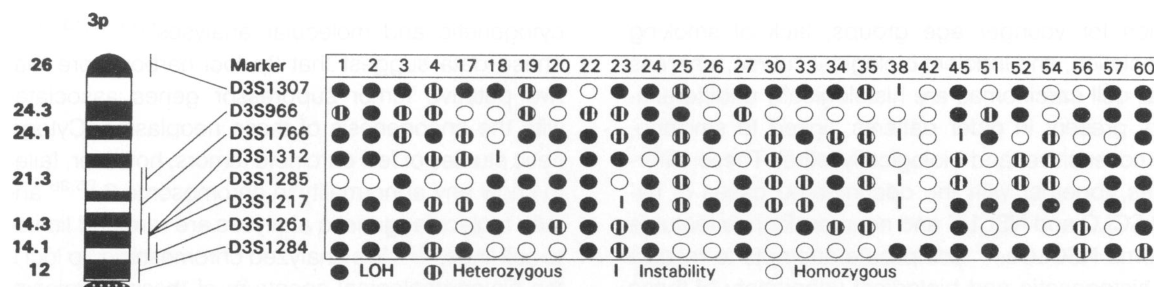

Figure 1. *Allelotyping of 3p microsatellite markers in altered SCLC and NSCLC cases.*

Table 2. *Clinical Features, Tumor Characteristics, and Microsatellite Alterations in SCLC and NSCLC*

Case	Age (years)	Sex	Size (cm)	Location	Histology	Grade	DI	PI	≥2 LOH	Instability	Stage
1	51	M	4.0	LL	SCLC	HG	1.95	15	Yes	No	I
2	72	M	1.7	RL	SCLC	HG	1.58	28	Yes	No	I
3	66	M	1.8	RL	SCLC	HG	NA	NA	Yes	No	I
4	87	M	2.8	LL	SCLC	HG	1.62	17	Yes	No	I
17	72	M	5.0	LL	SqCa	MD	1.59	26	No	No	II
18	71	M	5.0	LL	SqCa	PD	1.79	13	Yes	1	II
19	56	M	6.0	RL	SqCa	MD	1.72	16	Yes	No	I
20	73	M	4.0	RL	SqCa	PD	1.63	21	Yes	No	I
21	58	M	1.7	LL	SqCa	WD	1.00	5	No	No	I
22	61	M	3.5	LL	SqCa	PD	1.55	10	Yes	No	II
23	61	M	4.0	RL	SqCa	MD	1.00	23	Yes	1	II
24	63	M	7.5	LL	SqCa	MD	1.00	6	Yes	No	III
25	44	F	3.6	LL	SqCa	MD	0.89	6	Yes	No	I
26	63	M	7.0	LL	SqCa	MD	1.00	14	Yes	No	III
27	60	F	3.3	RL	SqCa	PD	1.00	31	Yes	No	I
28	70	M	12.5	RL	SqCa	MD	NA	NA	No	No	III
29	61	M	4.0	LL	SqCa	MD	1.00	5	No	No	I
30	35	F	1.8	RL	AdCa	PD	2.39	6	Yes	No	I
31	75	M	4.2	LL	AdCa	MD	1.95	5	No	No	I
32	70	M	2.5	LL	AdCa	PD	1.00	4	No	No	I
33	71	M	7.0	RL	AdCa	MD	1.96	5	Yes	No	III
34	51	F	2.8	LL	AdCa	PD	1.90	12	Yes	No	I
35	71	M	2.0	RL	AdCa	PD	1.85	8	Yes	No	I
36	74	M	4.8	LL	AdCa	MD	1.83	21	No	No	I
37	37	F	3.0	RL	AdCa	MD	3.15	22	No	No	I
38	74	M	5.5	LL	SCLC	HG	1.48	13	Yes	>2	II
39	65	M	1.2	LL	AdCa	WD	NA	NA	No	No	I
40	65	M	1.2	LL	AdCa	MD	NA	NA	No	No	I
41	53	M	1.5	RL	LCC	PD	1.78	9	No	No	I
42	75	M	6.0	RL	LCC	PD	1.70	20	Yes	No	II
43	77	F	3.0	RL	LCC	PD	3.38	27	Yes	No	I
44	57	M	1.6	RL	AdCa	MD	0.89	10	No	No	I
45	44	M	5.5	RL	LCC	PD	2.34	31	Yes	1	III
51	73	M	2.7	RL	SqCa	PD	1.30	11	Yes	No	I
52	72	M	6.5	RL	SqCa	PD	1.55	10	YES	No	III
53	44	F	2.5	RL	SqCa	MD	1.30	19	No	No	I
54	73	M	3.5	RL	AdCa	MD	1.19	5	Yes	No	I
55	36	F	6.2	RL	AdCa	MD	1.00	2	No	No	III
56	58	M	5.0	RL	SqCa	PD	1.00	6	Yes	No	II
57	70	M	4.0	RL	AdCa	PD	1.38	11	Yes	No	II
58	66	F	2.3	LL	AdCa	MD	1.58	21	No	No	I
59	64	F	2.3	RL	SqCa	MD	1.00	9	No	No	I
60	80	F	4.0	RL	SqCa	PD	1.70	14	Yes	No	I

