

Genetic progression and divergence in superficial esophageal squamous cell carcinoma by loss of heterozygosity analysis

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Abstract. Esophageal squamous cell carcinoma (SCC) is one of the most common fatal carcinomas worldwide and has some of the most malignant characteristics among gastrointestinal tumors. Although a high frequency of loss of heterozygosity (LOH) for various genes has been observed in esophageal SCCs, these findings do not provide any information regarding the genetic pathways that may underlie the development and progression of this type of tumor. To clarify the temporal and topographic pathways in the genetic evolution of esophageal SCC, we microdissected multiple foci from superficial mucosal invasive foci of tumors. We then carried out LOH analyses of the microdissected neoplastic foci. Sixteen superficial esophageal SCCs were examined. Three to six carcinoma foci from each superficial esophageal SCC were individually microdissected. We used 12 oligonucleotide primer pairs specific for the microsatellite markers for which frequent LOH in esophageal SCC has been reported. All tumors exhibited LOH of at least three microsatellite loci. A frequent homogeneous LOH pattern was detected for TP53 (60%), D16S518 (43%) and D3S1234 (29%), suggesting that the loss of these alleles is an early event in the development of esophageal SCC. A heterogeneous LOH pattern was detected for D13S325 (87%), D10S559 (73%), D3S1568 (58%), D3S1234 (57%) and D3S1621 (56%), suggesting that the loss of these alleles is a late event in the development of esophageal SCC. All tumors showed the LOH pattern of single clonal neoplasms with genetic progression and divergence. In conclusion, by extensive sampling of SCC lesions with microdissection and

LOH analysis of multiple chromosomal loci, we successfully demonstrated dynamic and successive accumulation of genetic alterations in early SCC.

Introduction

Esophageal squamous cell carcinoma (SCC) is one of the most common fatal carcinomas worldwide among gastrointestinal tumors and it shows some of the most malignant characteristics. Despite recent advances in the diagnosis and treatment of esophageal SCC, prognosis remains poor. To improve prognosis, it is important to clarify the molecular mechanisms involved in the development of this tumor. It has been reported that multiple genetic alterations, including the activation of oncogenes such as *cyclin D1*, *hst1*, *int-2*, *c-erbB* and *c-myc*; inactivation of tumor suppressor genes such as *p16* (1-3), *p53* (3-7), *RB1* (8,9), *BRCA2* (10), *DCC* (8), *APC/MCC* (8), and *ING1* (11); amplification of *myc* (12) and the human EGF receptor (12) and mutations in genes of the *ras* family (13), are involved in esophageal carcinogenesis. Furthermore, high frequencies of loss of heterozygosity (LOH), which suggest the existence of recessive tumor suppressor genes, have been observed for loci on human chromosomes 3p, 5q, 8p, 9p, 9q, 10p, 11p, 13q, 14q, 16q, 17p, 17q, 18p, 18q, 19q and 21q (4,10,14-31). However, these findings do not provide any information regarding the genetic pathways that may underlie development of esophageal carcinoma.

To clarify the temporal and topographic pathways in the genetic evolution of esophageal SCC, we microdissected multiple foci from superficial mucosal SCC and invasive foci of esophageal SCC. We then carried out LOH analyses of the microdissected neoplastic foci. We compared the LOH patterns and deduced the likely order of genetic changes in the evolution of individual tumors.

Patients and methods

Patients. Sixteen consecutive patients with superficial esophageal SCC were enrolled in the present study at Hiroshima University Hospital, Hiroshima, Japan, in 2004.

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Table I. The clinicopathological characteristics of 16 men with superficial esophageal squamous cell carcinoma.

