

# Loss of heterozygosity analysis of microsatellites on multiple chromosome regions in dysplasia and squamous cell carcinoma of the esophagus

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**Abstract.** The objective of this study was to characterize the molecular events in the carcinogenesis of esophageal squamous cell carcinoma (ESCC) and to identify biomarkers for early detection of the disease. Matched precancerous and cancerous tissues resected from 34 esophageal cancer patients from Chongqing, southern China, were compared to evaluate the extent of loss of heterozygosity (LOH). Sixteen microsatellite markers on chromosome regions 3p, 4p, 5q, 8p, 9p, 9q, 11p, 13q and 17p were used for PCR-based LOH analysis. The overall frequency of LOH at the 16 microsatellite loci was significantly increased as the pathological status of the resection specimens changed from low-grade dysplasia (LGD) to high-grade dysplasia (HGD) and SCC ( $P < 0.001$ ). A total of 8 markers showed LOH in the LGD samples. In addition, heterozygosity was regained at 4 loci in the SCC samples of 4 patients, respectively, in comparison to the results for these loci in the HGD samples. The overall rate of LOH increased significantly with the deterioration of the lesions, indicating that tumorigenesis of the esophageal squamous epithelia is a progressive process involving accumulative changes in LOH. The 8 loci showing allelic loss in the LGD samples may be involved in the early-stage tumorigenesis of ESCC, and LOH analysis at these loci may help improve the early detection of this disease. Regain of heterozygosity found in certain patients suggests the possibility of genetic heterogeneity in the tumorigenesis of esophageal cancer.

## Introduction

Esophageal cancer is a common type of malignant cancer in China. The 5-year survival rate of patients diagnosed with

esophageal cancer is less than 15%. Failure to make an early diagnosis is normally attributed to the absence of symptoms or the presence of mild symptoms during the early stages of the disease. Most of the patients are diagnosed in the intermediate or late stage of the disease. Although radical esophagectomy remains the primary treatment for esophageal cancer, it is quite difficult to treat patients with distant or lymph-node metastasis because of a tendency of postsurgical recurrence. Therefore, identification of biomarkers that improve the rates of early diagnosis of esophageal cancer or precursor lesions can significantly reduce mortality in esophageal cancer patients.

Similar to other solid tumors, chromosome loss is a common molecular defect in esophageal cancer. Chromosome regions with a high frequency of allelic loss indicate the presence of tumor-associated genes (such as tumor suppressors). A study of chromosome loss in dysplasia and early-stage esophageal cancer will aid in the discovery of major tumor-associated genes and will lead to the understanding of the tumorigenesis of esophageal cancer. They may also be used as potential biomarkers for early detection of esophageal cancer. Although extensive genomic instability has been found in patients with esophageal cancer, the molecular alterations that are closely related to the different stages of tumorigenesis have not been fully understood.

In order to further clarify the molecular alterations during the early-stage tumorigenesis of esophageal cancer, and to identify potential biomarkers for early detection of the disease, we analyzed allelic losses at a total of 16 microsatellite loci selected from chromosome regions 3p, 4p, 5q, 8p, 9p, 9q, 11p, 13q and 17p in matched squamous dysplasia and squamous cancer tissues resected from 34 esophageal cancer patients in Chongqing, southern China.

## Materials and methods

**Sample collection.** Resection specimens from a total of 34 esophageal cancer patients were obtained from the Department of Thoracic Surgery, South-West Hospital, Chongqing, China. The patients included 23 men and 11 women with an average age of 58.6 years (range 44-74). All of the patients were newly diagnosed with esophageal cancer,

