Comparative genomic hybridization analysis of genetic aberrations associated with development of esophageal squamous cell carcinoma in Henan, China

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AIM: To characterize cytogenetic alterations in esophageal squamous cell carcinoma (ESCC) and its metastasis.

METHODS: A total of 37 cases of primary ESCC and 15 pairs of primary ESCC tumors and their matched metastatic lymph nodes cases were enrolled from Linzhou, the high incidence area for ESCC in Henan, northern China. The comparative genomic hybridization (CGH) was applied to determine the chromosomal aberrations on the DNA extracted from the frozen ESCC and metastatic lymph node samples from these patients.

RESULTS: CGH showed chromosomal aberrations in all the cases. In 37 cases of primary ESCC, chromosomal profile of DNA copy number was characterized by frequently detected gains at 8q (29/37, 78%), 3q (24/37, 65%), 5p (19/37, 51%); and frequently detected losses at 3p (21/37, 57%), 8p and 9q (14/37, 38%). In 15 pairs of primary ESCC tumors and their matched metastatic lymph node cases, the majority of the chromosomal aberrations in both primary tumor and metastatic lymph node lesions were consistent with the primary ESCC cases, but new candidate regions of interest were also detected. The most significant finding is the gains of chromosome 6p with a minimum high-level amplification region at 6p12-6q12 in 7 metastatic lymph nodes but only in 2 corresponding primary tumors (P = 0.05) and 20p with a minimum high-level amplification region at 20p12 in 11 metastatic lymph nodes but only in 5 corresponding primary tumors (P < 0.05). Another interesting finding is the loss of chromosome 10p and 10q in 8 and 7 metastatic lymph nodes but only in 2 corresponding primary tumors (P < 0.05).

CONCLUSION: Using the CGH technique to detect chromosomal aberrations in both the primary tumor and its metastatic lymph nodes of ESCC, gains of 8q, 3q and 5p and loss of 3p, 8p, 9q and 13q were specifically implicated in ESCC in Linzhou population. Gains of 6p and 20p and loss of 10pq may contribute to the lymph node metastasis of ESCC. These findings suggest that the gains and losses of chromosomal regions may contain ESCC-related oncogenes and tumor suppressor genes and provide important theoretic information for identifying and cloning novel ESCC-related oncogenes and tumor suppressor genes.

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Key words: Comparative genomic hybridization; Genetic alterations; Esophageal squamous cell carcinoma; Metastatic lymph nodes

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INTRODUCTION

Advances in diagnostic and therapeutic modalities for malignancies have improved the survival of cancer patients. However, the mortality rate of patients with esophageal squamous cell carcinoma (ESCC) is still very high due to its highly invasive nature and potential to metastasize to lymph nodes and distant organs. ESCC is one of the leading causes of cancer-related death
in Linxian, Henan Province in northern China, with a mortality rate of 161/100,000 for male and 103/100,000 for female. Recent studies by us and other authors have indicated multiple genetic alterations underlying the multistage carcinogenesis of ESCC, such as p53-Rb pathway. However, the mechanisms of human esophageal multistage carcinogenesis in this area, especially the difference in genetic changes between primary ESCC and lymph node metastasis, are largely unknown.

Comparative genomic hybridization (CGH) analysis can provide comprehensive information about relative chromosomal losses and gains in malignant tumors in vitro. This technique can detect the changes of recurrent copy number and may highlight chromosomal regions containing genes that contribute to cancer development and/or progression. In this study, we used CGH analysis to examine 37 primary ESCC and 15 pairs of primary ESCC tumors and their corresponding metastatic lymph nodes, to elucidate the genetic pathway of carcinogenesis and to clarify the metastatic mechanisms of genetic aberrations in ESCC.

MATERIALS AND METHODS

The 37 primary tumor samples and 15 pairs of primary ESCC and their matched lymph node metastasis in this study were all from Linzhou, Henan, the high incidence area for ESCC. The primary specimen for each patient was collected at the time of surgical resection at the Department of Surgery, Yiaochun Hospital and Linzhon Hospital. All the patients underwent esophagectomy without preoperative radiotherapy and/or chemotherapy. Tumor tissue specimens were frozen in liquid nitrogen and kept in a freezer at -80°C until use.

Microdissection and DNA extraction

Tumor tissue was selected by histopathologic examination on the basis of estimated more than 80% cancer cells. The metastatic lymph nodes were embedded in OTC and cryosected into 15 μm serial slides under -20°C. For DNA extraction, we cut 22 serial 15 μm sections. The first and last ones were used for hematoxylin-cosin (HE) staining, and the remain 20 were lightly stained with hematoxylin. Under microscopic (MZ 12, Leica, Bensheim, Germany) observation, tumor tissues were microdissected manually from surrounding stromal tissues and normal cells with a disposable fine needle, and tissue fragments were collected and transferred into a microtue.

