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Loss of Heterozygosity in a Gene Coding for a Thyroid Hormone Receptor in Lung Cancers

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Summary

The ERBAβ gene codes for a DNA-binding thyroid hormone receptor (THR) and maps to chromosome 3p21-p25, overlapping a 3p deletion characterizing small-cell lung carcinoma (SCLC). A DNA clone detecting an RFLP at the ERBAβ locus has been used to probe a large number of lung tumors. Virtually all SCLC had lost heterozygosity, showing that the 3p deletion in SCLC includes this gene. A substantial but smaller proportion of non-small-cell carcinomas had lost heterozygosity at ERBAβ. Among all non-small-cell tumors some had lost heterozygosity at the proximal locus DNF15S2 (band 3p21) but not at ERBAβ, whereas none were found where the reverse was true. Therefore, the locus which plays a role in non-small-cell tumorigenesis probably lies closer to DNF15S2 than to ERBAβ and is almost certainly not the latter.

Introduction

Lung carcinoma is the most prevalent form of life-threatening tumor in Western societies and presents most commonly as one of four histological types: (1) squamous-cell, (2) small-cell (SCLC), and (3) large-cell carcinomas, and (4) adenocarcinoma. A frequently observed interstitial deletion of chromosome 3 between bands p14 and p23 (Whang-Peng et al. 1982*a,* 1982*b*) in SCLC has raised the possibility of recessive genetic changes being involved, at least for this tumor type. Karyotypically visible deletions in this region have been reported for non-SCLC tumors (Zech et al. 1985; Rey et al. 1987), but they are less frequent (Whang-Peng et al. 1982*a,* 1982*b*), and, when seen, occur in a minority of metaphases, at bands 3p21-3p22 (Zech et al. 1985; Rey et al. 1987). Molecular analyses of RFLPs in SCLC show that almost all the tumors lose heterozygosity at anonymous loci mapping in 3p14 and 3p21 (Brauch et al. 1987; Kok et al. 1987; Naylor et al. 1987; Yokota et al. 1987). Such a loss is also found in non-SCLC tumors (Kok et al. 1987; Naylor et al. 1987; Yokota et al. 1987), although not always at very high frequencies (Brauch et al. 1987). A gene coding for a thyroid hormone receptor, ERBAβ (Weinberger et al. 1986), has recently been shown to map to 3p21-3p25 (Dobrovic et al. 1988) and is genetically linked to both RAF1 (3p25) and the locus DNF15S2 (3p21) (B. Seizinger, personal communication). A preliminary survey using densitometric analysis has suggested the loss of at least one copy of this gene in all six SCLC tumors studied (Dobrovic et al. 1988). We now report the results of a comprehensive analysis of many lung tumors, using an ERBAβ genomic fragment which detects a *Hin*dIII polymorphism for which more than 40% of individuals are constitutionally heterozygous (Gareau et al. 1988).

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Material and Methods

Samples

All non-SCLC tumors were clinical samples obtained

at resection prior to chemotherapy, and SCLC tumors were obtained either at resection or at autopsy. Tumors and their normal counterparts were, after proper identification by the pathologist, rinsed in buffer and immediately frozen at −70°C. Cell lines of the Montreal collection were established either from a pleural effusion or from biopsy tissue from a lymph node (Dobrovic et al. 1988). Lines and tumors from the NCI Frederick, NCI Bethesda, and Groningen collections, as well as the analytical procedures used, were described elsewhere (Brauch et al. 1987; Kok et al. 1987; Naylor et al. 1987).

