



Microsatellite alterations in esophageal dysplasia and squamous cell carcinoma from laser capture microdissected endoscopic biopsies

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Abstract

Esophageal squamous cell carcinoma (ESCC), with a 5 year survival below 15%, is one of the most common fatal cancers worldwide. Significant reduction in mortality may be achieved by detecting and treating asymptomatic precursor lesions and curable early cancers. To explore this possibility and look for potential early detection markers, we examined alterations in 16 microsatellite markers in laser capture microdissected (LCM) endoscopic biopsies from the esophagus, including 15 dysplasias and 22 ESCCs, in patients from Shanxi Province, a region in north-central China. We found a significant increase in the total frequency of allelic loss with increasing disease severity. Allelic loss was seen in 2% of the markers in patients with low grade dysplasia (LGD), 15% of the markers in patients with high grade dysplasia (HGD), and 35% of the markers in patients with ESCC. Ten different markers (D3S4513, D5S2501, D8S1106, D9S118, D9S910, D13S1493, D13S894, D13S796, D15S655, and D17S1303) showed allelic loss in one or more of the premalignant lesions tested. The frequency of microsatellite instability (MSI) also increased with histological severity, from 22% in LGD to 33% in HGD and 59% in ESCC. These results indicate that the development of ESCC is associated with genetic instability, that this instability can be detected in endoscopic biopsies of recognized precursor lesions in patients without invasive cancer, and that these markers may be useful as predictive markers in the early detection of ESCC. Finally, we also report methodologic/technical modifications that enhance the use of LCM for screening endoscopic biopsies. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Allelic loss; Esophageal cancer; Precursor lesion; Microdissection

1. Introduction

Esophageal cancer is a common fatal malignancy throughout the world. Fewer than 15% of patients survive longer than 5 years after diagnosis, principally because most tumors are asymptomatic and go undetected until they present at an incurable stage. Significant reduction in esophageal cancer mortality will

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probably require new strategies to detect and treat asymptomatic precursor lesions and early cancers.

Molecular events associated with the initiation and progression of esophageal squamous cell carcinoma (ESCC) remain poorly understood. Chromosomal regions with frequent allelic loss may point to major susceptibility genes that will assist in understanding the molecular events involved in esophageal carcinogenesis and may serve as the basis for the development of markers for genetic susceptibility testing and screening for the early detection of this cancer. Our group has performed several studies of allelic loss in invasive ESCC patients from Shanxi Province, a region in north-central China with some of the highest esophageal cancer rates in the world [1,2]. An initial genome-wide scan identified 46 microsatellite markers from 14 different chromosomal regions with a very high frequency ($\geq 75\%$) of allelic loss [3]. These results were confirmed in a separate, larger study [4]. We followed these efforts up with a study of squamous precursor and invasive lesions from fully embedded esophageal resection specimens and further confirmed the high frequency of allelic loss at several selected markers on chromosomes 3p, 4p, 9q and 13q, and found evidence to suggest that allelic loss at these loci may be observed at early stages of tumor initiation and/or progression, and is more common in high-grade than in low-grade precursor lesions [5]. This increasing concentration of molecular alterations in high-grade precursor lesions is consistent with the findings of our prospective follow-up study in which increasing grade of precursor histologic abnormality were shown to predict increasing risk for the subsequent development of ESCC [6].

As of yet, the molecular changes identified in endoscopic biopsies from squamous epithelial dysplasia of the esophagus and early ESCC have not been reported. Identification of sensitive and specific markers of premalignancy should make possible screening of asymptomatic individuals from high risk populations for prevention strategies such as chemoprevention or local therapy. To explore this idea further, in this study we examined microsatellite alterations in laser capture microdissected endoscopic biopsies containing either squamous dysplasia or early ESCC using 16 microsatellite markers that showed frequent allelic loss in invasive ESCC in our previous studies.

2. Materials and methods

2.1. Patient selection

Between January and December 2000 over 400 outpatients underwent endoscopic examination at the Shanxi Cancer Hospital as part of their evaluation for upper gastrointestinal symptoms. One to two biopsies were routinely collected from each of these patients as part of the examination. A sample of 37 patients were selected for study from among those with biopsies that were of adequate size and orientation, including 15 with dysplasia of the esophagus and 22 with ESCC.

2.2. Biopsy processing and reading

Endoscopic biopsies were alcohol-fixed and paraffin-embedded, and obtained from the Pathology Tissue Bank of Shanxi Cancer Hospital in Taiyuan, Shanxi Province, China. For each biopsy, 5 μm thick serial sections were prepared and 6–7 sections were put on each uncoated slide in a ribbon. Routine haematoxylin and eosin-stained slides were evaluated by three pathologists (N.L., M.R., S.D.). Squamous dysplasia was defined as nuclear atypia (enlargement, pleomorphism and hyperchromasia), loss of normal cell polarity, and abnormal tissue maturation in the squamous epithelium, without invasion through the basement membrane. In low grade dysplasia (LGD), these abnormalities were confined to the lower half of the epithelium, whereas in high grade dysplasia (HGD) they also involved the upper half of the epithelium. In squamous cell carcinoma, malignant squamous cells had invaded through the basement membrane into the lamina propria or deeper layers [7].