Case no.	Age (years)	Tumor location ^a	Size (mm)	Macroscopic type ^b	Tumor depth	No. of foci dissected
1	65	Mt	15	0-IIc	sm1	3
2	54	Mt	20	0-IIc	m3	3
3	79	Mt	20	0-IIc	sm2	5
4	83	Mt	30	0-IIc	sm1	5
5	78	Mt	10	0-IIa	sm1	4
6	72	Mt	60	0-IIc	m3	4
7	58	Ut	10	0-IIa	m3	4
8	63	Mt	10	0-IIc	sm1	4
9	78	Lt	10	0-IIc	sm2	4
10	67	Lt	10	0-IIc	sm1	4
11	64	Lt	40	0-IIc	m3	4
12	78	Ae	10	0-I	sm1	5
13	53	Mt	100	0-IIc+IIa	m3	3
14	57	Mt	10	0-IIc	sm1	6
15	55	Mt	20	0-IIc	sm1	3
16	71	Mt	8	0-IIc	m3	3

^aDetermined according to the criteria of the Japanese Society for Oesophageal Disease. Ut, upper thoracic esophagus; Mt, middle thoracic esophagus; Lt, lower thoracic esophagus; Ae, abdominal esophagus. ^b0-I, superficial and protruding type; 0-IIa, slightly elevated type; 0-IIc, slightly depressed type.

None of the patients had multiple esophageal SCCs, and none had received radiotherapy or chemotherapy prior to surgical removal of the tumor. The study was approved by the local ethics committee (No. I-RIN-HI-106).

Histological examination. Sections (4 μ m) were prepared from formalin-fixed, paraffin-embedded esophageal tissues obtained by endoscopic mucosal resection. The sections were stained with H&E for histological examination. The depth of invasion of superficial esophageal SCC was classified as intramucosal (m) or submucosal (sm). The depth of invasion was also subclassified as m1 to m3 or sm1 to sm3 according to the grade of invasion (1, mild invasion; 2, moderate invasion; or 3, remarkable invasion).

DNA extraction and microdissection. Formalin-fixed, paraffin-embedded normal and tumor tissue blocks were obtained from each patient. The tissue sections (10 μ m) were placed on glass slides and stained with H&E, and then dehydrated in a graded series of ethanols and air-dried. Cancerous and normal tissues (2x2 mm) on the slides were scraped up with sterile needles as per the microdissection technique. DNA was extracted from tissues with 20 μ l extraction buffer (100 mM Tris-HCl; 2 mM EDTA, pH 8.0; 400 μ l/ml proteinase K) at 55°C overnight. Samples were boiled for 7 min to inactivate the proteinase K, and 2 μ l of each extract was used for polymerase chain reaction (PCR) amplification.

Three to six carcinoma foci were microdissected individually from each superficial esophageal SCC. A total

of 64 foci were microdissected from the 16 cases. Reference control tissue was microdissected from the adjacent non-malignant tissue.

Microsatellite assay. Twelve oligonucleotide primer pairs specific for microsatellites D3S1234 (chromosome 3p14.2), D3S1300 (chromosome 3p14.2), D3S1568 (chromosome 3p21.3), D3S1621 (chromosome 3p21.3), D4S2632 (chromosome 4p12-p14), D7S490 (chromosome 7q31-q35), D10S501 (chromosome 10p15), D10S559 (chromosome 10p15), D13S325 (chromosome 13q14.11), D13S1366 (chromosome 13q14.2), D16S504 (chromosome 16q23.3-q24.1), D16S518 (chromosome 16q23.3-q24.1) and TP53 (chromosome 17p13.1) were used. The microsatellite assay was performed as described previously (33). In brief, each 15 μ l reaction mixture containing 10-20 ng genomic DNA, 6.7 mM Tris-HCl (pH 8.8), 6.7 mM EDTA, 6.7 mM MgCl₂, 0.33 μ M primer labeled with [γ -³²P]dATP, 0.175 μ M unlabeled primer, 1.5 mM each dNTP and 0.75 U AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ) was amplified with 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 30 sec. PCR products were separated by electrophoresis on 6% polyacrylamide-8 M urea-32% formamide gels and subjected to autoradiography overnight at -80°C on Fuji RX film.

