Basic Research Paper

Application of multicolor fluorescence in situ hybridization to early diagnosis of esophageal squamous cell carcinoma

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Key words: esophageal tumor, neoplasm, squamous cell, early diagnosis, fluorescence in situ hybridization, chromosome aberrations

Background and Objective: The detection of chromosome aberrations has been used to diagnose some tumors. There are, however, no chromosomal markers for the diagnosis of esophageal carcinoma yet. This study analyzed the aberrations of some chromosomes in esophageal squamous cell carcinoma (ESCC) and its premalignant lesions, exploring the application of multicolor fluorescence in situ hybridization (M-FISH) for the early diagnosis and risk prediction of precursor lesions of ESCC.

Methods: Aberration statuses of chromosomes 3, 8, 10, 12, 17 and 20 were investigated in 124 esophageal tissue samples from 113 patients using M-FISH with chromosome-specific centromere DNA probes. The relationship between chromosome gains and clinicopathologic parameters was analyzed.

Results: Copy number gains on chromosomes 3, 8, 10, 12, 17 and 20 in ESCC were 80.9% (93/115), 81.0% (94/116), 70.5% (79/112), 75.9% (85/112), 68.7% (79/115) and 82.8% (48/58), respectively. No statistical relationships were found between chromosome aberrations and clinicopathologic parameters (p > 0.05). Polysomy rates of the six chromosomes in precursor lesions were 62.5% (5/8), 75.0% (6/8), 62.5% (5/8), 87.5% (7/8), 87.5% (7/8) and 100% (3/3); while those in early-stage ESCC were 80.0% (12/15), 93.8% (15/16), 71.4% (10/14), 64.3% (9/14), 75.0% (12/16) and 63.6% (7/11). Conclusions: Positive aneuploidy rates of chromosomes 3, 8, 10, 12, 17 and 20 are highly detected in both ESCC and its precursor lesions. M-FISH is helpful in the early diagnosis of ESCC, thus it may be used as a method to predict the risk to ESCC.

Esophageal carcinoma is one of the most common alimentary malignancies, and more than 90% of esophageal carcinomas in China are squamous cell carcinoma (ESCC). It is generally accepted that the oncogenesis of esophageal squamous cell includes the process from normal esophageal mucous membrane development to the hyperplasia of basal cells, to minor, moderate or severe dysplasia, to carcinoma in situ and finally, the invasive carcinoma, in which all dysplasia in different degrees is regarded as the precursor lesions.1 Because of the stealthy clinical feature of the early esophageal carcinoma, more than 50% of patients are diagnosed in an advanced stage where radical resection of the tumor becomes impossible or metastasis is observed by imaging examination.2 Therefore, the overall five-year-survival rate remains only 25–40% for patients of esophageal carcinoma post tumor resection.3 However, the five-year-survival for patients caught in the early stage exceeds 90% if they are treated in time.4 It is obvious that early diagnosis is an efficient way to improve the survival and decrease the mortality of esophageal carcinoma patients. Because problems exist in the methods of early diagnosis of esophageal carcinoma, researchers are seeking a diagnostic method that is safer, simpler and more efficient, and to develop a technique capable of evaluating the risk of esophageal oncogenesis.

Research has shown that molecular cytogenetic changes happen much earlier than cellular morphological changes during the process of epithelial oncogenesis.5 Based on worldwide research on chromosomes of esophageal carcinoma, changes of copy number of chromosomes exist not only in almost all cases of esophageal carcinoma, changes of copy number of chromosomes exist not only in almost all cases of esophageal carcinomas,6,7 but also in tissues neighboring the ESCC tumor.8,9 The development of esophageal carcinoma.