2.3. Laser capture microdissection and DNA extraction

Dysplasia or tumor and adjacent normal cells from the same biopsy were microdissected for each case under direct light microscopic visualization using methods described previously [8]. Briefly, unstained ethanol-fixed paraffin-embedded 5 μm thick histological tissue sections were prepared on glass slides, deparaffinized twice with xylene, rinsed twice with 95% ethanol, stained with eosin, and air-dried. Speci-

fic cells of interest were selected from the eosin-stained slides and microdissected by laser capture microdissection (LCM) (Pixcell 100, Arcturus Engineering, Mountain View, CA). The spot size was 15 μm , power was 60 mW, and duration was 5.5 ms. The CapSure™ film carrier was designed to be placed into a reagent tube immediately after LCM. The tube, containing 80–100 μl solution with 0.01 M Tris-HCL, 1 mM ethylene-diamine tetra-acetic acid (EDTA), 1% Tween-20, and 0.1 mg/ml proteinase K (pH 8.0), was incubated 48 h at 37 °C. The mixture was then boiled for 10 min to inactivate the proteinase K. The 260/280 ratio and concentration (ng/ μl) of LCM DNAs were measured using Biophotometer (Eppendorf AG, Hamburg, Germany). One drop of mineral oil was put on the top of the LCM products in the tubes, and the tubes were stored at -20 °C. Three to four microliters of this solution were used for each polymerase chain reaction (PCR). Each LCM DNA sample was tested by PCR using β -actin as a control and run on a 1.5% agarose gel before it was tested for microsatellite alterations. If the PCR products did not show clear bands on agarose gel, the LCM DNA was cleaned using either a Micro Bio-Spin chromatography column (BIO-RAD, Hercules, CA) or by adding more digestion solution in the mixture and incubating at 37 °C for another extract night (12–20 h). Finally, PCR reactions were

carried out again to make sure that the LCM DNA was amplifying properly.

2.4. Markers, PCR, and reading and interpretation of microsatellite alterations

Sixteen polymorphic radio-labeled microsatellite markers were selected to represent the marker loci identified during previous studies as having the highest frequencies of LOH in invasive ESCC [3,4]. They included D2S434 (2q), D3S4545 (3p), D3S1766 (3p), D4S2632 (4p), D4S2361 (4q), D5S2501 (5q), D6S1027 (6q), D8S1106 (8p), D9S1118 (9p), D9S910 (9q), D11S1984 (11p), D13S1493 (13q), D13S894 (13q), D13S796 (13q), D15S655 (15q), and D17S1303 (17p). Reported heterozygosity frequencies range from 0.64–0.82 for these markers (Research Genetics, Inc., Huntsville, AL).

DNA was extracted from dysplastic or tumor cells and adjacent normal squamous epithelium that was obtained by LCM from the endoscopic biopsies for each patient. PCR reactions were carried out using a 10 μl final volume containing 1.0 μl of 10 \times PCR buffer I (100 mM Tris-HCL, pH 8.3, 500 mM KCL, 15 mM MgCl₂), 1.0 μl of 1.25 mM deoxy nucleotide triphosphate, 4 μl of DNA extraction buffer, 0.6 μl of each primer, 0.10 μl of AmpliTaq DNA polymerase (Perkin Elmer), and 1 μCi of [α dCTP]. Typical

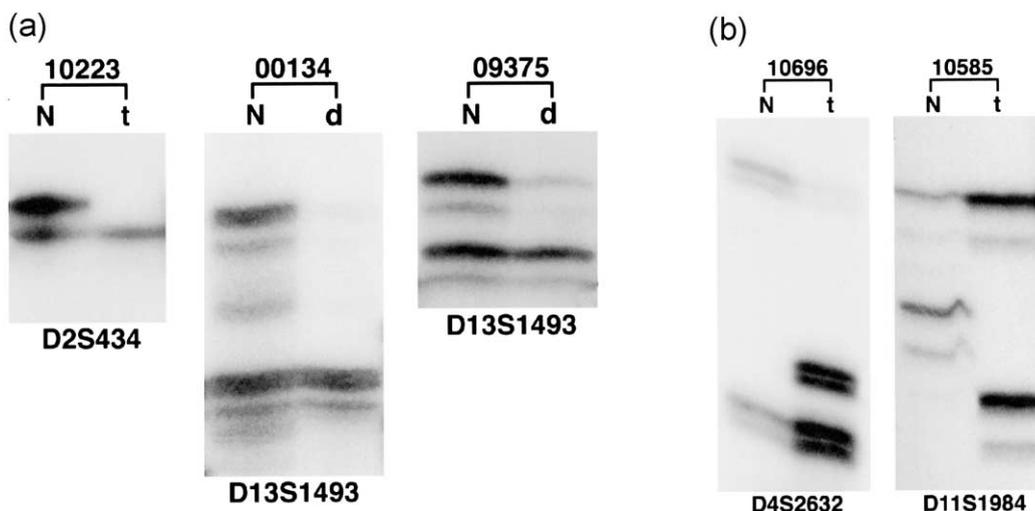


Fig. 1. Gel electrophoresis illustrating (a) allelic loss and (b) microsatellite instability.

