Consistent and Differential Genetic Aberrations between Esophageal Dysplasia and Squamous Cell Carcinoma Detected By Array Comparative Genomic Hybridization

Zhi-Zhou Shi, Li Shang, Yan-Yi Jiang, Jia-Jie Hao, Yu Zhang, Tong-Tong Zhang, De-Chen Lin, Shu-Guang Liu, Bo-Shi Wang, Ting Gong, Qi-Min Zhan, and Ming-Rong Wang

Abstract

Purpose: Our aim was to identify frequent genomic aberrations in both esophageal squamous cell carcinoma (ESCC) and esophageal dysplasia and to discover important copy number-driving genes and microRNAs (miRNA) in ESCC.

Experimental Design: We conducted array-based comparative genomic hybridization (array CGH) on 59 ESCC resection samples and 16 dysplasia biopsy samples. Expression of genes at 11q13.3 was analyzed by real-time PCR (RT-PCR) and immunohistochemistry (IHC). Integrated analysis was conducted to identify genes or miRNAs with copy number-expression correlations.

Results: Array CGH identified 11 amplifications and eight homozygous deletions in ESCC. Integrated analysis of array CGH data with matched gene expression microarray data showed that 90 overexpressed genes and 24 underexpressed genes were consistent with DNA copy number changes, including 12 copy number-driving miRNAs. In esophageal dysplasia, six gains, four losses, 12 amplifications, and four homozygous deletions were detected. Amplifications of 7p11.2 and 11q13.2–11q13.3 (CCND1) and homozygous deletion at 9p21.3 (CDKN2A) were consistent genomic changes in both dysplasia and carcinoma. ANO1 at 11q13.3 was overexpressed at the mRNA and protein levels in tumors, and higher mRNA expression was correlated with the copy number increase. In particular, ANO1 expression was elevated in moderate dysplasia compared with normal esophageal epithelium. IHC revealed that ANO1 overexpression was positively correlated with lymph node metastasis and advanced clinical stage. Knockdown of ANO1 significantly inhibited the proliferation of KYSE30 and KYSE510 cells.

Conclusion: Copy number aberrations in both esophageal dysplasia and ESCC may be useful as potential biomarkers for early detection. In addition, ANO1 may be a candidate target gene in esophageal tumorigenesis. Clin Cancer Res; 19(21); 5867–78. ©2013 AACR.
**Translational Relevance**

Early detection can greatly increase the chances of successful treatment of human cancer. In the present study, we carried out array-based comparative genomic hybridization (CGH) to compare genomic aberrations between esophageal dysplasia and squamous cell carcinoma (SCC) and identified amplifications of 7p11.2 and 11q13.2-11q13.3 (CCND1) and homozygous deletion at 9p21.3 (CDKN2A) as consistent genomic changes in both dysplasia and carcinoma. Copy number-driving genes/microRNAs were identified by integrative analysis. ANO1 on 11q13.3 was overexpressed as a result of amplification in esophageal squamous cell carcinoma (ESCC), particularly with an elevation in moderate dysplasia compared with normal esophageal epithelium. Overexpression of ANO1 was correlated with lymph node metastasis and advanced tumor stage. Knockdown of ANO1 significantly inhibited ESCC cell proliferation. These data revealed the molecular changes in precancerous lesions and ESCC. The genomic aberrations that were consistent or differed between dysplasia and ESCC may be useful as candidate biomarkers for early detection.

**Materials and Methods**

**Sample information**

Fresh tissues containing ESCCs and adjacent histologically normal epithelia from 233 patients with ESCC were collected by the Department of Pathology, Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China). Primary tumor regions and the corresponding normal mucosa from the same patients were separated by experienced pathologists and immediately stored at −70°C until use. All patients with ESCC were treated with radical operation, and none of them received any preoperative treatment.

Biopsy tissues were taken from symptom-free patients during endoscopic screening for esophageal cancer in Linzhou, China, which is a well-recognized high-risk area for ESCC. During endoscopy, the entire esophagus was visually examined and biopsies were taken from all focal lesions. If no focal lesions were detected, a standard site in the mid-esophagus was sampled.

All the samples used in this study were residual specimens after diagnostic sampling. All operated or endoscopically screened patients signed separate informed consent forms for sampling and molecular analysis. The clinicopathologic characteristics of patients used in the array CGH study are shown in Supplementary Tables S1 and S2.

