Int. J. Cancer: **78,** 568–575 (1998)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

MULTIFOCAL ACCUMULATION OF p53 PROTEIN IN ESOPHAGEAL CARCINOMA: EVIDENCE FOR FIELD CANCERIZATION

Defa TIAN[1], Zumei FENG[2], Nancy M. HANLEY[1], R. Woodrow SETZER[3], Judy L. MUMFORD[2] and David M. DEMARINI[1*]

[1] *Environmental Carcinogenesis Division, US Environmental Protection Agency, Research Triangle Park, NC, USA*
[2] *Human Studies Division, US Environmental Protection Agency, Research Triangle Park, NC, USA*
[3] *Research and Administrative Support Division, US Environmental Protection Agency, Research Triangle Park, NC, USA*

A systematic characterization of the cancerization field of esophageal carcinoma based on p53 protein accumulation has not been reported previously. The present report presents such a study based on 50 specimens of esophageal squamous-cell carcinoma from northern China. To gain insight into the etiology of this disease among the 50 subjects, DNA was analyzed for a polymorphism of the aldehyde dehydrogenase-2 (*ALDH2*) gene, which has been associated with increased risk for esophageal cancer among alcohol-consuming patients in Japan. However, the frequency of this polymorphism among our subjects, 30% (15/50), was within published control frequencies for this allele, suggesting that this allele may not play a role in the etiology of esophageal cancer in this northern Chinese population. Immuno-histochemical staining showed that 66% of the tumors were p53$^+$. Of 420 pieces near or adjacent to p53$^+$ tumors, p53$^+$ cells were present among 64% of basal-cell hyperplasia (BCH), 70% of dysplasia (DYS) and 88% of carcinoma *in situ* (CIS). Of 216 pieces near or adjacent to p53$^-$ tumors, p53$^+$ frequencies were 25% of BCH, 25% of DYS and 0% of CIS. The proportion of BCH cells that were p53$^+$ decreased at increasing distance from the tumor ($p = 0.006$). The sporadic distribution of p53$^+$ cells and the distribution and frequency of p53$^+$ precursor lesions support the view that accumulation of p53 protein is multifocal and occurs in precursor lesions in early stages of esophageal carcinogenesis. *Int. J. Cancer* 78:568–575, 1998.

Esophageal cancer is the 9th most common neoplasm in the world and the 2nd most common neoplasm in China, after stomach cancer (Blot, 1994). In China, esophageal cancer is associated with diets contaminated with mycotoxins and low in micronutrients and anti-oxidants, whereas in the United States, the disease is associated with tobacco and alcohol consumption (Blot, 1994). The incidence of esophageal cancer in the United States also varies among ethnic groups, with black men having 5 times the incidence relative to white men (Blot, 1994). Genetic factors, such as a polymorphism in the aldehyde dehydrogenase-2 (*ALDH2*) gene, along with alcohol consumption have been associated with an increased risk for esophageal cancer in Japan (Yokoyama *et al.* , 1996).

Although the etiology of this disease varies among regions and ethnic groups, some common molecular changes, such as mutations in the *p53* gene and alterations in p53 protein expression, have been observed in esophageal tumors (Stemmermann *et al.*, 1994; Bennett, 1995). However, even these biomarkers can vary depending on the geographical location of patients. For example, on average, 45% of esophageal carcinomas contain *p53* mutations (Greenblatt *et al.*, 1994); however, this frequency can vary in China from 20% to 55% depending on the province or region in which the patients live (Lung *et al.*, 1996). In addition, *p53* mutations are found more frequently in esophageal adenocarcinoma than in esophageal squamous-cell carcinoma (SCC) (Galiana *et al.*, 1995; Gleeson *et al.*, 1995; Liang *et al.*, 1995). GC → AT transitions are the predominant class of mutation detected in the *p53* gene of esophageal tumors, though exceptions have been found (Liang *et al.*, 1995). In general, mutations in the *p53* gene appear to be an early event in the development of esophageal cancer (Gao *et al.*, 1994; Greenblatt *et al.*, 1994; Stemmermann *et al.*, 1994; Bennett, 1995; Lung *et al.*, 1996).

Mutations in the *p53* gene result frequently in the synthesis of a mutant protein that has a longer half-life than that of the wild-type protein, causing an accumulation of altered p53 protein within the nuclei of affected cells (Bartek *et al.*, 1990). On this basis, studies using immuno-histochemical (IHC) staining have demonstrated that the presence of p53 protein is an early or intermediate event in esophageal cancer (Bennett *et al.*, 1992; Sasano *et al.*, 1992; Wang *et al.*, 1993, 1994, 1996; Jaskiewicz and De Groot, 1994; Stemmermann *et al.*, 1994; Bennett, 1995; Lung *et al.*, 1996; Ohashi *et al.*, 1997). As with *p53* mutations, the percentage of esophageal tumors with IHC-detectable p53 protein can vary depending on the geographical region in which the patients reside (Lung *et al.*, 1996). p53 protein also has been found in a variety of precursor lesions as well as histologically normal epithelium associated with the esophageal tumor (Bennett *et al.*, 1992; Sasano *et al.*, 1992; Wang *et al.*, 1993, 1994, 1996; Gao *et al.*, 1994; Jaskiewicz and De Groot, 1994; Ohashi *et al.*, 1997).

These studies have shown that esophageal cancer may develop at multiple sites within the tissue, which can be explained by either of 2 theories of tumorigenesis. Field cancerization theory postulates that an area of tissue becomes genomically unstable and predisposed to neoplasia due to prolonged exposure to carcinogens, resulting in multiple tumors (Slaughter *et al.*, 1953; Strong *et al.*, 1984). In contrast, monoclonal theory postulates that cells from a single neoplastic cell may spread to produce multiple tumors (Fialkow, 1976; Nowell, 1976). The data on *p53* mutations and p53 protein accumulation in esophageal cancer have provided evidence to support both theories (Bennett *et al.*, 1992; Chung *et al.*, 1993; Wang *et al.*, 1996), which are not mutually exclusive. However, these determinations have been made from small numbers of specimens involving limited areas of tissue adjacent to the tumors or biopsy material from symptom-free subjects.

To date, no systematic characterization of the cancerization field of esophageal carcinoma based on p53 protein accumulation has been reported. To address this issue, we present such a characterization of 50 SCCs of the esophagus from residents of northern China. p53 protein accumulation was determined for the tumors and surrounding precursor lesions at various distances from the primary tumor. The results are discussed in terms of the multifocal origin of esophageal cancer and the value of p53 protein accumulation as a biomarker for this neoplasm. In addition, we have determined among this group of Chinese patients the frequency of the polymorphism in the *ALDH2* gene mentioned above that has been associated with an increased risk for esophageal cancer among Japanese patients (Yokoyama *et al.*, 1996).

Grant sponsors: National Research Council; U.S. Environmental Protection Agency; University of North Carolina.

