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Genetic Progression and Heterogeneity Associated with the Development of Esophageal Squamous Cell Carcinoma

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ABSTRACT

Esophageal squamous cell carcinoma is a common fatal cancer, and Shanxi province, a region in north-central China, has some of the highest esophageal cancer rates in the world. Chromosomal regions with frequent allelic loss may point to major susceptibility genes that will assist us in understanding the molecular events involved in esophageal carcinogenesis and may serve as the basis for the development of markers for genetic susceptibility and screening for early detection of this cancer. This study was designed to identify events in the molecular progression of precursor and invasive lesions of squamous esophageal cancer. Twelve marker loci identified during our previous studies as having some of the highest rates of loss of heterozygosity (LOH) in invasive esophageal cancer were evaluated in laser-microdissected DNA obtained from low- and high-grade dysplastic lesions and invasive tumor foci from 10 fully embedded esophageal resection specimens. Each resection specimen contained a spectrum of disease, from epithelium that appeared histologically normal to invasive cancer, including a single dominant tumor surrounded by a region of precursor lesions (low- and high-grade dysplasia) and occasional "remote," nonadjacent precancerous foci. Using the 12 polymorphic markers, LOH was found in all of the three stages of disease. The frequency of LOH for all of the markers together increased with increasing disease severity. Among the informative low-grade dysplasia samples, LOH was detected with markers D3S1766 (3p), D4S2632 (4p), D9S910 (9q), and D13S1493 (13q), suggesting that LOH at these loci may be associated with early stages of tumor initiation and/or progression. LOH was detected among the informative high-grade (but not low-grade) dysplasia samples for the other eight markers tested, suggesting that LOH at these loci may occur later in the neoplastic process. In addition to the association between disease progression and these genetic changes, considerable genetic heterogeneity was found in each fully embedded resection specimen both between and within geographically separate neoplastic lesions.

INTRODUCTION

Esophageal SCC[3] is a common fatal cancer, and Shanxi province, a region in north-central China, has some of the highest esophageal cancer rates in the world (1, 2). Although epidemiological studies indicate that tobacco smoking and alcohol consumption are the major risk factors for squamous esophageal cancer in the low-risk regions of Europe and North America, the etiological agents in high-risk regions have yet to be convincingly identified. Within these high-risk regions, studies have shown a strong tendency toward familial aggregation, suggesting that genetic susceptibility, in conjunction with potential environmental exposures, may play a role in the etiology of this cancer (3–5). The potential for genetic susceptibility is further supported by the finding in tumors of frequent allelic deletions and other genetic abnormalities affecting individual tumor suppressor genes. Chromosomal regions with frequent allelic loss may point to major susceptibility genes that will assist us in understanding the molecular events involved in esophageal carcinogenesis and may serve as the basis for the development of markers for genetic susceptibility and screening for early detection of this cancer. Identifying the molecular events associated with early stages of esophageal SCC should be most helpful for identifying major susceptibility genes and carcinogenic mechanisms, but these events remain poorly understood.

Our group has performed two previous studies of allelic loss in esophageal SCC patients from Shanxi province. An initial genome-wide scan identified 46 markers covering 14 chromosomal regions with a very high frequency (≥75%) LOH (6). A subsequent study of esophageal SCC patients with and without family history of upper gastrointestinal tract cancer confirmed the frequent allelic loss in 14 chromosomal regions and successfully identified a locus on 13q (D13S894) where LOH was more common in patients with a family history of upper gastrointestinal cancer than in those without such a history and identified markers potentially related to metastasis (D6S1027 on 6q and D9S910 on 9q) and tumor grade (D4S2361 on 4p; Ref. 7).

The current study expands our efforts to understand the role of genetics in the etiology of SCC of the esophagus by attempting to identify molecular events associated with the development of precursor and invasive lesions. Twelve marker loci identified during the previous studies as having some of the highest rates of LOH in invasive esophageal cancer were evaluated in laser-microdissected DNA obtained from low- and high-grade dysplastic lesions and invasive tumor foci from 10 fully embedded esophageal resection specimens. The primary findings of the current study include a high level of genetic instability among the precursor lesions, the identification of four chromosomal regions (3p, 4p, 9q, and 13q) that undergo genetic change early in the neoplastic process, and a high level of intratumoral and preneoplastic genetic heterogeneity. This instability and heterogeneity exemplify the complexity of the genetic changes associated with the initiation and progression of esophageal SCC.