**Cell culture**

The human ESCC KYSE30 and KYSE510 cell lines were generously provided by Dr. Y. Shimada (Kyoto University, Kyoto, Japan). Cells were cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS.

**Genomic DNA extraction**

Genomic DNA was isolated from tumor tissues using the QiaGen DNaseasy Blood & Tissue Kit as described by the manufacturer (QiaGen). The tumor cell content of all the samples was more than 50% by hematoxylin and eosin (H&E) staining.

**Array CGH**

ESCCs were tested using a 44K human genome CGH microarray, and esophageal dysplasias were tested using a 60K microarray. Array CGH experiments were carried out using standard Agilent protocols (Agilent Technologies). Commercial human genomic DNA (Promega) was used as a reference. For each CGH hybridization, 500 and 400 ng of reference genomic DNA for the 44K and 60K microarrays, respectively, and the same amounts of tumor DNA were digested with the AluI and RsaI restriction enzymes (Promega). The digested reference DNA fragments were labeled with cyanine 3-dUTP and the tumor DNA was labeled with cyanine 3-dUTP (Agilent Technologies). After cleanup, labeled reference and tumor DNA were mixed as probes and hybridized onto an Agilent 44K/60K human genome CGH microarray (Agilent Technologies) for 40 hours/24 hours. Washing, scanning, and data extraction procedures were carried out following standard protocols.

**Total RNA extraction**

Total RNA was isolated from tissues using the RNeasy Mini Kit as described by the manufacturer (Qiagen) and used for gene/miRNA expression microarray assay and real-time PCR (RT-PCR) assay.

**Gene expression microarray detection**

Agilent Human Gene Expression Microarrays (Agilent Technologies) were used to determine the gene expression levels in paired tumorous and paracancerous tissues.
RNA was used as a template for cDNA synthesis using the One Color Low Input Agilent Quick Amp Labeling Kit, and the Spike-in Kit provided the positive controls. The Agilent One-Color Microarray-based gene expression analysis followed the manufacturer’s instructions, and passed Agilent’s quality control.

miRNA expression microarray detection

Agilent Human miRNA Microarrays (Agilent Technologies) were used to examine miRNA expression levels in paired tumorous and paracancerous tissues. The input for the Agilent miRNA labeling system was 100 ng of total RNA. After dephosphorylation and denaturation, the total RNA was labeled with cyanine 3-pCp and then hybridized to Agilent Human miRNA Microarray V2.0 using the miRNA Complete Labeling and Hyb Kit. Following hybridization for 20 hours, the slides were washed using the Gene Expression Wash Buffer Kit and scanned using an Agilent Scanner. The images were processed and analyzed with Agilent Feature Extraction Software. The raw data were normalized using quantile normalization and then analyzed in GeneSpring GX software (Agilent Technologies).

Microarray data analysis

Array CGH data were analyzed using Genomic Workbench (Agilent Technologies), BRB- CGHTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) and MD-SeeGH (www.filobox.com). Genomic Workbench was used to calculate the log₂ ratio for every probe and to identify genomic aberrations. The mean log₂ ratio of all probes in a chromosome region between 0.25 and 1.0 was classified as genomic gain, more than 1.0 as high-level DNA amplification, less than −0.25 as hemizygous loss, and less than −1.0 as homozygous deletion.

Gene/miRNA expression microarray data were analyzed by GeneSpring GX software (Agilent Technologies).

The array CGH data set is available at Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE46452.