Table 1
Description of characteristics and summary of marker results by patient^a

| No. | ID | Sex | Age | Diagnosis | LCM shots | LCM DNA Ratio(260/280) | LCM DNA Conc (ng/μl) | LCM DNA Vol (μl) | LOH (%) lost / #inform | MSI number |
|-----|--------|-----|-----|-----------|---------------|------------------------|----------------------|------------------|------------------------|------------|
| 1 | 10696 | M | 41 | SCC | T 3007 N 5931 | 1.17 1.24 | 10.2 10.8 | 90 90 | 7/10 (70) | 2 |
| 2 | 09680 | M | 68 | SCC | T 3388 N 6350 | 1.38 1.50 | 16.7 22.3 | 90 90 | 3/12 (25) | 1 |
| 3 | 09548 | F | 46 | SCC | T 4673 N 5393 | 1.45 1.38 | 17.7 16.6 | 90 90 | 8/13 (62) | 1 |
| 4 | 09637 | M | 71 | SCC | T 4333 N 4763 | 1.30 1.03 | 13.6 8.2 | 90 90 | 3/6 (50) | 1 |
| 5 | 09640 | M | 51 | SCC | T 3340 N 7420 | 1.26 1.33 | 11.8 13.5 | 80 90 | 1/10 (10) | 0 |
| 6 | 09681 | M | 52 | SCC | T 7156 N 6423 | 1.12 1.06 | 12.9 11.8 | 90 90 | 6/10 (60) | 1 |
| 7 | 10223 | M | 56 | SCC | T 3342 N 5697 | 1.37 1.57 | 15.5 22.9 | 80 90 | 2/10 (20) | 0 |
| 8 | 10228 | M | 70 | SCC | T 4931 N 4337 | 1.18 1.06 | 13.6 9.9 | 90 90 | 3/12 (25) | 0 |
| 9 | 10474 | M | 54 | SCC | T 4002 N 6766 | 1.30 1.39 | 13.4 16.8 | 80 90 | 1/4 (25) | 1 |
| 10 | 10727 | M | 47 | SCC | T 4075 N 5030 | 1.27 1.31 | 12.0 13.0 | 80 90 | 2/8 (25) | 3 |
| 11 | 10819 | M | 52 | SCC | T 3030 N 994 | 1.25 0.85 | 11.1 6.1 | 80 90 | 2/8 (25) | 0 |
| 12 | 09390 | M | 72 | SCC | T 3720 N 4078 | 1.48 1.38 | 19.6 16.2 | 90 90 | 3/9 (33) | 0 |
| 13 | 09426 | M | 52 | SCC | T 2561 N 2108 | 1.17 0.87 | 9.9 6.0 | 90 90 | 1/10 (10) | 1 |
| 14 | 09763 | M | 59 | SCC | T 1932 N 6709 | 0.99 1.01 | 10.0 9.9 | 90 90 | 5/10 (50) | 2 |
| 15 | 09799 | M | 58 | SCC | T 4330 N 5200 | 1.09 0.95 | 12.7 8.2 | 90 90 | 1/11 (9) | 1 |
| 16 | 10230 | M | 63 | SCC | T 5990 N 4268 | 1.05 0.96 | 10.0 8.2 | 90 90 | 5/11 (46) | 0 |
| 17 | 10329 | M | 65 | SCC | T 4670 N 5189 | 1.03 1.02 | 10.3 10.1 | 90 90 | 4/8 (50) | 2 |
| 18 | 10701 | F | 50 | SCC | T 2839 N 3787 | 1.02 1.16 | 8.7 10.1 | 90 90 | 2/11 (18) | 0 |
| 19 | 10585 | M | 55 | SCC | T 4720 N 2139 | 1.22 0.87 | 12.6 6.2 | 90 90 | 9/13 (69) | 1 |
| 20 | 09946 | M | 71 | SCC | T 4561 N 4309 | 1.10 1.33 | 11.0 14.9 | 90 90 | 2/16 (13) | 1 |
| 21 | 10941 | M | 68 | SCC | T 4706 N 3445 | 1.41 0.98 | 17.7 10.7 | 90 90 | 4/9 (44) | 1 |
| 22 | 09444 | M | 47 | SCC | T 2633 N 4185 | 0.86 0.94 | 9.0 11.8 | 90 90 | 3/8 (38) | 0 |
| 23 | 10781 | M | 61 | HGD | D 500 N 2614 | 0.93 1.21 | 7.2 20.8 | 90 90 | 0/11 (0) | 0 |
| 24 | 10699 | M | 57 | HGD | D 4366 N 5719 | 1.36 1.37 | 14.4 15.9 | 90 90 | 1/11 (9) | 0 |
| 25 | 10351 | F | 81 | HGD | D 4506 N 5620 | 1.25 1.35 | 12.5 16.4 | 90 90 | 1/13 (8) | 1 |
| 26 | 00074 | M | 59 | HGD | D 1774 N 1659 | 1.12 1.20 | 10.1 10.0 | 100 100 | 0/15 (0) | 1 |
| 27 | 00134 | M | 49 | HGD | D 1250 N 808 | 1.39 1.20 | 13.9 9.7 | 100 100 | 5/9 (56) | 0 |
| 28 | 09375 | M | 72 | HGD | D 7691 N 4832 | 1.43 1.45 | 20.9 18.1 | 90 90 | 4/12 (33) | 0 |
| 29 | 100091 | M | 70 | LGD | D 2587 N 4323 | 1.31 1.32 | 13.6 14.3 | 90 90 | 0/11 (0) | 0 |
| 30 | 000156 | M | 48 | LGD | D 1200 N 1433 | 0.99 1.18 | 7.4 10.2 | 100 100 | 0/13 (0) | 0 |
| 31 | 000269 | M | 56 | LGD | D 1620 N 2094 | 1.28 1.47 | 10.7 15.7 | 100 100 | 0/12 (0) | 0 |
| 32 | 000273 | F | 70 | LGD | D 3000 N 1342 | 1.47 1.40 | 15.7 13.8 | 100 100 | 0/10 (0) | 0 |
| 33 | 000282 | M | 51 | LGD | D 1463 N 763 | 1.37 1.37 | 12.8 14.6 | 100 100 | 0/9 (0) | 0 |
| 34 | 000286 | F | 53 | LGD | D 1165 N 1071 | 1.39 1.24 | 12.7 9.9 | 100 100 | 0/13 (0) | 0 |
| 35 | 000287 | F | 56 | LGD | D 1295 N 1568 | 1.24 1.37 | 10.7 14.1 | 100 100 | 1/9 (11) | 0 |
| 36 | 000299 | F | 44 | LGD | D 1188 N 1335 | 1.33 1.49 | 11.3 15.3 | 100 100 | 0/12 (0) | 1 |
| 37 | 009549 | F | 48 | LGD | D 5073 N 4383 | 1.37 1.32 | 12.5 9.6 | 90 90 | 1/10 (10) | 1 |