Fluorescence in situ hybridization (FISH), which is excellent in sensitivity, specificity, signal-noise ratio, and is characterized by the advantage being capable of identifying the abnormality of
both the structure and copy number of chromosomes, is one of the techniques most frequently used in modern cancer genetics. At present, detection on chromosome aberration by FISH has been widely used for the diagnoses of gene abnormalities of a variety of tumors, such as hematological malignancies, breast cancer and bladder transitional cell carcinoma, and therefore guides both the treatment and prognosis on a chromosome or gene level. However, no diagnostic chromosome marker has yet been developed for esophageal carcinoma.

In order to further investigate the cytogenetic features of esophageal carcinoma to find the possible non-random chromosome aberrations correlating to oncogenesis of ESCC, we adopted multicolor fluorescence in situ hybridization (M-FISH) to detect the heteroploid in chromosomes 3, 8, 10, 12, 17 and 20 of ESCC and various degrees of precursor lesions respectively, and discussed the feasibility of M-FISH to be applied to early diagnosis of ESCC and risk prediction of precursor lesions.

Materials and Methods

Collection of the samples. One hundred and twenty-four samples of esophageal diseases were collected from 113 ESCC patients, including 76 males and 37 females, aged from 43 to 79 years with a median age of 58 years. The 113 patients included 45 in stage I or II and 68 in stage III; 21 highly differentiated, 59 moderately differentiated, and 33 poorly differentiated; as well as eight cases of precursor lesion (including basal cell hyperplasia), 16 early ESCCs and 100 advanced ESCCs. All cases were proved by pathology, and no patients underwent radiotherapy or chemotherapy before the surgery.

The samples came from three sources as follows: (1) 10 cases of ESCC fresh tissue sampled from biopsy by Endoscopy Department of Cancer Institute & Hospital, Chinese Academy of Medical Sciences from April to May 2006, included two cases of early ESCC. The cancer tissue detected by endoscopy was sampled by biopsy forceps. (2) 42 cases of ESCC fresh tissue resected by Cancer Institute & Hospital, Chinese Academy of Medical Sciences from November 2006 to September 2007, included one ESCC in early stage and 41 in advanced stage. Each resected sample was cut open along the longitudinal axis of the esophagus and then the specimen was sampled directly from the tumor. (3) 72 cases of -70°C frozen specimen of ESCC with its adjacent iodine-staining positive lesion, which were resected by Cancer Hospital of Linzhou City, Henan Province from October to November 2003, including eight precursor lesions of ESCC (three basal cell hyperplasias, one moderate dysplasia, two severe dysplasias, and two carcinoma in situ); 13 ESCCs in early stage and 51 in advanced stage. The resected specimen was cut open along the longitudinal axis, outspread adequately, fixed, stained with iodine, and then observed the staining status of the esophageal mucous membrane. Sampling was carried out from the tumor and the iodine-staining negative area respectively.

Preparation of the specimen. The specimen was cut into pieces with a surgical scissor, treated with hypotonic solution (0.075 mol/L KCL), fixed with 3:1 methanol/acetic acid (Glacial), and then prepared into a single-cell suspension, which was kept in a 4°C refrigerator and available for routine smears aging overnight under ambient temperature for laboratory examination.

Selection and labeling of probes. Based on the results of comparative genomic hybridization (CGH) of esophageal carcinoma in the preliminary study as well as the frequent aberrant chromosomes as proved by previous report, centromere probes of chromosomes 3, 8, 10, 12, 17 and 20 were selected. Fluorescin was used to directly label the probes according to the instructions of BioPrime DNA Labeling System (Invitrogen).

In situ hybridization. Preparation of the glass slice, the glass slices were treated with RNase (Sigma, 0.1 g/L) and then Pepsin (Sigma, 0.1/L) for 40 min and 20 min respectively, fixed with 10 g/L Paraformaldehyde for ten min, washed with PBS buffer, denaturalized with 70% Formamide/2x SSC (pH 7.0) under 72°C for three min, precooled 2x SSC under 4°C twice for three min for each, dehydrated with ethanol with gradient concentration of 75%, 85% and 100% successively, and then dried under ambient temperature.