MATERIALS AND METHODS

Patient Selection. Patients presenting in 1998 to the Shanxi Cancer Hospital in Taiyuan, Shanxi province, People's Republic of China who were diagnosed with esophageal SCC and were considered candidates for curative surgical resection were identified and recruited to participate in this study. The study was approved by the Institutional Review Boards of the Shanxi Cancer Hospital and the United States National Cancer Institute. For this study, a total of 10 patients (8 males and 2 females) who underwent esophagectomy for SCC were selected. None of the patients had prior therapy, and Shanxi was the ancestral home for all of them. After obtaining informed consent, patients were interviewed to obtain information on demographic and cancer lifestyle risk factors. Data were also recorded concerning the clinical/pathological characteristics of the tumors.

Biological Specimen Collection and Processing. A 10-ml sample of venous blood was drawn from each patient before surgery, and genomic DNA was extracted and purified. The entire esophageal resection specimen obtained during surgery was fixed in ethanol (Fig. 1*A*) and then cut longitudinally into 0.4-cm wide columns and transversely into 2.0-cm long rows suitable for

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[3] The abbreviations used are: SCC, squamous cell carcinoma; LOH, loss of heterozygosity; LGD, low-grade squamous dysplasia; HGD, high-grade squamous dysplasia.

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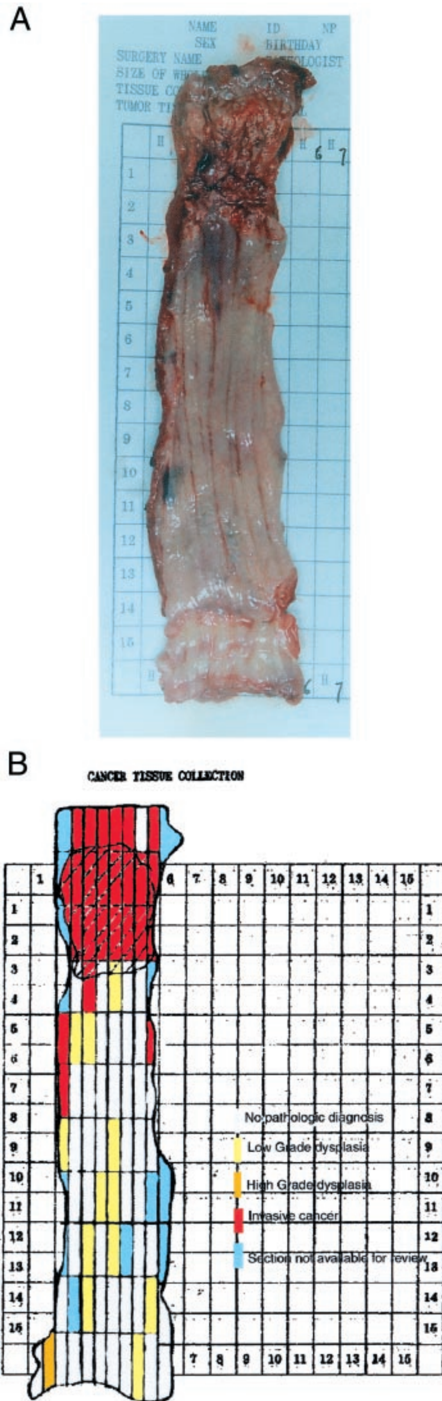

Fig. 1. *A*, esophageal resection specimen opened longitudinally to reveal mucosal surface, gastroesophageal junction (*bottom*), and erosive cancer (*top*). *B*, graphic illustration of the sectioning used to fully submit the specimen for histological review and the multifocality of disease present within each specimen. The histological grade of the worst epithelial change within each section is indicated (*gray*, no pathological diagnosis; *yellow*, low-grade dysplasia; *orange*, high-grade dysplasia; *red*, invasive tumor).