*Correspondence to: US EPA (MD-68), Research Triangle Park, NC 27711, USA. Fax: (919) 541–0694. E-mail: demarini.david@epa.gov

Received 25 March 1998; Revised 10 June 1998

MATERIAL AND METHODS

Patients and samples

Surgically resected specimens of esophageal carcinoma from 50 patients were collected from Anyang Oncology Hospital in Anyang City, Henan Province, People's Republic of China. None of the patients had received either radiation or chemotherapy prior to surgery. The clinical stage of the tumor at the time of surgical resection was defined according to the pTNM classification based on pathological observation. The resected segment of the esophagus was opened longitudinally and flattened. Gross inspection included measurement of tumor size across the longest length and width as well as inspection of the mucosa surrounding the tumor. Tumors were classified as medullary, fungating, ulcerative or scirrhous. Special attention was paid to areas where the mucous membrane was slightly depressed, elevated or eroded in order to identify evidence of early esophageal cancer.

Specimens were fixed in 10% formalin and then cut serially along the entire length into 6 to 8 segments. Each segment was then cross-cut into 3 to 4 pieces. A total of approximately 1,200 pieces were processed by standard methods prior to embedding in paraffin blocks. Serial sections (5 μ m thick) were prepared and stained by hematoxylin and eosin (HE). A systematic survey was made of these sections to identify the following lesions: basal-cell hyperplasia (BCH), dysplasia (DYS), carcinoma *in situ* (CIS) and invasive carcinoma. Tumors were classified histologically as either well, moderately or poorly differentiated, and the distance between various precursor lesions and the main tumor was measured.

To characterize further the potential multifocal origin of the precursor lesions, we selected several blocks containing lesions that could have arisen by spreading of cancer cells from a tumor to another area in the epithelium. Blocks containing precursor lesions were cut whole, and adjacent blocks were reverse re-embedded; both blocks were then cut into sections 5 μ m thick. These were used to determine the continuity, length, width and area of the lesions.

IHC staining

Accumulation of p53 protein was determined using a mouse monoclonal antibody to the *p53* gene product BP53–12–1 (Biogenex, San Ramon, CA) and an avidin-biotin-peroxidase complex (ABC) method (Top *et al.*, 1995) with modifications. Briefly, tissue sections were dewaxed in xylene and rehydrated in graded alcohol, and the endogenous peroxidases were blocked by immersing the sections in 3% hydrogen peroxide in water for 5 min. Sections were treated at 97 ° C for 20 min with target-retrieval solution (DAKO, Carpinteria, CA), and non-specific binding was blocked by incubation in normal horse serum (2% v/v in 0.1% BSA/PBS) for 20 min. Sections were incubated at 4 ° C overnight in a humidified chamber with a 1:800 dilution of the primary anti-p53 MAb. Localization of the primary antibodies was obtained by use of the ELITE ABC mouse IgG Detection Kit (Vector, Burlingame, CA). After washing with PBS, sections were incubated with a biotinylated anti-mouse IgG antibody and ABC reagent. The color was developed with diaminobenzidine (Stable DAB; Research Genetics, Huntsville, AL). Finally, the nuclei were counterstained with hematoxylin. An intense brown color within the nucleus was considered positive for accumulation of p53 protein. The extent of the p53 nuclear reactivity was classified into 4 grades: no reactivity (–); focal presence of positive cells, *i.e.*, 1– 10% tumor cells (+); heterogeneous nuclear reactivity, *i.e.*, 10– 50% tumor cells (++); and homogeneous intense nuclear reactivity, *i.e.*, 50– 100% tumor cells (+++). The p53 nuclear reactivity in the precursor lesions was estimated in the same manner.

ALDH2 genotyping

DNA was isolated by standard methods using phenol/chloroform/isoamyl alcohol from portions of the tumor samples and analyzed by a mismatch PCR/restriction fragment length polymorphism (RFLP), described previously (Yokoyama *et al.*, 1996) with the following modifications. A master mix minus the Taq polymerase was made containing non-radioactive primers (10 pmoles of each primer/30- μ l PCR), 200 μ M of each dNTP and 3.3 mM of $MgCl_2$. This master mix and each DNA sample (suspended in 10 μ l of water) were incubated separately at 90 ° C for 5 min. The Taq (1 U/reaction) was then added to the master mix, and 20 μ l of the mix were added to each DNA sample. The reaction tubes were then transferred to a Perkin-Elmer (Norwalk, CT) 9600 thermal cycler equilibrated at 85 ° C, and after 5 min of incubation, the reactions were subjected to 35 cycles at 94 ° C for 15 sec, 56 ° C for 90 sec and 72 ° C for 30 sec. Samples were then precipitated by ethanol, digested by MboII, concentrated by vacuum centrifugation to 5 to 10 μ l and loaded onto ethidium bromide/agarose gels (4%) made from Metaphor agarose (FMC BioProducts, Rockland, ME). Samples were electrophoresed at 100 v for 3 hr. An example of this modified analysis is shown in Figure 1.

RESULTS

Of the 50 patients, 34 were male (average age 52, ranging from 39 to 70 years) and 16 were female (average age 53, ranging from 44 to 64 years) (Table I). Histo-pathological diagnosis indicated that all 50 cases were invasive SCC. The location of the tumors was determined by gross inspection during surgery: 8% (4/50) were in the upper third, 60% (30/50) were in the middle third and 32% (16/50) were in the lower third of the esophagus (Table I). Gross inspection of the mucosa surrounding the tumors showed that the most common abnormalities were a thickening of the mucosa and of the longitudinal and transverse rugae, which were also wide and irregular in shape. In some cases, the mucosa was depressed or eroded, and histological data showed that most of the DYS and CIS were present in these areas.

Among the tumors, 44% (22/50) were well differentiated, 48% (24/50) were moderately differentiated and 8% (4/50) were poorly differentiated (Table I). A total of 36 showed invasion of the primary tumor into the muscularis propria (PT2), 9 showed invasion into adventitia (PT3) and 4 showed metastasis of regional lymph nodes (PN1). The frequency of $p53^+$ tumors was 68% (15/22) among the well-differentiated cases, 66% (16/24) among the moderately differentiated cases and 75% (3/4) among the poorly differentiated cases. The frequency of $p53^+$ tumors was 67% (23/34) among stage PT2, 63% (7/11) among stage PT3 and 60% (3/5) among stage PN1. No significant correlations were found between the frequency of $p53^+$ tumors and histological grading or TNM stage.

The *ALDH2* genotypes of the patients are also shown in Table I. There was no obvious association between the presence of the polymorphism (heterozygous subjects) and any histological features of the tumors. The frequency of the heterozygous genotype was 30% (15/50) among this sample of esophageal cancer patients

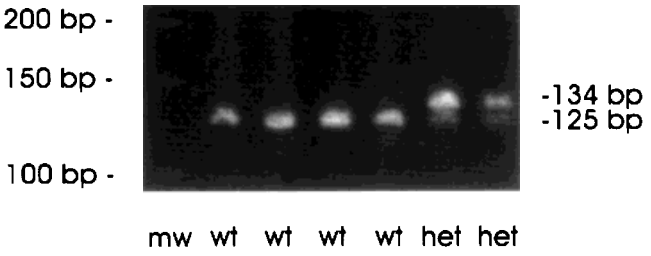

FIGURE 1 – Agarose gel of PCR/RFLP analysis of esophageal samples for *ALDH2* polymorphism. The mismatch PCR creates an *MboII* restriction site in the region of the polymorphism. Lane 1, 50-bp m.w. DNA ladder marker (Life Technologies, Grand Island, NY); lanes 2– 5 show the 125-bp band from subjects 9, 26, 31 and 32, respectively, indicating that they are *ALDH2*1/ALDH2*1* (wild-type); lanes 6 and 7 show the 125- and 134-bp bands for subjects 34 and 35, respectively, indicating that they are *ALDH2*1/ALDH2*2* (heterozygous).