Preparation of probes. 2 μL of each probe was taken, together with 4 μL salmon sperm DNA (Sigma), 2 μL Human Cot-1 DNA (Infirton), 42 μL H2O, 5 μL 3 M NaAc and 110 μL anhydrous alcohol (-20°C) to prepare a deposition system, which was mixed even, kept under -70°C for at least 30 min, centrifuged with 14,000 g for ten min with the supernatant removed, and then washed with 75% ethanol (-20°C). The deposit obtained was dried in ambient temperature, added with 7 μL centromere probe hybridization liquid (containing 3.5 μL Formamide deionized, 1.75 μL 40% Dextran Sulfate, 0.7 μL 20x SSC and 1.05 μL double distilled water), dissolved for more than 30 min in a dark place under 37°C, denaturalized in a 72°C-water bath for eight min, and then renaturalized in a 37°C-water bath for 20–30 min.

Hybridization. the specimen on the slice was added with probe, covered by the cover glass to prevent bubbles, sealed with rubber cement, kept in a moisturizing box and then hybridized for about 24 h in darkness in a 37°C thermostat.

Washing of the slice. The sealing rubber was removed and the slice was put into a solution of 50% formamide/2x SSC for washing under 43°C for 15 min (while being rocked to avoid the non-specific hybridization), washed with tossing twice for three min for each under ambient temperature, dehydrated with ethanol with gradient concentrations, dried in darkness under ambient temperature, added with 10 μL Diamino-phenylindole (DAPI, sigma) for re-staining and then sealed.

The fluorescein used in this research included Cy3, FITC or Spectrum Green and Cy5, two or three probes labeled with different fluoresceins might be transused for simultaneous hybridization on a same slice. The labeled probe was firstly hybridized with metaphase chromosomes of healthy human peripheral lymphocytes in order to prove both the specificity and reliability of the probe, and at least one sample obtained from healthy human peripheral lymphocytes was adopted as the control for each hybridization.

Signal counting and determination criteria. Images were observed under a fluorescent microscope and then videoed by a CCD controlled by the MetaMorph® Imaging System (Universal Imaging Corporation). At least 50–400 interphase nuclei were...
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FISH result of ESCC interphase nucleus. Aberration of chromosome quantity in ESCC tissue manifested mainly as the chromosome gains, while rare loss was observed. The rate of copy number gains of chromosomes 3, 8, 10, 12, 17 and 20 were respectively 80.9% (93/115), 81.0% (94/116), 70.5% (79/112), 75.9% (85/112), 68.7% (79/115) and 82.8% (48/58) in ESCC, with the highest rate observed in chromosomes 20, 8 and 3 (Table 1 and Fig. 2). In addition, monosomy appeared in chromosomes 10 and 17 in two and one case(s) of ESCC respectively (Fig. 2H), with obvious chromosome loss with the rate of positive cell up to 72.6%.

FISH result of interphase nucleus of precursor lesion adjacent to ESCC tumor. Copy gains of the foresaid 6 chromosomes were also observed with fairly high rates in the ESCC precursor lesion, which were 62.5% (5/8), 75.0% (6/8), 62.5% (5/8), 87.5% (7/8), 87.5% (7/8) and 100% (3/3) respectively (Table 1 and Fig. 3).

FISH result of both ESCC and the precursor lesion adjacent to the tumor in the same case. The difference of chromosome aberration was analyzed between individual tumor tissue and its adjacent precursor lesion in three cases, as well as between early/advanced ESCC tissues from the same patient in four cases. The result showed that the degree of the chromosome aberration became more and more obvious along with the pathological progression (Fig. 3A and B), while the chromosome aberration might not be completely the same between different locations with the same pathological grade (Fig. 3E and F). However, roughly the same chromosome aberration was observed between early and advanced ESCC tissue in the same case.

Statistics. SPSS 13.0 package was used, and \( \chi^2 \) was adopted to analyze the correlation between the copy gains of chromosomes and clinical-pathologic parameters. \( p < 0.05 \) was considered as statistical significance.

