

Identification of putative target genes for amplification within 11q13.2 and 3q27.1 in esophageal squamous cell carcinoma

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Abstract

Background Genomic aberration is a common feature of human cancers and also is one of the basic mechanisms that lead to overexpression of oncogenes and underexpression of tumor suppressor genes. Our study aims to identify frequent genomic changes and candidate copy number driving genes in esophageal squamous cell carcinoma (ESCC).

Methods We used array comparative genomic hybridization to identify recurrent genomic alterations and screened the candidate targets of selected amplification regions by quantitative and semi-quantitative RT-PCR.

Results Thirty-four gains and 16 losses occurred in more than 50 % of ESCCs. High-level amplifications at 7p11.2, 8p12, 8q24.21, 11q13.2-q13.3, 12p11.21, 12q12 and homozygous deletions at 2q22.1, 8p23.1-p21.2, 9p21.3 and 14q11.2 were also identified. 11q13.2 was a frequent amplification region, in which five genes including CHKA, GAL, KIAA1394, LRP5 and PTPRCAP were overexpressed in tumor tissues than paracancerous normal tissues.

The expression of ALG3 at 3q27.1 was higher in ESCCs, especially in patients with lymph node metastasis.

Conclusions Target gene identification of amplifications or homozygous deletions will help to reveal the mechanism of tumor formation and explore new therapy method.

Keywords Array CGH · Esophageal squamous cell carcinoma · Amplification · Deletion · Lymph node metastasis

Introduction

Esophageal squamous cell carcinoma (ESCC) is the fourth most common malignant tumor in China, and prevalent throughout the world. Although diagnostic method and cancer treatment have been improved in recent years, the prognosis remains poor because of widespread lymph node metastasis and relatively frequent distant metastasis. Therefore, understanding of molecular changes underlying esophageal carcinogenesis and metastasis may reveal the mechanism of tumor formation and provide new therapy method.

ESCC formation is a multi-step process, and chromosomal abnormality was a characteristic attribute. Several studies have reported that gains of 3q26-qter, 5p15, 7p, 7q, 11q13.3, 8q24.3-qter and losses of 16p13.3 and 18q22-qter were the most frequent genomic changes in ESCC [1–3]. Previous observations also showed that gains of 12p, 11q13.2, 8q24.21 (*MYC*) and loss of 3p14.2 (*FHIT*) were associated with poor prognosis, and overexpression of *CPT1A* in 11q13.2 was an independent prognosticator [3, 4]. Sakai et al. [5] found that amplification of 2q12-q14, 3q24-q26 and 7q21-q31 was associated with lymph node metastasis, and amplification of all these three

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chromosomal regions reflected worse survival. Qin et al. [6] found that gains of 6p, 20q and losses of 10p and 10q were more common in metastatic lymph nodes than primary lesions. The relationships between gain of 8q, loss of 4p and nodal metastasis, between gain of 2p, losses of 4pq, 11q14-qter and distant organ metastasis were revealed [7]. P53 mutation was associated with poorer response to chemotherapy and poorer prognosis. However, p53 immunohistochemistry staining did not correlate with response to chemotherapy or prognosis [8]. Lower the long interspersed nucleotide element-1 (LINE-1) methylation levels were detected in ESCC in comparison with matched normal esophageal mucosa. And the LINE-1 hypomethylation was significantly associated with disease-free survival and cancer-specific survival [9]. Especially, many genes were amplified more frequently in ESCC than esophageal adenocarcinoma (EAC) such as *VEGFA*, *ERBB2*, *PIK3CA* and *FGFR1*, and inactivating mutations of *NOTCH1* were only detected in ESCC [10, 11].

Genomic aberration is one of the mechanisms that can result in gene dysfunction and contribute to the carcinogenesis and tumor progression. *GAL*, *GSTP1*, *MRPL11*, *MRPL21*, *SF3B2*, *YIF1A* at gain region 11q13.1-q13.3 and *GALR1* at loss region 18q23 have been shown to be expression-dysregulated due to DNA copy number changes in ESCC. And the galanin signaling pathway is also altered because of genomic changes, which regulates oncogenesis in human squamous cell carcinoma [12].