TABLE I – CLINICAL CHARACTERISTICS AND GENOTYPIC ANALYSES OF 50 ESOPHAGEAL SCC AND THEIR PRECURSOR LESIONS

Patient number	Age/ sex	Tumor ($L^1/G^2/S^3$)	p53 (P/T)[4]	Percentage (frequency) of $p53^+$ lesions[5]			*ALDH2*[6]
				BCH	DYS	CIS	
1	52/M	M/M/T2	+/−	40 (4/10)	22 (2/9)	0 (0/8)	Het.
2	51/M	M/M/N1	+/+	67 (4/6)	100 (2/2)	100 (1/1)	W.T.
3	63/F	M/W/T4	+/+	62 (8/13)	67 (4/6)	67 (2/3)	W.T.
4	57/M	M/M/T2	+/+	67 (8/12)	50 (3/6)	67 (2/3)	W.T.
5	48/M	L/M/T2	+/−	38 (3/8)	50 (2/4)	0 (0/2)	W.T.
6	54/F	L/P/N1	+/+	71 (5/7)	67 (2/3)	0 (0/0)	Het.
7	58/M	M/M/T4	+/+	64 (7/11)	100 (8/8)	100 (5/5)	W.T.
8	45/M	M/W/T2	+/+	80 (8/10)	100 (3/3)	100 (2/2)	W.T.
9	50/F	M/M/T3	+/+	75 (3/4)	67 (2/3)	83 (5/6)	W.T.
10	44/M	M/M/T2	−/−	0 (0/11)	0 (0/4)	0 (0/2)	Het.
11	47/F	L/M/T4	−/−	0 (0/9)	0 (0/7)	0 (0/2)	Het.
12	61/M	M/M/T2	+/+	83 (10/12)	100 (6/6)	100 (2/2)	W.T.
13	70/M	M/W/T2	+/+	50 (4/8)	50 (1/2)	0 (0/0)	Het.
14	52/M	M/M/T2	+/+	73 (8/11)	40 (2/5)	0 (0/0)	W.T.
15	62/F	L/M/N1	+/−	29 (2/7)	50 (3/6)	0 (0/2)	Het.
16	39/M	L/W/T4	+/+	71 (5/7)	50 (2/4)	0 (0/0)	W.T.
17	41/M	L/M/T2	+/−	33 (2/6)	50 (2/4)	0 (0/0)	W.T.
18	45/F	L/W/T3	+/−	33 (3/9)	33 (1/3)	0 (0/0)	W.T.
19	59/M	L/W/T2	+/+	75 (3/4)	63 (5/8)	0 (0/0)	W.T.
20	40/M	M/P/T2	+/−	30 (3/10)	33 (1/3)	0 (0/1)	W.T.
21	66/M	L/W/T2	+/+	29 (2/7)	20 (1/5)	100 (2/2)	Het.
22	51/M	L/W/T2	+/+	86 (6/7)	71 (5/7)	0 (0/0)	W.T.
23	44/F	M/W/T3	−/−	0 (0/8)	0 (0/2)	0 (0/1)	W.T.
24	60/M	M/W/T2	+/−	80 (4/5)	67 (2/3)	0 (0/1)	W.T.
25	50/M	M/M/T2	+/+	40 (2/5)	71 (5/7)	100 (1/1)	W.T.
26	57/M	M/W/T2	+/−	100 (3/3)	33 (1/3)	0 (0/1)	W.T.
27	42/M	M/M/T2	+/+	63 (5/8)	88 (7/8)	67 (2/3)	W.T.
28	46/F	M/M/T4	+/+	67 (4/6)	100 (2/2)	0 (0/2)	W.T.
29	47/M	M/M/T2	+/+	75 (3/4)	33 (1/3)	100 (1/1)	Het.
30	54/M	L/W/T3	+/+	89 (8/9)	60 (3/5)	100 (1/1)	W.T.
31	65/M	L/M/T2	+/−	80 (4/5)	50 (1/2)	0 (0/0)	W.T.
32	44/F	U/M/T2	+/+	57 (4/7)	100 (5/5)	100 (3/3)	W.T.
33	60/M	U/W/N1	+/−	33 (2/6)	33 (2/6)	0 (0/0)	W.T.
34	46/F	L/P/T3	+/+	56 (5/9)	38 (3/8)	80 (4/5)	Het.
35	64/F	M/W/T4	−/−	0 (0/8)	0 (0/4)	0 (0/3)	Het.
36	69/M	M/M/T2	+/+	50 (3/6)	75 (3/4)	100 (2/2)	Het.
37	53/M	U/W/T2	+/+	80 (4/5)	40 (2/5)	100 (2/2)	W.T.
38	60/F	M/W/T3	+/+	40 (2/5)	67 (2/3)	100 (2/2)	W.T.
39	52/M	U/W/N1	−/+	75 (3/4)	67 (2/3)	0 (0/0)	W.T.
40	60/F	M/W/T2	+/+	60 (3/5)	100 (2/2)	100 (1/1)	W.T.
41	60/F	M/M/T2	+/+	71 (5/7)	100 (2/2)	0 (0/0)	W.T.
42	41/M	L/M/T2	+/+	38 (3/8)	0 (0/0)	0 (0/0)	Het.
43	50/M	L/W/T2	+/+	80 (4/5)	100 (4/4)	67 (2/3)	Het.
44	43/M	M/M/T4	−/−	0 (0/6)	0 (0/3)	0 (0/3)	W.T.
45	42/M	M/P/T2	+/+	60 (3/5)	67 (2/3)	100 (0/0)	W.T.
46	50/M	L/M/T3	+/+	50 (1/2)	0 (0/0)	0 (0/0)	Het.
47	49/F	M/W/T2	+/+	75 (3/4)	33 (1/3)	0 (0/0)	W.T.
48	54/F	H/M/T2	−/−	0 (0/5)	0 (0/4)	0 (0/1)	W.T.
49	58/M	M/W/T2	+/+	43 (3/7)	100 (4/4)	100 (2/2)	W.T.
50	42/M	M/W/T3	−/−	0 (0/3)	0 (0/2)	0 (0/0)	Het.

[1]L, location; L, lower; M, middle; U, upper.–[2]G, grade; W, well-differentiated; M, moderately differentiated; P, poorly differentiated.–[3]S, stage; T2, tumor invading muscularis propria; T3, tumor invading adventitia; T4, tumor invading adjacent structures; N1, metastasis of regional lymph nodes.–[4]P, precursor lesion; T, tumor.–[5]Percentage was calculated from the ratio of $p53^+$ lesions/total lesions as defined by HE staining.–[6]Het., heterozygous (*ALDH2*1/ALDH2*2*); W.T., wild-type (*ALDH2*1/ALDH2*1*).

from northern China; no homozygous mutants were detected among this group of samples.

Based on HE straining and clear histological features, a total of 636 pieces containing precursor lesions were selected for IHC staining. The characteristic brown color indicative of p53 protein accumulation ($p53^+$) was found almost exclusively in nuclei; however, 3 specimens exhibited this color in both nuclear and cytoplasmic areas. The results of IHC staining from at least 2 sections per specimen for tumors and precursor lesions for all 50 patients are shown in Table I, and examples of stained precursors and tumors are shown in Figure 2.