^a N, normal; D, dysplasia; T, tumor

PCR conditions were as follows: 10 min of denaturation at 94 °C, then 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% bromophenol 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.

Loss of heterozygosity (LOH) was defined as either complete or nearly complete loss of a band in the dysplasia or tumor sample relative to the corresponding normal DNA (Fig. 1). There was no convincing evidence of a homozygous deletion in any dysplasia or tumor samples at any of the 16 markers employed. Microsatellite instability (MSI) was defined as the presence of one additional band in the DNA from dysplasia or tumor that was not present in the normal sample (Fig. 1). The results were reviewed independently by two investigators (N.L. and N.H.). Discrepant cases were re-evaluated and repeated if necessary, and the data were accepted and included in the analysis only if the two reviewers agreed on the results.

3. Results

3.1. Patient characteristics

We studied 15 patients with epithelial dysplasia of the esophagus, including nine with LGD (four men and five women) and six with HGD (five men and one woman), who ranged in age from 44 to 81 years. We also studied 22 patients with ESCC, 20 men and two women, whose age ranged from 41 to 72 years. (Table 1).

3.2. LCM efficiency

Table 1 summarizes the LCM DNA data for all 37 patients studied. The number of LCM shots required varied from 500 to 7691 for each histologic type, depending on the size of the target tissue (for example, see Fig. 2). The range for the 260/280 ratio was 0.85–1.57, and the LCM DNA concentration ranged from 6.1–20.9 ng/µl. The estimated total amount of LCM DNA varied from 549 to 1881 ng per sample. Approximately 24–50 ng LCM DNA, equivalent to about 100 cells, was used for each PCR reaction.

3.3. LOH analysis

A total of 16 microsatellite markers were evaluated

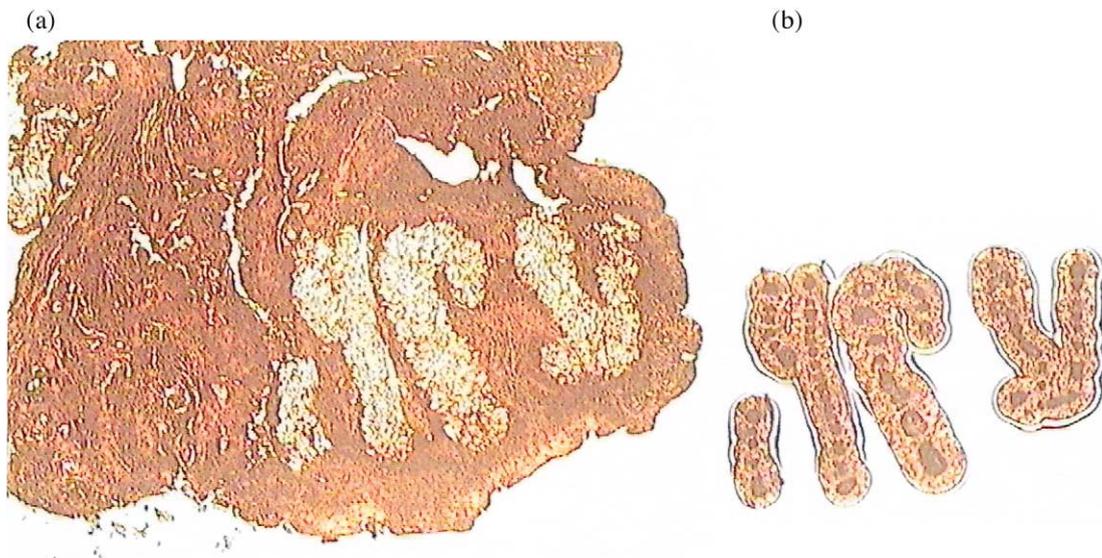


Fig. 2. Photomicrograph of a tumor (a) after microdissection and (b) showing microdissected tissues.