To clarify the recurrent genomic changes in ESCC, we analyzed 20 ESCCs by array comparative genomic hybridization (array CGH). And we further screened the candidate targets of 11q13.2, and found that five genes were overexpressed in tumor tissues. *ALG3* at amplification region 3q27.1 was overexpressed, which was associated with lymph node metastasis.

Materials and methods

Patients

Freshly resected tissues from 77 ESCC patients were collected by the Department of Pathology, Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. All the ESCC patients were treated with radical operation and none of them received any treatment before surgery. Representative tumor regions were excised by experienced pathologists and immediately stored at -70°C until used. All the samples used in this study were residual specimens after diagnosis sampling. And every patient signed separate informed consent forms for sampling and molecular analysis. Clinical characteristics of patients used in the array CGH study are shown in Table 1.

Table 1 Clinical characteristics of patients used in the array CGH study

No.	Sex	Age	pT	pN	pM	Stage	Grade
1	Female	49	T1	N0	M0	I	G3
2	Male	33	T2	N0	M0	IIA	G2
3	Male	65	T3	N0	M0	IIA	G1
4	Male	69	T3	N0	M0	IIA	G2
5	Male	70	T3	N0	M0	IIA	G2
6	Male	49	T3	N0	M0	IIA	G2
7	Female	55	T3	N0	M0	IIA	G2
8	Male	58	T3	N0	M0	IIA	G2
9	Male	80	T3	N1	M0	III	G3
10	Male	60	T3	N1	M0	III	G3
11	Female	55	T3	N1	M0	III	G3
12	Female	51	T3	N1	M0	III	G2
13	Male	49	T3	N1	M0	III	G1
14	Male	66	T3	N1	M0	III	G2
15	Male	70	T3	N1	M0	III	G3
16	Male	67	T3	N1	M0	III	G3
17	Male	27	T3	N1	M0	III	G2
18	Male	39	T3	N1	M0	III	G1
19	Male	58	T3	N1	M0	III	G1
20	Male	59	T3	N1	M0	III	G1

Genomic DNA extraction

Genomic DNA was isolated from tumor tissues using the Qiagen DNeasy Blood & Tissue Kit as described by the manufacturer (Qiagen, Hilden, Germany). Tumor cell content of all the samples was greater than 50 % by HE staining.

Array-based CGH

ESCCs were detected by 60 K human genome CGH microarray. Array CGH experiments were performed using standard Agilent protocols (Agilent Technologies, Santa Clara, CA). Commercial human genomic DNA (PROMEGA, Warrington, UK) was used as reference. For each CGH hybridization, 400 ng of reference genomic DNA and the same amount of tumor DNA were digested with *Alu I* and *RSA I* restriction enzyme (PROMEGA, Warrington, UK). The digested reference DNA fragments were labeled with cyanine-5 dUTP and the tumor DNA with cyanine-3 dUTP (Agilent Technologies, Santa Clara, CA). After clean-up, labeled reference and tumor DNA as probes were mixed and hybridized onto Agilent 60 K human genome CGH microarray (Agilent) for 24 h. Washing, scanning and data extraction procedures were performed following standard protocols.

Microarray data analysis

Array CGH data were analyzed using Genomic Workbench (Agilent), BRB-CGHtools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) and MD-SeeGH (<http://www.flintbox.com>). Genomic Workbench was used to calculate \log_2 -ratio for every probe and to identify genomic aberrations. Mean \log_2 -ratio of all probes in a chromosome region between 0.25 and 1.0 was classified as genomic gain, >1.0 as high-level DNA amplification, <-0.25 as hemizygous loss, and <-1.0 as homozygous deletion.

Total RNA extraction

Total RNA was isolated from tissues using the RNeasy Mini Kit as described by the manufacturer (Qiagen, Hilden, Germany) and used for reverse transcription PCR and real-time PCR assay.

Reverse transcription PCR

Total RNA was used to synthesize the first strand of cDNA using SuperScript II RT 200 units/ μ l (Invitrogen, San Diego, CA). And 10 μ l of each PCR product were resolved by 2 % (w/v) agarose gel electrophoresis. The primers of detected genes are listed in Table S1.