RT-PCR

RT-PCR was used to detect the copy number and mRNA expression of ESCC and precancerous lesions. The PCR reactions were carried out in a total volume of 20 μl, including 10 μl of 2×Power SYBR Green PCR Master Mix (Applied Biosystems), 2 μl of cDNA/DNA (5 ng/μl), and 1 μl of primer mix (10 μmol/L each). PCR amplification and detection were conducted in a LightCycler 480 II (Roche Applied Science) as follows: an initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. The relative gene expression and copy number of the target gene was calculated using the comparative ∆∆CT method (9). The gene expression and copy number of the target gene were normalized to an endogenous reference [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], and relative to the calibrator were given by the formula 2^−∆∆CT. ∆CT was calculated by subtracting the average GAPDH CT from the average CT of the gene of interest. The ratio defines the level of relative expression and copy number status of the target gene to that of GAPDH. The primers for the examined genes are listed in Supplementary Table S3.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded esophageal tumors were placed on the tissue microarray. For each case, the cancer tissues were repeated three times and adjacent morphologically normal tissues were repeated two times. The slides were deparaffinized, rehydrated, immersed in a 3% hydrogen peroxide solution for 10 minutes, heated in citrate buffer (pH 6) for 25 minutes at 95°C, and cooled for 60 minutes at room temperature. The slides were blocked with 10% normal goat serum for 30 minutes at 37°C and then incubated with rabbit polyclonal antibody against ANO1 (Abcam) and mouse monoclonal antibody against MCM7 (Santa Cruz Biotechnology) overnight at 4°C. After washing with PBS, the slides were incubated with biotinylated secondary antibody (diluted 1:100) for 30 minutes at 37°C, followed by streptavidin-peroxidase (1:100 dilution) incubation for 30 minutes at 37°C. Immunolabeling was visualized with a mixture of 3,3’-diaminobenzidine (DAB) solution. Tissues were counterstained with hematoxylin.

Expression level was determined on the basis of staining intensity and the percentage of immunoreactive cells. Negative expression (score = 0) was considered no, or faint staining, or moderate to strong staining in less than 25% of cells. Weak expression (score = 1) was considered moderate or strong staining in 25% to 50% of cells. Strong expression (score = 2) was considered strong staining in more than 50% of cells.

Transfection assay

siRNAs specific to ANO1 (Silencer Select siRNA; s30185) and a control siRNA (Silencer Select Negative Control no. 1) were purchased from Ambion. Cell transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Western blot analysis

Cells were washed with ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] containing phenylmethylsulfonylfluoride (PMSF; 1 mmol/L) and protease inhibitors (2 g/ml; Protease Inhibitor Cocktail Set III; Calbiochem) on ice for 30 minutes. The lysates were clarified by centrifugation at 13,000 g for 30 minutes at 4°C. The total protein concentration was estimated using a Protein Assay Kit (Bio-Rad). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), blocked, and probed with antibodies against ANO1 (1:1,000; Santa Cruz Biotechnology). After washing, blots were incubated with horseradish peroxidase–conjugated secondary antibodies and visualized using super enhanced chemiluminescence (ECL) detection reagent (Applygen).
Cell proliferation assay
A Cell Counting Kit8 (CCK-8; Dojindo Laboratories) was used to evaluate cell proliferation. At 24 hours after transfection, the cells were seeded into 96-well plates with 1,000 cells per well and cultured for 24, 48, 72, 96, and 120 hours. Then, 10 μl of CCK-8 solution was added to each well and incubated for 1 hour. Optical density was examined at a wavelength of 450 nm.

Statistical analysis
The correlation between genomic aberrations and clinicopathologic parameters was analyzed in three steps. First, we identified candidate genomic aberrations with different frequencies among clinicopathologic statuses using MD-CGH software. We then narrowed the regions of these candidates using the aberration data produced by Genomic Workbench. Finally, we analyzed the correlations between the genomic aberrations and clinicopathologic parameters using Pearson χ² test. The correlations between the copy number and mRNA expression levels of CCND1, ANO1, FADD, and FGF19 were analyzed using the Spearman rank correlation test. Other statistical analyses were conducted using Student t tests with the statistical software SPSS 15.0. Differences were considered statistically significant when the corresponding two-sided P value was less than 0.05.

