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Loss of Heterozygosity for Genes on 11p and the Clinical Course of Patients with Lung Carcinoma[1]

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ABSTRACT

Forty-five primary human lung carcinomas were evaluated for the loss of heterozygosity for genes on the short end of chromosome 11. Of 40 evaluable heterozygous cases, loss of the 11p genes c-H-*ras* and insulin was documented in nine cases (22%). The clinical parameters investigated for each patient included the disease stage at presentation, the presence of metastatic disease in either bronchial or mediastinal lymph nodes, and the presence of positive parietal pleural margins in the surgically resected specimen. There were no differences found with respect to these indicators when patients exhibiting the loss of heterozygosity were compared with those who did not have such genetic loss. In addition, when the clinical courses of the two patient groups were compared, there was no difference in survival. We conclude that the loss of heterozygosity for c-H-*ras* and insulin on 11p is a common finding in primary non-small cell human lung carcinomas but does not confer a more aggressive phenotype on these tumors. Although this genetic lesion may be important in the initial transformation of the cells to carcinoma, the available data for lung carcinoma are insufficient to prove causality.

INTRODUCTION

Much evidence has accumulated to suggest that, in many tumors, the genetic lesion which causes cellular transformation is the inactivation or loss of genes which normally act to control cellular growth (1). Thus, many human tumors exhibit the nonrandom loss of genetic material. For example, in retinoblastomas there is a consistent deletion on the arm of chromosome 13 (2). Among the more common solid tumors of adulthood, there are numerous examples of nonrandom accumulation of genetic deletions (3). In each of these cases, investigators reported a frequent nonrandom loss of genetic material in the tumor determined by either restriction fragment length polymorphisms or cytogenetic analysis. These genetic deletions, coupled with a second event which inactivates the remaining allele, cause loss of function of critical regulatory proteins. Such genetic aberrations are thought to be important in the pathogenesis of tumors.

However, there have been very few reports where an attempt was made to determine how the loss of these genes affects the biological aggressiveness of the tumor. Such a study could help determine if the loss of genetic heterozygosity is a late event which enables a clone of previously transformed cells to exhibit greater biological aggressiveness, or whether this genetic event occurs early and is a step in the actual cellular transformation process.

Bronchogenic carcinoma is the most commonly fatal tumor affecting adults in the United States (4), with more than 140,000 deaths expected to occur in 1990 from the disease. Recently, investigators have reported that there is a frequent loss of genetic material from the short arm of chromosome 11 in human lung carcinomas (5), as well as in other tumor systems (6, 7). We have investigated a near-consecutive series of bronchogenic carcinomas for the loss of 11p genetic material. This was done by using the insulin, β-globulin, and c-H-*ras* (H-*ras*) oncogene genetic probes which have well-characterized restriction fragment length polymorphisms. The goal of the study was to investigate the relationship between the loss of heterozygosity at these loci and the aggressiveness of the tumor as determined by the clinical course of the disease.

MATERIALS AND METHODS

Patients. A nearly consecutive series of 45 patients with operable bronchogenic carcinoma of the lung were treated by thoracotomy and curative pulmonary resection between December 1984 and April 1987. Criteria for entry into this study were the availability of satisfactory tumor and normal lung tissue for preparation of high-molecular-weight genomic DNA. Patients were excluded only when satisfactory tissue was unavailable. Follow-up data were obtained through the Durham Veterans Administration Hospital Medical Center Tumor Registry or by review of hospital charts. Additional information was obtained by telephone calls to treated patients. All patients dying during follow-up were assumed to be dead with lung cancer. The median follow-up was 22 mo including those who died. Follow-up was complete in 42 patients; 3 patients were lost in follow-up prior to death.