Figure 2*a* illustrates the uniform staining intensity found for $p53^+$ cells in CIS; however, $p53^+$ cells were generally distributed in a diffuse pattern throughout the layers of the epithelium. Figure 2*b* illustrates the variable distribution of $p53^+$ cells in different precursor lesions. As in Figure 2 *a*, the CIS contained mostly $p53^+$ cells in a diffuse pattern that extended on the right to the entire thickness of the epithelium. In the center, however, $p53^+$ cells were present in the DYS from the lower to the upper layers of the epithelium. On the left, $p53^+$ cells were distributed sporadically in the basal-cell zone or along the papilla. Thus, the proportion of positively staining cells varied depending on the degree of DYS. In BCH or nearly normal epithelium, $p53^+$ cells were not distributed evenly and appeared sporadically in the mucosa surrounding the tumor (Fig. 2*c*). Only a few, sporadically distributed $p53^+$ cells were found among the proliferative basal cells or along the papilla

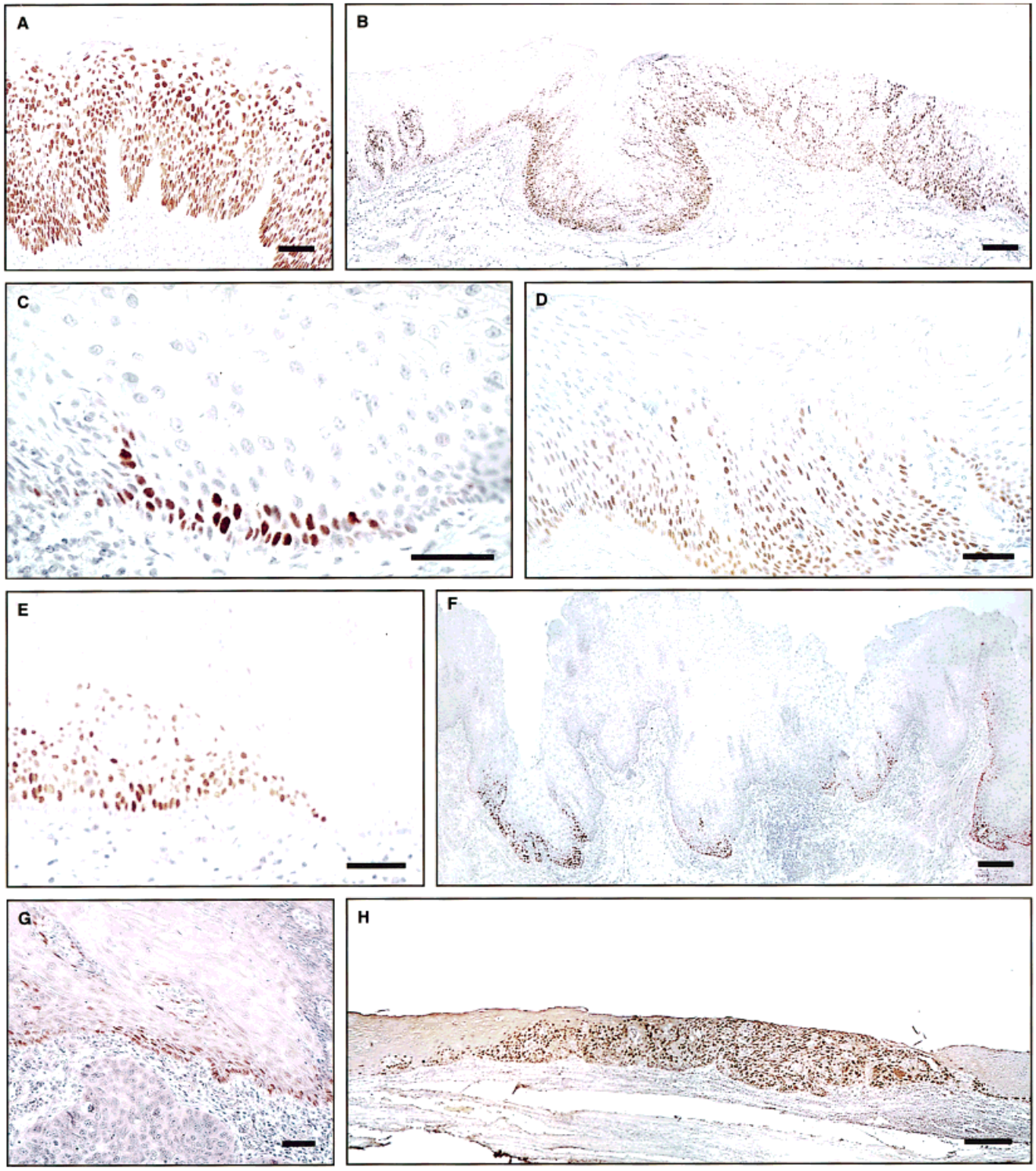

FIGURE 2 – Examples of $p53^+$ staining. (*a*) $p53^+$ cells in CIS exhibit uniform staining intensity and a diffuse distribution pattern throughout the layers of epithelium. (*b*) Distribution of $p53^+$ cells is variable in different precursor lesions. CIS contain mostly $p53^+$ cells in a diffuse pattern that extends on the right to the entire thickness of the epithelium; in the center, $p53^+$ cells in the DYS are in the lower as well as upper layers of the epithelium; on the left, $p53^+$ cells are distributed sporadically in the basal-cell zone or along the papilla. (*c*) Sporadic distribution of $p53^+$ cells in BCH of mucosa surrounding the tumor. Only a few $p53^+$ cells are present among the proliferative basal cells. (*d*) $p53^+$ cells are distributed sporadically among the basal cells or along the papilla in BCH of mucosa. (*e*) $p53^+$ cells located above the basal-cell zone. A large number of parabasal cells are positive, but the single basal-cell layer is negative. (*f*) The multifocal origin of $p53^+$ cells is illustrated by the presence of several small $p53^+$ foci interspersed within hyperplastic epithelium. All $p53^+$ cells are proliferative basal or parabasal cells. (*g*) Discordant p53 protein accumulation in hyperplastic epithelia and solid tumor. Basal and parabasal cells in BCH are $p53^+$, but the tumor is $p53^-$. (*h*) A single, independent focus of CIS, 2 cm from the tumor margin, is surrounded by normal epithelia. This illustrates that multifocal precursor lesions arose independently throughout the esophageal specimen. (*a, d, e* and *g)* Bar: 100 µ m. (*b, f* and *h)* Bar: 200 µ m. (*c*) Bar: 50 µ m.

in BCH of the mucosa (Fig. 2*d*). However, in some cases (Fig. 2*e*), the $p53^+$ cells were distributed above the parabasal-cell layers and the single basal-cell layer remained $p53^-$.

In many cases, the $p53^+$ cells were concentrated heterogeneously in small clusters, or a few $p53^+$ cells were located above the basement membrane, which was surrounded by normal cells. Thus, a single precursor lesion often contained several small $p53^+$ foci interspersed within BCH or DYS areas (Fig. 2*f*), illustrating the multifocal origin of $p53^+$ cells among proliferative basal or parabasal cells. Figure 2*g* shows discordant p53 protein accumulation in hyperplastic epithelia and solid tumor tissue. Basal and parabasal cells in BCH were $p53^+$, but the solid tumor was $p53^-$. Evidence that multifocal precursor lesions arose independently throughout the esophageal specimens is shown in Figure 2*h*. In this case, a single, independent focus of CIS, 2 cm from the tumor margin, was surrounded by normal epithelia.