Real-time PCR

The PCR reactions were performed in a total volume of 20 μ l, including 10 μ l of 2XPower SYBR[®] Green PCR Master Mix (Applied Biosystems, Warrington, UK), 2 μ l of cDNA/DNA (5 ng/ μ l), 1 μ l of primer mix (10 μ M each). The PCR amplification and detection were carried out in a 7300 Real Time PCR System (Applied Biosystems) for 40 cycles, each with 15 s at 95 °C, 1 min at 60 °C, and initial denaturation with 10 min at 95 °C. The relative gene expression (relative copy number) was calculated using the comparative CT Method. The copy number of the target gene normalized to an endogenous reference (GAPDH or β -actin), and relative to calibrator was given by the formula $2^{-\Delta\Delta C_t}$. ΔC_t was calculated by subtracting the average GAPDH (β -actin) CT from the average CT of the gene of interest. The ratio defines the level of relative expression (relative copy number) of the target gene to that of GAPDH or β -actin.

Fluorescence in situ hybridization

Tumor tissues were minced and then digested with 0.2 % type II collagenase (Sigma, St. Louis, MO). Cells were incubated in 0.075 M KCL hypotonic buffer for 60 min at 37 °C and fixed in methanol/acetic acid (3:1, v/v) at 4 °C.

Single-cell suspensions were dropped on cool wet slides. After air-drying overnight, slides were sequentially treated with RNase and pepsin. Denaturation was performed in 70 % formamide, 2XSSC (pH 7) for 3 min at 75 °C. BAC clones covering GAL (RP11-569N5), KIAA1394 (RP11-389P13) and ALG3 (RP11-631P04) were labeled by random primer technique with cyanine-3 dUTP or fluorescein isothiocyanate dUTP. The probe mixture was denatured for 7 min at 75 °C and then hybridized to denatured slides for 48 h at 37 °C. Post-hybridization washes were carried out in 50 % formamide for 15 min and twice in 2XSSC. Slides were counterstained with 4, 6-diamidino-2-phenylindole (DAPI). Gray images were captured with Cascade 512 B CCD camera (Protometrics, Tucson, AZ) equipped with an Olympus fluorescence microscope. The images were analyzed using the MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA).

Statistical analysis

Student's *t* test and Chi square test were performed with the statistical software SPSS 15.0. The differences were judged as statistically significant when the corresponding two-sided *P* value were <0.05 .

Results

Recurrent copy number changes in ESCC detected by array CGH

Array CGH detected 34 gains and 16 losses in ESCC with frequency over 50 %. The five most frequent gains were 8q24.3, 3q29, 7q21.3-q22.1, 3q26.32-q28 and 20q11.21-q11.23, and the five most common losses, at 18q21.33-q23, 9p21.1-p21.3, 3p12.1-p13, 18q12.1-q12.3 and 4p12-p13 (Fig. 1a; Table S2). High-level amplifications at 7p11.2, 8p12, 8q24.21, 11q13.2-q13.3, 12p11.21, 12q12 and homozygous deletions at 2q22.1, 8p23.1-p21.2, 9p21.3 and 14q11.2 were also identified (Table 2). GISTIC analysis, which scores the significance of recurrent gains or losses and detects peak regions likely to contain the driver gene(s), revealed that gains of 7p12.1, 7p11.2 (*EGFR*), 8q24.21 (*MYC*), 11q13.2-q13.4 (*ANO1*, *FADD* and *CTTN*), 11q22.1-q22.3 (*MMP7*, *MMP3*) and losses of 9p21.3 (*MTAP*, *CDKN2A* and *CDKN2B*) and 11q11 (*SPRYD5*, *OR5W2*) were the most common genomic changes (Fig. 1c; Table 2). Among them, amplifications of 7p11.2, 8q24.21, 11q13.2-q13.3 and homozygous deletion of 9p21.3 were both found by GISTIC and Genomic Workbench (Agilent). By analyzing the numbers of genomic aberrations, we found that 25 % of ESCCs had more than 100 genomic aberrations (Fig. 1b).