Table 2
Description of results by marker

| Marker No. | Locus | Location on chromosome | LOH lost/ informative (%) | | | MSI (#cases with MSI) | | |
|------------|----------|------------------------|---------------------------|-------------|----------------|-----------------------|-----|-------|
| | | | LGD (N = 9) | HGD (N = 6) | Tumor (N = 22) | LGD | HGD | Tumor |
| 1 | D2S434 | 2q35 | 0/5 (0) | 0/2 (0) | 6/12 (50) | 0 | 0 | 3 |
| 2 | D3S4545 | 3p24.3-p25 | 1/6 (17) | 0/5 (0) | 8/12 (67) | 0 | 0 | 3 |
| 3 | D3S1766 | 3p14-p21 | 0/4 (0) | 0/3 (0) | 8/12 (67) | 1 | 0 | 2 |
| 4 | D4S2632 | 4p12-p14 | 0/7 (0) | 0/4 (0) | 2/17 (12) | 0 | 1 | 1 |
| 5 | D4S2361 | 4q21.3-q22 | 0/8 (0) | 0/4 (0) | 6/14 (43) | 0 | 0 | 1 |
| 6 | D5S2501 | 5q21-q23.3 | 0/7 (0) | 1/2 (50) | 2/7 (29) | 0 | 0 | 2 |
| 7 | D6S1027 | 6q21 | 0/6 (0) | 0/6 (0) | 1/15 (7) | 0 | 0 | 0 |
| 8 | D8S1106 | 8p21.2-p23.3 | 0/4 (0) | 1/4 (25) | 5/16 (31) | 0 | 0 | 0 |
| 9 | D9S1118 | 9p11-q11 | 0/8 (0) | 1/5 (20) | 4/18 (22) | 1 | 1 | 3 |
| 10 | D9S910 | 9q22.3-q31 | 0/7 (0) | 1/5 (20) | 3/15 (20) | 0 | 0 | 0 |
| 11 | D11S1984 | 11p15.3-p15.5 | 0/7 (0) | 0/6 (0) | 4/14 (29) | 0 | 0 | 1 |
| 12 | D13S1493 | 13q11 | 0/6 (0) | 2/5 (40) | 7/14 (50) | 0 | 0 | 0 |
| 13 | D13S894 | 13q12.3-q14.2 | 0/6 (0) | 1/6 (17) | 6/13 (46) | 0 | 0 | 0 |
| 14 | D13S796 | 13q32-q34 | 0/7 (0) | 1/5 (20) | 7/17 (41) | 0 | 0 | 2 |
| 15 | D15S655 | 15q22-q23 | 1/5 (20) | 1/5 (20) | 5/14 (36) | 0 | 0 | 0 |
| 16 | D17S1303 | 17p11.2-p12 | 0/6 (0) | 2/4 (50) | 3/9 (33) | 0 | 0 | 1 |
| | | | 2/99 (2) | 11/71(15) | 77/219 (35) | 2 | 2 | 19 |

in 15 epithelial dysplasias of the esophagus and 22 ESCCs (Table 1). Overall, allelic loss at one or more of these 16 loci was detected in two of nine LGDs (22%), four of six HGDs (67%), and all 22 ESCCs (100%). For all 16 markers combined, the frequency of LOH increased with increasing disease severity: 2% of markers in LGD, 15% of markers in HGD, and 35% of markers in ESCC (Table 2). The differences between LGD vs. HGD, LGD vs. ESCC, and HGD vs. ESCC were all statistically significant (Fisher's exact test, $P = 0.02$, $P < 0.001$, and $P = 0.007$, respectively).

When considered by individual marker, only two markers showed LOH in one or more patient with LGD (13%; includes markers on chromosome arms 3p and 15q) while nine markers showed LOH in at least one of the HGD patients (56%; includes markers on chromosome arms 5q, 8p, 9p, 9q, 13q, 15q, and 17p), and all 16 markers showed LOH in the ESCC patients (100%) (Table 2).

