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Gene Amplification and Microsatellite Instability Induced in Tumorigenic Human Bronchial Epithelial Cells by Alpha Particles and Heavy Ions

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Gene amplification and microsatellite alteration are useful markers of genomic instability in tumor and transformed cell lines. It has been suggested that genomic instability contributes to the progression of tumorigenesis by accumulating genetic changes. In this study, amplification of the carbamyl-P-synthetase, aspartate transcarbamylase, dihydro-orotase (*CAD*) gene in transformed and tumorigenic human bronchial epithelial (BEP2D) cells induced by either α particles or ^{56}Fe ions was assessed by measuring resistance to *N*-(phosphonacetyl)-L-aspartate (PALA). In addition, alterations of microsatellite loci located on chromosomes 3p and 18q were analyzed in a series of primary and secondary tumor cell lines generated in nude mice. The frequency of PALA-resistant colonies was 1–3 × 10^{-3} in tumor cell lines, 5–8 × 10^{-5} in transformed cells prior to inoculation into nude mice, and less than 10^{-7} in control BEP2D cells. Microsatellite alterations were detected in all 11 tumor cell lines examined at the following loci: D18S34, D18S363, D18S877, D3S1038 and D3S1607. No significant difference in either PALA resistance or microsatellite instability was found in tumor cell lines that were induced by α particles compared to those induced by ^{56}Fe ions.

INTRODUCTION

Lung cancer is considered to be a disease caused mainly by exposure to environmental carcinogens, of which high-LET radiation in the form of α particles emitted from radon progeny has been shown to play an important etiological role (*1*). Similarly, astronauts traveling in space are likely to be bombarded by a battery of charged particles of varying LET (*2*). The potential cancer risk for lung and hematopoietic tissue as well as other organ sites associated with exposure of astronauts to heavy ions in space has become a major concern of space agencies. While radiation has been established to be a human carcinogen, the mechanisms of radiation carcinogenesis remain elusive. To gain a better understanding of radiation-induced bronchial carcinogenesis, a human papillomavirus-immortalized human bronchial epithelial (BEP2D) cell transformation model has been developed (*3, 4*). Although BEP2D cells are immortal, they are anchorage-independent and do not form tumors in immunosuppressed host animals. After exposure to a single low dose of either α particles or ^{56}Fe ions, transformed cells arise through a series of sequential stages including an altered growth pattern, resistance to serum-induced terminal differentiation, and agar-positive growth before becoming tumorigenic in nude mice (*3, 5*). Several lines of tumor cells including those of primary, secondary and metastatic origins have subsequently been established from nude mice based on these earlier studies. However, further study is needed for a better understanding of the genetic processes involved in the malignant transformation of BEP2D cells by a low dose of high-LET radiation.

Carcinogenesis is a progressive multistage process. Genomic instability induced by a single low dose of high-LET radiation may contribute to clonal selection with accumulating genetic changes and ultimately lead to malignant conversion. The consistent presence of chromosomal aberrations such as translocations, insertions, deletions, aneuploidy, mutations, gene amplification and loss of heterozygosity in transformed and malignant cells suggest a causal relationship between genetic instability and carcinogenesis (*6, 7*). Genomic instability is an important manifestation of tumor cell heterogeneity as well as the induction of metastatic and drug-resistant phenotypes (*8, 9*). It has been demonstrated that increased amplification of the *CAD* gene results in acquired resistance to the chemotherapeutic drug PALA [*N*-(phosphonacetyl)-L-aspartate] among tumor cell lines compared with their normal counterparts (*10–12*). In addition, changes in microsatellite repeats have been shown to be reliable markers for genetic instability in hereditary non-polyposis sporadic colon carcinoma (*13, 14*) and in a variety of other tumors (*15–19*). Microsatellite alterations at chromosomes 2p, 3p, 3q, 9p, 11p, 11q, 13q, 17p and 18q have also been detected in pathology samples of lung cancers (*20–23*).