Genomic DNA was extracted from tumor specimens by proteinase K/sodium dodecyl sulfate digestion followed by phenol/chloroform/alcohol extraction. Normal reference DNA was prepared from peripheral blood lymphocytes of healthy donors.

Slide preparation

Metaphase chromosome spreads were prepared from peripheral blood leukocytes of healthy donors. Blood cells were cultured for 72 h in RPMI1640 containing 15% fetal bovine serum and penicillin-streptomycin (PHA 5 μg/mL). Blood cells were harvested by arresting with Colcemid (0.05 g/L) for 1h, followed by hypotonic treatment in KCl (0.075 mmol/L) for 20 min on ice and fixation in cold methanol: acetic acid (3:1).

Comparative genomic hybridization (CGH)

CGH was performed essentially as described previously. Briefly, genomic DNA from a tumor sample and a sex-matched normal reference was labeled directly with Spectrum Green-dUTP and SpectrumRed-dUTP (Vysis, Downers Grove, IL, USA) by nick translation. Two hundred nanograms of labeled tumor DNA and normal DNA probes were used in a 10 μL hybridization mixture (containing 55% formamide, 2 × SSC), and 10 μg human Cot1 DNA, which was denatured at 75°C for 5 min. The slide containing normal metaphase spreads was treated with RNase (100 mg/L) at 37°C for 1 h and then denatured at 75°C in 70% formamide, and 2 × SSC for 5 min. Hybridization with probes was then carried out at 37°C in a moist chamber for 72 h. The slide was then washed in 0.4 × SSC/0.3% NP-40 at 75°C for 2 min and then in 2 × SSC/0.1% NP-40 at room temperature for 2 min. After washing, the slide was counterstained with 1 mg/L DAPI in an antifade solution.

Digital image analysis

The hybridized metaphase chromosomes were analyzed using a digital image analysis system containing a Zeiss Axioskop microscope equipped with a Metachrome II cool-charged device camera (Photometrics, AZ). Three images of each metaphase were captured using filter wheel-mounted, single band excitation Rhodamine, FITC, and DAPI filters. The image analyses were carried out using Quips CGH Analysis software (Vysis). Five metaphases were analyzed to generate fluorescence ratio profiles in each case. Interpretation of the profiles was performed according to the program guidelines. The thresholds used for interpretation of gains and losses of a DNA sequence copy number was defined as a tumor/reference ratio greater than 1.25 or less than 0.75, respectively, by both the standard and the reverse hybridization methods.

Statistical analysis

We analyzed the genetic aberrations of ESCC using the Fisher exact test for independence. Differences with a P value less than 0.05 were considered statistically significant.

RESULTS

CGH analysis

A total of 230 DNA copy number gains and 212 DNA copy number losses were found in 37 ESCC samples, with an average of DNA copy number gains of 6.22 and DNA copy number losses of 5.73 per patient. In ESCC, the gain was most frequently detected on chromosome arms 8q (29/37, 78%), 3q (24/37, 65%) and 5p (19/37, 51%), (the chromosomal aberrant frequency on chromosome 6q, 7p and 7q was similar, all 38%), which was followed by (from high to low) 18p (12/37, 32%), 1q (11/37, 30%), 11q (10/37, 27%), 20q (10/37, 27%), 12p (9/37, 24%), 13q (6/37, 16%) and 18q (6/37, 16%). There were 20 cases
with high copy number amplifications (tumor/reference ratio > 1.5), which were located on chromosome 1pter-1q31, 1p11-1q22, 2q24.1-2q31, 3q24-3qter, 5p, 5q21-qter, 6p21-6q15, 7q11-7q22, 8q12-8qter, 12q12-12q13, 14q22-qter, with an average of 6.8 and 6.0, respectively, and 110 gains and 142 losses were found in 15 lymph nodes metastasis of 6.0/case).

The other three high copy-number amplifications were located on chromosome 6p21-6q15, 8q (11 of 15, 73%), 1q (11 of 15, 73%), 12p, 18q12, 18p and 20p. The chromosomal profile of DNA copy number losses was characterized as follows: the most frequently detected loss was on 3p (21/37, 57%), which was followed in turn (from high to low) by 8p (14/37, 38%), 9q (14/37, 38%), 11q (13/37, 35%), 13q (13/37, 35%), 5q (12/37, 32%), 1p (12/37, 32%), 4p (11/37, 30%), 8q (11/37, 30%), 16q (10/37, 27%), 1q (10/37, 27%) and 9p (10/37, 27%). A summary of genetic aberrations detected by CGH in ESCC is provided in Table 1 and Figure 1.