Results
Recurrent copy number alterations in ESCC by array CGH
In ESCCs, 19 gains and nine losses were detected in more than 50% of cases (Fig. 1A and Supplementary Table S4). Among them, the most common gain was detected at 7q21.3-q22.1 (70%), whereas the most frequent region of loss was 3p22.2-p24.3 (60%). Eleven regions including 3q27.1 (EIF2BS2), 7p11.2, 8p12-p11.21, 8q24.21 (MYC), 11q13.2-q13.3 (CCND1), 12q14.3-q15, 13q22.1, 14q11.2 (PARP2), and 19q13.11-q13.12 showed high-level amplifications, and eight homozygous deletions were identified at 4p16.1-p15.1, 4q34.3-q35.1, 6p22.1, 9p21.3 (CDKN2A), 1p15.4, 13q14.2 (RB1), 14q12, and 22q13.1 (Table 1). Thirteen genes located in these amplification regions, including AP2M1, ALG3, PSM2D2, EIF4EBP1, CTN1, PPFIA1, ANO1, APEX1, USF2, FXYD3, LRFN3, HAUS5, and C14orf128, were overexpressed in esophageal tumors compared with paracancerous tissues (Table 1). Nearly half of ESCCs had 51 to 75 genomic changes (Fig. 1B). GISTIC analysis, which scores the significance of recurrent gains or losses and detects peak regions likely to contain driver gene(s), revealed that gains of 7p11.2, 7q21.3, 8q24.21, 11q13.2-q13.4, 12q14.3-q21.1, and 19q13.11-q13.12 and losses of 9p21.3 and 11p15.4 were the most significant recurrent genomic aberrations (Fig. 1C; and Supplementary Table S5).

Frequency plot comparison analysis was applied to identify genetic alterations associated with clinicopathologic parameters. The results showed that 11q was lost more frequently in patients with lymph node metastasis and that loss of 9p was more common in those at earlier stages (I and II; Supplementary Fig. S1A and S1B). Detailed frequency and statistical analyses confirmed that the 11q14.3 loss was more frequent in patients with lymph node metastasis (P < 0.001). In addition, loss of 9p21.2-p21.1 was significantly associated with earlier clinical stage in ESCC (P < 0.001).

Integrated analysis of copy number and gene/miRNA expression data
To explore the genes and miRNAs that were differentially expressed between tumorous and paracancerous tissues and also consistent with genomic changes, we further analyzed eight and four ESCC samples (also measured by array CGH) by cDNA expression microarray and miRNA expression microarray, respectively. In total, 159 genes (170 probes) were identified when the cutoffs were a fold change more than 5.0 and a P value less than 0.01. Among them, 135 genes (141 probes) were overexpressed and 24 (29 probes) were underexpressed in tumor tissues compared with paracancerous tissues (Supplementary Table S6). In addition, 90 overexpressed genes (67% of all overexpressed genes including MCM7, MTDH, STAT2, MMP14, and RELB) and nine underexpressed genes (38%) were consistent with their DNA copy number changes. We further validated the mRNA and protein levels of the candidate gene MCM7. At the mRNA level, MCM7 was overexpressed in 64% (9 of 14) of ESCCs. At the protein level, MCM7 overexpression occurred in 88% (7 of 8) and 65% (13 of 20) of ESCCs examined by Western blotting and immunohistochemistry (IHC), respectively (Supplementary Fig. S2).

Twenty-seven miRNAs were identified when the cutoff was a fold change more than 2.0. Among them, 22 miRNAs were overexpressed and five were underexpressed in tumor tissues (Supplementary Table S7). Overexpression of miR-1280, miR-574-5p, miR-595, iR-548d-5p, miR-483-5p, miR-1260, miR-342-3p, miR-940, miR-21, miR-23a, and miR-766 and underexpression of let-7c were consistent with the gains and losses, respectively. We then compared the selected genes/miRNAs with published data (Supplementary Fig. S3) and found that COL1A1 and an additional 21 genes, including MMP1 and SSIP, were selected in two studies (10, 11). In studies by Kimura, Ogawa, and in our study, miR-16, miR-107, and miR-21 were overexpressed in esophageal tumor tissues. In addition, both we and Sato found that let-7c was underexpressed in ESCC (12, 13).

Differentially expressed genes were further analyzed using the Gene Ontology (GO) option of GeneSpring GX 11.5, yielding 22 significant GO terms (corrected P < 0.05). Detailed information on these GO terms is provided in Supplementary Table S8. The top five GO terms were as following: (i) epidermis development (corrected P = 0.0052); (ii) nuclear envelope (corrected P = 0.0053); (iii) mRNA export from the nucleus (corrected P = 0.0249); (iv) mRNA transport (corrected P = 0.0249); and (v) tissue development (corrected P = 0.0249). Pathway enrichment analysis showed that six pathways were significantly changed in ESCC including the Alpha6Beta4Integrin, TNF-α/NE-
κB, interleukin (IL-6), EGFR1, IL-4, and IL-3 pathways (Supplementary Table S9).