In this study, we examined whether genomic instability

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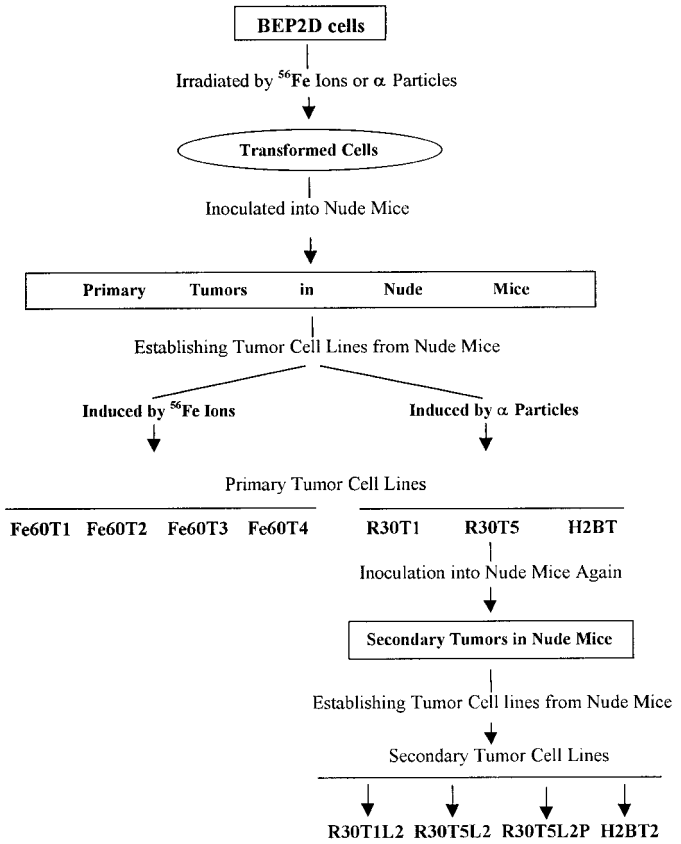

FIG. 1. Schematic diagram showing the lineage of the various tumor cell lines used in the study.

is functionally involved in the malignant transformation of bronchial epithelial cells induced by a low dose of high-LET radiation using the well-established BEP2D cells as a model. Since radiations of different qualities but similar LET have been shown to have different biological effects in mammalian cells (*24, 25*), we further examined these changes in cells transformed by either α particles or ^{56}Fe ions, both of which have an LET of ~150 keV/μm. Amplification of the *CAD* gene and microsatellite instability at genomic markers of chromosomes 3p and 18q were selected for the present study based on their frequent alterations in lung cancer. Analyses were performed on a total of 11 tumor cell lines derived from BEP2D cells irradiated with either a single 30-cGy dose of α particles or a single 60-cGy dose of ^{56}Fe ions. We show herein that gene amplification and microsatellite instability are detected in all the tumor cell lines examined and that there is no significant difference in these two end points among BEP2D cells transformed by either α particles or heavy ions.

MATERIALS AND METHODS

Origin of Tumor Cell Lines and Culture

BEP2D cells were irradiated with a single 30-cGy dose of α particles accelerated with the van de Graaff accelerator at the Radiological Research Accelerator Facility of Columbia University or with a single 60-cGy dose of 1 GeV/nucleon ^{56}Fe ions accelerated with the Alternating Gradient Synchrotron (AGS) at the Brookhaven National Laboratory. The irradiation procedure has been described in detail elsewhere (*3–5*). Briefly, 4–6 × 10^6 exponentially growing BEP2D cells plated in either 60-mm-diameter stainless rings with a Mylar base (α-particle irradiation) or T-25 tissue culture flasks (^{56}Fe ions) were irradiated at room temperature. After irradiation, cells were trypsinized, counted, and replated for both survival and expression of transformed phenotypes as described (*3, 4*). Over a period of 14–16 weeks of continuous culture, irradiated cells acquired a series of phenotypic changes including an altered growth pattern, resistance to serum-induced terminal differentiation, agar-positive growth, tumorigenicity and metastasis. Irradiated cells were injected into nude mice with 5–7 × 10^6 cells per site. Animals were maintained under sterile conditions for 4–7 months and examined for tumor development once a week. Tumors that were larger than 1 cm in diameter were resected to establish primary tumor cell lines. Secondary tumor cell lines were established by injecting cells from primary tumor cell lines into nude mice again. Figure 1 shows the lineage of tumor cell lines used in the present study. All control and tumor BEP2D cells were cultured in serum-free LHC-8 medium supplemented with growth factors as described previously (*3, 5*).