M, male; F, female; HG, high grade; PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated; DI, DNA index; PI, proliferative index; NA, not available; LL, left lung; RL, right lung; SqCa, squamous carcinoma; AdCa, adenocarcinoma; LCC, large-cell carcinoma.

tures, differ in many other phenotypic, epidemiological, and biological aspects.[1–5] Carcinoid tumors are characterized by organoid cytoarchitecture, predilection for younger age groups, lack of smoking association, and indolent biological course, whereas small-cell carcinomas are histologically undifferentiated, present in older patients, linked to smoking, chemosensitive, and biologically lethal. These differences, together with the documented cases of hybrid SCLC and NSCLC and neuroendocrine features in some NSCLCs,[6,7] complicate attempts to resolve the histogenetic and biological uncertainty of these neoplasms.

Studies of small-cell and non-small-cell lung carcinomas have consistently demonstrated genotypic alterations at the short arm of chromosome 3 by both cytogenetic and molecular analyses[6,13–16,19,21–24]. These data suggest that 3p loci harbor more than two putative tumor suppressor genes associated with the oncogenesis of these neoplasms. Cytogenetic studies of few carcinoid tumors, however, failed to show any abnormality in chromosome 3,[25,26] and their molecular genetic analyses are rare and limited to cell lines.[23,27] We analyzed chromosome 3p loci in the histopathological spectrum of these neoplasms in an attempt to determine their underlying molecular

Table 3. *Relationship between LOH at 3p Microsatellite Loci and Clinicopathological and Tumor Characteristics of NSCLC*

Characteristic	LOH	No LOH	*P* value
Grade			
Well and moderately differentiated	7	14	0.001
Poorly differentiated	15	2	
Type			
Squamous carcinoma	13	6	
Adenocarcinoma	7	8	0.36
Large-cell carcinoma	3	1	
Stage			
I and II	17	14	0.67
III	5	2	
DNA ploidy			
Diploid	5	5	0.44
Aneuploid	17	8	
Proliferative fraction			
≤10%	7	7	0.29
>10%	15	6	

alterations and to define potential diagnostic and biological markers.

Our results show lack of alterations at 3p markers in carcinoid tumors (typical and atypical). All small-cell and the majority of non-small-cell carcinomas showed LOH at multiple markers on 3p loci. Such findings indicate that both SCLC and NSCLC share common 3p alterations and differ from carcinoid tumors. This, together with the absence of p53 and Rb gene alteration in previous studies of carcinoid tumors, highlights the fundamental molecular divergence between these tumors and favors the concept of a separate origin or an early stochastic derivation from a common progenitor for their evolution.[5,28–31]

Our results are inconsistent with those of a recent study of carcinoid tumor cell lines in which LOH at 3p loci paralleled those of SCLC.[32] In that study, however, corresponding normal tissues were not available for analysis and LOH was determined by comparing the results of pooled frequencies of heterozygosity at these loci to those of the general population. Concurrent normal tissue analysis, however, is critical to the definitive interpretation of the results as PCR product anomalies due to slippage or recombination of truncated products are not uncommonly encountered during amplification of repetitive sequences.[33–35]