Extensive serial sectioning of the precursor lesions showed that the lesions were multifocal and did not result from spreading. For example, block 57C3 contained the isolated CIS shown in Fig. 2*h*; the CIS was 2 cm from the tumor margin and was 3 mm long. After 320 slices, the CIS was no longer visible. An adjacent block, 57C2, was aligned with block 57C3 and reverse re-embedded. After 440 slices, the CIS was no longer visible. Thus, this precursor lesion was observed in a total of $320 + 440 = 760$ contiguous slices, making the width of the lesion $760 \times 5\ \mu m = 3{,}800\ \mu m$ or 3.8 mm; the area was $3 \times 3.8\ mm = 11.4\ mm^2$. Similar observations were made for several other pairs of blocks; all analyses supported a multifocal origin for the lesions.

Analyses of the data in Table I indicated that 66% (33/50) of the patients had $p53^+$ tumors and 34% (17/50) had $p53^-$ tumors (Table II). Among those patients with $p53^+$ tumors, 100% (33/33) of the precursor lesions were $p53^+$; among those with $p53^-$ tumors, 43% (7/17) and 57% (10/17) of the associated precursor lesions were $p53^+$ and $p53^-$, respectively (Table II). In addition, the majority (69%, 290/420) of the precursor lesions associated with the $p53^+$ tumors were also $p53^+$, whereas only 22% (47/216) of precursor lesions associated with $p53^-$ tumors were $p53^+$ (Table II). From a total of 636 precursor lesions examined by HE staining, 53% (337/636) contained $p53^+$ cells (Tables II, III). Among the $p53^+$ tumors, the frequency of $p53^+$ precursor lesions increased from 64% for BCH to 70% for DYS to 88% for CIS. Among the $p53^-$ tumors, the frequency of $p53^+$ precursor lesions decreased from 25% for both BCH and DYS to 0% for CIS (Table II).

Table III shows the proportion of $p53^+$ lesions of a particular type (BCH, DYS or CIS) relative to the total number of lesions of that type within a specific distance from the tumor. Application of a 1-sided Cochran-Armitage-Yates trend test for the combined lesions (total) showed a significant negative trend ($p = 0.004$), indicating that significantly fewer precursor lesions were $p53^+$ at increasing distance from the tumor. However, a trend test of the individual precursor lesions indicated that significance was found only for BCH ($p = 0.006$); the other lesions had p values of 0.16 (DYS) and 0.44 (CIS). Thus, the data showed strong evidence that the probability of a BCH being $p53^+$ decreased significantly at increasing distance from the tumor; however, this was not the case for the other two precursor lesions.

TABLE II – ASSOCIATION BETWEEN p53 PROTEIN ACCUMULATION IN TUMORS AND PRECURSOR LESIONS

Tumor		Percentage (frequency) of $p53^+$ precursor lesions			
p53 status	% (number)	BCH	DYS	CIS	Total
+	66 (33/50)	64 (146/228)	70 (98/140)	88 (46/52)	69 (290/420)
−[1]	34 (17/50)	25 (30/120)	25 (17/69)	0 (0/27)	22 (47/216)

[1]Among the 17 $p53^-$ tumors, 41% (7/17) had associated precursor lesions that were completely $p53^-$; the remaining 59% (10/17) had associated precursor lesions in which 43% were $p53^+$ and 57% were $p53^-$.

A 2-sided Cochran-Armitage-Yates trend test was applied to determine if there were significant changes in the proportion of each class of lesion at increasing distance from the tumor, regardless of p53-staining status. This analysis showed that among the total precursor lesions the proportion of BCH increased significantly with increasing distance from the tumor ($p = 0.009$). In contrast, the proportion of CIS decreased significantly with increasing distance from the tumor ($p = 0.005$). There was no significant trend for DYS ($p = 0.41$).

DISCUSSION

Classical studies of the morphological features of esophageal cancer have shown that *(i)* multifocal regions of transformed cells may coalesce to form a single neoplasm, *(ii)* multicentric carcinomas exist and may be connected by transformed mucosa and *(iii)* tumors may develop in a multifocal manner in various parts of the mucosa (Shao-Ch'ien and Hsia, 1962; Gluckman *et al.*, 1980). For example, evaluation of 100 specimens of surgically resected esophageal SCC showed that 22 had precursor lesions at the periphery of the tumor or at the resected margin (Shao-Chi'ien and Hsia, 1962), epithelia adjacent to the carcinomas being always hyperplastic to some degree. The development of epithelial hyperplasia from minimal to extensive and finally to early carcinoma appeared to be a gradual process. Along with ours, other studies have extended these morphological observations to the molecular level and have provided additional evidence for the applicability of field cancerization theory to esophageal cancer.

Several studies have shown that p53 protein detected by IHC reflects the accumulation of p53 protein due to conformational changes in the protein as a consequence of missense mutations in the *p53* gene and not to over-expression of the gene (Bartek *et al.*, 1990; Gannon *et al.*, 1990; Sasano *et al.*, 1992). In addition, esophageal precursor lesions or tumors that have accumulated p53 protein tend to contain *p53* mutations, whereas those that have not tend not to contain *p53* mutations (Bennett *et al.*, 1991, 1992; Gao *et al.*, 1994; Lung *et al.*, 1996; Wang *et al.*, 1996). Consequently, the frequency and pattern of p53 protein accumulation detected in the present study likely reflect the frequency and pattern of mis-sense mutations in the *p53* gene.

Viewed in this light, the data presented here strongly support a multifocal origin of esophageal cancer, and the pattern of p53 protein accumulation is consistent with the field theory of cancerization (Slaughter *et al.*, 1953; Strong *et al.*, 1984). As first articulated (Slaughter *et al.*, 1953), this theory derived from a study of serial sections of a set of tumors of the oral cavity that were ≤ 1 cm in diameter in which separate foci of CIS or isolated islands of invasive SCC were present, including in the mucosa surrounding the large tumor. Based on the pattern of p53 protein accumulation in the precursor lesions and tumors described here, a model consistent with this view for esophageal cancer is illustrated in Figure 3.

The model shows regions in which BCH and DYS are adjacent; regions in which DYS and CIS are adjacent; and separate regions of BCH, DYS or CIS. The spatial relationship of these regions to each other as well as to the tumor is random. The model postulates that tumor development is multifocal and sporadic within the overall region (the cancerization field) and that p53 protein accumulation may be discordant between precursor lesions and the tumor. This is illustrated, *e.g.*, in Figure 2*e*, which shows the accumulation of p53 protein in parabasal cells but not in adjacent basal cells in esophageal precursor lesions, and in Figure 2*g*, which shows the accumulation of p53 protein in basal and parabasal cells in BCH but not in the adjacent tumor. Extensive serial sectioning of precursor lesions provided additional evidence that the lesions were multifocal in origin and not due to spreading. Although we cannot exclude the possibility that some small proportion of the lesions may have resulted from spreading, we found no evidence to support this view.