3.4. MSI analysis

The number of patients with MSI at one or more of the 16 markers increased with increasing disease severity: 22% for LGD (two of nine), 33% for HGD (two of six), and 64% for ESCC (14 of 22); but these

differences were not statistically significant ($P > 0.10$ for each of the two-way comparisons). The number of loci with MSI among patients who demonstrated any MSI also appeared to vary by disease severity. The two patients each with LGD and HGD had MSI only at a single locus (Tables 1 and 2), while among the 14 ESCC patients with MSI, one (#10727) had MSI at three loci (D3S1766, D4S2361, and D9S1118), three had MSI at two loci (#10696 had MSI at D2S434 and D4S2632; #09763 had MSI at D2S434 and D13S796; #10329 had MSI at D5S2501 and D9S1118), and ten had MSI at a single locus (Tables 1 and 2). The loci where more than one patient was found to have MSI included markers on chromosome arms 2q, 3p, and 9p (three patients each), and 3p, 5q, 13q (two patients each) (Table 2).

4. Discussion

Numerous studies have documented genetic alterations in esophageal cancer [9,10], however, changes in precursor lesions of the esophagus are less well understood. In this initial study, we examined dysplasia of the esophagus and ESCC from endoscopic biopsies using 16 microsatellite markers that showed a very high frequency of LOH in invasive ESCC from

our previous studies [3,4]. We found that the frequency of LOH increased significantly with increased histologic severity, distinguishing LGD, HGD, and ESCC. To our knowledge, these are the first data reporting LOH results from LCM DNA obtained from endoscopic biopsies of squamous dysplasia of the esophagus and ESCC, although molecular alterations of Barrett's esophagus in microdissected endoscopic biopsies have been previously published [11]. Our findings support the idea that an accumulation of genetic alterations is associated with the progression of normal mucosa to precursor lesions and cancer [12–16]. Our findings are also consistent with the previous reports that increased histologic severity of precursor lesions predicted higher risk for ESCC [6].

The present study identified LOH at ten different markers on chromosome arms 3p, 5q, 8p, 9p, 9q, 13q, 15q and 17p in precursor lesions, suggesting that genetic alterations in these regions may be involved in the early stages of tumor development. These results are consistent with previous studies [5,17] as several known genes related to cancer are located in these regions (e.g., adenomatous polyposis coli gene (*APC*) on chromosome 5q; *CDK2 A/CDK2B* on 9p; aneuploidy gene (*ANXI*) on 9q; retinoblastoma gene (*RBI*), breast cancer 2 gene (*BRCA2*), and inhibitor of growth gene (*ING1*) on 13q; and tumour protein 53 gene (*TP53*) on 17p). Several studies have reported frequent LOH on 9p and mutation and homozygous deletion of cyclin-dependent kinase gene (*CDK2 A/CDK2B*) in esophageal cancer [18,19]. Recent proteomic analysis showed a loss of *ANXI* protein expression in ESCC compared with normal tissues from the same patients [20]. Frequent LOH in the absence of mutations in *RBI* [8], low mutation rates but high frequency of intragenic allelic loss in *BRCA2* [21], and low mutation frequency for *ING1* [22], suggest that other unknown gene(s) on chromosome 13q may be involved in the development of ESCC. Frequent mutation in *TP53* has been reported in many cancers, including ESCC from Shanxi, China [23]. Taken together, these data suggest that markers at loci showing LOH in precursor lesions of the esophagus and ESCC may be potential predictive markers for screening, if the results reported here can be confirmed in future studies with larger sample sizes.

The overall frequency of LOH in the endoscopic

biopsies in this study was lower than that found in our previous studies of surgical resection specimens [3–5]. This difference may in part have been due to the more advanced stage of the resection specimens, which may have allowed them to accumulate a greater number of genetic alterations [5]. The smaller endoscopic lesions may also have been more homogeneous in their cellular content and, thus, contain a simpler genetic profile. Furthermore, in the current study adjacent histologically normal appearing epithelium, rather than venous blood, was used to represent somatically unaffected DNA for comparison with histologically abnormal tissue (e.g. dysplastic epithelium or tumor). Given that molecular changes can be detected in histologically normal appearing epithelia, the 'normal epithelium' in these biopsies may have contained molecular alterations that resulted in an incorrect homozygous or non-informative classification at some markers.

We found MSI not only in ESCC, but also in LGD and HGD, and the frequency increased with increased histological severity of the disease. These results appear to differ from that of previous studies (including our own) in which very low frequency of MSI was found in ESCC [3,4,25]. Recently, however, Kagawa et al. reported MSI in 42% of ESCC cases and 59% of all lesions [26]. Although MSI is linked to defects in the DNA mismatch repair system and occurs in hereditary nonpolyposis colon cancer [27] and other cancers [28], little is known about the role of MSI in esophageal carcinogenesis. Despite the relatively high frequency of patients with MSI in at least one marker, only 4% of all markers tested here showed MSI, a figure comparable to the 0.8% we observed in an earlier study [3]. Differences in the present and previous study regarding MSI frequency are quite possibly due to chance, but alternative explanations include differences in the type of samples studied (endoscopic biopsies here vs. resection specimens before), differences in the method of microdissection used (LCM here vs. manual microdissection before), potential differences in tumor stage, or simply the fact that different cases were evaluated in the two studies.