DNA Isolation and Southern Blotting

Tumor tissue and normal pulmonary tissue were homogenized mechanically. Proteinase K and SDS were added to disrupt cellular membranes (Dobrovic et al. 1988). DNA was extracted according to a standard procedure (Dobrovic et al. 1988) and was analyzed, before digestion by gel electrophoresis, for evidence of degradation. Only samples with no trace of degradation were included in this study. DNA was digested to completion with *Hin*dIII (BRL). Ten micrograms of digested DNA was then size fractionated by electrophoresis in a 0.7% agarose gel and transferred to a nylon membrane (Gene Screen Plus™). Membranes were crosslinked by UV irradiation for 3 min, dried, and prehybridized for 6 h at 65°C in 5 × SSC, 5 × Denhardt's solution, 20 mM Tris pH 7.5, 10% dextran sulfate, 1% SDS, 250 μg salmon sperm DNA/ml, and 50 μg human liver DNA/ml (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate, and 1 × Denhardt's 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, and 0.02% BSA). The blot was subsequently hybridized in the same mixture at 65°C overnight to 100 ng DNA probe which had been ^{32}P labeled by nick-translation to 3 × 10^8 cpm/μg (Maniatis et al. 1982). The filters were washed in 0.1 × SSC, 0.5% SDS at 65°C for three periods of 30 min and were exposed to X-ray film.

DNA probes.—The plasmids pH3H2 and pBH302 were maintained in *E. coli* HB101 and carry, respectively, an anonymous 2-kb *Hin*dIII DNA fragment (Carritt et al. 1986) and a 2-kb *Bam*HI fragment from the 5′ untranslated region of the ERBAβ sequence (Gareau et al. 1988).

Blot analysis.—To quantitate hybridization intensity of Southern blot bands in informative cases, densitometry tracings were made of matched normal and tumor lanes by using an Ultroscan XL™ laser densitometer. Relative intensities of the bands corresponding to the two alleles in the tumor DNA were calculated by normalizing to the intensities of the normal DNA bands. Tracings were made on films exposed to give intensities in the intermediate range. In some cases where intensities were different in the normal and tumor lanes (perhaps owing to copy-number differences), this required that two different exposures be analyzed.

Results

Four lung tumor collections (summarized in table 1) have been analyzed. Three, based at NCI (Bethesda and Frederick) and Groningen, have been described (Brauch et al. 1987; Kok et al. 1987). The fourth, in Montreal, consists of 45 primary non-SCLC operable lung tumors obtained at resection and 14 SCLC samples. Nine of the latter were established as cell lines at Kingston, Ontario, or at Montreal, and the remaining five were clinical samples obtained either at autopsy

Table 1

Summary of Sources and Types of Tumor Material Analyzed

	No. of Tumors					
	SCLC				Non-SCLC	
	Cell Lines		Tumors			
Collection	Matched and Informative	Unmatched	Matched and Informative	Unmatched	Cell Lines (all unmatched)	Tumors (all matched and informative)
Montreal	2	4	2	2	0	12
NCI	7	38	2	0	23	6
Groningen	0	0	4	0	0	7
Total	9	42	8	2	23	25

Table 2

Loss of Heterozygosity at ERBAβ in SCLC from Informative Patients

Collection	Tumor Number or Cell Line	Tumor Genotype[a]
NCI	Tumor 3	2
	Tumor 7	1
	NCI-H209	2
	NCI-H1184	1
	NCI-H1436	1
	NCI-H1173	1
	NCI-H1339	1
	DMS114	1,2
	UMC15	2
Montreal	MM1	2
	RB	2
	LGI	1
	SCT14	2
Groningen	86-8315	1
	86-575	2
	86-9926	2
	86-00916	1

[a] All matched normal samples gave Southern blot bands corresponding to alleles 1 (7.0 kb) and 2 (5.5 kb). The number shown indicates the remaining allele.

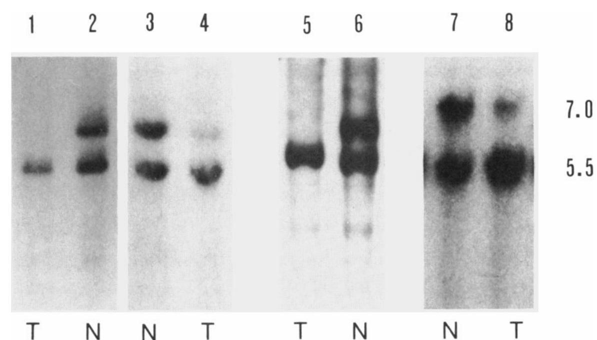

Figure 1 Southern blots of *Hin*dIII-digested DNA from SCLC samples and matching normal tissue probed with BH302 (Gareau et al. 1988). DNA was extracted and digested with *Hin*dIII according to the instructions of the supplier, Boehringer-Mannheim. Samples were fractionated by gel electrophoresis, blotted, and hybridized to radioactively labeled DNA of fragment BH302 (Maniatis et al. 1982) by standard procedures. Lanes 1 and 2, 86-575; lanes 3 and 4, 86-9926; lanes 5 and 6, MM-1; lanes 7 and 8, RB. T = Tumor sample; N = normal samples.