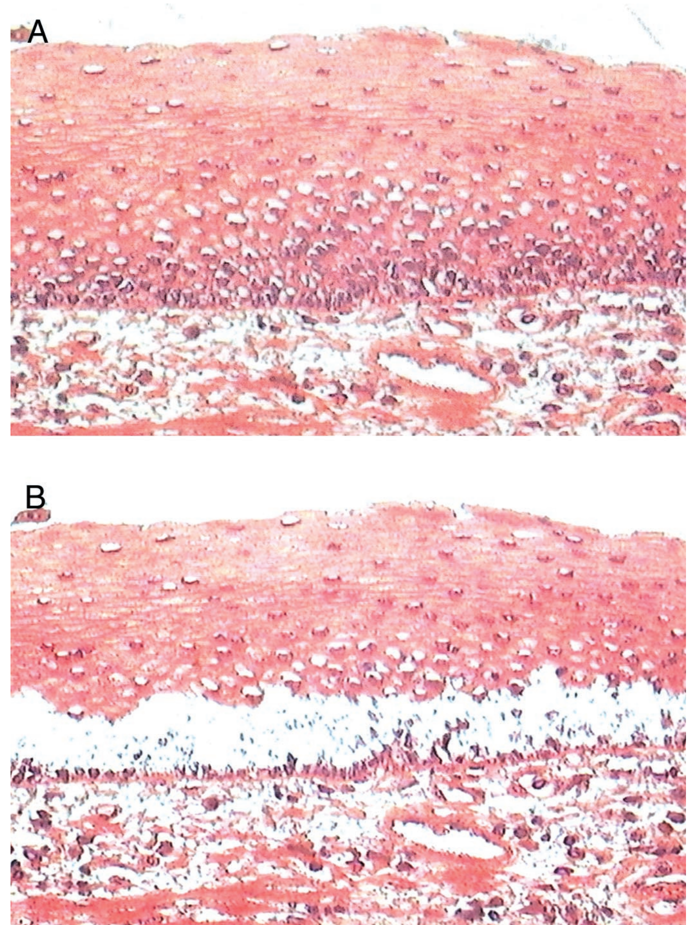

Fig. 2. Photomicrograph of a precursor lesion before (*A*) and after (*B*) microdissection.

paraffin embedding and histological processing (Fig. 1*B*). Each section was histologically reviewed and given a diagnosis based on the worst epithelial change present, according to criteria described previously (low-grade dysplasia = mild dysplasia; high-grade dysplasia = moderate or severe dysplasia; Ref. 8).

One section each of LGD, HGD, and invasive SCC were initially selected from each case based on the ease with which the lesional tissue could be microdissected and analyzed. With one exception, all of the sections used for testing were categorized based on the highest histological grade contained in that section. In one case, the cancer so extensively involved each section of the specimen that foci of LGD and HGD had to be microdissected from blocks with a worst histological diagnosis of invasive carcinoma. Normal control DNA was obtained from whole blood.

After our initial results identified several cases in which a different allele (upper *versus* lower) was lost among the three disease categories from the same patient, we returned to our mapped resection specimens and selected two additional sections of tumor from each of these patients for inclusion in a set of confirmatory LOH analyses. We then included these additional tumor sections along with the corresponding original precursor and invasive sections in a repeat analysis (n = 30 blocks total) using the specific polymorphic marker where a discrepancy was identified. To rule out artifactual allelic loss, these repeat studies also included the microdissection and analysis of adjacent lymphocytes and/or stromal cells from within each histological block for use, in conjunction with the blood DNA, as a normal control. In a few cases (n = 3), these additional analyses were performed more than once, for a total of up to three repeats.

Laser Capture Microdissection and Extraction of DNA. LGD, HGD, and invasive tumor cells were microdissected under direct light microscopic visualization using methods described previously (9). Briefly, unstained ethanol-fixed, paraffin-embedded 5-μm histological tissue sections were prepared on glass slides, deparaffinized twice with xylene, rinsed twice with 95% ethanol, stained with eosin, and air-dried. Specific cells of interest were selected from eosin-stained slides and microdissected by laser capture microdissection (Pixcell 100; Arcturus Engineering, Mountain View, CA; Fig. 2). Procured cells were immediately resuspended in a 50-μl solution containing 0.01 M Tris-HCl, 1 mM EDTA, 1% Tween 20, and 0.1 mg/ml proteinase K (pH 8.0) and incubated 2 nights at 37°C. The mixture was then boiled for 5 min to inactivate the proteinase K. Two μl of this solution were used for each PCR reaction.

Markers, PCR, and LOH Reading and Interpretation. This study included 12 polymorphic radiolabeled microsatellite markers that were selected

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to represent the marker loci identified during the previous studies as having the highest frequencies of LOH in invasive cancer. They included D3S1766, D3S4545, D4S2632, D8S1106, D9S1118, D9S910, GATA62F03, D11S1984, D13S1493, D13S894, D13S796, and D17S1303 from chromosomes 3p, 4p, 8p, 9p, 9q, 11p, 13q, and 17p, with heterozygosity frequencies ranging from 0.64–0.82. All but one of these markers contain tetranucleotide repeats.