TABLE 1
Microsatellite Markers Used in the Study

Marker	Chromosomal location
D18S-877	18q11.1-q11.2
D18S-34	18q12.2-q12.3
D18S-535	18q12.3
D18S-454	18q12.3-q21.1
D18S-474	18q21.1
D18S-46	18q21.1
D18S-363	18q21.1
DCC	18q21.1-q21.2
D18S-858	18q21.2
D18S-38	18q21.1-q21.3
D18S-58	18q22.3-q23
D3S-1284	3p13-p14
D3S-1289	3p21.1-p14.3
D3S-1067	3p21.1-p14.3
D3S-1038	3p25
D3S-1611	3p21.3

Determination of Frequency of PALA-Resistant Colonies

Exponentially growing control, transformed and tumor cells were plated into 100-mm-diameter dishes at a density of 2 × 10^3 each in medium containing PALA (Drug and Synthesis Branch, Division of Cancer Treatment, National Cancer Institute). The concentrations of PALA used ranged from 130–180 m*M* and corresponded to nine times the LD_{50} of the cells (*8*). Cultures were maintained in the selective medium for 12–14 days with fresh medium replenished every 3 days. They were then fixed and stained, and the number of PALA-resistant colonies was counted. The frequencies of resistant colonies were calculated as the ratio of plating efficiency in the presence or absence of PALA.

Analysis of Microsatellite Instability

The presence of microsatellite instability and loss of heterozygosity among tumor cells were analyzed by PCR as described previously (*26*). High-molecular-weight DNA from tumor cell lines and control BEP2D cells was isolated using a standard extraction method and subjected to PCR amplification using primer pairs for the various polymorphic markers (Table 1) obtained from Research Genetics (Huntsville, AL). The GenePhor System (Pharmacia Biotechnology, Piscataway, NJ) was used in electrophoresis in the analysis of microsatellite alterations. Samples

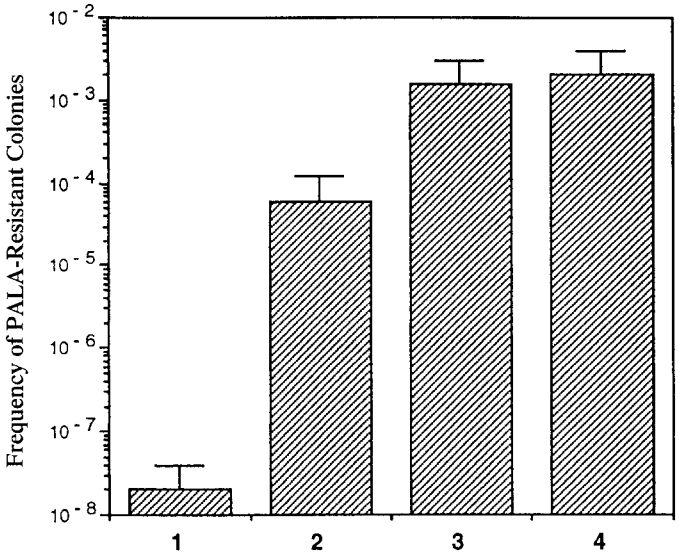

FIG. 2. Frequency of PALA-resistant colonies in BEP2D cells determined at a concentration of PALA that corresponds to nine times the LD_{50}. 1: Control BEP2D cells; 2: transformed cells from populations irradiated with 60 cGy ^{56}Fe ions prior to inoculation into nude mice; and tumor cell lines induced by exposure to 60 cGy ^{56}Fe ions (3) and to 30 cGy α particles (4). Data are pooled from three independent experiments. Bars represent ± SD.

which consisted of 3 ml of PCR products and 2 ml of denaturing solution (50 m*M* NaOH, 1 m*M* EDTA) were heated at 50°C for 10 min and then were placed on ice immediately to prevent reannealing of the single-strand DNA. Two milliliters of loading buffer was added to each sample before the DNA was fractionated by electrophoresis using GeneGel clean 15/14 agarose (Pharmacia). The gel was rehydrated for 3 h in 13 ml of supplied gel buffer containing 7 *M* urea, run at 200 V for 2 h at 55°C (*27*), and stained using a DNA silver staining kit (Pharmacia). Alterations of bands relative to the control (extra bands, band deletions and expansions) were analyzed.

RESULTS

In this study, genomic instability was analyzed by measuring the frequency of resistance to PALA in tumor cells, in transformed cells prior to inoculation into nude mice, and in control BEP2D cells. The frequency of colony growth was less than 10^{-7} in control BEP2D cells (Fig. 2). In contrast, among the transformed, nontumorigenic cells, the frequency of colony growth ranged from 5–8 × 10^{-5} (Table 2 and Fig. 2). As transformed cells acquired the tumorigenic phenotype, the frequency of PALA-resistant growth increased such that among the α-particle-induced tumorigenic cell lines, the frequency was 1.5 ± 0.3 × 10^{-3} in the primary tumor cell lines and 2.8 ± 0.8 × 10^{-3} in the secondary tumor lines. Among the four primary tumor cell lines induced by heavy ions, the frequency of PALA-resistant growth was 1.9 + 0.4 × 10^{-3} (Table 2).