Preparation of Genomic DNA and Southern Hybridization. Tumor tissue and normal tissue were collected by snap freezing in liquid nitrogen directly after gross dissection in the pathology laboratory. Tissue was stored prior to use at −70°C. Tissues were pulverized frozen, and DNA was prepared by phenol extraction (8). DNA (5 μg) was cleaved with appropriate restriction endonucleases according to conditions recommended by the supplier. To detect polymorphisms in the VTR[3] of H-*ras*, both *Bam*HI, which cuts on either side of the gene and results in fragments between 6.7 and 8.7 kilobases, and the isoschizomer pair *Msp*I/*Hpa*II, which cuts the gene in multiple sites outside the VTR, were used. Length polymorphisms on the 5′ side of the insulin gene were detected by *Bgl*II digestion and in β-globin by *Hin*dIII digestion. Following digestion, DNA was separated in 0.8% agarose gels and transferred to nitrocellulose paper (9). Hybridization was performed using DNA probes labeled with [^{32}P]dCTP by nick translation (10). Hybridization was carried out under stringent conditions in 50% formamide at 42°C for 14 h. The probe used to detect H-*ras* sequences was the full length genomic clone pbc-N1 contributed to the American Type Culture (11). This probe was used to assay the multiallelic polymorphisms which have been previously defined (12). The probe used to detect length polymorphisms at the 5′ end of the human insulin gene was the 1.9-kilobase insert of the pUC 13/hINS plasmid released by digestion with *Bam*HI and *Bgl*II provided by P. Rotwein (13). This probe spans the area of the 5′ length polymorphism. The β-globin probe was a 3.4-kilobase *Hin*dIII-*Eco*R1 fragment spanning the G-γ sequences and including polymorphic *Hin*dIII sites. Absence of the *Hin*dIII site results in a 9-kilobase genomic fragment, whereas the presence of the site generates two bands 8.2 kilobases and 0.8 kilobases in size (14). This probe was supplied by R. Kaufman, Department of Medicine, Duke University Medical Center. In each case, tumor DNA and normal DNA were electrophoresed in adjacent lanes. Homozygos-

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[3] The abbreviation used is: VTR, tandemly repeated region.

ity was scored by the presence of a single hybridizing band of correct size in both normal DNA and tumor DNA. Heterozygosity was scored by the presence of two appropriately sized bands in the genomic DNA. Because of incomplete digestion, partial transfer, or contaminating normal cellular DNA in tumors, deletion of an allele was scored only when equal amounts of DNA from tumor tissue and normal tissue were loaded in adjacent lanes and when one of the expected bands in the tumor tissue was clearly missing or markedly reduced in intensity.

Clinical Information and Outcome. Histological slides were reviewed, and the morphological type was assigned by R. V. Staging was done according to the tumors-nodes-metastases method devised by the American Joint Committee for Cancer Staging and End Results Reporting (4). The Yates-corrected χ^2 test or Fisher's exact test was used to determine statistical significance of categorical data. Survival data were analyzed by the Kaplan-Meier method.

RESULTS

In this study, the DNA obtained from 45 primary human lung carcinomas was evaluated for the loss of heterozygosity of genetic markers on the short end of chromosome 11. The polymorphic genes used in this analysis were H-*ras* (45 tumors), insulin (44 tumors), and β-globin (16 tumors). The H-*ras* and insulin genes were chosen because genetic loss in other tumor systems spans these two closely linked loci. The human β-globin gene, more proximally located on 11p, was chosen to define the extent of the deletion. Representative Southern blots, demonstrating the absence of these alleles in the tumor tissue when compared with the normal tissue, are presented in Fig. 1. In our analysis, there were 40 patients who were heterozygous at one of these 3 loci, while 5 patients were homozygous. The DNA from the homozygous patients could not be evaluated for loss of genetic material on 11p. Overall, we found that 9 of 40

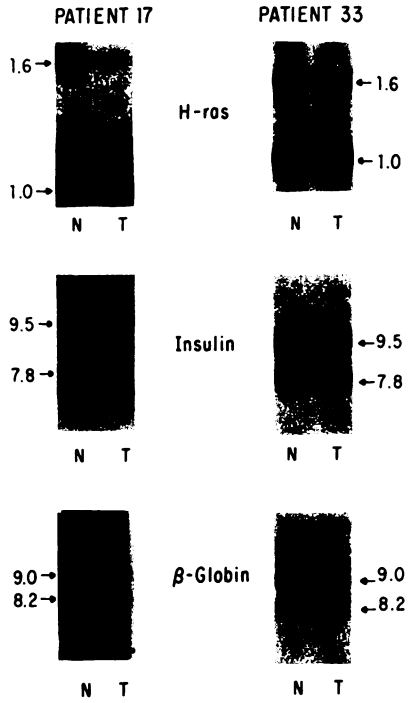

Fig. 1. Representative Southern blots for Patients 17 and 33 demonstrating the allelic status of the three genetic loci investigated. The DNA from the normal tissue is in the *lane* labeled *N*, while tumor DNA is in *lane T*. Both patients demonstrate hemizygosity at the H-*ras* locus, and Patient 17 exhibits the loss of the 7.8-kilobase insulin allele as well. Use of the isoschizimer pair *MspI*/*HpaII*, which cuts on either side of the VTR in H-*ras*, results in many small fragments within the gene which are too small to be visualized in well-digested samples. The faint band at 1.6 kilobases in Patient 33 must be explained by DNA from nonmalignant elements in the tumor.