DNA extracted from tumor cells microdissected from the resection specimen and genomic DNA from venous blood were used for each patient. PCR reactions were run in duplicate and carried out using a 10-μl final volume containing 1.0 μl of 10 × PCR buffer I [100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 15 mM $MgCl_2$], 1.0 μl of 1.25 mM deoxynucleotide triphosphate, 2 μl of DNA extraction buffer, 0.6 μl of each primer, 0.09 μl of AmpliTaq DNA polymerase (Perkin-Elmer), and 1 μCi of [α dCTP]. Typical PCR conditions were as follows: 10 min of denaturation at 94°C and then for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. An elongation step at 72°C for 10 min was added to the final cycle. The PCR products were mixed with 5 μl of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol), denatured for 6 min at 95°C, and chilled on ice until loaded onto a 6% polyacrylamide gel. Samples were electrophoresed at 60 W for 1–3 h and radiographed for 1–2 days using Kodak BioMax MR films.

LOH Reading and Interpretation. LOH was defined as either complete or near complete loss of a band in the tumor sample relative to the corresponding normal DNA. Convincing evidence of a homozygous deletion in a tumor sample was not observed at any of the 12 markers used. The results were reviewed independently by three investigators (M. R., N. H., and M. E. B.). Differences in interpretation were resolved during a group review.

Calculation of the Frequency of Allelic Loss. The frequency of allelic loss at each chromosome locus was calculated separately for LGDs, HGDs, and invasive tumors, using only the results from the initial analysis unless otherwise noted. In each histological category, the frequency was calculated as the number of lesions with allelic loss at that locus divided by the number of informative lesions at that locus.

RESULTS

Patient Characteristics. The patients included eight men and two women, ranging in age from 44 to 73 years. The location and characteristic of the tumors and the patients' histories of selected cancer risk factors are given in Table 1. Two (20%) of the patients had a family history of upper gastrointestinal cancer (*i.e.,* one had a mother with esophageal cancer and a father with cancer of the gastric cardia, and one had a maternal aunt's daughter with esophageal cancer).

Summary of the Esophageal Histological Diagnoses. Ten resection specimens (Table 2), consisting of an average of 68 (range, 40–100) surgical blocks each, were histologically reviewed. Each resection specimen contained a spectrum of disease, from normal epithelium to invasive cancer, including a single dominant tumor surrounded by a region of precursor lesions (LGD and/or HGD) and occasional "remote," nonadjacent precancerous foci (Fig. 1*B*). Half (50%) of the sections contained invasive cancer as their worst histological diagnosis, followed in prevalence by nonprecancerous epithelium (*i.e.,* normal epithelium or esophagitis, 31%; LGD, 12%; and HGD, 5%) lesions. Although each histological section was classified by its worst histological disease, many sections contained multiple microscopic foci of less severe histological findings. Thus, although almost every section from case 352 had a worst histological diagnosis of invasive carcinoma, foci of LGD and HGD could also be identified within some of these sections.

Table 2 *Esophagectomy specimens, including the worst epithelial histology/block*

Pathology no.	Nonprecancerous lesions[a]	LGD	HGD	SCC	Unsatisfactory for histological evaluation	Total no. of blocks
326	5 (12)	5 (12)	2 (5)	27 (68)	1 (2)[b]	40
337	47 (54)	9 (10)	11 (13)	17 (20)	3 (3)	87
339	9 (16)	12 (21)	3 (5)	32 (57)	0 (0)	56
341	1 (2)	3 (6)	7 (14)	40 (78)	0 (0)	51
342	44 (54)	13 (16)	1 (1)	23 (28)	0 (0)	81
344	16 (25)	16 (25)	2 (4)	29 (45)	2 (3)	65
345	24 (49)	10 (20)	2 (4)	13 (26)	0 (0)	49
347	42 (42)	6 (6)	6 (6)	43 (43)	3 (3)	100
352 [c]	0 (0)	0 (0)	0 (0)	82 (99)	1 (1)	83
4001	22 (32)	11 (16)	1 (2)	34 (50)	0 (0)	68
Total	210 (31)	85 (12)	35 (5)	340 (50)	10 (2)	680

[a] Nonprecancerous lesions included normal epithelium and esophagitis.
[b] No. of blocks (row percentage).
[c] Foci of LGD and HGD for genetic analysis were selected from within sections containing invasive cancer.

Frequency of LOH. Using the 12 polymorphic markers, LOH was found in all of the three stages of disease (Table 3). The total frequency of LOH for all of the markers in all of the cases increased with increasing disease severity: 9% for low-grade dysplasia (LOH seen with 4 markers), 41% for high-grade dysplasia (LOH seen with all of the 12 markers), and 57% for invasive cancer (LOH seen with all of the 12 markers), resulting in a statistically significant difference between low-grade dysplasia and both the high-grade dysplasia and invasive lesions in the frequency of LOH among the informative cases for all of the 12 markers combined (χ^2; $P < 0.001$). The frequency of LOH for individual markers also increased with increasing disease severity for all but one marker, D3S1766, which showed a slightly higher frequency of LOH among the HGD lesions (83%) than the invasive cancers (67%), but these differences were not statistically significant.

