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<td>Author(s)</td>
<td>An, JY; Fan, ZM; Gao, SS; Zhuang, ZH; Qin, YR; Li, JL; He, X; Tsao, GSW; Wang, LD</td>
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Loss of heterozygosity in multistage carcinogenesis of esophageal carcinoma at high-incidence area in Henan Province, China

Ji-Ye An, Zong-Min Fan, Shan-Shan Gao, Ze-Hao Zhuang, Yan-Ru Qin, Ji-Lin Li, Xin He, George Sai-Wah Tsao, Li-Dong Wang

Abstract

AIM: Microsatellites are the repeated DNA sequences scattered widely within the genomes and closely linked with many important genes. This study was designed to characterize the changes of microsatellite DNA loss of heterozygosity (LOH) in esophageal carcinogenesis.

METHODS: Allelic deletions in 32 cases of matched precancerous, cancerous and normal tissues were examined by syringe microdissection under an anatomic microscope and microsatellite polymorphism analysis using 15 polymorphic markers on chromosomes 3p, 5q, 6p, 9p, 13q, 17p, 17q and 18q.

RESULTS: Microsatellite DNA LOH was observed in precancerous and cancerous tissues, except D9S1752. The rate of LOH increased remarkably with the lesions progressed from basal cell hyperplasia (BCH) to squamous cell carcinoma (SCC) (P<0.05). Three markers, D9S171, D13S260 and TP53, showed the highest incidence of LOH (>60%). LOH loci were different in precancerous and cancerous tissues. LOH in D3S1234 and TP53 was the common event in different lesions from the same patients.

CONCLUSION: Microsatellite DNA LOH occurs in early stage of human esophageal carcinogenesis, even in BCH. With the lesion progressed, gene instability increases, the accumulation of this change may be one of the important mechanisms driving precancerous lesions to cancer.
this method, 90% purified cells could be collected
proteinase K (50
tpg were divided into BCH, DYS, CIS and SCC
According to cell morphologic changes, the esophageal epithelia
hematoxylin-eosin (HE) for histopathological diagnosis.
extraction and LOH analysis. Five slides were stained with
from cancer and adjacent parts were frozen and cut into
were divided into two parts: One was fixed with 85% ethanol
and paraffin embedded for histopathological diagnosis, and
were taken from cancer and adjacent tissues. Various tissues
in a -80
DNA was chosen as the criteria for LOH status. Individual
results were classified into three categories: LOH (loss of
allelic loss), and uninformative (homozygous alleles)

MATERIALS AND METHODS

Precancerous and cancerous tissues
Thirty-two surgically resected squamous cell carcinoma
(SCC) specimens were collected from Linxian Country, a
high-incidence area of EC in Henan Province, China. Of
the EC patients, 18 were males and 14 were females with
an average age of 59 years (range 44-73 years). All the
SCC patients were not treated by either chemotherapy or
radiotherapy before surgery. Surgically resected specimens
were divided into two parts: One was fixed with 85% ethanol
and paraffin embedded for histopathological diagnosis, and
the other was stored in liquid nitrogen and then transferred
in a -80 °C freezer for further use. Ten of thirty-two cases
were taken from cancer and adjacent tissues. Various tissues
from cancer and adjacent parts were frozen and cut into
5-μm thick sections and 30-60 slides were used for DNA
extraction and LOH analysis. Five slides were stained with
hematoxylin-eosin (HE) for histopathological diagnosis.
According to cell morphologic changes, the esophageal epithelia
were divided into BCH, DYS, CIS and SCC[3].

Syringe microdissection under anatomic microscope and DNA
extraction
According to the distribution of cells in HE-stained sections,
we dropped glycerol in matched spots, separated precancerous,
cancerous and normal cells by a 5-ml syringe under an anatomic
microscope, put them into 180 μL lysis buffer, added 20 μL
proteinase K (50 μg/μL), and kept overnight at 56 °C. By
this method, 90% purified cells could be collected[4]. DNA
was extracted according to the protocols of the QIAGEN
DNA Mini Kit.