or upon resection of a metastasis. Altogether, 17 informative SCLC samples were tested, together with another 44 for which matching normal tissue was not available. A total of 25 informative non-SCLC tumors were available, in addition to 23 unmatched non-SCLC lines.

Reduction to homozygosity was observed within the ERBAβ locus in nearly all informative SCLC tumors studied (table 2; results of some blots are shown in figure 1), the only exception being DMS114. This cell line was also informative at DNF15S2, a locus in band 3p21 at which another *Hin*dIII polymorphism exists (Carritt et al. 1986). Blot analysis using the DNF15S2 probe (Carritt et al. 1986) showed bands corresponding to both alleles at this locus, albeit at different relative intensities (results not shown). Subclones of this line were tested and gave similar results. It is possible that this line is trisomic for chromosome 3, and the pattern observed corresponds to dosage differences of the alleles. In any event, loss of heterozygosity was not seen at either ERBAβ or DNF15S2 for this cell line. We also tested a total of 42 SCLC lines and two SCLC tumors for which matching tissue was not available. One cell line, H1185, was found to be heterozygous at the ERBAβ locus. The number of heterozygotes expected among a group this size, if 43% heterozygosity (which is our experience) is assumed, is 19, with 95% probability of there being at least 12. Thus we can state with confidence that the overall frequency of loss of heterozygosity at the ERBAβ locus in SCLC is more than 90%.

Non-SCLC tumor DNA was also assessed (table 3). Those tumors with matched normal DNA were all clinical samples, which are frequently contaminated with some normal tissue (Brauch et al. 1987; Kok et al. 1987). Thus, allele loss is frequently manifested in reduction in intensity of one band on a Southern blot rather than in complete loss, and this may be difficult to interpret, especially since in extreme cases normal tissue may make up most of the sample. Nevertheless, of a total of 25 informative cases, 7 clearly lost heterozygosity of ERBAβ, with at least a threefold difference in hybridization intensity between the two bands (table 3, fig. 2). Thus, a conservative estimate of the frequency of loss among non-SCLC tumors is about 30% (7/25). However, densitometry of the blots of many of the samples gave intensity ratios for the two alleles of between 0.35 and 0.6, and a substantial proportion of these probably had lost heterozygosity. To arrive at better estimates of frequencies for both retention and loss of heterozygosity, all slides of nine of the tumors in the Montreal collection were subjected to a double-blind reexamination, and semiquantitative estimates of normal stroma and inflammation were made (table 4). Three tumors already scored as homozygous (T20, T26, and T33) had

Table 3

Loss of Heterozygosity at ERBAβ in Non-SCLC Tumor Tissue from Informative Patients[a]

Collection	Tumor Number	Tumor Genotype	
		ERBAβ	DNF15S2
NCI Frederick	31-40386	1	1
	31-39689	1,2	1,2
	31-39943	1,2	1,2
	31-40385	1,2	1,2
	31-39995	1,2	1,2
	31-39542	1,2	1,2
Montreal	T2	1,2	1,2
	T18	1,2	1,2
	T20	1	1
	T26	1	N. inf.
	T33	2	2
	T37	1,2	1,2
	T42	1,2	1,2
	T44	1,2	1,2
	T49	1,2	1,2
	T52	1,2	1,2
	T53	1,2	1,2
	T55	1,2	N. inf.
Groningen	86-9309	2	2
	86-8718	1,2	1,2[b]
	86-5680	1,2	1,2[b]
	86-1245	1	N. inf.
	86-6505	2	N. inf.
	86-5181	1.2	1,2[b]
	86-11956	1,2	1

Note.—N. inf. = noninformative (homozygous in normal tissue).