Among the informative low-grade dysplasia samples, LOH was detected with markers D3S1766, D4S2632, D9S910, and D13S1493, suggesting that LOH at these loci may be associated with early stages of tumor initiation and/or progression. LOH was detected among the informative high-grade (but not low-grade) dysplasia samples for the other eight markers tested, suggesting that LOH at these loci may occur later in the neoplastic process.

Genetic Heterogeneity. Within this analysis, there were 40 instances in which LOH was found in a lesion less severe than invasive cancer, and a more severe lesion was also analyzed with the same

Table 1 *Demographics, clinical/pathological characteristics, and lifestyle cancer risk factors of the esophageal cancer patients*

Case no.	Age/sex	Tumor location	Pathological grade	Stage	Lymph node metastasis (no. positive/ no. examined)	Tobacco use	Alcohol consumption	Pickled vegetable consumption	Hot food consumption	Family history of gastrointestional cancer
326	57/M	Lower	G2	III	Y (2/7)	Y	Y	No	Never	No
337	67/M	Middle	G2	III	N (0/9)	N	N	No	Daily	No
339	44/M	Lower	G2	III	N (0/10)	N	N	Unknown	Weekly	Yes
341	51/F	Middle	G3	III	Y (11/19)	N	N	Unknown	Seldom	No
342	73/M	Upper	G1	II	N (0/4)	N	Y	No	Seldom	No
344	49/M	Middle	G2	III	Y (1/8)	Y	Y	Unknown	Daily	No
345	66/M	Middle	Unknown	III	N (0/13)	Y	N	Unknown	Daily	No
347	63/M	Middle	G2	III	Y (1/22)	Y	Y	No	Daily	No
352	60/F	Lower	G2	II	N (0/6)	N	N	Unknown	Daily	Yes
4001	66/M	Middle	G2	III	Y (2/12)	Y	Y	No	Seldom	No

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Table 3 *LOH frequencies of 12 polymorphic microsatellite markers at three stages of esophageal squamous neoplasia*

Marker no.	Name	Chromosome location	Low-grade dysplasia[a]	High-grade dysplasia[a]	Invasive cancer[a]
1	D3S1766	3p14-p21	2/6 (33)	5/6 (83)	4/6 (67)
2	D3S4545	3p24.3-p25	0/5 (0)	2/6 (33)	5/6 (83)
3	D4S2632	4p12-p14	1/9 (11)	3/10 (30)	6/10 (60)
4	D8S1106	8p21.2-p23.3	0/7 (0)	1/7 (14)	3/7 (43)
5	D9S1118	9p11-q11	0/9 (0)	3/9 (33)	5/9 (56)
6	D9S910	9q22.3-q31	2/2 (100)	2/2 (100)	2/2 (100)
7	GATA62F03	9p23-p24	0/7 (0)	5/7 (71)	5/7 (71)
8	D11S1984	11p15.3-p15.5	0/9 (0)	3/9 (33)	3/9 (33)
9	D13S1493	13q11	2/9 (22)	5/10 (50)	6/10 (60)
10	D13S894	13q12.3-q14.2	0/4 (0)	1/4 (25)	2/4 (50)
11	D13S796	13q32-q34	0/6 (0)	1/6 (17)	3/6 (50)
12	D17S1303	17p11.2-p12	0/7 (0)	3/7 (43)	3/7 (43)
Total[b]			7/80 (9)	34/83 (41)	47/83 (57)

[a] No. of cases with LOH/number of informative cases (percentage).

[b] There is a statistically significant difference between low-grade dysplasia and both the high-grade dysplasia and invasive lesions in the frequency of LOH among the informative cases for all of the 12 markers combined (χ^2; $P < 0.001$). However, the differences in LOH frequency among the histological grades for each marker were not statistically significant.

marker (Table 4). Thirty-one times (78%) the more severe lesion showed LOH of the same allele as that seen in the less severe lesion. In one instance (2%), a LOH pattern changed to a heterozygous pattern. Eight times (20%) LOH (one allele lost) changed to LOH of the complementary allele. In case 326, marker D3S1766 showed LOH in high-grade dysplasia but a heterozygous pattern (no LOH) in the invasive tumor from the same patient (Table 4). Examples of changes from LOH of one allele to LOH of the complementary allele were seen in case 326 with marker D9S910 (Fig. 3) and case 337 with markers D4S2632 and D17S1303 (Table 4). These pattern differences suggest a lack of clonality between the less and more severe lesions or genetic heterogeneity within the evolution of a single clone.