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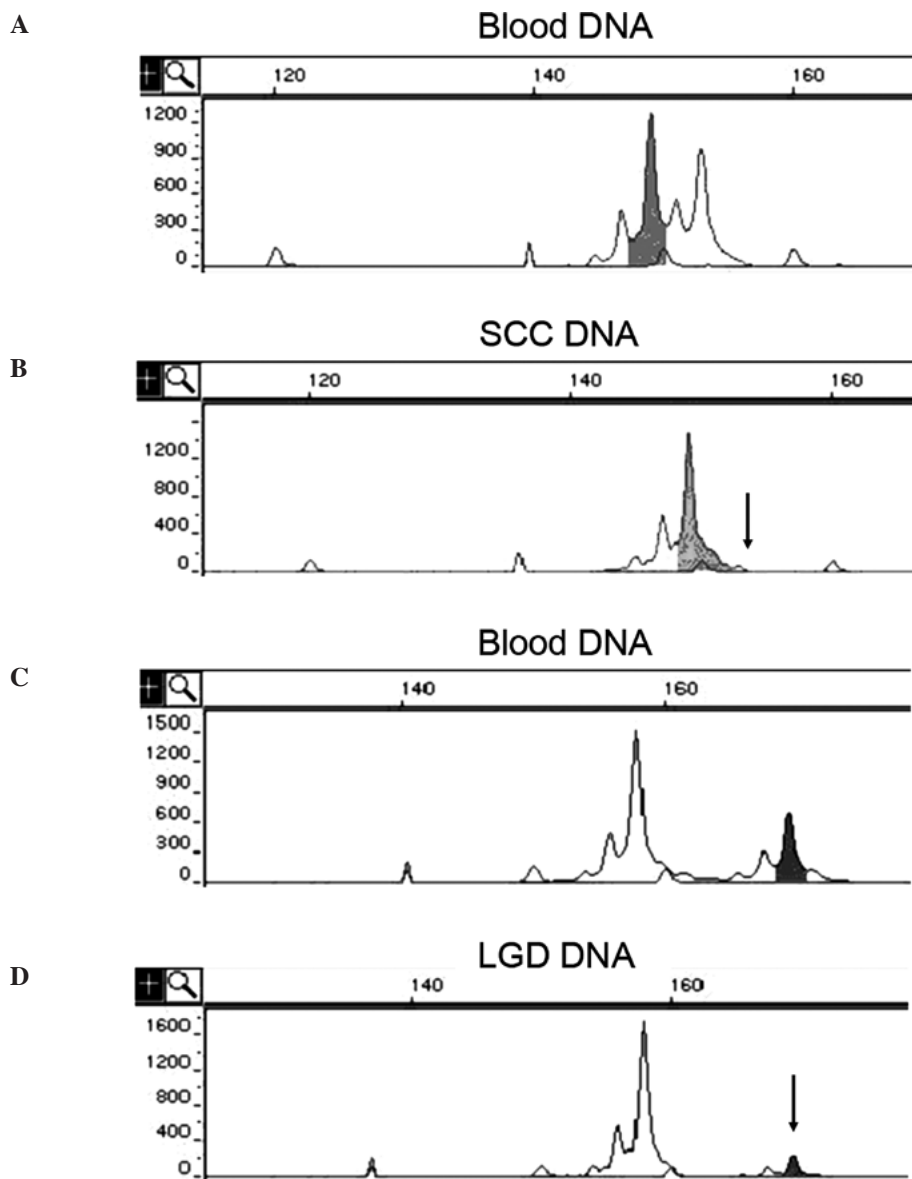


Figure 1. X-coordinate represents the size of the fragment and Y-coordinate represents the fluorescence intensity. (A and B) Allelotyping results of the D3S2452 locus from the peripheral blood samples and cancer tissue samples of a patient. DNA from the peripheral blood sample shows heterozygosity at the D3S2452 locus with fragments of two sizes, 149 and 153 bp, whereas DNA from the tumor tissue only shows a single fragment, 149 bp (arrowhead indicates the loss of a 153-bp fragment). These results indicate that LOH occurred at the D3S2452 locus in the cancer tissue. (C and D) Allelotyping results of the D4S174 locus from the peripheral blood and LGD-tissue samples of a patient. Both the peripheral blood and LGD samples comprise segments of two sizes, 157 and 169 bp, at the D4S174 locus; the fluorescence intensity at the 169-bp segment (arrowhead) in the LGD sample was reduced by at least 30% of that in the peripheral blood sample. The results highlight the presence of LOH at the D4S174 locus in the LGD sample. SCC, squamous cell carcinoma; LGD, low-grade dysplasia, including mild and medium dysplasia (in this study).