Table 1 *Incidence of allelic loss for markers on 11p in lung cancer*

No. of patients	11p status
31	Heterozygous (alleles present)
9	Heterozygous (alleles absent)
5	Homozygous

Table 2 *Histological diagnosis and allelic loss of 11p markers*

Histological diagnosis	No.	11p absent	%
Squamous cell	27	4	15
Adenocarcinoma	13	4	31
Large cell undifferentiated	3	0	0
Bronchoalveolar	1	1	100
Small cell	1	0	0

Table 3 *Allelic status and disease stage*

Stage	No. of patients	11p markers absent	%
I	34	5	15
II	3	2	66
III	8	2	25

(21%) evaluable tumor specimens demonstrated the loss of genetic material in this region of chromosome 11 (Table 1). All nine hemizygous patients exhibited H-*ras* allelic loss; in two of these cases, both alleles at the insulin locus were present. The 16 patients analyzed at the β-globin locus included 8 of the 9 patients with missing alleles at either insulin or *ras*. All 16 patients were heterozygous at the β-globin locus, and both alleles were present in tumor DNA and normal DNA. Therefore, allelic loss in the region of *ras* and insulin, located on the distal end of 11p, was a common finding in human lung carcinomas. Retention of β-globin alleles indicates that the chromosomal loss in these eight patients was confined to regions farther toward the end of the short arm of chromosome 11.

Each histological specimen was microscopically reviewed in order to verify the proper pathological diagnosis. Of the 45 primary lung tumors obtained, there were 27 squamous cell carcinomas (60%), 13 adenocarcinomas (29%), 3 large cell undifferentiated carcinomas (7%), and one each of bronchoalveolar carcinoma and small cell carcinoma. The incidence of loss of heterozygosity for 11p markers in each of these pathological groups is shown in Table 2. We discovered the absence of genetic material of chromosome 11 in 4 of 27 squamous cell carcinomas (15%), 4 of 13 adenocarcinomas (31%), and in one bronchoalveolar carcinoma (100%), when tumor DNA was compared with the DNA obtained from normal tissue. With only one small cell carcinoma studied, no conclusion regarding loss of genes on 11p can be made. While the loss of heterozygosity tended to be more frequent in adenocarcinomas when compared with squamous cell carcinomas, this was not statistically significant ($P = 0.16$; Fisher's exact test). The frequency of 11p genetic loss in non-small cell lung cancer does not appear to be related to the histological type.

The possible relationship between the loss of heterozygosity for selected regions on 11p and the biological aggressiveness of primary lung carcinoma was also evaluated. At the time of diagnosis and surgical resection, 34 patients had Stage I disease, 3 patients had Stage II disease, and there were 8 Stage III patients. As shown in Table 3, we found no significant correlation between the absence of genetic material of chromosome 11 and the disease stage at the time of diagnosis when patients with Stage I disease were compared with patients with Stage II or III disease ($P = 0.10$; Fisher's exact test). Similarly, when

11p allelic status was correlated with the other commonly used parameters of tumor invasiveness, such as pleural involvement at the time of resection or positive bronchial and mediastinal lymph nodes, there was no correlation found (data not shown).

Finally, the biological aggressiveness of primary non-small cell human lung carcinomas which have lost heterozygosity at one of the loci on 11p was compared with those not demonstrating the loss of this genetic material by comparing the clinical outcomes of the two patient groups following surgical resection. The survival curves for these data are shown in Fig. 2. In this analysis, the patients who died perioperatively were excluded. Clinical follow-up was obtained in 6 patients who had the absence of genetic material in chromosome 11 and in 32 patients who did not demonstrate the absence of this genetic material. In a mean follow-up of nearly 2 yr, there is no significant difference in survival between the two groups. The mean survival was 26 mo in patients who had the loss of heterozygosity and 23 mo in the patients who maintained heterozygosity at 11p. Thus, this genetic lesion did not appear to significantly affect the biology of this group of non-small cell primary lung carcinomas.

DISCUSSION

The importance of genes which act recessively to induce carcinoma has been most convincingly demonstrated in the case of the embryonal tumor, retinoblastoma (2). In this tumor system, it has been shown that the absence of both alleles of the retinoblastoma gene results in the development of the neoplasm (15). Presumably, this gene normally functions to regulate cell growth. In its absence, however, the retinal cells undergo uncontrolled mitosis, and a retinoblastoma results. Similarly, the Wilms' tumor gene, which is located on chromosome 11, apparently acts in the same fashion (16, 17). Indeed, the nonrandom loss of genetic material has been discovered in a number of tumor systems, including breast cancers (6, 18), gastric carcinoma (19), and colorectal carcinoma (20, 21). Previous investigators have also shown the frequent absence of genetic material from chromosomes 3, 13, 17, and 11 in primary lung carcinomas (5, 22–25). Thus, it is probable that there is a class of tumor-suppressor genes whose presence in normal cells is required to prevent the emergence of tumors.