LOH was identified in tumors when only one major band was detected in the DNA isolated from the cancerous tissue, whereas two major bands were present in the normal tissue specimen from the same sample. Moreover, LOH was classified as LOH α when the upper band was retained and

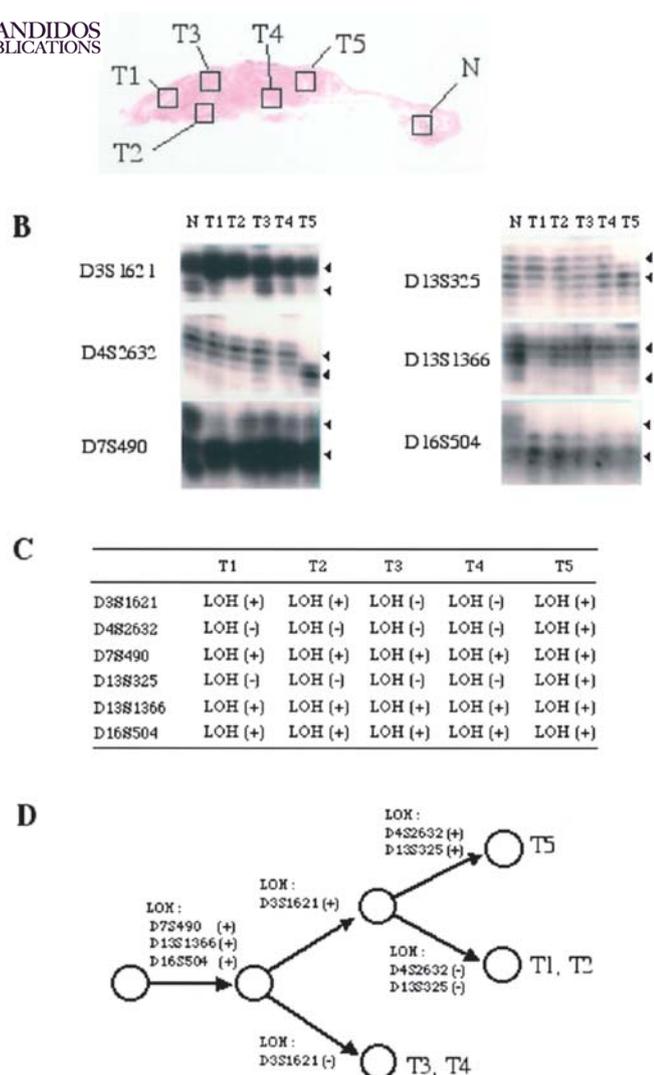


Figure 1. Patient 4. (A) H&E-stained section of esophageal tissue containing both tumor and normal tissue. T1, 2, 3, 4, 5, cancerous lesion; N, normal tissue. (B) Genetic analysis. Arrowheads indicate 2 major bands in each microsatellite locus. (C) Summary of LOH at each locus. (D) Proposed genetic pathway. Arrows indicate LOH.