had no previous history of any other malignant cancer and had not undergone previous radiotherapy or chemotherapy. The patients were Han Chinese who had lived in Chongqing for more than 20 years and did not have any direct kinship with each other. The resected samples were placed in liquid nitrogen immediately after the surgical removal and were preserved at  $-80^{\circ}\text{C}$  before use. In addition to the cancerous foci, surrounding tissues were also collected from the 34 patients. The tissue samples were pathologically diagnosed with mild, medium and severe dysplasia and squamous cell carcinoma (SCC) (1). This study classified mild and medium dysplasia as low-grade dysplasia (LGD), and severe dysplasia as high-grade dysplasia (HGD). In addition, peripheral blood samples were collected prior to surgery. Informed consent was obtained

from all of the study subjects in accordance with the standards established by the local institutional review boards.

**Tissue cell acquisition using microdissection.** The frozen sections of squamous cancer tissue and dysplastic tissue ( $5\ \mu\text{m}$ ) were stained with H&E and subjected to microdissection under a  $\times 40$  dissecting microscope to ensure that the proportion of tumor cells and dysplastic cells was  $>80\%$ , which significantly reduced the rates of false positive or negative results during the loss of heterozygosity (LOH) analysis.

**Extraction of genomic DNA.** Genomic DNA was extracted from the patient blood samples and tissues using the DNeasy Blood and Tissue kit (Qiagen).

Table I. Frequencies of LOH at 16 microsatellite loci in the squamous dysplastic tissues and ESCC tissues.

Marker	Location	LGD sample (%) <sup>a</sup>	HGD sample (%) <sup>a</sup>	SCC sample (%) <sup>a</sup>
D3S1597	3p25	4/21 (19.0)	12/22 (54.5)	18/22 (81.8)
D3S2452	3p21-p14	7/25 (28.0)	20/25 (80.0)	18/25 (72.0)
D3S1285	3p14	3/19 (15.8)	14/21 (66.7)	16/21 (76.2)
D4S174	4p14-p13	2/27 (7.4)	7/27 (25.9)	12/27 (44.4)
D5S409	5q14-q15	0/16 (0.0)	7/16 (43.7)	8/16 (50.0)
D5S2501	5q21-q23.3	1/13 (7.7)	7/13 (53.8)	7/13 (53.8)
D8S261	8p22-p21.3	0/18 (0.0)	5/18 (27.8)	6/18 (33.3)
D9S157	9p23-p22	0/23 (0.0)	16/23 (69.5)	15/23 (65.2)
D9S111	9q12-q21.1	0/25 (0.0)	9/25 (36.0)	15/25 (60.0)
D9S125	9q34-q34	9/27 (33.3)	21/27 (77.8)	23/27 (85.2)
D11S1338	11p15.5	0/29 (0.0)	10/29 (34.5)	11/29 (37.9)
D13S175	13q13	0/22 (0.0)	10/23 (43.5)	12/23 (52.2)
D13S153	13q14.2	4/24 (16.7)	10/24 (41.7)	13/24 (54.2)
D13S173	13q32-q34	0/24 (0.0)	5/24 (20.8)	11/24 (45.8)
D17S786	17p13.1	5/23 (21.7)	13/23 (56.5)	16/23 (69.5)
D17S261	17p11.2	0/22 (0.0)	10/22 (45.4)	11/22 (50.0)
Total <sup>b</sup>		35/358 (9.8)	176/362 (48.6)	212/362 (58.5)

<sup>a</sup>LGD, low-grade dysplasia, including mild and medium dysplasia (in this study); HGD, high-grade dysplasia, i.e., severe dysplasia; SCC, squamous cell carcinoma. <sup>b</sup>If  $P < 0.001$ , then the difference is statistically significant.