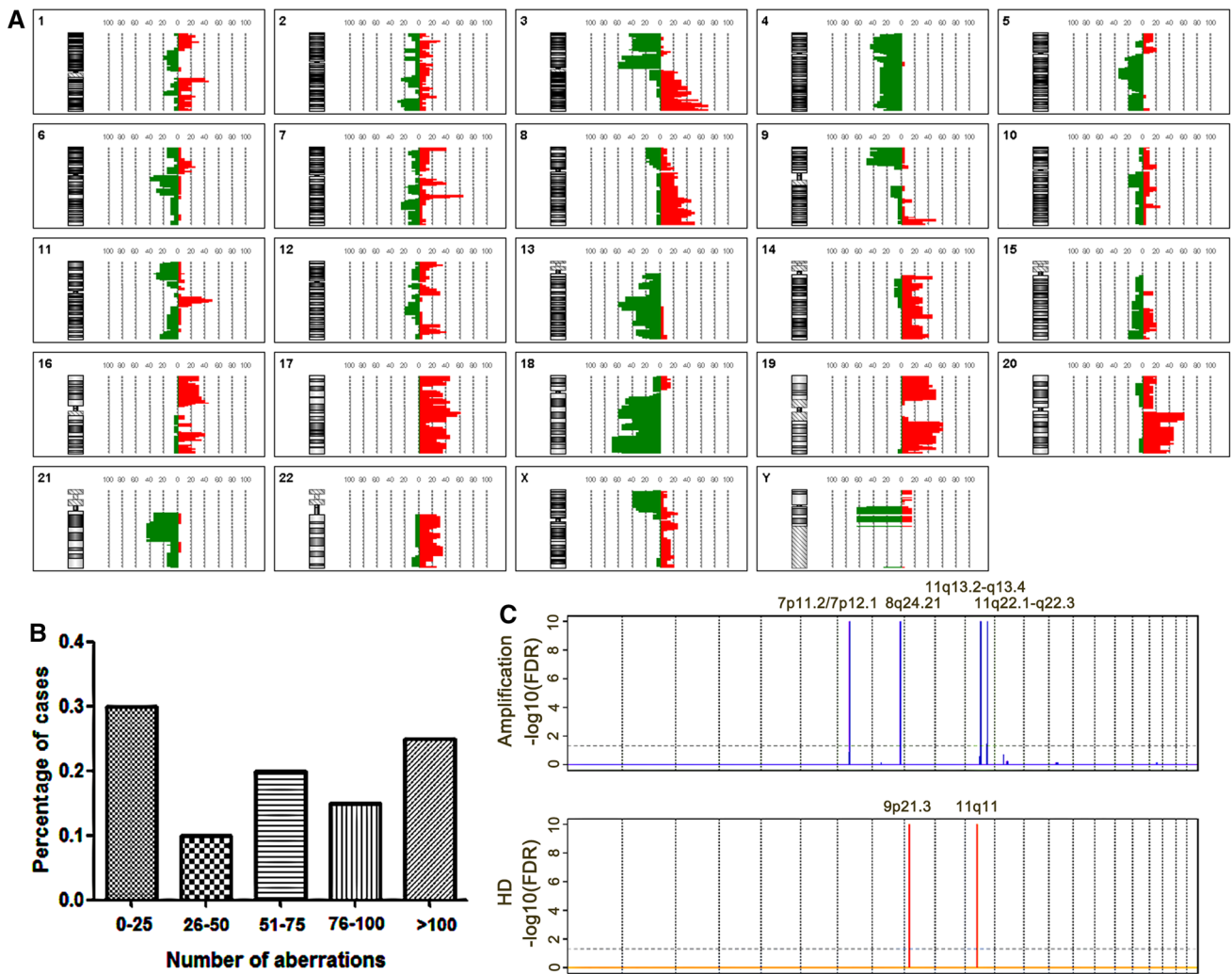


Fig. 1 Genomic aberration in ESCC. **a** Genome-wide frequency plot of ESCC by array CGH analysis. *Right*: gain; *left*: loss. **b** Numbers of aberrations in ESCC. *X* Number of aberrations, *Y* Number of cases. **c** Amplifications and homozygous deletions (HDs) identified by GISTIC

We further validated the three most frequent gains and losses (Table S2) using real-time PCR in eight independent ESCCs. The results showed that gains of 8q24.3, 3q29 and 7q22.1 occurred in 3, 2 and 2 cases, respectively (Fig. 2a). Losses of 9p21.3 and 3p13 were detected in 3 cases, and 18q21.33 in one case (Fig. 2b). We analyzed the correlation between the profiling of genomic aberrations and clinico-pathological parameters. Gains of 3q, 8q, 12p, 16p, 17q and loss of 18q were more frequently detected in poorly differentiated tumors (Fig. 2c).