The high incidence of 3p loci alterations in our SCLC and NSCLC cases are in agreement with

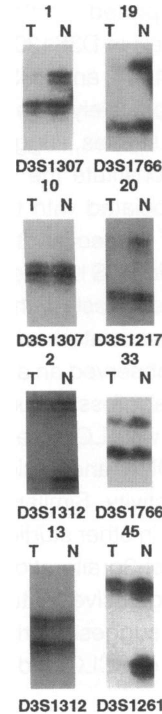

Figure 2. *Representative autoradiographs of selected cases with and without LOH at microsatellite loci tested in lung carcinoid and carcinomas (SCLC and NSCLC). Case 1, LOH in SCLC; case 10, lack of alteration at the same locus in carcinoid tumor; case 2, LOH in another SCLC and the lack of alterations at the same locus in carcinoid 13. Cases 19, 20, 33, and 45 are representative of NSCLC cases with LOH.*

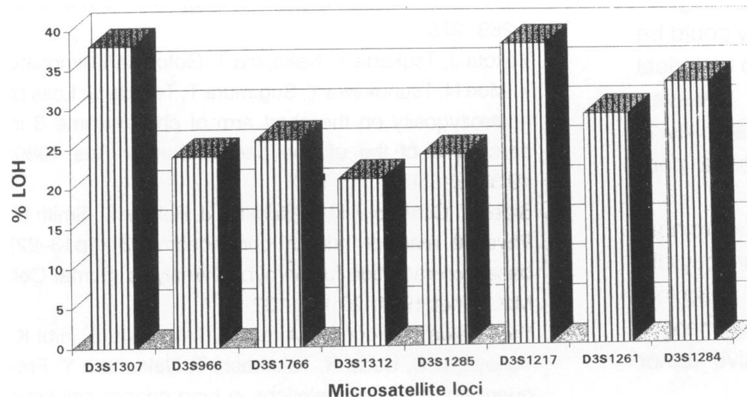

Figure 3. *Cumulative representation of LOH at the microsatellite markers used in lung carcinomas.*

those previously reported.[1-5,15,21-24] The most frequent LOH was noted in D3S1307 (3p26; telomeric) and D3S1217, D3S1284, and D3S1261 (3p14, 3p12, and 3p13) loci, respectively. These results, together with those of earlier studies, suggest that at least two separate regions constitute the sites of tumor suppressor genes associated with the development of lung carcinomas.[22] Moreover, the finding of high LOH at the telomeric D3S1307 locus may also indicate, as previously suggested, that gene(s) at these regions may be associated with tumor development.[36,37] We also observed an association between 3p alterations and aggressive tumor characteristics in NSCLC. Tumors with LOH were more poorly differentiated, were DNA aneuploid, and manifested high proliferative activity. Similar associations have been demonstrated in other studies.[38,39] The consistent demonstration of 3p alterations in SCLC and its association with aggressive features in NSCLC observed in this study suggest a primary or early event in the development of SCLC and tumor progression in NSCLC.

Our data lend credence to previous studies showing differences in the incidence of LOH between squamous cell carcinoma and adenocarcinoma[40]; squamous cell carcinomas manifested a higher incidence of LOH than adenocarcinomas. The pathobiological implications of this finding, however, are currently unclear. We also noted discontinuous alterations at 3p loci in the same tumor in few of our small-cell and non-small-cell carcinomas. This suggests that either two independent recombination events occur in the same cell clone or temporally separate events occurred in different cell populations leading to genetic heterogeneity.[41,42] The clinical and biological significance of this observation remain to be investigated.

Microsatellite instability was noted in only one SCLC (1.7%) in our cohort. Although this incidence of instability is distinctly lower than that reported in some SCLC and NSCLC studies,[24,43] it is in agreement with those of others.[44] Such variability could be attributed to differences in cohort size or to technical and/or interpretative factors. Our results, however, suggest a minor role for microsatellite instability in the development and progression of these neoplasms.[45]

In conclusion, our study indicates that carcinoid tumors lack 3p loci alterations and are distinctly different from SCLC and NSCLC. Alterations at 3p loci may constitute an early event in the development of SCLC and are associated with aggressive tumor characteristics in NSCLC.

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