**Analysis of loss of heterozygosity.** A total of 16 microsatellite loci were chosen from the 3p, 4p, 5q, 8p, 9p, 9q, 11p, 13q and 17p chromosome regions. The resected samples and paired blood samples from the 34 esophageal cancer patients were analyzed for LOH at the 16 loci. Information concerning these markers and primer sequences is available on the Genome Database (<http://www.gdb.org/>) and the NCBI genome database (<http://www.ncbi.nlm.nih.gov/>). The primers were obtained as labeled primers with fluorescent dye at the 5-terminus (MWG Biotech, Ebersberg, Germany).

LOH analysis was performed as described in our previous study (2). In brief, the 5- $\mu$ l volume of microsatellite DNA amplification included 40 ng of DNA template, 1X Buffer, 200  $\mu$ M dNTPs, 250 nM microsatellite primer, 2.5 mM  $MgCl_2$  and 0.25 units of *Taq* DNA polymerase. The PCR conditions were as follows: pre-denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 55-60°C for 30 sec, 72°C for 1 min and a final elongation step at 72°C for 4 min. PCR products were loaded on a fluorescent sequencer gel (ABI PRISM 377). The images were captured, converted and analyzed using GeneScan software, and the size of each allele fragment was automatically calculated.

When the peripheral blood DNA of a patient showed heterozygosity at a certain locus, the locus was marked as informative. The tissue sample of the patient was considered to show LOH when the DNA from the sample showed homozygosity at the informative locus or when the fluorescence intensity of one of the two alleles was <30% of that in the blood DNA of the patient (Fig. 1). The frequency of LOH at each locus was defined as the total number of tissue samples with LOH at this locus/the total number of informative samples.

**Statistical analysis.** The LOH frequencies at each locus between the different groups were compared using the  $\chi^2$  test, and a P-value <0.05 was considered statistically significant.

## Results

The results of the present study are shown in Table I. The overall frequencies of LOH at the 16 microsatellite loci significantly increased as the pathological status of the resection samples changed (from LGD to HGD and to SCC). The overall frequency of LOH in the LGD samples (9.8%) was significantly lower than that in the HGD (48.6%) and SCC (58.5%) samples ( $P < 0.001$ ). Although the LOH frequency at each locus showed an increasing trend with increasing disease severity, the difference was not statistically significant. Eight loci (D3S1597, D3S2452, D3S1285, D4S174, D5S2501, D9S125, D13S153 and D17S786) showed LOH in the informative LGD samples, and another 8 loci were found to present LOH in the informative HGD specimens. All 16 loci were found to have LOH in the SCC samples. Furthermore, by comparing the occurrence of LOH in samples with different pathological statuses from the same patient, we found regain of heterozygosity at loci D3S2452, D4S174, D9S125 and D17S261, in the SCC samples of 4 patients, respectively, compared to the HGD specimens which showed LOH at the corresponding loci.

## Discussion

A total of 16 highly polymorphic microsatellite markers from 9 chromosome regions with a high frequency of allelic loss in esophageal cancer were selected. Surgically resected squamous dysplasia and SCC samples from 34 esophageal

cancer patients were subjected to LOH analysis at these loci. The results showed that the overall frequencies of LOH at the 16 microsatellite loci significantly increased as the pathological status of the resection specimens deteriorated (i.e., from LGD to HGD and to SCC). These results indicated that tumorigenesis of esophageal squamous epithelia is a progressive process involving a series of molecular alterations. As the alterations accumulate to a certain degree, the cell morphology and behavior undergo a radical change, leading to malignancy (3). This finding was consistent with previous studies involving other tumor types, such as prostate, colon and breast cancer (4-7). Moreover, the results were also in accordance with those of a previous study showing that increasing grades of dysplasia were associated with an increased risk of developing ESCC (8).