TABLE III – PROPORTION OF $p53^+$ LESIONS OF A PARTICULAR TYPE RELATIVE TO THE TOTAL NUMBER OF LESIONS OF THAT TYPE WITHIN A SPECIFIC DISTANCE FROM THE TUMOR

Precursor lesion	Percentage (frequency) of $p53^+$ lesions				
	0–1 cm	1.1–2 cm	2.1–3 cm	3–4 cm	Total
BCH	57 (47/82)	55 (67/122)	47 (40/86)	38 (22/58)	51 (176/348)
DYS	61 (37/61)	53 (38/72)	55 (27/49)	48 (13/27)	55 (115/209)
CIS	60 (21/35)	55 (12/22)	62 (8/13)	56 (5/9)	58 (46/79)
Total	59 (105/178)	54 (117/216)	51 (75/148)	42 (40/94)	53 (337/636)

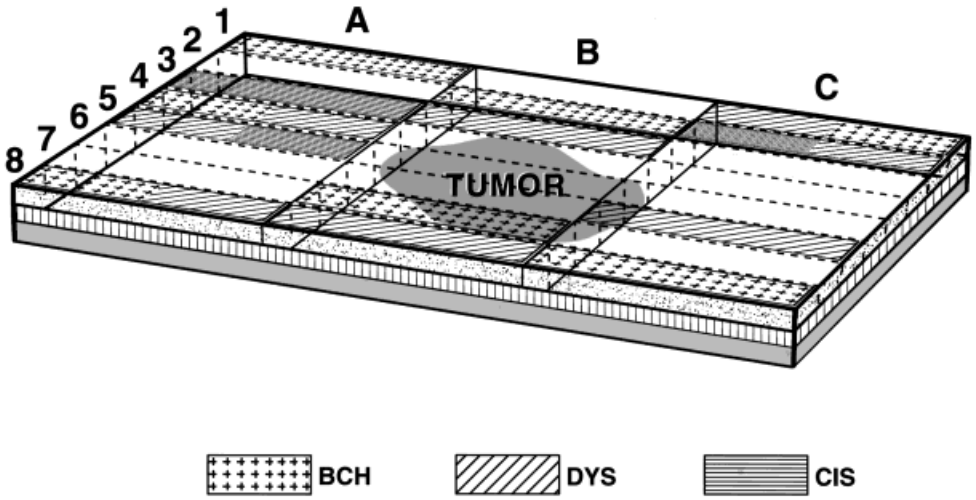

FIGURE 3 – Model illustrating the multifocal origin of precursor lesions and tumors throughout the esophageal cancerization field. The model includes regions of adjacent BCH and DYS, regions of adjacent DYS and CIS, and regions of separate BCH, DYS or CIS. These regions are randomly located relative to each other and the tumor. See text for further details.

Our analysis showed that among the $p53^-$ tumors, 22% (47/216) of the precursors lesions were $p53^+$ (Table II). Although no comparable study has been performed on esophageal tumors to which these data could be compared, one example of a $p53^-$ esophageal tumor with an adjacent $p53^+$ mucosa has been reported (Sasano *et al.*, 1992). An additional study (Nakanishi *et al.*, 1995) showed that among 12 cases of $p53^+$ squamous epithelium surrounding multicentric SSC, 75% (9/12) of the tumors were $p53^+$ but 25% (3/12) were $p53^-$. Although it is likely that the cells within a particular tumor are monoclonal, the model predicts that multiple tumors within a region would not necessarily be monoclonal and would, in contrast, likely be independent in origin.

Mutation data in the *p53* gene combined with p53 protein accumulation have provided additional evidence for the independent origin of multiple tumors within the esophagus. Analysis of pairs of biopsies (one from the middle and one from the lower third of the esophagus) from 55 symptom-free subjects found that 93% (51/55) of the patients exhibited p53 protein accumulation in both pairs of biopsies (Wang *et al.*, 1996). In one subject, the *p53* mutation in the middle-third biopsy (codon 161, GCC → GAC) was different from the mutation present in the lower-third biopsy (codon 159, GCC → CCC); only a single mutation was found in 4 other patients. Similarly, analysis of pre-invasive DYS or CIS adjacent to surgically resected SCC of the esophagus from 3 patients showed that all had p53 protein accumulation, and the *p53* gene mutations in one patient were different in the DYS compared with the invasive tumor (Bennett *et al.*, 1992). The second patient had a *p53* gene mutation in the invasive carcinoma, but the adjacent pre-invasive lesions contained wild-type *p53*; the *p53* gene was not mutated in the third patient.

Similar observations have been made for head-and-neck cancers, also supporting field cancerization for these tumors. In one study (Chung *et al.*, 1993), 21/31 subjects showed discordant *p53* mutations in the secondary *vs.* the primary tumor and single mucosal biopsies often contained several small $p53^+$ foci interspersed within hyperplastic or inconspicuous areas, suggesting that these tumors arose independently and were not monoclonal. This study (Chung *et al.*, 1993) provided the first molecular evidence for field cancerization of the upper aerodigestive tract. In a study of squamous and respiratory epithelia either adjacent to or at a significant distance from a set of primary head-and-neck tumors, p53 protein accumulation was found to be multifocal, and different *p53* mutations were found in different-tumor-distance epithelia from the same patients (Nees *et al.*, 1993). Finally, compelling molecular evidence for field cancerization of non-melanoma skin cancer of the head and neck has been provided by the finding of multiple and different *p53* mutations in tumor and adjacent non-malignant skin samples from 8 patients (Kanjilal *et al.*, 1995). Collectively, these studies confirm the presence of discordant mutations among non-neoplastic mucosa, primary cancers and secondary primary lesions, strongly indicating that *p53* mutations are multifocal within the region of cancerization for head-and-neck and aerodigestive tract tumors.

The decline in the proportion of $p53^+$ BCH at increasing distance from the tumor noted here is consistent with a field-cancerization model for esophageal SCC. Our data suggest that BCH is an early precursor lesion that likely converts to DYS and then to CIS. Thus, a greater proportion of BCH is $p53^+$ at regions close to the tumor than farther away from the tumor. In contrast, DYS and CIS are more advanced lesions that, presumptively, have had a greater probability of incurring a mutation in the p53 gene. Therefore, the frequency of $p53^+$ DYS and CIS lesions is not significantly different among such lesions whether they are close to or distant from the tumor. In other words, the data suggest that $p53^+$ staining is closely associated with DYS and CIS precursor lesions regardless of the distance of such lesions from the tumor. In

contrast, BCH are more likely to be $p53^+$ when located close to the tumor.

Consistent with this notion was our finding that the proportion of BCH increased significantly with increasing distance from the tumor, whereas the proportion of CIS decreased with increasing distance from the tumor; the proportion of precursor lesions that were DYS was not significantly different at various distances from the tumor. These data indicate that BCH is an early lesion, whereas DYS is in equilibrium regardless of distance from the tumor. The proportion of precursor lesions that are CIS is highest at distances closest to the tumor, suggesting that this lesion is a late event in tumorigenesis.