[a] Genotypes in DNF15S2 are taken from refs 4 and 5 for the NCI and Groningen collections, respectively. In all cases a threefold (or greater) difference in intensity of hybridization between bands of alleles 1 and 2 was scored as homozygosity.

[b] Allele 1:allele 2 ratios were 0.6, 1.5, and 0.6 for 86-8718, 86-5680, and 86-5181, respectively (Kok et al. 1987).

very little contaminating normal tissue, as expected. Two samples (T44 and T49) had a proportion of normal material which corresponded quite closely to the respective residual intensity of the allele present in reduced amount, so these tumors had also probably undergone loss of heterozygosity. One tumor, T52, clearly had not, since it had only about 15% contamination and no loss of alleles. The three other cases, T2, T37, and T53, could not be explained simply by normal tissue contamination, and some other effect, such as trisomy or tumor heterogeneity, is probably involved. In any event, we consider a minimum of one of these nine tumors to have retained heterozygosity and between three and five to have lost heterozygosity.

Additional information was obtained by comparing the genotypic changes at ERBAβ with those at DNF15S2 among the 21 non-small-cell individuals who were heterozygous at both loci. Four of these were scored as losing heterozygosity at ERBAβ, and all four were equally clearly homozygous at DNF15S2 (table 3). Among the remainder, one tumor, 86-11956, had lost heterozygosity at DNF15S2 (results presented in Kok et al. 1987), with no residual band corresponding to the lost allele. This tumor is heterozygous at ERBAβ (fig. 2, lane 8). Three other tumors were scored as having lost one allele at DNF15S2 (Kok et al. 1987) but were heterozygous at ERBAβ (allele 1:allele 2 ratios were about 1:1; table 3, fig. 2). The level of contaminating normal tissue was relatively high in these samples, giving allele ratios of about 1.5:1, but the heterozygosity at ERBAβ served as an internal control to indicate that trisomy was not a confounding factor. In no tumor was heterozygosity lost at ERBAβ but retained at DNF15S2.

We have also probed many of these tumors' DNA samples with cloned DNA fragments detecting RFLPs on other chromosomes (C. Hajj, unpublished data). As has been reported elsewhere (Brauch et al. 1987; Naylor et al. 1987; Yokota et al. 1987), frequency of loss on chromosomes other than 3p is relatively rare, suggesting that the loss on 3p is specific.

Discussion

Our results show that a substantial proportion of non-SCLC tumors lose heterozygosity at ERBAβ, with a frequency of at least 9/25, including the two for which normal contaminating tissue was the probable cause of the visibility of the lost allele. On the other hand, we can establish an upper limit on the frequency of this loss, since a certain number were shown to maintain heterozygosity. Besides the four tumors above (table 3) which retained both alleles even though the locus DNF15S2 was reduced to homozygosity, contaminating normal tissue was clearly ruled out as a confounding factor in a fifth case (T52; table 4). The results of probing 23 unmatched non-SCLC lines (about 10 of which should be from informative individuals) were consistent with these estimates, with two lines, H460 and H1373, showing heterozygosity at ERBAβ (results not shown). Since these were cloned cell lines, normal tissue was not a problem.

The large size of the tumor collections which were surveyed in this work allows us to make some generalizations from the results. First, all major lung tumor types are characterized, at least in some proportion,