Of the 16 loci, 8 loci were found to show LOH in LGD, indicating that LOH at these 8 loci may be involved in the early-stage tumorigenesis of ESCC, and that these specific loci may be used as markers for the screening of esophageal cancer. At present, certain tumor-associated genes have been identified near these loci, such as *FHIT* (3p), *RASSF1A* (3p), *APC* (5q), *ANXI* (9q), *DEC1* (9q), *RBI* (13q), *BRCA2* (13q), *ING1* (13q) and *TP53* (17p). The expression of *ANXI* in chromosome 9q has been reported to be closely related to tumor progression in patients with breast cancer and possibly plays an important role in the early stage of tumorigenesis (9). Study concerning this gene in esophageal cancer showed that its expression at the protein level in cancer tissues was significantly lower than that in normal tissues (10). Another candidate gene, *DEC1* at chromosome 9q, has been reported to be down-regulated in esophageal cancer, and transfection of the cDNA of *DEC1* may inhibit the proliferation of various types of cancer cells, suggesting that it may participate in the development of esophageal cancer (11). The chromosome 13q region has a high frequency of allelic loss in esophageal cancer. However, the identified candidate genes within this region, such as *RBI*, *BRCA2* and *ING1*, seldom show mutations in esophageal cancer, implying that other unknown tumor-suppressor genes within this region may participate in the tumorigenesis of this cancer (12). The chromosome 17p region involving the *P53* locus also shows a high frequency of allelic loss in esophageal cancer. Studies of the *P53* gene in esophageal cancer have shown that LOH and mutations are the leading causes of its inactivation, which is in accordance with the 'two-hit' model of tumorigenesis indicating that *P53* is a major tumor suppressor in esophageal cancer (13).

In addition, 8 other loci were found to show LOH in the HGD samples, indicating that LOH at these loci may be involved in the late stages of tumorigenesis (such as invasion and metastasis) in esophageal cancer.

This study also compared the occurrence of LOH in samples of different pathological statuses from the same patient. Notably, in 4 patients LOH was found at certain loci in the HGD samples, whereas heterozygosity was regained at the same loci in matched SCC samples. The regain of heterozygosity at some loci in tumor tissues indicated that the tumorigenesis of esophageal cancer may show genetic heterogeneity, i.e., the HGD and SCC samples of the same patient may have been derived from different tumor-cell clones. Cells with different molecular defects may have distinct tumorigenesis processes, leading to different tumor-cell clones. A similar

result has been found in adenocarcinoma of the esophagus; the molecular defects found in the severe dysplastic lesions were not detected in the invasive adenocarcinoma lesions of the same patient (14). A study involving patients with oral squamous cancer has also shown that most of the molecular alterations found in the precursor lesions were not revealed in the matched tumor tissues (15). Similar observations have been reported in studies on other cancers, such as breast and prostate cancer (16,17).

Finally, it is worth mentioning that in several LGD samples from our subjects, there were already multiple loci exhibiting allelic loss, indicating that the earliest molecular event in esophageal cancer may occur in histologically normal squamous epithelia. In a recent study on esophageal cancer, 19% of the histologically normal epithelia within the abnormal mucosal region (unstained with iodine) already showed LOH alterations, suggesting that the histologically normal epithelia adjacent to the tumor tissues may have early-stage LOH alterations, which may be necessary for the tumorigenesis of esophageal cancer (18). Although, at present, histological examination remains an effective method for the early detection of cancer, molecular tests, such as LOH analysis of certain specific loci at the early stage, can be a promising and rational strategy for the early diagnosis or prediction of esophageal cancer.

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