LOH analysis
According to the results based on our previous work in
esophageal carcinogenesis at high-incidence area for EC
in Henan Province, China[8], we chose 15 microsatellite DNA
loci for LOH analysis using a circulating p53-Rb system.
These loci represented D3S966 (RASSF1A), D3S1234 and
D3S1300 (FHIT), D5S82 (DPI), D5S346 (APC), D6S497
(HLA, Waf1), D9S1752 (INK4a), D9S171 (INK4b), D13S260
(BRCA2), D13S321 and D13S233 (Rb1), TP53 and D17S786
(p53), D17S855 (BRCA1) and D18S858 (DCC).

Microsatellite polymorphism analysis
Each locus was amplified from 40 to 50 ng of template
DNA by PCR using paired primers, of which the forward
primer was 5’ end-labeled with [γ\(^{32}\)P] ATP. Eight percent
of nondenaturing polyacrylamide gel electrophoresis was
performed at 1 650 V for 2-3 h. After electrophoresis, the
gel was transferred to a 3 MM chromatography paper and
dried in a vacuum gel dryer at 80 °C for 1 h. The dried gel
was placed into an X-ray film cassette with an intensifying
screen and exposed to a Kodak film at -70 °C or at room
temperature for 2-48 h.

Result determination
Three individuals examined the relative intensities of
polymorphic alleles in different lesions. LOH status was
established if the intensity of one allele in the tumor was
significantly reduced as compared with its corresponding
allele in constitutive DNA. When the decision was not
unanimous, the density of alleles was determined by quantitative
densitometry. Reduction of 30% intensity of one allele in
tumor DNA compared to its matching allele in the constitutive
DNA was chosen as the criteria for LOH status. Individual
results were classified into three categories: LOH (loss of
one allele), heterozygosity retained (no allelic loss), and
uninformative (homozygous alleles)[9].

Statistical analysis
The data were analyzed by SPSS10.0 statistical software.
LOH frequency was performed by χ² test. P<0.05 was
considered statistically significant.

RESULTS

Relationship between LOH frequency and lesions (Figures 1
and 2, Table 1)
Microsatellite DNA LOH was observed in precancerous
and cancerous tissues, except D9S1752. The rate of LOH
increased remarkably with the lesion developed from BCH
to SCC (P<0.05). At least on LOH locus was 41% (7/17)
in BCH, 82% (14/17) in DYS, 100% (17/17) in CIS and
97% (31/32) in SCC. Three samples from SCC and one
sample from CIS were found to have deletions in 18 loci.

Distribution of microsatellite DNA LOH in different lesions
(Figure 3)
LOH loci were different in precancerous and cancerous
tissues. In BCH, DYS and SCC, the highest LOH loci
were TP53 (20%), D13S260 (33.3%) and TP53 (68.2%)
respectively. In CIS, the highest LOH markers had three
loci, D13S260, D9S171 and D3S966 (50%). Three markers,
D9S171, D13S260 and TP53, showed the highest incidence
of LOH (>60%). LOH in D3S1234 and TP53 was the
common event in different lesions of the same patients.