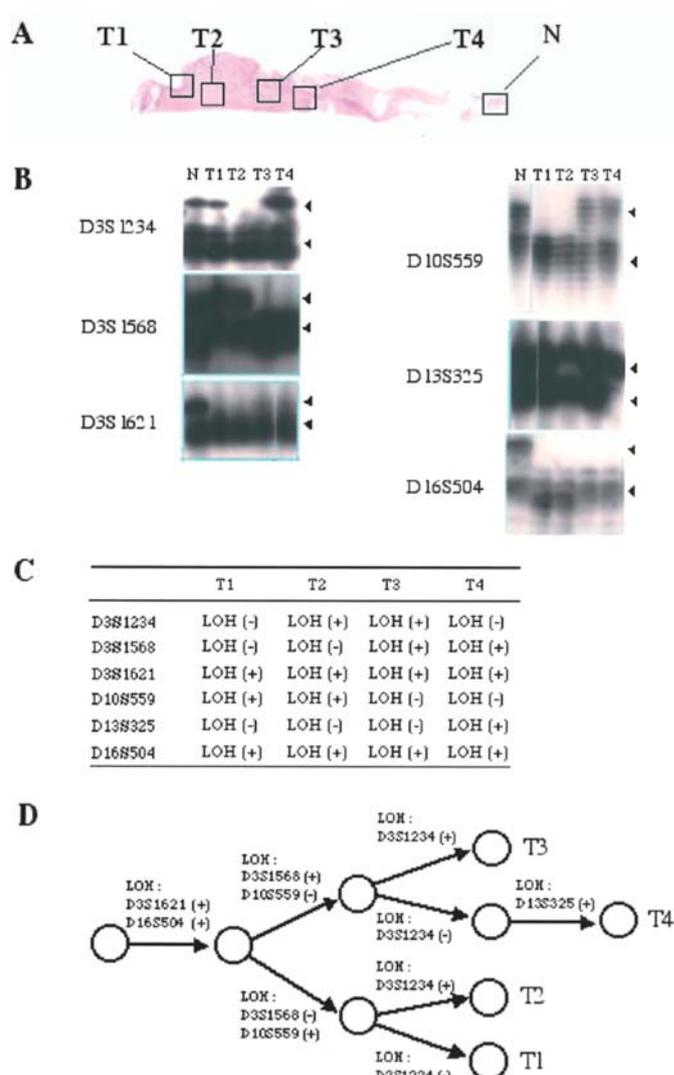


Figure 2. Patient 6. (A) H&E-stained section of esophageal tissue containing both tumor and normal tissue. T1, 2, 3, 4, cancerous lesion; N, normal tissue. (B) Genetic analysis. Arrowheads indicate 2 major bands in each microsatellite locus. (C) Summary of LOH at each locus. (D) Proposed genetic pathways. Arrows indicate LOH.

LOHb when the lower band was retained. Microsatellite instability was identified when additional bands not present in the normal tissue DNA were detected.

LOH was detected homogeneously or heterogeneously in the microdissected foci for each microsatellite locus. When homogeneous LOH of several microsatellite loci was detected throughout the microdissected foci, the lesion was considered to be the result of the expansion of a single clone. Heterogeneous LOH was detected as the tumor progressed or diverged genetically. During genetic progression, in addition to the early and homogeneous LOH events, LOH of some alleles was detected in only a subset of microdissected tumor foci, indicating linear genetic progression of the neoplastic clone. During genetic divergence, in addition to early homogeneous LOH events, different LOH patterns for several microsatellite loci were detected in different parts of the tumor, indicating that a single-neoplastic clone had diverged in two or more directions. The patterns of LOH were classified as follows: i) Single clonal neoplasms with

homogeneous genetic changes characterized by homogeneous loss of one or more microsatellite loci throughout the tumor, ii) single clonal neoplasms with genetic progression characterized by the identical loss of one or more microsatellite loci throughout the tumor, iii) single clonal neoplasms with genetic progression and divergence characterized by LOH of additional loci.

Results

Patients. The 16 superficial esophageal SCC patients were all male, with a mean age of 67.2±10.1 years (range, 54-83 years). The clinicopathological characteristics including age, sex, tumor location and size, macroscopic type and depth of the tumor are shown in Table I.

Patterns of LOH in esophageal SCC. The microdissected foci and representative genetic analysis for patients 4 and 6 are shown in Figs. 1 and 2, respectively. For example, in patient 4

Table II. LOH pattern of each microsatellite locus in individual tumors.