In another set of experiments, the 11 primary and secondary tumor cell lines and control BEP2D cells were analyzed for genomic instability using a total of 16 microsatellite markers. Eleven of these markers are on chromosome 8q and 5 are on chromosome 3p. Table 3 lists the tumor cell lines analyzed and the incidence of microsatellite alterations detected at the various markers. Genomic instability in loci D18534, D18538, D185474 and D351038 was detected in all the tumor cell lines examined. In addition, alterations in loci D185877 and D351067 were detected only in the 3 secondary tumor cell lines (R30T1L2, R30T5L2, H2BT2). Extra bands were revealed in most of the tumor cell lines, while band expansion characterized by a change in allelic intensity was observed in only some of the tumors (Fig. 3). Neither deletions nor LOH were found (data not shown). Furthermore, no significant difference in either the incidence of PALA resistance or microsatellite instability was observed between tumor cell lines induced by either α particles or ^{56}Fe ions.

TABLE 2
Frequencies of PALA-Resistant Colony Growth among Control and Tumorigenic BEP2D Cells

Cells	Treatment	Frequency of colony growth in 9× LD_{50} PALA	Average
BEP2D	Control	$<10^{-7}$	
Transformed, non-tumorigenic	Irradiated with ^{56}Fe ions or α particles	5–8 × 10^{-5}	
Primary tumor cells	Irradiated with α particles		
R30T1		1.2 × 10^{-3}	
R30TS		1.8 × 10^{-3}	1.5 ± 0.3 × 10^{-3}
H2B		1.4 × 10^{-3}	
Secondary tumor cells	Irradiated with α particles		
R30T1L2		2.5 × 10^{-3}	
R30T5L2		2.8 × 10^{-3}	
R30T5L2P		3.4 × 10^{-3}	2.8 ± 0.8 × 10^{-3}
H2BT2		2.5 × 10^{-3}	
Primary tumor cells	Irradiated with ^{56}Fe ions		
Fe60T1		1.0 × 10^{-3}	
Fe60T		2.1 × 10^{-3}	
Fe60T3		1.5 × 10^{-3}	1.9 ± 0.4 × 10^{-3}
Fe60T4		2.9 × 10^{-3}	

TABLE 3
Microsatellite Alterations in Tumor Cell Lines Examined

	Tumor cell lines induced by α particles							Tumor cell lines induced by ^{56}Fe particles			
Marker	R30T1	R30T1L2	R30T5	R30T5L2	H1AT	H2BT	H2BT2	Fe60T1	Fe60T2	Fe60T3	Fe60T4
D18S877	−	+	−	+	−	+	+	−	−	−	−
D18S34	+	+	+	+	+	+	+	+	+	+	+
D18S535	−	−	−	−	−	−	−	+	−	−	−
D18S454	−	−	−	−	−	−	−	−	−	−	−
D18S474	+	+	+	+	+	+	+	+	+	+	+
D18S46	−	−	−	−	−	−	−	−	−	−	−
D18S363	+	+	+	+	+	+	+	+	+	+	+
DCC	−	−	−	−	−	−	−	−	−	−	−
D18S858	−	−	−	−	−	−	−	−	−	−	−
D18S38	+	+	+	+	+	+	+	+	+	+	+
D18S58	−	−	−	−	−	−	−	−	−	−	−
D3S1284	−	−	−	−	−	−	−	−	−	−	−
D3S1289	−	−	−	−	−	−	−	−	−	−	−
D3S1067	−	+	−	+	−	+	−	+	−	−	−
D3S1038	+	+	+	+	+	+	+	+	+	+	+
D3S1611	−	−	−	−	−	−	−	−	−	−	−

DISCUSSION

The correlation between radiation exposure and elevated incidence of cancer is well known (*28*). However, it is not clear how a single exposure to a low dose of radiation can induce development of cancer. The early effects of radiation result in a variety of directly and indirectly induced DNA lesions, including DNA base alterations, single- and double-strand breaks, repair of damaged DNA, activation of gene transcription, and cell cycle-specific growth arrest, among others (*29–31*). Carcinogenesis is a late effect induced by ionizing radiation, and genomic instability may play an important role in the evolution from early to late effects among irradiated cells. High-LET radiation has enhanced relative biological effectiveness which is thought to result in part from the increased multiplicity of damaged sites confined to small regions of DNA. Lesion complexity at these areas of clustered damage may severely challenge cellular repair and may contribute to the onset of genomic instability (*32*). In addition, genomic instability can be initiated by primary alterations in DNA replication, DNA repair, chromosome segregation, and gene amplification. There is evidence to suggest that genomic alterations can also be transferred through cell division to successive generations, which may lead to the development of cancer (*8, 9*).