Differences in copy number alterations between 15 pairs of primary ESCC and their matched lymph node metastasis

A total of 102 gains and 90 losses were found in 15 primary ESCC samples, with an average of gains and losses per patient of 6.8 and 6.0, respectively, and 110 gains and 142 losses were found in 15 lymph nodes metastasis samples, with an average of gains and losses per patient of 7.4 and 9.5. More genetic changes (252) were found in lymph node metastasis than in the corresponding primary lesions (192), especially the losses (9.5 case vs 6.0 case). The copy-number changes for the entire genome detected in these 15 primary ESCC and their corresponding metastatic lesions are summarized in Table 2 and Figure 2. In primary tumors, the most frequently detected sites of chromosome gain were 3q (38 of 15, 25%), 8q (11 of 15, 73%), 1q (11 of 15, 73%), 12p (8 of 15, 53%), 18q (6 of 15, 40%), 5p (7 of 15, 47%), 6p (7 of 15, 47%), 20p (11 of 15, 73%). High copy-number amplification with a minimum gain 3q24-qter was detected in two primary tumors and six metastatic lymph nodes. The other three high copy-number amplifications of 6p21-6q12 were detected only in metastatic lymph node tumors but not in their corresponding primary tumors. The high copy-number amplification of 20p was detected in metastatic lymph node lesions but not in their corresponding primary tumor. In primary tumors, the chromosomal profile of ESCC...
DNA copy number losses were 3p (10 of 15, 67%), 10p (2 of 15, 13%), 10q (2 of 15, 13%), 4q (6 of 15, 40%), 4p (7 of 15, 47%), 19p (8 of 15, 53%), 13q (4 of 15), 1p (7 of 15), 17p and 18q (4 of 15, 27%). In metastatic lymph nodes, the chromosomal profiles of DNA copy number losses were 3p (12 of 15, 80%), 10p (8 of 15, 53%), 10q (7 of 15, 47%), 4q (10 of 15, 67%), 4p (9 of 15, 60%), 19p (10 of 15, 67%), 13q (8 of 15, 53%), 1p (7 of 15, 47%), 17p and 18q (7 of 15, 47%).

Statistically, in DNA copy number gains on chromosome 6p and 20p, there were significant differences between primary ESCC lesions and their corresponding lymph nodes ($P < 0.05$). In DNA copy number losses on chromosome 10pq, differences were significant between primary ESCC lesions and their corresponding lymph nodes ($P < 0.05$), (Table 3).

**DISCUSSION**

We have identified a genome-wide map of genetic alterations in ESCC, and compared primary tumor and metastatic lymph nodes in this study. The majority of the chromosomal aberrations in both primary tumor and metastatic lymph node lesions were consistent with the previous reports. For example, the gains of 3q, 5p, 8q23-ter and 20q and deletions of 3p25, 4p, 6q21, 9q22.3-q31, 9p, 11q22-qter, 13q12-13, 19q22.3, and 19q had been detected as frequent chromosomal alterations in ESCC in at least one of the previous CGH studies$^{[3-6]}$. By comparing the primary ESCC with metastatic lymph nodes, we did detect new candidate regions of interest, such as 6p, 20p and 10pq, which may harbor the genes involved in lymph node metastasis.