**Recurrent copy number alterations in esophageal dysplasia by array CGH**

To explore the early genomic changes in esophageal carcinogenesis, we conducted array CGH on 16 precancerous lesions. The most frequent copy number alterations in dysplasia with a frequency above 30% were gains of 3q11.2-q29, 5p15.2-15.33, 7q21.3-q22.3, 8q11.21-q24.3, 11q13.2-q13.3, and 22q13.33 and losses of 3p11.2-p26.3, 9p24.3-p34.2, 13q21.1-q21.2, and 13q32.2-q32.3 (Fig. 2A and Supplementary Table S10). Twelve high-level amplifications at 1q25.2 (RASAL2), 3q33-q34.1, 6p21.31, 7p11.2, 7q21.3-q22.2, 8p12, 9p22.2-p21.3, 9p21.2-p21.1, 11q13.2-q13.3 (CCND1), 14q21.1, 14q21.3-q23.1, and 18q11.2 (RBBP8) and four homozygous deletions at 2q22.1 (LRP1B), 7p14.1 (CDC2L5), 9p21.3 (CDKN2A), and 20p12.1 were identified (Table 1). GISTIC analysis identified an 11q13.2-q13.3 gain and losses of 3q26.1 and 11q11 in esophageal dysplasia (Fig. 2C Supplementary Table S5).

By comparing the genomic profiles of ESCC and dysplasia, we found that amplifications of 7p11.2 and 11q13.2-11q13.3 and homozygous deletion at 9p21.3 were consistent genomic changes and that 11q13.2-
q13.3 amplification was the only identified aberration that was common in both dysplasia and ESCC by GISTIC (Table 1 and Supplementary Table S5). A frequency plot comparison analysis revealed that 3q, 5p, 7q, 8q, and 11q13 and losses of 3p and 9p were shared by dysplasia and cancer. Loss of 9q was more frequent in dysplasia, but gains of 16p, 17q, 19p, 19q, 20p, 20q, and 22q and losses of 4p, 4q, 5q, 8p, 18q and 21q were more frequent in ESCC (Fig. 2D). As the data obtained using all methods showed that 11q13.2-13.3 was frequently amplified in both dysplasia and cancer, we validated the results using FISH (Supplementary Fig. S4A). The number of genomic aberrations in ESCC was significantly higher compared with esophageal dysplasia (Supplementary Fig. S4B).

Pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was applied to the array CGH data, and we found that 14 and 11 pathways were enriched for genes with genomic changes in ESCC and esophageal dysplasia, respectively (Supplementary Table S11). Interestingly, three pathways including cytokine–cytokine receptor interaction, the Toll-like receptor signaling pathway, and regulation of autophagy were enriched in genes with copy number changes in both ESCC and dysplasia.

Table 1. Amplification and homozygous deletion in squamous cell carcinoma and dysplasia of esophagus

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<th>Aberration</th>
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<th>Region</th>
<th>No of cases</th>
<th>Differentially expressed genes*</th>
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<th>Region</th>
<th>No of cases</th>
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NOTE: The number of ESCC and dysplasia in array CGH study are 59 and 16, respectively. Abbreviations: Amp, amplification; HD, homozygous deletion. In gray: the amplifications or homozygous deletions are shared by both groups. *Genes with different expression level between ESCC tissues and paracancerous tissues in our microarray assay. †The cancer genes are identified by Cancer Gene resource http://cbio.mskcc.org/CancerGenes/.
Figure 2. Genomic aberrations detected in esophageal dysplasia. A, genome-wide frequency plot of esophageal dysplasia by array CGH analysis. Line on the right of 0%-axis, gain; line on the left of 0%-axis, loss. B, number of aberrations in esophageal dysplasia. X, number of aberrations; Y, number of cases. C, amplifications and homozygous deletions (HD) identified by GISTIC. D, frequency plot comparison of esophageal dysplasia with ESCC. The presentation is per array probe: gains are represented by the lines on the right and losses by the left. The vertical line represents 100% of the samples.
Candidate target genes in the sequence from esophageal dysplasia to cancer