We have used LCM routinely to obtain morphologically homogenous tissue from surgical resection specimens for molecular genetic studies in our laboratory since 1998. Using endoscopic biopsies in the present study required modifications to our usual

LCM techniques, including reduction in spot size for biopsies to half the size used for resection specimens, and reduction in shot duration because of the shallower depth of lesions (especially premalignant lesions) in biopsies. Several studies have suggested that PCR artifacts are frequently found when using paraffin-embedded tissues, especially when the number of cells analyzed is small [24]. To minimize the potential for artifactual allelic imbalance resulting from LCM of tiny biopsies, we used a relatively large number of shots during LCM to procure a large number of cells. We estimated that 25–40 ng of LCM DNA, or DNA from a minimum of 100 cells, was used in each PCR reaction. For example, in case #10871, 500 shots were taken using medium spot size (15 μm) and duration (5.5 ms). This produced an estimated total of 2500 cells (5 cells/per shot) which, in 90 μl of mixed digestion solution, contained an estimated 648 ng of DNA. Each 4 μl of mixture used per PCR reaction, therefore, contained approximately 111 cells (28 ng LCM DNA).

In this study, we used a modified processing method to optimize the DNA obtained from the endoscopic biopsies. We found that the quality of the DNA (260/280 ratios from 0.85–1.57), was lower than that of DNA extracted from venous blood (1.75–1.80), probably because some undigested materials were found in the mixture. Although the LCM DNA from most patients worked well, the undigested material in this mixture appeared to affect the efficiency of amplification. We used two modifications to reduce the amount of undigested materials in the LCM mixture, including extending the digestion time from two to three nights (with addition of extra digestion solution), and cleaning of the LCM DNA using a Micro Bio-Spin chromatography column. With these modifications nearly all samples produced PCR products. Thus it is our experience that using LCM on endoscopic biopsies requires care but can provide sufficiently good quality DNA to be useful for testing numerous molecular markers.

In summary, the results of this study suggest that the development of ESCC is associated with genetic instability, including LOH and MSI, and that this instability occurs in recognized precursor lesions. Our results also identify several microsatellite markers that may be useful as predictive markers in the early detection of ESCC. Finally, we present a modified

LCM protocol for the effective PCR-based molecular analysis of endoscopically obtained biopsies.

References

- [1] M.H. Li, P. Li, P.J. Li, Recent progress in research on esophageal cancer in China, *Adv. Cancer Res.* 33 (1980) 173–249.
- [2] J.Y. Li, Epidemiology of esophageal cancer in China, *NCI Monogr.* 62 (1982) 113–120.
- [3] N. Hu, M.J. Roth, M. Polymeropolous, Z.Z. Tang, M.R. Emmert-Buck, Q.H. Wang, A.M. Goldstein, S.S. Feng, S.M. Dawsey, T. Ding, Z.P. Zhuang, X.Y. Han, T. Reid, C. Giffen, P.R. Taylor, Identification of novel regions of allelic loss from a genome-wide scan of esophageal squamous-cell carcinoma in a high-risk Chinese population, *Genes Chromosomes Cancer* 27 (2000) 217–228.
- [4] N. Hu, M.J. Roth, M.R. Emmert-Buck, Z.Z. Tang, M. Polymeropolous, Q.H. Wang, A.M. Goldstein, X.Y. Han, S.M. Dawsey, T. Ding, C. Giffen, P.R. Taylor, Allelic loss in esophageal squamous cell carcinoma patients with and without family history of upper gastrointestinal tract cancer, *Clin. Cancer Res.* 5 (1999) 3476–3482.
- [5] M.J. Roth, N. Hu, M.R. Emmert-Buck, Q.H. Wang, S.M. Dawsey, G. Li, W.J. Guo, Y.Z. Zhang, P.R. Taylor, Genetic progression and heterogeneity associated with development of esophageal squamous cell carcinoma, *Cancer Res.* 61 (2001) 4098–4104.
- [6] S.M. Dawsey, K.J. Lewin, F.R.C. Path, G.Q. Wang, F.S. Liu, R.K. Neiberg, Y. Yu, J.Y. Li, W.J. Blot, B. Li, P.R. Taylor, Squamous esophageal histology and subsequent risk of squamous cell carcinoma of the esophagus: a prospective follow-up study from Linxian, China, *Cancer* 74 (1994) 1686–1692.
- [7] S.M. Dawsey, K.J. Lewin, S. F, G.Q. Liu, Q. Wang, Esophageal morphology from Linxian, China: squamous histologic findings in 754 patients, *Cancer* 73 (1994) 2027–2037.
- [8] M.R. Emmert-Buck, R.F. Bonner, P.D. Smith, R.F. Chuaqui, Z. Zhuang, S.R. Goldstein, R.A. Weiss, L.A. Liotta, Laser capture microdissection, *Science* 274 (1996) 998–1001.
- [9] T. Aoki, T. Mori, X. Du, T. Nishihira, T. Matsubara, Y. Nakama, Allelotype study of esophageal carcinoma, *Genes Chromosomes Cancer* 10 (1994) 177–182.
- [10] R. Montesano, M. Hollstein, P. Hainaut, Genetic alterations in esophageal cancer in their relevance to etiology and pathogenesis: a review, *Int. J. Cancer* 69 (1996) 225–235.
- [11] S. Romagnoli, M. Roncalli, D. Graziani, B. Cassani, E. Roz, L. Bonavina, A. Peracchia, S. Bosari, G. Coggi, Molecular alterations of Barrett's esophagus on microdissected endoscopic biopsies, *Lab. Invest.* 18 (2001) 241–247.
- [12] P.C. Nowell, The clonal evolution of tumor cell populations, *Science* 194 (1976) 23–28.
- [13] P. O'Connell, V. Pekkel, S.A. Fuqua, C.K. Osborne, G.M. Clark, D.C. Allred, Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci, *J. Natl. Cancer Inst.* 90 (1998) 697–703.
- [14] T.T. Wu, T. Watanabe, R. Heitmiller, M. Zahurak, A.A. Forastiere, S.R. Hamilton, Genetic alterations in Barrett