The highest frequency of DNA gain in ESCC occurred at chromosome 8q (78%), the chromosomal region at 8q harbors MYCC gene (8q24.1) which has been identified with a high frequency of amplification in ESCC$^{[7,8]}$. Gains of 3q were commonly seen in ESCC$^{[9]}$. Possible candidate genes involved in tumor development include the genes for ribosomal protein L22 (RPL22), butyrylcholinesterase (BCHE), glucose transporter 2 (SLC2A2), transferring receptor (TFRC), thrombopoietin (THPO) and the phosphatidylinositol-3 kinase catalytic α-polypeptide (PIK3CA). Deletion of 9p and gain of 5p were seen...
Deletion of chromosome 3p is one of the most frequent allelic imbalances in ESCC detected by CGH. In our study, loss of 3p was detected in 65% in ESCC. Possible candidate tumor suppressor genes on these region were FHIT (fragile histidine triad)\cite{13}, catenin (CTNNB1)\cite{14}, and von Hippel-Landau gene\cite{17}. Our results of proximal 3p loss mainly in ESCC may indicate a specific tumor suppressor gene at this locus involved in ESCC in high-risk areas. Loss of 3p was found in 38% of ESCC cases. Deletion of 8p22-pter has been reported in patients with ESCC\cite{18}. Mutations of the Fas-calculation and elongation protein zeta-1 (PEZI) gene at 8p22 were found in patients with ESCC\cite{19}, and mutations of two other genes in these regions, tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2), were found in patients with metastatic breast carcinoma\cite{20}. Loss of 17p was detected in 37% of ESCC. One potentially relevant gene at 17p is TP53, whose product contributes to the control of cell proliferation and malignant transformation. Inactivation of TP53 is the most common defect found in human cancer including ESCC from Henan, China. Loss of 1p occurred in 32%. The minimal common region of deletion encompasses the most distal band 1p36-pter in ESCC. Loss of chromosome band 1p36 is frequently found in many malignancies, including gastric cardia carcinoma, colon cancer, and ESCC\cite{21}. In 15 pairs of ESCC and their corresponding metastatic lymph nodes, the most interesting finding in this study is the gain of 6p12-6q21 detected in seven metastatic lymph node lesions but only in two corresponding primary tumors (13% vs 47%; P = 0.05). This suggests that 6p may harbor a putative oncogene that plays an important role in the ESCC progression, especially in the lymph node metastasis of the tumor.
of ESCC. Heselmeyer et al demonstrated that gains of 6p and 20p were connected with advanced-stage cervical carcinomas. Hu et al demonstrated the over-expression of CDC25B gene in ESCC. Possible candidate gene on 20p includes CDC25B and proliferating cell nuclear antigen (PCNA), each of which plays an important role at specific stages of cell cycle progression.

Loss of 10p was seen in eight metastatic lymph nodes but only in two corresponding primary tumors. The difference between primary tumors and their metastatic lymph node lesions of ESCC was significant (13% vs 53% P = 0.05). Loss of 10q was detected in seven metastatic lymph nodes but only in two corresponding primary tumors.

Table 3 Chromosomal aberrations with primary ESCC and lymph node metastasis

| Chromosomal changes | Primary tumor (%) | Lymph node metastasis % | P value |
|---------------------|------------------|-------------------------|---------|
| Gain of 6p          | 2/15 (13)        | 7/15 (47)               | 0.05    |
| Gain of 20p         | 5/15 (40)        | 11/15 (73)              | 0.03    |
| Loss of 10p         | 2/15 (13)        | 8/15 (53)               | 0.05    |
| Loss of 10q         | 2/15 (13)        | 7/15 (47)               | 0.05    |
tumors. The difference between primary tumors and their metastatic lymph node lesions of ESCC was significant (13% vs 47% P = 0.05). These alterations had also been found in other carcinoma metastases, such as the head-neck squamous cell carcinoma (HNSC) and Spitzoid malignant melanoma. A role of the 10q deletion in tumor progression is however conceivable since additional germine mutations in the PTEN gene (on chromosome 10q23) have been shown to be present in Crowden’s disease.

In this study, more genetic changes were found in lymph node metastasis than in their corresponding primary lesions, especially the losses (9.5/case vs 6.0/case). Previous studies showed that DNA copy number losses were more common in the metastases than in the primary larynx tumors and HNSCC. In ESCC, DNA losses seem to be more closely associated with metastases than DNA amplification.

In conclusion, using the CGH technique to detect chromosomal aberrations in both the primary tumor and its metastatic lymph nodes of ESCC, gains of 8q, 3q, 5p and losses of 3p, 8p, 9q and 13q were specifically implicated in ESCC in Linzhou population. Furthermore, gains of 6p, 20p and loss of 10pq may contribute to the lymph metastasis of ESCC. These findings suggest that the gains and losses of chromosomal regions may contain ESCC-related oncogenes and tumor suppressor genes. The gains and losses of chromosomal regions identified in this study provide important theoretic information for identifying and cloning novel ESCC-related oncogenes and tumor suppressor genes. Finally, this study provides a practicable model to detect specific genetic change related to tumor metastasis by comparing the primary tumor with its corresponding metastatic tumor using the CGH technique.

## Applications

These loci provide important theoretic information for identifying and cloning novel ESCC-related oncogenes and tumor suppressor genes. Further studies are necessary to identify specific genes of these chromosomal regions and their functions. The target tumor susceptibility genes will be further characterized by mutation analysis. Single strand conformation polymorphism (SSCP) and sequencing analysis will be used to screen the mutation.

## Terminology

Comparative genomic hybridization (CGH); Single strand conformation polymorphism (SSCP).

## Peer review

This is a nicely written and presented paper with 37 cases of primary esophageal cancer and matched metastatic tissues. Although the number of studied cases is small, it is a useful contribution to the literature.

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