Target screening of 11q13.2 amplification region

11q13 was one of the most common high-level amplification regions, and the frequency of gain and amplification was more than 40 and 10 %, respectively (Fig. 3a, b). Most of the attention was paid to 11q13.3 and target *CCND1*, but the driving gene of 11q13.2 was still unclear.

Therefore, we conducted a target-screening assay to 37 genes at 11q13.2 using RT-PCR. The top ten genes including *GAL*, *KIAA1394*, *TBX10*, *GPR152*, *CHKA*, *MGRPRD*, *LRP5*, *PTPRCAP*, *CDK2AP2* and *CLCF1* were further analyzed in 8 ESCCs by real-time PCR. The results showed that the expression of *CHKA*, *GAL*, *KIAA1394*, *LRP5* and *PTPRCAP* was significantly higher in ESCC tissues than in paracancerous tissues (Fig. 3c). We analyzed the copy number status of the five genes, and found that 67 % (4/6) of patients with *LRP5* overexpression had copy number increase, and the rates for *KIAA1394*, *GAL*, *CHKA* and *PTPRCAP* were 60 % (3/5), 57 % (4/7), 50 % (3/6) and 40 % (2/5), respectively. However, the expression of *TBX10*, *GPR152*, *MGRPRD*, *CDK2AP2* and *CLCF1* was not significantly different between tumor tissues and paracancerous tissues (Fig. S1). We also applied FISH to validate the copy number increase of *GAL* and *KIAA1394* in 8 ESCCs, and found

Table 2 Genomic aberrations in ESCC by array CGH

GISTIC Analysis ($\log_2\text{ratio} >0.25$ or <-0.25 ; FDR <0.05)		Genomic Workbench ADM-2 ($\log_2\text{ratio} >1.0$ or <-1.0)		
Aberration	Cytoband	Region	Gene symbol	
Aberration	Cytoband	Region	No. of cases	
Aberration	Cytoband	Region	Candidate genes	
Gain	7p12.1	53605427-53724423		
	7p11.2	54924763-55274812	EGFR	
	8q24.21	128401403-128828456	POU5F1B, LOC727677, MYC	
	11q13.2-q13.4	68454441-71286776	IGHMBP2, MRGPRD, MRGPRF, TPCN2, MYEOV, CCND1, ORAOV1, FGF19, FGF4, FGF3, ANO1, FADD, PPF1A1, MIR548K, CTTN, SHANK2, MIR3664, DHCR7, NADSYN1, KRTAP5-7, KRTAP5-8, KRTAP5-9, KRTAP5-10, KRTAP5-11, FAM86C, DEFB108B, LOC100133315	
	11q22.1-q22.3	100000125-103060917	ARHGAP42, MIR548G, TMEM133, PGR, TRPC6, MIR3920, ANGPTL5, KIAA1377, C11orf70, YAPI, BIRC3, BIRC2, TMEM123, MMP7, MMP20, MMP27, MMP8, MMP10, MMP1, MMP3, MMP12, MMP13, DCUN1D5, DYNC2H1	
				Amp
				7p11.2 54892257-55242365 EGFR
				8p12 37782033-37911894 GPR124, BRF2, RAB11FIP1, GOT1L1
				8q24.21 127839514-128897697 POU5F1P1, POU5F1B, LOC727677, MYC, PVT1
				11q13.2-q13.3 68804860-70182767 MYEOV, CCND1, ORAOV1, FGF19, FGF4, FGF3, ANO1, FADD, PPF1A1, CTTN, SHANK2
Loss				
				12p11.21 31248212-31746017 FAM60A, FLJ13224, DENND5B, C12orf72, AMN1
				12q12 40373082-40958582 GLT8D3, YAF2
				2q22.1 141622629-141753424 LRP1B
				8p23.1-p21.2 1094834-2546738 DLGAP2, CLN8, ARHGEF10, KBTBD11, MYOM2
				9p21.3 21957548-21999029 C9orf53, CDKN2A, CDKN2BAS, CDKN2B
				11q11 55375127-55438414 SPRYD5, OR5W2
				14q11.2 19323579-19446107 OR4N2, OR4K2

Amp amplification, HD homozygous deletion, bold font: genes shared by GISTIC and Genomic Workbench ADM-2 analysis