Case no.	D3S1234	D3S1300	D3S1568	D3S1621	D4S2632	D7S490	D10S559	D13S325	D13S1366	D16S504	D16S518	TP53
1	MSI		□			MSI	□	□		△	△	
2	MSI	●	●	△	△	■	□	●	●	△	△	
3	△	△	□	MSI		△	□	△	□	△	△	
4	△	●	●	□	□	■		□	■	■	△	
5	□	●	□	□	■	●	■	□	●	■	●	
6	□	●	□	■	△	●	□	□	●	■	△	
7	■	●	■			MSI	●	□				□
8	●	●	●	□	□	●	□	●		□	△	
9	△	□	●	△	△	□	●	□	MSI	●	■	△
10	△	●	□	●	●	□	□	□	□	□	■	△
11	□	●	□	●	△	●	■	□	■	●	△	■
12	△	●	□	□	●	●	□	□	△	●	●	△
13	△	●		□	●	●	□	□	●	■	■	■
14	□	■		■	□	□	□	□	□	●	□	□
15						●	□	□		●		■
16	■					□	□	□		●		●

●, LOH (-); ■, homogeneous LOH; □, heterogeneous LOH; △, homozygous alleles; blank space, not informative; MSI, microsatellite instability.

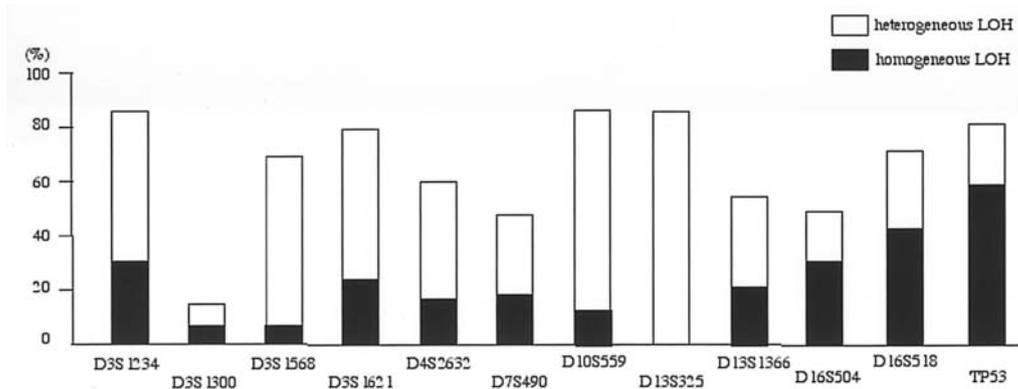


Figure 3. Frequencies of homogeneous and heterogeneous LOH patterns for each microsatellite locus.

(Fig. 1), LOH of D7S490, D13S1366 and D16S504 was detected in all microdissected areas. LOH of D3S1621 was detected in areas 1, 2 and 5. LOH of D4S2632 and D13S325 was detected in area 5. These findings suggest that LOH of D7S490, D13S1366 and D16S504 may occur as early events in the development of esophageal SCC. In patient 6 (Fig. 2), LOH of D3S1621 and D16S504 was detected in all microdissected areas. LOH of D3S1568 was detected in areas 3 and 4, LOH of D3S1234 was detected in areas 2 and 3, LOH of D10S559 was detected in areas 1 and 2, and LOH of D13S325 was detected in area 4. These findings suggest that LOH of D3S1621 and D16S504 may occur as early events and that LOH of D3S1568, D3S1234, D10S559 and D13S325 may be late events in the development of esophageal SCC. All cases showed pattern C (single clonal neoplasms with genetic progression and divergence).

There was no relation between the location of the tumor and genetic progression or divergence.

LOH of each microsatellite locus in esophageal SCC. The frequency and pattern of LOH at each microsatellite marker are shown in Table II and Fig. 3. A homogeneous LOH pattern was frequently observed at TP53 (60%), D16S518 (43%) and D3S1234 (29%), and the heterogeneous LOH pattern was frequently observed at D13S325 (87%), D10S559 (73%), D3S1568 (58%), D3S1234 (57%), D3S1621 (56%) and D4S2632 (43%).