To further explore this pattern of genetic heterogeneity, we reanalyzed the original precursor lesions and tumor sections in the cases with "atypical" LOH patterns and included two additional tumor sections from each case. For 2 of the 10 cases (cases 352 and 326), there were differences in the patterns of allelic loss in the three separate sections of tumor. With marker D9S1118, both the first and second analyses of the original tumor section from case 352 showed LOH with retention of the upper allele (1^1); whereas a second area from the same tumor showed a heterozygous pattern, and a third area showed LOH with retention of the lower allele (1^2; Fig. 4). Similar differences were also seen in this case with polymorphic marker GATA62F03 and in case 326 with marker D9S910 (data not shown).

The repeat analysis included microdissected lymphocytes and/or adjacent stroma as an intrasection control. In all of the instances, identical LOH results were obtained with the normal control blood

Table 4 *LOH analysis of individual microsatellite markers in three stages of esophageal squamous neoplasia*[a]

Marker	326	337	339	341	342	345	347	344	352	4001
D3S1766										
LGD	2	0	1^2	0	1^2	0	2	2	2	0
HGD	1^1	0	1^2	0	1^2	0	1^1	1^2	2	0
Tumor	2	0	1^2	0	2	0	1^1	1^2	1^2	0
D3S4545										
LGD	–	0	0	2	0	2	2	2	2	0
HGD	2	0	0	2	0	2	1^2	2	1^1	0
Tumor	1^1	0	0	2	0	1^2	1^2	1^2	1^2	0
D4S2632										
LGD	–	2	2	1^1	2	2	2	2	2	2
HGD	2	1^2	2	1^1	1^1	2	2	2	2	2
Tumor	1^1	1^1	2	1^1	1^1	2	1^2	2	2	1^1
D8S1106										
LGD	0	0	2	2	2	2	2	0	1^2	2
HGD	0	0	2	2	2	2	2	0	1^{1^2}	2
Tumor	0	0	2	1^2	1^1	2	2	0	1^2	2
GATA62F03										
LGD	0	0	2	2	2	0	2	2	2	2
HGD	0	0	2	2	1^1	0	1^1	1^1	1^1	1^2
Tumor	0	0	2	2	1^1	0	1^1	1^1	1^2	1^2
D9S1118										
LGD	2	0	2	2	2	2*	2	2	2	2
HGD	2	0	2	2	2	1^2*	2	2	1^2	1^1
Tumor	2	0	2	2	2	1^1*	1^2	1^1	1^1	1^1
D9S910										
LGD	1^2	0	0	0	0	1^2	0	0	0	0
HGD	1^2	0	0	0	0	1^2	0	0	0	0
Tumor	1^1	0	0	0	0	1^1	0	0	0	0
D11S1984										
LGD	2	2	2	2	0	2	2	2	2	2
HGD	1^1	2	2	2	0	2	2	1^2	1^1	2
Tumor	1^1	2	2	2	0	2	2	1^2	1^1	2
D13S894										
LGD	0	0	0	0	2	2	2	2	0	0
HGD	0	0	0	0	2	2	2	1^2	0	0
Tumor	0	0	0	0	2	2	1^1	1^2	0	0
D13S1493										
LGD	–	2	2	2	1^1	2	2	1^1	2	2
HGD	1^1	2	2	1^1	1^1	2	1^1	1^1	2	2
Tumor	1^1	2	2	1^1	1^1	2	1^1	1^1	1^1	1^1
D13S796										
LGD	2	0	2	2	0*	2	0	0	2	2
HGD	2	0	2	2	0*	2	0	0	1^2	2
Tumor	1^1	0	2	1^2	0*	2	0	0	1^2	2
D17S1303										
LGD	0	2	2	2	2	0	2	2	2	0
HGD	0	1^1	2	2	2	0	1^1	1^1	2	0
Tumor	0	1^2	2	2	2	0	1^1	1^1	2	0

[a] 1, LOH (+); 1^1 or 1^2; retention of allele 1 or allele 2; 2, heterozygous; 0, homozygous; *, initial analysis failed and results shown are from a repeat analysis; –, no results.