The short end of chromosome 11 has been implicated in the development of tumors from several different organs (6, 7).

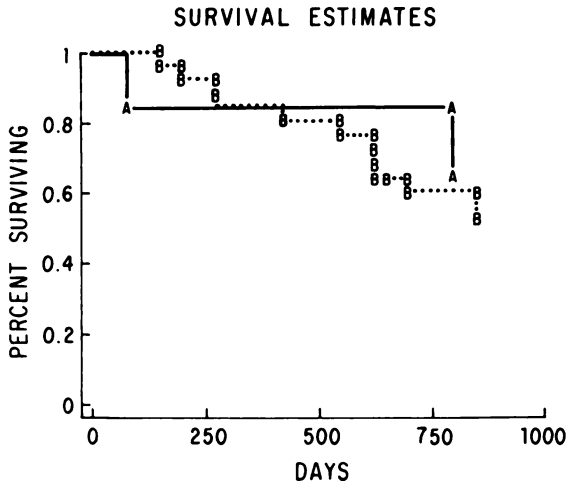

Fig. 2. Survival curves. The *A curve* depicts the fraction surviving of lung cancer patients exhibiting the loss of 11p genetic material. *Curve B* represents the data from patients demonstrating no such genetic loss.

Indeed, the first tumor system investigated for the loss of heterozygosity was bladder carcinoma, where nonrandom losses were found on 11p (26). More recently, authors have demonstrated that there is an absence of 11p genetic material in as many as 42% of human lung carcinomas (5). A number of *in vitro* studies have suggested that this region of the human genome contains genes which are important in tumorigenesis. For example, genes involved in Wilms' tumor are known to reside on 11p (16), and if a normal chromosome 11 is introduced into a Wilms' tumor cell line, the cells become phenotypically less aggressive as measured in nude mouse tumor studies (27). Furthermore, elegant studies with human somatic cell hybrids have demonstrated that genes present on chromosome 11 regulate the expression of the tumorigenic phenotype in Wilm's tumor (28). For these reasons, we elected to study the importance of 11p genetic material in determining the biological aggressiveness of primary human lung carcinomas.

In this study, Southern analysis was used to assay for the H-*ras*, insulin, and β-globin genetic alleles in tumor tissue and normal tissue from each patient. When digested with the appropriate restriction endonucleases, each of these genes demonstrates appropriate restriction fragment length polymorphisms as indicated in "Materials and Methods." Thus, at a particular locus for which the patient is heterozygous, two alleles will be normally present. The absence of one allele in the tumor tissue indicates that the loss of genetic material has occurred. We found this hemizygosity to be a common event in non-small cell human lung carcinomas. Of 45 tumors evaluated, 5 were homozygous at the H-*ras* and insulin loci. Of the remaining 40 tumors, 9 demonstrated the absence of either H-*ras* or insulin or both genes. Consequently, the genetic material closely linked to these genes may be important in the etiology of as many as 20% of non-small cell human lung carcinomas.

A unique aspect of the current study was the correlation of 11p hemizygosity in bronchogenic carcinoma tissue with the commonly used prognostic indicators in primary lung carcinoma. As shown in Table 3, we found no significant correlation between the loss of heterozygosity on 11p and the clinical parameters which portend a more aggressive tumor, such as increased stage or the presence of metastasis in bronchial or mediastinal lymph nodes. Furthermore, when the two patient groups were directly compared with survival curve analysis, as shown in Fig. 2, no difference was found. Results from patients in Japan were somewhat different. In a study of 45 patients, Shiraishi *et al.* (5) found 17 patients with loss of heterozygosity on 11p. In contrast to the current study, four of these patients with gene deletions had small cell carcinomas of the lung. Furthermore, only one of the patients with genetic loss presented with Stage I disease (6%) compared with 11 of 24 cases (46%) retaining heterozygosity which were Stage I at presentation. However, no data on survival after treatment were presented. Follow-up data from Japan may increase the number of patients with loss of heterozygosity and provide additional survival information.

These data suggest that, in primary human lung carcinoma, genes located on the short end of chromosome 11 are not important in determining the biological aggressiveness of the tumors. Although 11p genetic abnormalities have been implicated in the initiation of human tumors and are frequently found in lung cancer, we have been unable to link these genetic events to the biological aggressiveness of this tumor. However, genetic alterations which are important in tumorigenesis may be distinct from those which are useful predictors of clinical behavior. We conclude that, if such recessively acting genes

located on chromosome 11 are important in lung cancer, they function in the early stage of carcinogenesis and in the actual transformation of the cell rather than providing a measure of clinical aggressiveness.

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