Result

FISH result of healthy human peripheral lymphocytes. A slice of metaphase chromosome of healthy human peripheral blood was used for hybridization and each centromere probe manifested as two hybridization signals (Fig. 1).
Relationship between chromosome aberration in ESCC and clinicopathologic parameters. No correlation with statistical significance was found between copy gains of chromosomes under investigation and the clinicopathologic parameters such as gender, age, T stage, lymph node metastasis, or pathologic stage/grade, with \( p > 0.05 \).

Discussion

It has been proven by genetic research that numerical chromosome aberrations frequently occur in human malignancies, and that the quantity of abnormalities of chromosomes in the malignancies occupies a rate much higher than that in the benign diseases. The chromosomes under investigation in this research all have high frequency of quantity aberration, especially the chromosomes 20, 8 and 3, implying their possible significance in the oncogenesis and development of esophageal carcinoma.

The presence of a certain rate of chromosome aberration in precursor lesion, early and advanced ESCC, as discovered in our research, suggests that changes of polyploid status of the foresaid six chromosomes has happened as early as the early stage of ESCC and even the precursor lesion. However, aberration rate of chromosomes 3, 8 and 10 in precursor lesions increased significantly along with the pathological progression, as compared with early ESCC, while the result of chromosomes 12, 17 and 20 (the three chromosomes with the highest rate of aberration in the precursor lesion) was quite contrary, which suggests that copy number gains of chromosomes 12, 17 and 20 (especially 12 and 20) occurring earlier than the other three chromosomes in the precursor lesion may be the early event of chromosome aberration. This has possible significance concerning the research on early diagnosis of precursor aberration. The aberration rate of chromosomes 12 and 20 was higher in the advanced ESCC than in the early ESCC. However, the aberration rate of chromosome 8 was very high (up to 93.8%), which suggests that this abnormality may be a relatively earlier genetic alteration during the oncogenetic process of ESCC. Because there were less samples of the precursor lesion and early ESCC, no conclusion can be made as to which chromosome aberration acts as the key role in oncogenesis and development of ESCC until further investigation is carried out.

The significant loss of 10 p and 10 q in the metastatic tumor, as showed by the CGH investigation concerning the primary tumor of ESCC and the lymph node metastasis, implies that the gene responsible for ESCC metastasis may exist on 10 p and 10 q. In this research, similarly, we also found the presence of monosomy of chromosome 10 in two cases of ESCC, either of which was complicated with lymph node metastasis. Therefore, it can be inferred that significant correlation may exist between the loss of chromosome 10 and the lymph node metastasis of ESCC.

The fact that the aberrant degree of the same chromosome became more and more obvious along with the pathological progression, as shown in this research on the lesions with different pathological grades in the same patient, strongly supports the multi-stage progressive process of oncogenesis of esophageal epithelial carcinoma.

No unified result is available as to the relationship between the chromosome aberration and clinicopathologic parameters of ESCC, and no statistical significance was found between copy number gains of chromosome and the clinicopathologic parameters. Therefore, no acceptable conclusion can be made as to whether a more valuable prognostic marker can be discovered through research on genetic classification until further investigations are implemented.

At present, standards of evaluation on FISH signal have not been unified. Based on previous reports and the results obtained from our preliminary study, we adopted a stricter standard of signal determination in order to find vital chromosome variations, and to make it helpful for the early diagnosis of esophageal carcinoma.

Iodine staining was used in this research as an adjuvant method for sampling the precursor lesion. As found by clinical observation, non-iodine staining areas often exist in the tissue adjacent to the resected tumor of esophageal carcinoma and are proved to be precursor lesion with various grades in a status with high risk of establishment of malignancy can be screened by FISH from the population with minor, moderate dysplasia or even basal cell hyperplasia, the ultra-early diagnosis of ESCC can be accomplished.