We next screened the candidate targets for 11q13.2-q13.3 amplification. RT-PCR showed that the expression levels of FGF19 and ANO1 were significantly higher in tumor tissues than in paracancerous tissues, with a P value below 0.05 and fold change above 2.0 (Fig. 3A). Therefore, we chose FGF19 and ANO1 to examine the correlation between copy number increase and mRNA overexpression in ESCC and their mRNA expression status in normal esophageal epithelium, esophagitis, mild dysplasia, and moderate dysplasia. We also analyzed CCND1 and FADD because their overexpression in ESCC has been previously reported. ANO1 and FADD were overexpressed in ESCC with amplification (Fig. 3B), and increased mRNA expression of ANO1 and FADD was significantly correlated with copy number increases, according to a Spearman correlation analysis (Supplementary Table S12). CCND1, ANO1, and FADD were significantly overexpressed in moderate dysplasia compared with normal epithelium, but there was no difference in the expression of these genes between normal epithelium and esophagitis (Fig. 3C). The IHC results further showed that ANO1 was overexpressed in 25% (n = 88) of ESCCs and that higher expression of ANO1 was significantly correlated with lymph node metastasis and advanced tumor stage (Fig. 4A; Supplementary Table S13). In vitro studies revealed that ANO1 knockdown in the ESCC KYSE30 and KYSE510 cell lines, which have increased ANO1 expression, inhibited cell proliferation (Fig. 4B and C and Supplementary Figs. S5, S6A, and S6B).

Discussion

Genomic aberrations can contribute to carcinogenesis and tumor progression. Earlier reports have identified multiple abnormal regions in ESCC, including amplifications at 1p34, 3q, 5p, 7p12, 8q, 11q13, 12p, 17q12, and 22q as well as deletions at 2q, 3p, 4q, 5q13-q21, 9p21.3, and 13q (5, 7, 8, 14–21). Our study further narrowed these altered chromosome regions and identified candidate amplification- or homozygous deletion–associated genes. The known cancer genes EIF2B5, MYC, CCND1, and PARP2 were amplified, and CDKN2A and RB1 were homozygously deleted in ESCC. Importantly, amplification of CCND1 and homozygous deletion of CDKN2A were observed in both ESCC and dysplasia. It has been reported that CCND1 is expressed in 20% of severe esophageal dysplasias and significantly amplified and overexpressed in ESCC (22, 23). CDKN2A is deleted in ESCC and underexpressed in 80% of esophageal...
dysplasias and 93% of ESCCs (23, 24). These findings indicate that CCND1 and CDKN2A may play important roles in esophageal carcinogenesis and that copy number change is one mechanism leading to the dysregulation of these two genes. In addition, the gene expression showed that AP2M1 (3q27.1), ALG3 (3q27.1), PSMD2 (3q27.1), EIF4EBP1 (8p12), CTNN (11q13.3), PPIA1 (11q13.3), ANO1 (11q13.3), APEX1 (14q11.2), USF2 (19q13.12), FXYD3 (19q13.12), LRFN3 (19q13.12), and HAUS5 (19q13.12) were amplified and overexpressed in ESCC and that C14orf128 at 14q12 were homozygously deleted and underexpressed.

ESCC arises from defined histopathologic lesions, including mild, moderate, and severe dysplasia. A followup study by Wang and colleagues indicated that in the high-risk rural population in Linzhou, China, the rates of mild, moderate, and severe dysplasias becoming ESCC after 13.5 years were 23.7%, 50%, and 73.9%, respectively (25). Therefore, squamous dysplasia is a major risk factor and a precursor to ESCC. To date, many molecular changes have been