Discussion

Cancer research studies have revealed that cancer is caused by the accumulation of alterations in cancer-associated genes. Several genetic alterations have been reported in



al SCC (33-40). Although the molecular events with the initiation and progression of esophageal SCC are not well-understood, various chromosome regions harboring putative tumor suppressor genes have been identified by the detection of LOH. One candidate tumor suppressor gene for esophageal SCC, *DICE1*, is located on chromosome 13q14. Another candidate tumor suppressor gene, *KLF6*, is located on chromosome 10p15. The *hTR*-repression-related gene is also located on chromosome 10p15. Therefore, we chose microsatellite loci D13S325 (chromosome 13q14.11) and D13S1366 (chromosome 13q14.2) for *DICE1* and D10S501 (chromosome 10p15) and D10S559 (chromosome 10p15) for *KLF6* and the *hTR*-repression-related genes, respectively. In addition, Li *et al* (37) reported high frequencies of LOH of D13S325 (71%) and D13S887 (75%). Wang *et al* (38) reported that 20% of esophageal SCCs showed LOH of D7S490. Kuroki *et al* (16) reported that 70% of cases showed LOH of D16S518, 55% of cases showed LOH of D3S1234 and D3S1300 and 55% of cases showed LOH of D3S1568 and D3S1621. We previously reported that 17% of esophageal SCCs showed LOH of D10S501 and that 50% of esophageal SCCs showed LOH of D10S559 (31). We chose to examine these loci in the present study.

Carcinomas, in general, have been postulated to progress from early neoplastic lesions to invasive foci through the sequential accumulation of multiple genetic changes (41-48). When genetic alterations are examined in multiple individually microdissected pre-invasive pathological foci, the temporal changes in the clonal neoplasms become evident. Fujii *et al* (42) described the genetic progression and divergence in the clonal evolution of breast cancer by studying the allelic loss in ductal *in situ* carcinoma and the invasive components of breast cancers. In the present study, we found genetic heterogeneity in all 16 cases of superficial SCC, suggesting genetic progression and/or divergence. During genetic progression, there was a linear and gradual accumulation of LOH. These heterogeneous patterns of allelic loss were consistent with either genetic progression or genetic divergence occurring during the clonal evolution of these neoplasms. In the present study, the clonal neoplastic process started with early and homogenous LOH of TP53 (60%), D16S518 (43%) and D3S1234 (29%) with late and heterogeneous LOH of D13S325 (87%), D10S559 (73%), D3S1568 (58%), D3S1234 (57%), D3S1621 (56%) and D4S2632 (43%).

TP53, for which homogenous LOH was frequently observed, is located in the field of the *p53* gene. *p53* is frequently mutated not only in esophageal carcinoma but also in gastric carcinoma (21%) (49) and colorectal carcinoma (46%) (50). The results of our present study are consistent with those of previous investigations. It has been reported that there is a high frequency of LOH of D16S518 and D3S1234 in various carcinomas including prostate carcinoma (51), pulmonary carcinoma (52), intrahepatic cholangiocarcinoma (53), uterine cervix carcinoma (54), oral carcinoma (55), breast carcinoma (56) and head and neck carcinoma (57). D16S518 is located in the *WWOX* gene (16), and D3S1234 is located adjacent to the fragile histidine triad (*FHIT*) gene. Our present findings indicate that *WWOX* and

FHIT may be related to the carcinogenesis of superficial SCC. In contrast, D13S325 and D10S559, for which heterogeneous LOH was frequently observed, are located adjacent to the *DICE1* and *PTEN/MMAC1* genes (37,58). These genes are thought to be related to the progression, not the initiation, of superficial SCC. However, there are various combination patterns, and therefore, it is likely that SCC progresses from various courses.

Concomitant LOH α and LOH β of a microsatellite marker in different neoplastic foci from a tumor sample were present in six cases. If DNA was isolated only from the whole tissue of a cancerous lesion, LOH would not have been detected because both alleles would be amplified. This fact supports the usefulness of microdissection in LOH analysis. Studies of LOH of microsatellite loci in DNA from the whole tissue without microdissection may lead to an underestimation of the frequencies of LOH.

In conclusion, extensive sampling of SCC lesions by microdissection and LOH analysis of multiple chromosomal loci revealed that dynamic and successive accumulation of genetic alterations occurs in superficial SCC.

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