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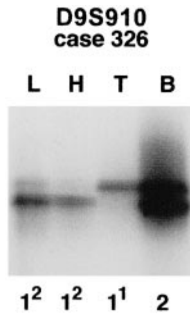

Fig. 3. Gel electrophoresis illustrating a change in pattern from LOH of one allele to LOH of the complementary allele for case 326 with marker D9S910. Both low-grade (*L*) and high-grade (*H*) dysplasias show loss of the upper allele and retention of the lower allele (1^2), whereas the invasive cancer (*T*) shows an opposite pattern with loss of the lower and retention of the upper allele (1^1). The control DNA from blood (*B*) shows a heterozygous pattern (*2*).

and the corresponding lymphocytes and/or stroma, suggesting that preferential specific amplification of an allele was not responsible for the genetic heterogeneity seen among a patient's lesions.

DISCUSSION

Several studies report finding a high frequency of genetic changes in SCC of the esophagus (10–13). The development of this and many other tumors is believed to be the result of a multistep process involving a series of genetic events and the subsequent evolution of a clonal population of cells with an accumulation of genetic errors (14–18). Given the apparent high number of these molecular changes found in invasive tumors, it is necessary to analyze precursor lesions to identify the changes most closely associated with initiation and/or progression of esophageal SCC.

In our study, we used 12 polymorphic microsatellite genetic markers identified previously (6, 7) in this population as having a high rate of LOH, located on 8 chromosome arms, to analyze a set of low- and high-grade precursor lesions and invasive tumors selected from 10 fully embedded esophageal resection specimens. The resection specimens were remarkable for their diversity and extent of disease, a pattern that has been referred to previously (19, 20) as field cancerization, which is thought to result from prolonged exposure of the entire upper aerodigestive tract to the same carcinogen(s). This study used the latest laser microdissection techniques, with a spot size of approximately two cells, to harvest DNA from specific cellular foci in an effort to identify the genetic changes associated with the initiation and progression of precursor lesions.

We found that the number of markers showing any LOH and the frequency of LOH seen in these 12 markers increased with the histological severity of the disease. These findings support the idea that an accumulation of genetic changes is associated with the progression of normal mucosa to precursor lesions to cancer and are consistent with previous studies on the genetic progression of Barrett esophagus to adenocarcinoma and studies of the development of prostate, lung, colon, head and neck, and breast cancers (14–18, 21–24). They are also consistent with the progressive increase in relative risk for developing SCC of the esophagus found with increasing histological severity of these precursor lesions (25) and the hypothesis that a threshold of genetic alterations may need to be surpassed before invasive cancer develops (26).

Our study identified LOH among the LGD lesions at four loci, on chromosomes 3p14-p21, 4p, 9q, and 13q. This finding suggests that genetic changes in these regions may be involved in the earlier stages of tumor initiation and/or progression. LOH on 3p (27, 28) and 9q (28) have been reported previously in precursor lesions of the esophagus. Damage to chromosomes 3 and 9 has also been detected in squamous metaplasia and hyperplasia of the bronchus, lesions believed to occur early in the development of lung tumors (17, 29, 30). The significance of these early genetic changes may be related to known genes of interest located in these chromosomal regions, including the fragile histidine triad gene (*FHIT*) on 3p14.2 (31–34), the retinoblastoma gene (*Rb1*) on 13q14.2 (35, 36), and the breast cancer susceptibility locus 2 (*BRCA2*) on 13q12.1 (37, 38). Also of interest is the annexin I gene located at 9q11-q22 (39). This gene produces a calcium- and phospholipid-binding protein that has been implicated in regulation of cell differentiation and growth (40). Annexin I expression is believed to correlate with malignant breast cancer progression and is most likely involved at an early stage of breast cancer development (41). Recent proteomic analysis of tumor specimens from the Shanxi region showed a loss of annexin I protein expression in tumors compared with normal tissues from the same patients (42). Thus, annexin I may play an important role in the development of esophageal cancer.

In our patients, eight markers on chromosomes 3p24.3-p25, 8p, 9p, 11p, 13q, and 17p were first found to show LOH in HGD, suggesting that these loci may be involved in the later stages of tumor development, including invasion.

In addition to the association between disease progression and these genetic changes, each fully embedded resection specimen was found to contain abundant evidence of genetic heterogeneity. This included instances of apparent reversion from LOH to heterozygosity or change in the allele that was lost (*i.e.,* upper *versus* lower band on gel electrophoresis) among geographically distinct lesions of varying histological severity and among separate regions taken from the same tumor. Such differences in LOH pattern among geographically distinct lesions may represent separate primary growth of different neoplastic clones, but the differences seen among the separate sections of the same gross tumor mass probably reflect genetic heterogeneity within the evolution of lesions that started out as single clones.