DISCUSSION

Inactivation of tumor suppressor genes appears to be one
of the genetic mechanisms involved in the development of
esophageal cancer. This process includes mutation of one
allele, followed by a deletion of the remaining one (LOH)
or homozygous deletion of both alleles. Allelic deletions
detected as LOH have been proved useful for mapping
regions of DNA that contain tumor suppressor genes[7].
LOH at specific chromosomal regions strongly suggested
the existence of tumor suppressor genes at the relevant segments. We performed deletion mapping analyses in 32 cases of matched precancerous, cancerous and normal tissues using 15 microsatellite markers on chromosomes 3p, 5q, 6p, 9p, 13q, 17p, 17q and 18q and found that microsatellite DNA LOH occurred in early precancerous stage and in BCH. With the development of the disease, the rate of LOH increased, indicating that the genetic changes occurred in the early stage of EC. With the lesion progressed, gene instability increased, the accumulation of this change may be one of the important mechanisms transforming precancerous lesions to cancer. LOH loci were different even at the same stages (such as DYS), indicating that there existed different molecular changes in precancerous lesions with a similar morphology. These changes might be the key factors to decide precancerous lesions developing in different directions, especially in those with a similar morphology. Esophageal carcinogenesis is a multistep progressive process characterized by multiple genetic changes (accumulation and overlap). The changes of p15INK4b, BRAC2 and p53 genes are common molecular events in esophageal carcinogenesis. LOH in D3S1234 and TP53 is the common event in different lesions of patients, indicating that the alternations of fragile histidine triad (FHIT) and p53 might be the key points to induce mild precancerous lesions to cancer. RASSF1A - one of the candidate TSGs - might be Figure 1 Allelic deletion patterns in TP53 microsatellite loci. Arrows indicate LOH in different lesions. B: basal cell hyperplasia (BCH), D: dysplasia (DYS), C: carcinoma in situ (CIS), S: squamous cell carcinoma (SCC), N: normal tissue, A: LOH in BCH; B: LOH in DYS; C: LOH in CIS; D: LOH in SCC.

Figure 2 Allelic deletion patterns of various microsatellite markers in ESCC. Paired tumor DNA (T) and non-neoplastic DNA (N) were examined for each case. Arrows indicate LOH in tumor samples. A: D3S966 LOH; B: D6S497 LOH; C: D9S1752 LOH; D: D13S233 LOH; E: TP53 LOH; F: D18S858 LOH.
involved in the esophageal carcinogenesis in Henan Province, China.

The p15\textsuperscript{INK4b} gene is an inhibitor of cyclin-dependent kinase 4, which has been identified in 95% genome sequence homogeneity with p16\textsuperscript{INK4a}. They encode two important cyclin-dependent kinase inhibitors, which could negatively regulate G1-S transition of the proliferating cells by contributing to the maintenance of pRb in an active state\[9\]. Xing \textit{et al} \[10\], reported that both p15\textsuperscript{INK4b} and p16\textsuperscript{INK4a} genes were frequently inactivated in EC, but the inactivation of p15\textsuperscript{INK4b} and p16\textsuperscript{INK4a} involved different mechanisms, with p16\textsuperscript{INK4a} predominantly affected by aberrant methylation and p15\textsuperscript{INK4b} by deletion.

We analyzed the allelic loss of p15\textsuperscript{INK4b} and p16\textsuperscript{INK4a} using D9S1752 and D9S171 microsatellite markers, and found that the LOH frequency of p15\textsuperscript{INK4b} was much higher than that of p16\textsuperscript{INK4a} in precancerous and cancerous lesions. Our results support the speculation of Xing \textit{et al} \[10\], and suggest that the deletion of p15\textsuperscript{INK4b} gene might be involved in the multistage development of EC at the high-incidence area in Henan Province, China. LOH of p16\textsuperscript{INK4a} might not be an important event in the esophageal carcinogenesis in the area.

BRCA1 and BRCA2 are tumor suppressor genes in familial breast-ovarian carcinoma syndrome and are located in different chromosomes. The BRCA2 gene is located on chromosome 13q12\[11\]. Extensive genetic and biochemical characterization has shown that BRCA2 is involved in the maintenance of chromosomal stability. It could serve as a critical mediator of DNA repair through direct interactions with Rad51 and might play an important role in recombination-mediated double-strand DNA break repair\[12\]. Harada \textit{et al} \[13\], performed a fine deletion mapping on 13q by analyzing 60 EC patients with 18 polymorphic markers and found the frequent loss at D13S260 (43.7%). Up to now, to our knowledge, there are no other reports on the changes of BRCA2 in precancerous tissues. In our experiment, BRCA2 gene alternations were detected as 0% in BCH, 33% in DYS, 50% in CIS, and 61.5% in SCC, indicating that the deletion of BRCA2 might be involved in the development of EC and is one of the common molecular events in the esophageal carcinogenesis.