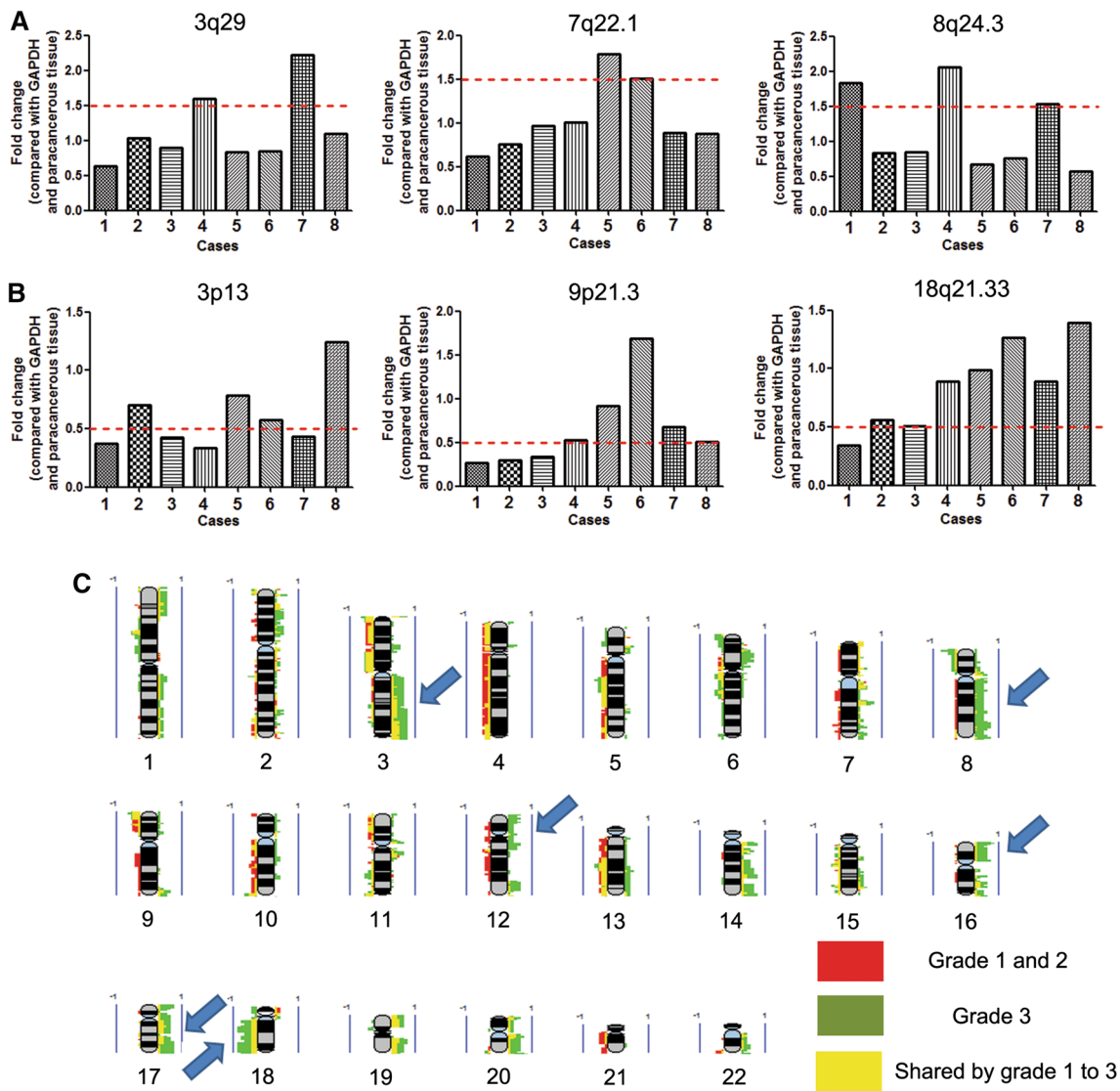


Fig. 2 Validation of genomic aberration and tumor grade associated chromosomal changes. **a** Validation of gains in ESCC by real-time PCR. *X* case number, *Y* fold change of copy number (compared with GAPDH and paracancerous tissues); *dashed line* the definition of gain is above 1.5. **b** Validation of losses in ESCC by real-time PCR. *X* case number; *Y* fold change of copy number (compared with GAPDH and

paracancerous tissues); *dashed line* the definition of loss is below 0.5. **c** Frequency plot comparison of ESCC with different tumor grade. 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

that *GAL* and *KIAA1394* were co-amplified in five cases ($n = 8$, Fig. 3d).