Several studies have reported similar findings of genetic heterogeneity in precursor lesions and invasive cancers, consistent with the simultaneous development of separate clones or the presence of genotypic divergence during the clonal evolution of esophageal cancer. For instance, Shimada *et al.* (28) found differences in the specific allele lost in foci of mild dysplasia, severe dysplasia, and contiguous cancer in cases of esophageal SCC. Such diversity among separate neoplastic growths has also been reported in Barrett esophagus containing foci of glandular dysplasia and adenocarcinoma (17, 43). In these reports, high-grade lesions had genetic abnormalities not found in the synchronous invasive cancer. A study of paired dysplastic

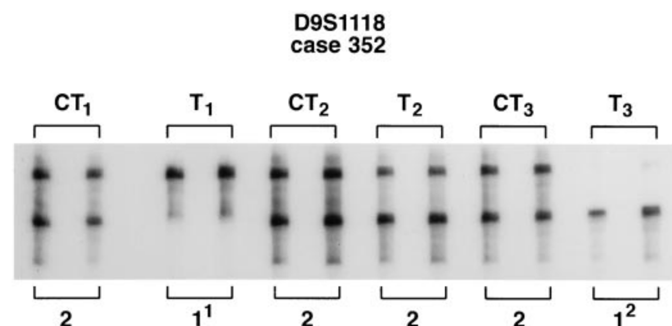

Fig. 4. Gel electrophoresis illustrating the differences in patterns of allelic loss among three areas of the same tumor mass. With marker D9S1118, the original tumor section (T_1) from case 352 showed LOH with retention of the upper allele (1^1); whereas a second area (T_2) showed a heterozygous pattern (*2*), and a third area (T_3) showed LOH with retention of the lower allele (1^2). This analysis included microdissected lymphocytes and/or adjacent stroma as an intrasection control (CT_{1-3}), all of which showed a heterozygous pattern (*2*).

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lesions and invasive oral SCCs also showed that a majority of the dysplastic lesions had genetic alterations that were not present in the concurrent cancers, including a case of paired samples that showed loss of different alleles (24). Other studies of the aerodigestive tract show that multiple primary tumors and original and second primary cancers may contain different TP53 mutations (44, 45), and analysis of multiple noninvasive foci of SCC of the lung reported that more than half of the neoplastic lesions may have arisen as independent clonal events (18). Similar reports (15, 46, 47) of unrelated clones among geographically separate precursor lesions and primary tumor foci have also been reported in breast and prostate.

The intralesional genetic heterogeneity found in our study is consistent with the idea that genetic changes acquired at an early stage of carcinogenesis are probably shared by most tumor cells, but changes acquired at later stages of tumor evolution may be restricted to particular tumor subclones (48, 49). The high frequency and common finding of such heterogeneity in our lesions may reflect the high specificity of our laser microdissection approach. This technique may have increased our ability to identify small, genetically dissimilar subclones within the same histological lesion.

The degree of genetic instability, including the frequency of LOH and the amount of genetic heterogeneity, seen in our precursor lesions is striking. Even our low-grade dysplasias showed multiple genetic changes, implying that the earliest genetic changes associated with tumor initiation and promotion might possibly occur in histologically normal epithelium. Clonal TP53 mutations have been identified with a relatively high frequency in histologically normal tissues of the upper aerodigestive tract, especially in patients with multiple tumors (50), and allelic loss has been found in samples of normal bronchial epithelium samples from squamous cell lung cancer and breast cancer patients (18, 51, 52). Additional studies examining histologically normal mucosa from patients with esophageal neoplastic lesions are warranted.

In conclusion, our study describes an extensive array of histological changes associated with the development of esophageal SCC in patients from a high-risk region in China, a pattern compatible with the notion of a carcinogen field effect. It shows that the development of esophageal SCC is associated with a high degree of genetic instability and that this instability occurs even among some of the earliest histologically recognizable precursor lesions. It identifies several specific chromosomal regions that appear to undergo genetic change early (3p14–21, 4p, 9q, and 13q) or late (3p24.3-p25, 8p, 9p, 11p, 13q, and 17p) in the evolution of SCC. Finally, it suggests that this cancer develops in a background of multiple geographically separate and clonally independent foci of neoplastic tissue, and it contains genetically distinct subclones within individual precursor and invasive lesions.

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Genetic Progression and Heterogeneity Associated with the Development of Esophageal Squamous Cell Carcinoma

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