- esophagus and adenocarcinomas of the esophagus and esophago-gastric junction region, *Am. J. Pathol.* 153 (1998) 287–294.
- [15] M. Barrett, C.A. Sanchez, L. Prevo, D.J. Wong, P.C. Galipeau, T.G. Paulson, P.S. Rabinovitch, B.J. Reid, Evolution of neoplastic cell lineages in Barrett oesophagus, *Nat. Genet.* 22 (1999) 106–109.
- [16] I.I. Wistuba, C. Behrenes, S. Michgrub, D. Bryant, J. Hung, J.D. Minna, A.F. Gazdar, Sequential molecular abnormalities are involved in the multistage development of squamous cell lung carcinoma, *Oncogene* 18 (1999) 643–650.
- [17] T. Mori, A. Yanagisawa, Y. Kato, K. Muria, T. Nishihira, S. Mori, Y. Nakamura, Accumulation of genetic alterations during esophageal carcinogenesis, *Hum. Mol. Genet.* 3 (1994) 1969–1971.
- [18] T. Mori, K. Miura, T. Aoki, T. Nishihira, S. Mori, Y. Nakamura, Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/Cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma, *Cancer Res.* 54 (1994) 3396–3397.
- [19] X.L. Zhou, L. Tarmin, J. Yin, H.Y. Jiang, H. Suzuki, M.G. Rhyu, J.M. Abraham, S. Meltzer, The MTS1 gene is frequently mutated in primary human esophageal tumors, *Oncogene* 9 (1994) 3737–3741.
- [20] M.R. Emmert-Buck, J.W. Gillespie, C.P. Paweletz, D.K. Ornstein, V. Basrur, F. Appella, Q.H. Wang, J. Huang, N. Hu, P. Taylor, E.F. Petricoin III, An approach to proteomic analysis of human tumors, *Mol. Carcinog.* 27 (2000) 158–165.
- [21] N. Hu, G. Li, W.J. Li, C.Y. Wang, A.M. Goldstein, Z.Z. Tang, M.J. Roth, S.M. Dawsey, J. Huang, Q.H. Wang, T. Ding, C. Giffen, P.R. Taylor, M.R. Emmert-Buck, Infrequent mutation in the BRCA2 gene in esophageal squamous cell carcinoma, *Clin. Cancer Res.* 8 (2002) 1121–1126.
- [22] L. Chen, N. Matsubara, T. Yoshino, T. Nagasaka, N. Hoshizima, Y. Shirakawa, Y. Naomoto, H. Isozaki, K. Riabowol, N. Tanaka, Genetic alterations of candidate tumor suppressor INGI in human esophageal squamous cell cancer, *Cancer Res.* 61 (2001) 4345–4349.
- [23] H. Hu, J. Huang, M.R. Emmert-Buck, Z.Z. Tang, M.J. Roth, C.Y. Wang, S.M. Dawsey, G. Li, W.J. Li, Q.H. Wang, X.Y. Han, T. Ding, C. Giffen, A.M. Goldstein, P.R. Taylor, Frequent inactivation of the TP53 gene in esophageal squamous cell carcinoma from a high-risk population in China, *Clin. Cancer Res.* 7 (2001) 883–891.
- [24] M. Yamano, H. Fujii, T. Takagaki, N. Kadowaki, H. Watanabe, T. Shirai, Genetic progression and divergence in pancreatic carcinoma, *Am. J. Pathol.* 156 (2000) 2123–2133.
- [25] F. Muzeau, J.F. Flejou, J. Belghiti, G. Thomas, R. Hamelin, Infrequent microsatellite instability in oesophageal cancers, *Br. J. Cancer* 75 (1997) 1336–1339.
- [26] Y. Kagawa, K. Yoshida, T. Hirai, T. Toge, H. Yokozaki, W. Yasui, E. Tahara, Microsatellite instability in squamous cell carcinoma and dysplasia of the esophagus, *Anticancer Res.* 20 (2000) 213–217.
- [27] C.R. Boland, S.N. Thibodeau, S.R. Hamilton, D. Sidransky, J.R. Eshleman, R.W. Burt, S.J. Meltzer, M.A. Rodrigues-Bigas, R. Fodde, G.N. Ranzani, S.A. Srivastava, National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer, *Cancer Res.* 58 (1998) 5248–5257.
- [28] S.B. Atkin, Microsatellite instability, *Cytogenet. Cell Genet.* 92 (2001) 177–181.