The p53 tumor suppressor gene is located on chromosome

<p>| Table 1 | Comparison between frequency of microsatellite LOH in different lesions of esophagus |</p>
<table>
<thead>
<tr>
<th>LOH frequency n (%)</th>
<th>BCH</th>
<th>DYS</th>
<th>CIS</th>
<th>SCC</th>
<th>p</th>
</tr>
</thead>
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<tr>
<td>D3S1234</td>
<td>1/14 (7.1)</td>
<td>3/12 (25)</td>
<td>5/15 (33.30)</td>
<td>9/23 (39.1)</td>
<td>0.042</td>
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<tr>
<td>D3S1300</td>
<td>0/11 (0)</td>
<td>2/10 (20)</td>
<td>4/15 (40)</td>
<td>10/26 (38.5)</td>
<td>0.024</td>
</tr>
<tr>
<td>D3S966</td>
<td>0/13 (0)</td>
<td>3/12 (23.1)</td>
<td>8/15 (50)</td>
<td>11/20 (55.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>D5S82</td>
<td>2/12 (16.7)</td>
<td>2/12 (16.7)</td>
<td>6/15 (40)</td>
<td>8/20 (40.0)</td>
<td>0.010</td>
</tr>
<tr>
<td>D5S346</td>
<td>2/12 (16.7)</td>
<td>2/12 (16.7)</td>
<td>4/10 (40)</td>
<td>14/25 (56.0)</td>
<td>0.011</td>
</tr>
<tr>
<td>D6S497</td>
<td>0/10 (0)</td>
<td>0/13 (0)</td>
<td>0/12 (0)</td>
<td>9/26 (34.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>D9S1752</td>
<td>1/15 (6.7)</td>
<td>2/12 (16.7)</td>
<td>3/15 (20)</td>
<td>5/25 (20.0)</td>
<td>0.315</td>
</tr>
<tr>
<td>D9S171</td>
<td>0/13 (0)</td>
<td>5/16 (31.3)</td>
<td>7/14 (50)</td>
<td>14/23 (60.8)</td>
<td>0.000</td>
</tr>
<tr>
<td>D13S233</td>
<td>0/11 (0)</td>
<td>3/13 (23.1)</td>
<td>6/16 (37.5)</td>
<td>10/21 (47.6)</td>
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<tr>
<td>D13S321</td>
<td>0/14 (0)</td>
<td>3/12 (25)</td>
<td>6/14 (42.9)</td>
<td>11/25 (44.0)</td>
<td>0.006</td>
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<tr>
<td>D13S260</td>
<td>0/16 (0)</td>
<td>4/12 (33.3)</td>
<td>7/14 (50)</td>
<td>16/26 (61.5)</td>
<td>0.000</td>
</tr>
<tr>
<td>D17S855</td>
<td>2/13 (15.4)</td>
<td>4/14 (28.6)</td>
<td>6/15 (40)</td>
<td>14/26 (53.8)</td>
<td>0.014</td>
</tr>
<tr>
<td>TP53</td>
<td>3/15 (20)</td>
<td>4/13 (30.8)</td>
<td>7/16 (43.8)</td>
<td>15/22 (68.2)</td>
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<tr>
<td>D17S786</td>
<td>0/14 (0)</td>
<td>3/12 (25)</td>
<td>6/14 (42.9)</td>
<td>12/22 (54.5)</td>
<td>0.001</td>
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<tr>
<td>D18S858</td>
<td>0/13 (0)</td>
<td>3/16 (18.8)</td>
<td>4/12 (33.3)</td>
<td>10/18 (55.6)</td>
<td>0.000</td>
</tr>
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MSM: microsatellite marker; LOH: loss of heterozygosity; BCH: basal cell hyperplasia; DYS: dysplasia; CIS: carcinoma \textit{in situ}; SCC: squamous cell carcinoma; -: not done.

Figure 3 | Distributions of microsatellite DNA-LOH in different precancerous lesions of esophagus FAL: fractional allelic loss for each tumor ○ retention of heterozygosity ■ loss of heterozygosity Ø uninformative, X not done.