Figure 4. Overexpression of ANO1 in ESCCs and effect of ANO1 on cell proliferation. A, IHC assay of ANO1. N, paracancerous tissues; T, ESCC tissues. B and C, knockdown of ANO1 inhibited the proliferation of KYSE30 (B) and KYSE510 cells (C). The absorbance at 450 nm was measured.
identified in esophageal dysplasia. Yang and colleagues found that 21% of precancerous lesions and 38% of ESCCs express PTCH1, a target of hedgehog signaling, by IHC and suggested that hedgehog signaling plays an important role in esophageal carcinogenesis (26). Considering PTCH1 located in the commonly deleted region 9q, further study should be conducted to analyze the correlation between deletion or no expression of PTCH1 and clinical factors in ESCC. Another study showed that a panel of four genes (AHRPR, P16INK4a, MT1G, and CLDN3) in esophageal balloon cytology specimens had a sensitivity and specificity of 50% and 68%, respectively, in detecting esophageal dysplasia (27). However, the understanding of genomic aberrations in esophageal dysplasia was quite limited. The present study not only confirmed at the molecular level that esophageal dysplasia was the precursor lesion of ESCC but also revealed alterations of some genes in the precancerous lesions of the esophagus. Six gains, four losses, 12 amplifications, and four homozygous deletions were identified in dysplasia. In particular, amplification of 7p11.2 and 11q13.2-q13.3 and homozygous deletion at 9p21.3 were detected both in dysplasia and ESCC. Interestingly, three pathways including cytokine–cytokine receptor interaction, the Toll-like receptor signaling pathway and regulation of autophagy, were enriched in genes with genomic changes in both ESCC and dysplasia. Future research could focus on the contribution of these three pathways to esophageal carcinogenesis. In addition, the copy numbers of 3q26.1 and 11q11 have often been reported to be increased in tumors, including ESCC (28–30). Interestingly, we identified 3q26.1 and 11q11 as novel regions of common loss in esophageal dysplasia, which may suggest that genes located in these two regions play different roles in dysplasia formation than in ESCC. We also found that loss of 9q was more frequent in dysplasia than in ESCC, and further studies should be conducted to explore the tumorigenic role of 9q loss.

By comparing our results with the CGH data presented on the Progenetix website (31, 32), we found that most genomic aberrations were consistent. However, there were some differences. For example, gains of chromosomes 16, 17, 19, and 22 were more common than their losses in our study, whereas the frequencies of gain and loss in chromosomes 16 and 17 were similar, and the loss of chromosomes 19 and 22 was more dominant in the Progenetix database. In our study, gain of 9q34.11-34.3 was detected in 53% of ESCCs, whereas the frequency of 9q gain is reported as less than 20% in the Progenetix database. Considering the possible experimental artifacts, a large number of ESCCs need to be detected to validate the differences between our results and the Progenetix data.

11q13.2-q13.3, which was amplified in both ESCC and dysplasia in the CGH assay, is a commonly amplified region in many types of malignancies including esophageal, breast, liver, and ovarian cancers (8, 33–35). Hu and colleagues have reported that gains of PSCA1, CCND1, CTTN, PPHHA1, and SHANK2 at 11q13 are correlated with increased RNA expression in ESCC (36). Sugimoto and colleagues have indicated that GAL is the target of 11q13 amplification in ESCC (37). Our results showed that ANO1 was significantly overexpressed as a result of amplification in esophageal cancer tissues and that overexpression of ANO1 was linked to lymph node metastasis and advanced tumor stage. In particular, ANO1 knockdown significantly inhibited the proliferation of ESCC cells. On the basis of the elevated expression in moderate dysplasia compared with normal esophageal epithelium, ANO1 may play an oncogenic role in esophageal carcinogenesis. ANO1 was recently identified as a Ca(2+)-activated Cl(−) channel. Overexpression of ANO1 has been reported in prostate carcinoma and squamous cell carcinoma of the head and neck (HNSCC). In addition, in prostate cancer, higher ANO1 expression has been correlated with clinical tumor—node—metastasis stage and Gleason score (38, 39). Inhibition of ANO1 expression significantly reduces the proliferation, metastasis, and invasion of prostate cancer and HNSCC cells. Moreover, in HNSCC, ANO1 promotes anchorage-independent growth by increasing extracellular signal–regulated kinase (ERK)1/2 activation and cyclin D1 induction (40). However, the role of ANO1 in ESCC remains unclear. It will be important to investigate the mechanisms underlying the involvement of ANO1 in esophageal carcinogenesis.

Overall, our study identified multiple copy number-altered chromosome regions and differentially expressed genes and miRNAs. These findings provide important insights into the molecular alterations associated with ESCC. Further studies should be conducted to explore the possible tumorigenic roles of these candidate genes.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Z.-Z. Shi, Y. Zhang, D.-C. Lin, M.-R. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z.-Z. Shi, L. Shang, Y.-Y. Jiang, J.-J. Hao, D.-C. Lin, S.-G. Liu, T. Gong
Writing, review, and/or revision of the manuscript: Z.-Z. Shi, M.-R. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-Y. Jiang, T.-T. Zhang, B.-S. Wang, T. Gong
Study supervision: Q.-M. Zhan, M.-R. Wang

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Zhi-Zhou Shi, Li Shang, Yan-Yi Jiang, et al.


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