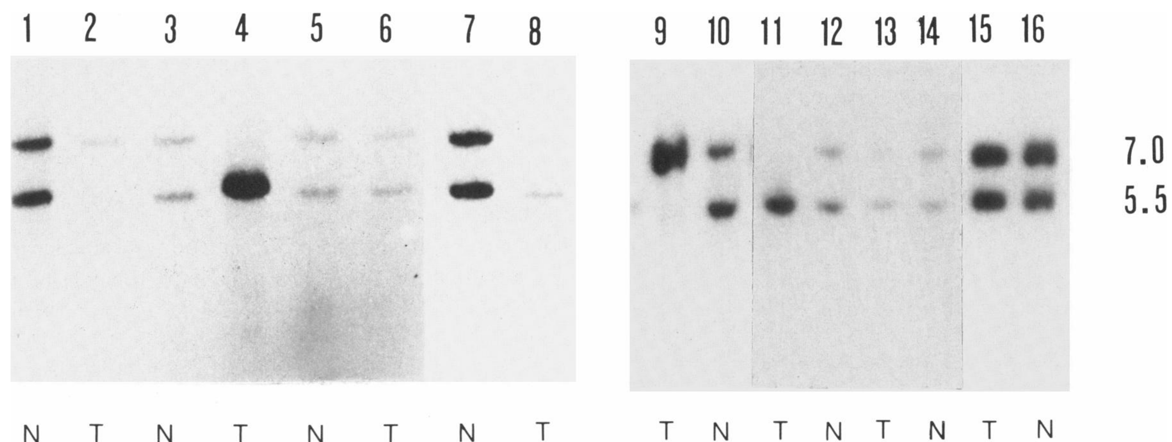

Figure 2 Southern blots of *Hin*dIII-digested DNA from non-SCLC samples and from matching normal tissue probed with BH302. Lanes 1 and 2, sample 86-1245; lanes 3 and 4, 86-6505; lanes 5 and 6, 86-5181; lanes 7 and 8, 86-11956; lanes 9 and 10, T20; lanes 11 and 12, T33; lanes 13 and 14, T 44; lanes 15 and 16, T52.

by loss of genetic material at the ERBAβ locus. Second, SCLC differs from the other types in that a particularly high proportion have undergone loss of one allele of this gene. Of all 61 SCLC analyzed (17 matched), only two, DMS114 and H1185, retained heterozygosity at ERBAβ, and one of the two is clearly an exceptional case since retention was also observed at DNF15S2. This result is of interest in two respects. First, as a physical marker distal to the loci in 3p21 which have been used so far in analyzing the 3p deletion in SCLC, the probe BH302 helps to define the extent of this deletion. Our results therefore further confirm the original cytogenetic studies (Whang-Peng et al. 1982*a*, 1982*b*). Second, the locus is a gene which codes for a DNA-binding thyroid hormone receptor. Thyroid hormones play a major role in development and growth control (Di Liegro and Cestelli 1987), and the loss of one copy of this gene in SCLC may have some biological effect on the phenotype of the tumor cells. Further, the undeleted allele may have undergone mutations undetectable by this analysis. If this were so, ERBAβ would be a candidate for the recessive oncogene in SCLC.

The demonstration that ERBAβ was at least occasionally reduced to homozygosity in non-SCLC tumors is also of interest. The frequency of this loss was clearly not as high as in SCLC. This may reflect an important molecular difference between chromosome 3p deletions in SCLC and non-SCLC. A further point worth considering is the comparison with genotypic change in band 3p21. Loss of heterozygosity in this band in non-SCLC has previously been shown in 4/15 (Brauch et al. 1987) and 14/14 tumors (Kok et al. 1987) in the NCI and Groningen collections, respectively. In addition, 5/8 non-SCLC tumors in another study lost heterozygosity in 3p21 (Yokota et al. 1987). We report here that allele loss also occurred at ERBAβ in non-SCLC, but the frequency is probably lower than for the 3p21 region. In particular, among individuals heterozygous at both loci studied, four tumors had undergone reduction to homozygosity at DNF15S2 but not at ERBAβ

Table 4

Analysis of Nine Non-SCLC Tumors from Patients Informative at Both ERBAβ and DNF15S2

Tumor Number	% Normal Tissue[a]	Genotype[b] Intensity Ratio (allele 1:allele 2)
T2	30	1:.7
T20	11	1:.1
T26	7	.22:1
T33	16	.15:1
T37	12	1:.50
T44	27	.35:1
T49	60	1:.49
T52	15	.92:1
T53	7	.35:1

[a] % Stroma and % inflammation were separately assessed for several sections of each tumor, and the sum of these percentages is presented in the table. About eight slides from each tumor were examined.

[b] Densitometry scans were performed on blots of normal and tumor DNA hybridized with the BH302 probe, and intensities were normalized (higher-intensity band = 1.0).

(table 3), whereas none were found for which the reverse was true. This almost certainly rules out ERBAβ as a candidate for the essential non-SCLC gene and is consistent with this gene's lying closer to DNF15S2 than to ERBAβ. It remains to be determined whether this gene is the same as or different from that involved in SCLC.

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