The presence of a high rate of numerical chromosome aberrations in both ESCC and its precursor lesions, as shown by this research, suggests that the chromosome aberration happens as early as the early oncogenesis of esophageal carcinoma, and that M-FISH is helpful for the early diagnosis of ESCC and may be adopted as a method for risk prediction of oncogenesis.

Table 1 Chromosome aberrations in 124 esophageal lesions

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of lesions</th>
<th>Chromosome 3 (%)</th>
<th>Chromosome 8 (%)</th>
<th>Chromosome 10 (%)</th>
<th>Chromosome 12 (%)</th>
<th>Chromosome 17 (%)</th>
<th>Chromosome 20 (%)</th>
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<tbody>
<tr>
<td>Precursor lesions</td>
<td>8</td>
<td>62.5(5/8)</td>
<td>75.0(6/8)</td>
<td>62.5(5/8)</td>
<td>87.5(7/8)</td>
<td>87.5(7/8)</td>
<td>100(3/3)</td>
</tr>
<tr>
<td>ESCC</td>
<td>116</td>
<td>80.9(93/115)</td>
<td>81.0(94/116)</td>
<td>70.5(79/112)</td>
<td>75.9(85/112)</td>
<td>68.7(79/115)</td>
<td>82.8(48/58)</td>
</tr>
<tr>
<td>Early stage ESCC</td>
<td>16</td>
<td>80.0(12/15)</td>
<td>93.8(15/16)</td>
<td>71.4(10/14)</td>
<td>64.3(9/14)</td>
<td>75.0(12/16)</td>
<td>63.6(7/11)</td>
</tr>
<tr>
<td>Late stage ESCC</td>
<td>100</td>
<td>81.0(81/100)</td>
<td>79.0(79/100)</td>
<td>70.4(69/98)</td>
<td>77.6(76/98)</td>
<td>67.7(67/99)</td>
<td>87.2(41/47)</td>
</tr>
</tbody>
</table>
Figure 2. Chromosome aberrations in interphase nuclei of early-stage and late-stage ESCC detected by FISH (X630). (A) Polysomy of chromosome 8 (red) and trisomy of chromosome 17 (green) in early-stage ESCC under endoscopy; (B) Trisomy of chromosome 8 (red) and disomy of chromosome 17 (green) in early-stage ESCC under endoscopy; (C) Disomy of chromosomes 12 (red), 17 (yellow) and 20 (green) in late-stage ESCC; (D) Trisomy of chromosomes 10 (red), 8 (yellow) and 3 (green) in late-stage ESCC; (E) Disomy of chromosomes 3 (green), 17 (yellow), but trisomy of chromosome 8 (red) in late-stage ESCC; (F) Trisomy of chromosomes 10 (red), 3 (green) and polysomy of chromosome 8 (yellow) in late-stage ESCC; (G) Trisomy of chromosome 3 (red) and polysomy of chromosome 8 (green) in late-stage ESCC; (H) Monosomy of chromosome 10 in late-stage ESCC; (I–K) Polysomy of chromosomes 12 (red), 20 (green) and tetrasomy of chromosome 17 (yellow) in late-stage ESCC in the same visual field; (L) The combined image of (I–K).
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Figure 3. Chromosome aberrations in interphase nuclei of esophageal precursor lesions detected by FISH (X630). (A) Trisomy of chromosomes 8 (green) and 17 (red) in basal cell hyperplasia; (B) Tetrasomy of chromosome 8 (green) and trisomy of chromosome 17 (red) in late-stage ESCC from the same case with (A); (C and D) Disomy of chromosomes 3 (red), 10 (green), but gain of chromosome 8 in moderate dysplasia; (E) Tetrasomy of chromosomes 10 (green) and 3 (red) in severe dysplasia from the same case with (E); (F) Tetrasomy of chromosomes 10 (green) and 3 (red) in severe dysplasia; (G and H) Polysomy of chromosome 8 and trisomy of chromosome 10 (green), tetrasomy of chromosome 3 (red) in in situ carcinoma from the same case with (C and D).

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References