Locus Localization | BCH | DYS | CIS
<table>
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<tr>
<td>D3S1234</td>
<td>3p21.1-p14.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D3S1300</td>
<td>3p21.1-3p14.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D3S966</td>
<td>3p21.1-3p23.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D5S82</td>
<td>5p23-5p15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D5S346</td>
<td>5p21-5p22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D6S497</td>
<td>6p21.2-21.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D9S1752</td>
<td>9p21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D9S171</td>
<td>9p21-9p21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D13S233</td>
<td>13p14.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D13S321</td>
<td>13p31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D13S260</td>
<td>13q12.3-13q12.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D17S855</td>
<td>17p13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP53</td>
<td>17p13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D17S86</td>
<td>17p22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D18S858</td>
<td>17p22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAL (%)</td>
<td>25 0 0 17 0 14 0 0 17 33 20 0 0 40 0 0 0 83 0 14 29 29 43 0 67 0 71 14 50 50 50 17 50 43 100 14 50 43 86 14 43 29 67 40 67 50 50 29 60 86 50</td>
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</table>
17p13.1 and encodes a 53 ku nuclear phosphoprotein that binds to DNA and blocks the progression of the cell cycle in response to DNA damage and mediates apoptosis[14]. Allelic loss of p53 gene on chromosome 17p13.1 has been demonstrated to be one “hit” of inactivation of p53 gene. It was reported that the frequencies of LOH ranged from 65% to 83.8%, and often co-existed with mutations (“two hits”) in EC[13]. In our experiments, the deletion of p53 occurred in the early stage of BCH (20%), indicating that the alternations of p53 might be the key points to induce mild precancersous lesions to cancer.

Frequent allele loss has been observed on chromosome 3p in EC and its premalignant lesions, indicating that inactivation of putative tumor suppressor genes on 3p may be involved in early stages of esophageal carcinogenesis. It has been reported that FHIT gene is a novel tumor suppressor gene located on chromosome 3p14.2[16]. Highly frequent abnormal transcripts of FHIT gene have been found in a variety of human cancers, including cancers of the digestive tract, lung, breast, and head and neck[17-19]. Point mutations of the FHIT gene were also seen in gastric and breast carcinomas, but very rarely. A review of the literature showed that different results were obtained by different researchers[18-20].

Zou et al[16], reported that the deletions of FHIT were involved in 11 of 50 (22%) EC samples. On the other hand, Mori et al[21], observed that the LOH frequency of the FHIT gene was 76%. In our experiment, loss of FHIT was detectable in 7% BCH, 20% DYS, 33% CIS and 30% SCC. Although the LOH frequency of FHIT gene was low in our results, the change occurred in each stage of esophageal carcinogenesis. Exposure to different environmental carcinogens might be one of the reasons why there existed differences in different areas.

RASSF1A is a novel tumor suppressor gene that was isolated recently from the lung tumor suppressor locus 3p21.3[22]. The presence of a Ras associated domain in RASSF1A suggested that this protein might function as an effector of Ras signaling in normal cells. Its protein structure also suggested that this protein might function as an effector of Ras signaling in normal cells. Its protein structure also suggested that RASSF1A might participate in the DNA damage response or in DNA damage-induced regulation of other cell signaling events[11,23]. Chan et al[24], demonstrated the methylation status of RASSF1A and the frequency of LOH in 3p21.3 region in bladder cancer and found that the frequency of LOH and methylation of RASSF1A were 57.9% and 47.5% respectively, showing that RASSF1A might be inactivated in accordance with the two-hit inactivation model, involving deletion of one allele and hypermethylation of the other[25]. Up to now, much research work has been done in promoter hypermethylation of RASSF1A. In the present study, we observed that the LOH frequency of D3S96 was 23.1% in DYS, 50% in CIS and 55% in SCC, indicating that RASSF1A might be involved in the esophageal carcinogenesis at the high-incidence area in Henan Province, China. To our knowledge, this report is the first to identify allelic loss of RASSF1A during esophageal carcinogenesis in Henan Province, China.

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