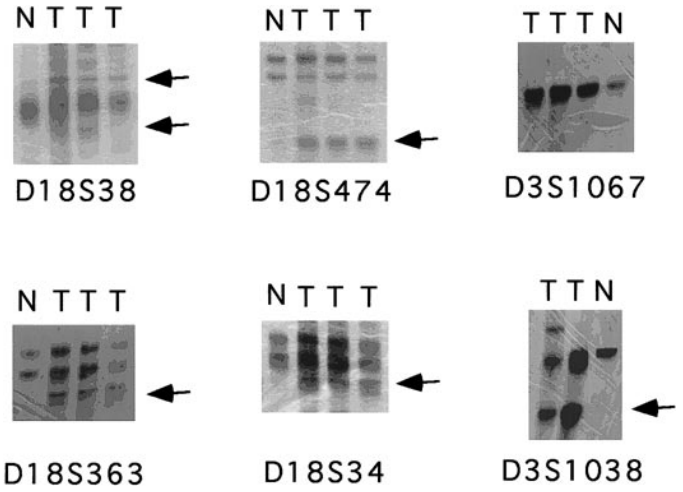

FIG. 3. Representative patterns in microsatellite alterations detected in parental BEP2D cells (N) and in three different tumor cell lines (T). Four microsatellite markers for the long arm of chromosome 18 and two markers for the short arm of chromosome 3 are shown. Arrows indicate novel bands in D18S38, D18S363, D18S474, D18S34 and D3S1038 whereas band expansion is shown in D3S1067.

As markers of genomic instability, microsatellites are frequently observed in human lung cancers (*20–23*). In this study, we used an *in vitro* model to analyze microsatellite alterations occurring in tumor cell lines derived from immortalized human bronchial epithelial (BEP2D) cells irradiated with high-LET radiation such as α particles and ^{56}Fe ions. Our results are consistent with those from clinical pathological studies of lung cancers. BEP2D cells are immortalized by HPV-18, which disturbs normal TP53 and RB function. The TP53 dysfunction due to the E6 oncoproteins of HPV-18 may have already contributed to genomic instability in these cells. However, they are nontumorigenic even in late passages (*2, 3*). The persistence of microsatellite alterations in tumor cell lines induced by α particles and heavy ions but not in control BEP2D cells strongly supports the notion that additional genetic changes are needed for the tumorigenic conversion of BEP2D cells.

Although the initial molecular events leading to gene amplification are not known, the analysis of DNA from PALA-resistant colonies by Southern blotting confirmed that this resistance is due to the amplification of the *CAD* gene (*8*). There is evidence to suggest that chromosomal breakage followed by formation of acentric fragments, which harbor the target gene, may play a role (*32*). Our present findings

that alterations at 5 microsatellite loci were observed in primary tumors induced by α particles while 7 were detected in secondary tumors among the 16 loci examined are consistent with the notion that increased genomic instability correlates with the malignant phenotype. The observations that the frequency of PALA-resistant colonies was less than 10^{-7} in control BEP2D cells, 5–8 $\times$ 10^{-5} in transformed cells, 1.5 $\times$ 10^{-3} in primary tumors, and 2.8 $\times$ 10^{-3} in secondary tumors suggest that the stepwise neoplastic transformation process induced by high-LET radiation is associated with an increase in genomic instability. While mammalian cells irradiated with α particles may have different initial effects than those exposed to ^{56}Fe ions, both radiation types induce malignant transformation in BEP2D cells. Although different cytogenetic and molecular alterations may be induced by α particles and ^{56}Fe ions, no significant differences in amplification of the *CAD* gene or changes in microsatellite instability induced by the two types of radiation are detected in tumorigenic cells. These findings suggest that gene amplifications and microsatellite alterations are associated only with tumorigenicity and not with radiation quality.

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