Studies on the frequency of $p53^+$ cells within certain lesions are also consistent with a field-cancerization theory for esophageal cancer. For example, the frequency of $p53^+$ cells among DYS adjacent to the tumor was higher than in the adjacent normal mucosa and controls (Jaskiewicz and De Groot, 1994). Based on IHC staining and analysis of esophageal tumors and biopsies, the proportion of $p53^+$ cells decreased among the precursor lesions as follows: CIS > DYS > BCH (Wang *et al.*, 1993). The accumulation of p53 protein also has been observed to increase with increasing grade of DYS in esophageal cancer. For example, the percentage of normal or DYS regions with p53 protein accumulation increased from 0% to 9% to 55% for specimens classified as normal, low- and high-grade DYS, respectively (Top *et al.*, 1995).

The accumulation of p53 protein has been associated with the proliferative status of esophageal tumors. Markers of cell proliferation and differentiation, such as Ki-67, EGF and TGF- α , were elevated along with p53 protein in esophageal DYS, and all but TGF- α were elevated in the tumor (Jaskiewicz and De Groot, 1994). We (Fig. 2*b*,*d*) and others (Wang *et al.*, 1993) have observed p53 protein accumulation along the papillae, which have been shown to exhibit a marker for abnormal cell proliferation in esophageal tumors (Yang and Lipkin, 1990). Increased p53 protein accumulation and apoptosis (based on *in situ* DNA nick end-labeling) have been associated with the onset of early carcinomatous invasion of esophageal tumors (Ohashi *et al.*, 1997). Thus, in addition to being an early event in the initiation and progression of esophageal SCC, p53 protein accumulation appears to coincide with increased proliferation, invasion and apoptosis in this organ.

As noted previously, the environmental carcinogens associated with esophageal cancer vary according to geographical region and may include agents as diverse as alcohol, tobacco, mycotoxins, nitrosamines and viruses (Blot, 1994). Nonetheless, the molecular basis for this disease holds features in common among patients regardless of their geographical origin. Among these features is the early accumulation of p53 protein. In addition, 6 codons within the *p53* gene are mutated frequently in esophageal carcinomas among patients from various regions in China (Lung *et al.*, 1996), suggesting a common environmental carcinogen(s).

Genetic factors may also play a role, such as a polymorphism of the *ALDH2* gene (Yokoyama *et al.*, 1996). We found the frequency of *ALDH2* heterozygotes to be 30% (15/50) among the esophageal cancer patients studied here (Table I). This is within the reported range of frequencies of heterozygotes among the Chinese population: 28%, 36% or 41% (Thomasson *et al.*, 1991; Goedde *et al.*, 1992; Muramatsu *et al.*, 1995). Thus, our results in a Chinese population differ from those in a Japanese population, in which 61% of the esophageal cancer patients were heterozygous at *ALDH2* compared to 43% of the controls (Yokoyama *et al.*, 1996). The results from the Japanese population suggested that the *ALDH2* polymorphism, combined with alcohol consumption, would lead to the accumulation of the carcinogen acetaldehyde, which could play a role in the etiology of the tumor. Although we did not have matched controls, our finding suggests that the *ALDH2* polymorphism may not play a role in the etiology of esophageal cancer in the population studied here. This is consistent with epidemiological studies showing that dietary factors other than alcohol consumption are associated with esophageal cancer among northern Chinese populations (Blot, 1994). We are studying this issue further with esophageal cancer patients and matched controls from northern China whose drinking habits are known.

In conclusion, the pattern and frequency of p53 protein accumulation in precursor lesions and tumors of the esophagus are consistent with the field theory of cancerization. The independent, multifocal origin of p53 protein accumulation supports a view in which primarily the middle and lower parts of the esophagus are exposed to carcinogenic agents that induce mutations throughout the exposed region. The lack of accumulated p53 protein in some tumors but its presence in at least 50% of BCH + DYS combined indicates that p53 protein accumulation is an early but not essential event in the genesis of esophageal cancer. The potential value of p53 protein accumulation as a biomarker for esophageal cancer is strengthened by the demonstration of circulating anti-p53 antibodies in most esophageal cancer patients with core domain mutations in the *p53* gene (Bennett *et al.*, 1996). Support for the potential field effect of cigarette smoking has been provided based on metaplastic changes in the lung (Mao *et al.*, 1997). Extensions of similar studies to esophageal cancer (Bennett *et al.*, 1992) should clarify the molecular basis of this disease.

ACKNOWLEDGEMENTS

We thank Drs. H.-H. Yang, G.-F. Zhao and P. Xiao for collecting the esophagus samples. D.T. was supported by a post-doctoral fellowship from the National Research Council; Z.F. was supported by a post-doctoral fellowship under a co-operative agreement between the US EPA and the Department of Epidemiology at the University of North Carolina at Chapel Hill. This manuscript was reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

REFERENCES

BARTEK, J., IGGO, R., GANNON, J. and LANE, D.P., Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene*, **5,** 893–899 (1990).

BENNETT, W.P., p53 alterations in progenitor lesions of the bronchus, esophagus, oral cavity, and colon. *Cancer Detect. Prevent.*, **19,** 503–511 (1995).

BENNETT, W.P., HOLLSTEIN, M.C., HE, A., ZHU , S.M., RESAU, J.H., TRUMP, B.F., METCALF, R.A., WELSH, J.A., MIDGLEY, C., LANE, D.P. and HARRIS, C.C., Archival analysis of p53 genetic and protein alterations in Chinese esophageal cancer. *Oncogene*, **6**, 1779–1784 (1991).

BENNETT, W.P., HOLLSTEIN, M.C., METCALF, R.A., WELSH, J.A., HE, A., ZHU, S.-M., KUSTERS, I., RESAU, J.H., TRUMP, B.F., LANE, D.P. and HARRIS, C.C., *p53* mutation and protein accumulation during multistage human esophageal carcinogenesis. *Cancer Res.*, **52**, 6092–6097 (1992).

BENNETT, W.P., LIANG, L., HE, A.-G., ZHU, S.M., TURSZ, T., JANIN, N. and TRIVERS, G.E., Circulating anti-p53 antibodies in esophageal cancer patients are found predominantly in individuals with p53 core domain mutations in their tumors. *Cancer Res.*, **56**, 4917–4921 (1996).

BLOT, W.J., Esophageal cancer trends and risk factors. *Semin. Oncol.*, **21,** 403–410 (1994).

CHUNG, K.Y., MUKHOPADHYAY, T., KIM, J., CASSON, A., RO, J.Y., GOEPFERT, H. and HONG, W.K., Discordant *p53* gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. *Cancer*, **53**, 1676–1683 (1993).

FIALKOW, P.J., Clonal origin of human tumors. *Biochem. biophys. Acta*, **458,** 283–321 (1976).

GALIANA, C., LOZANO, J.-C., BANCEL, B., NAKAZAWA, H. and YAMASAKI,

H., High frequency of Ki- *ras* amplification and *p53* gene mutations in adenocarcinomas of the human esophagus. *Mol. Carcinogen.*, **14,** 286–293 (1995).

GANNON, J.V., GREAVES, R., IGGO, R. and LANE, D.P., Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J.*, **9**, 1595–1602 (1990).

GAO, H., WANG, L.-D., ZHOU, Q., HONG, J.-Y., HUANG, T.-Y. and YANG, C.S., *p53* tumor suppressor gene mutation in early esophageal precancerous lesions and carcinoma among high-risk populations in Henan, China. *Cancer Res.*, **54,** 4342–4346 (1994).

GLEESON, C.M., SLOAN, J.M., MC GUIGAN, J.A., RITCHIE, A.J. and RUSSELL, S.E.H., Base transitions at CpG dinucleotides in the *p53* gene are common in esophageal adenocarcinoma. *Cancer Res.*, **55,** 3406–3411 (1995).

GLUCKMAN, J.L., CRISSMAN, J.D. and DONEGAN, J.O., Multicentric squamous-cell carcinoma of the upper aerodigestive tract. *Head Neck Surg.*, **3**, 90–96 (1980).

GOEDDE, H.W. and 15 OTHERS, Distribution of ADH_2 and ALDH2 genotypes in different populations. *Hum. Genet.*, **88**, 344–346 (1992).

GREENBLATT, M.S., BENNETT, W.P., HOLLSTEIN, M. and HARRIS, C.C., Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.*, **54,** 4855–4878 (1994).

JASKIEWICZ, K. and DE GROOT, K.M., *p53* gene mutant expression, cellular proliferation and differentiation in oesophageal carcinoma and non-cancerous epithelium. *Anticancer Res.*, **14,** 137–140 (1994).

KANJILAL, S., STROM, S.S., CLAYMAN, G.L., WEBER, R.S., EL -NAGGAR, A.K., KAPUR, V., CUMMINGS, K.K., HILL, L.A., SPITZ, M.R., KRIPKE, M.L. and A NANTHASWAMY, H.N., *p53* mutations in nonmelanoma skin cancer of the head and neck: molecular evidence for field cancerization. *Cancer Res.*, **55**, 3604–3609 (1995).

LIANG, Y.-Y., ESTÈVE, A., MARTEL-PLANCHE, G., TAKAHASHI, S., LU, S.-H., MONTESANO, R. and HOLLSTEIN, M., *p53* mutations in esophageal tumors from high-incidence areas of China. *Int. J. Cancer*, **61,** 611–614 (1995).

LUNG, M.L., CHAN, W.C., ZONG, Y.S., TANG, C.M.C., FOK, C.L., WONG, K.T., CHAN, L.K. and LAU, K.W., *p53* mutational spectrum of esophageal carcinomas from five different geographical locales in China. *Cancer Epidemiol. Biomarkers Prevention*, **5,** 277–284 (1996).

MAO, L. and 14 OTHERS, Clonal genetic alterations in the lungs of current and former smokers. *J. nat. Cancer Inst.*, **89,** 857–862 (1997).

MURAMATSU, T., ZU-CHENG, W., YI -RU, F., KOU -BAO, H., HEQIN, Y., YAMADA, K., HIGUCHI, S., HARADA, S. and KONO, H., Alcohol and aldehyde dehydrogenase genotypes and drinking behavior of Chinese living in Shanghai. *Hum. Genet.*, **96,** 151–154 (1995).

NAKANISHI, Y., NOGUCHI, M., MATSUNO, Y., SAIKAWA, M., MUKAI, K., SHIMOSATO, Y. and HIROHASHI, S., p53 expression in multicentric squamous cell carcinoma and surrounding squamous epithelium of the upper aerodigestive tract. *Cancer*, **75,** 1657–1662 (1995).

NEES, M., HOMANN, N., DISCHER, H., ANDL, T., EBDERS, C., HEROLD-MENDE, C., SCHULMANN, A. and BOSCH, F.X., Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: a possible molecular basis for the development of multiple tumors. *Cancer Res.*, **53,** 4189–4196 (1993).

NOWELL, P.C., The clonal evolution of tumor cell populations. *Science*, **194,** 23–28 (1976).

OHASHI, K., NEMOTO, T., EISHI, Y., MATSUNO, A., NAKAMURA, K. and HIROKAWA, K., Proliferative activity and p53 protein accumulation correlate with early invasive trend, and apoptosis correlates with differentiation grade in oesophageal squamous cell carcinomas. *Virchows Arch.*, **430,** 107–115 (1997).

SASANO, H., GOUKON, Y., NISHIHIRA, T. and NAGURA, H., *In situ* hybridization and immunohistochemistry of p53 tumor suppressor gene in human esophageal carcinoma. *Amer. J. Pathol.*, **141,** 545–550 (1992).

SHAO-CH'IEN, S. and HSIA, W., Squamous cell carcinoma of the esophagus: early carcinomatous changes of the epithelium adjacent to the principal lesion. *Chin. med. J.*, **81,** 557–567 (1962).

SLAUGHTER, D.P., SOUTHWICK, H.W. and SMEJKAL, W., ''Field cancerization'' in oral stratified squamous epithelium: clinical implications of multicentric origin. *Cancer*, **6,** 963–968 (1953).

STEMMERMANN, G., HEFFELFINGER, S.C., NOFFSINGER, A., HUI, Y.Z., M ILLER, M.A. and FENOGLIO-PREISER, C.M., The molecular biology of esophageal and gastric cancer and their precursors: oncogenes, tumor suppressor genes, and growth factors. *Hum. Pathol.*, **25,** 968–981 (1994).

STRONG, M.S., INCZE, J. and VAUGHAN, C.W., Field cancerization in the aerodigestive tract— its etiology, manifestation, and significance. *J. Otolaryngol.*, **13,** 1–6 (1984).

THOMASSON, H.R., EDENBERG, H.J., CRABB, D.W., MAI, X.-L., JEROME, R.E., LI, T.-K., WANG, S.-P., LIN, Y.-T., LU, R.-B. and YIN, S.-J., Alcohol and aldehyde dehydrogenase genotypes and alcoholism in Chinese men. *Amer. J. Hum. Genet.*, **48,** 677–681 (1991).

TOP, B., MOOI, W.J., KLUVER, S.G., BOERRIGTER, L., WISMAN, P., ELBERS, H.R.J., VISSER, S. and RODENHUIS, S., Comparative analysis of *p53* gene mutations and protein accumulation in human non-small-cell lung cancer. *Int. J. Cancer*, **64,** 83–91 (1995).

WANG, L.D., HONG, J.-Y., QIU, S.-L., GAO, H. and YANG, C.S., Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. *Cancer Res.*, **53,** 1783–1787 (1993).

WANG, L.D., SHI, S.T., ZHOU, Q., GOLDSTEIN, S., HONG, J.-Y., SHAO, P., QIU, S.-L. and YANG, C.S., Changes in p53 and cyclin D1 protein levels and cell proliferation in different stages of human esophageal and gastric-cardia carcinogenesis. *Int. J. Cancer*, **59,** 514–519 (1994).

WANG, L.D., ZHOU, Q., HONG, J.-Y., QIU, S.-L. and YANG, C.S., p53 protein accumulation and gene mutations in multifocal esophageal precancerous lesions from symptom free subjects in a high incidence area for esophageal carcinoma in Henan, China. *Cancer*, **77,** 1244–1249 (1996).

YANG, K. and LIPKIN, M., AE1 cytokeratin reaction patterns in different differentiation states of squamous cell carcinoma of the esophagus. *Amer. J. clin. Pathol.*, **94,** 261–269 (1990).

YOKOYAMA, A., MURAMATSU, T., OHMORI, T., HIGUCHI, S., HAYASHIDA, M. and ISHII, H., Esophageal cancer and aldehyde dehydrogenase-2 genotypes in Japanese males. *Cancer Epidemiol. Biomarkers Prevention*, **5,** 99–102 (1996).