Overexpression of *ALG3* at 3q27.1 in ESCC

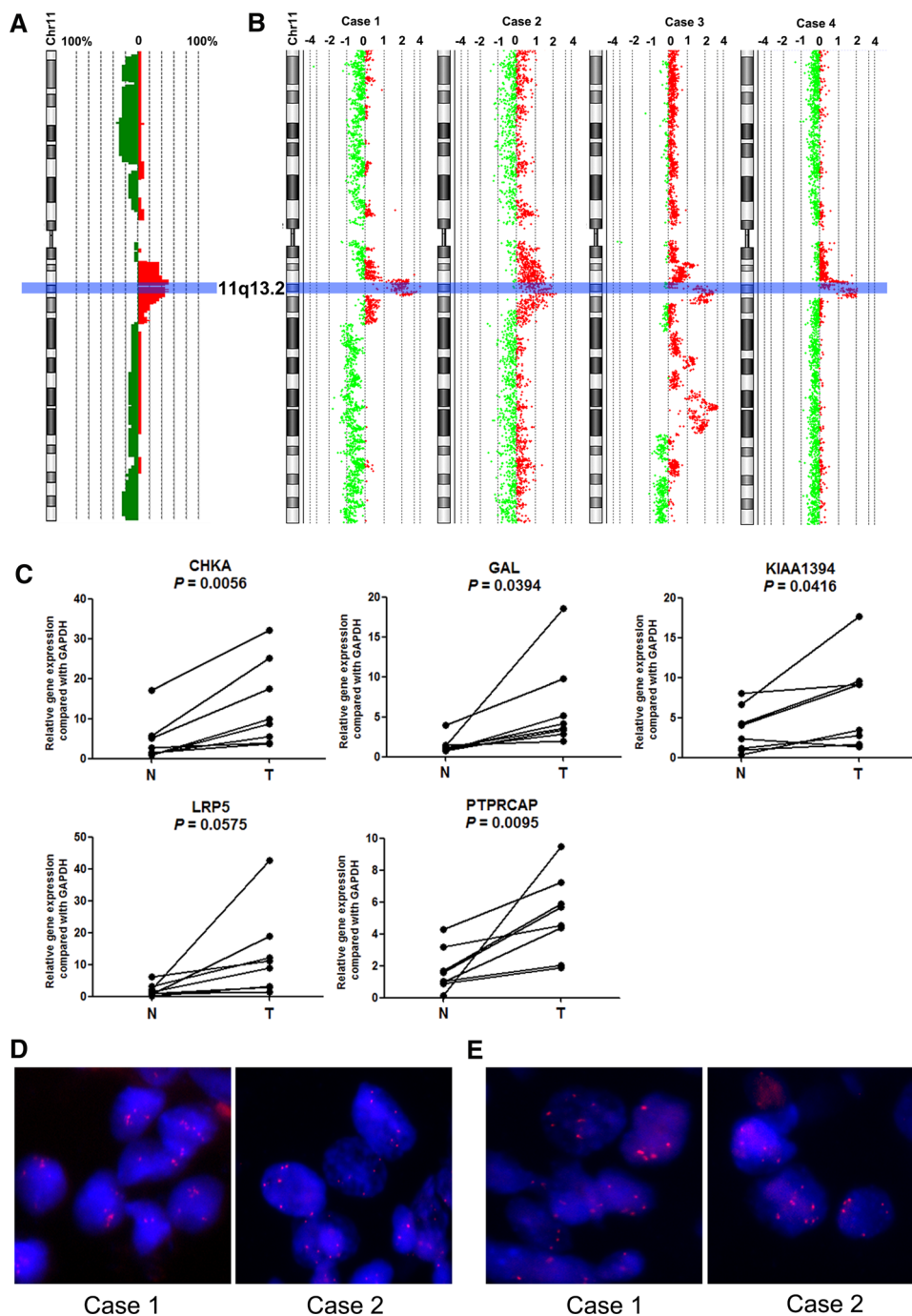
Copy number gain at 3q was very common in ESCC and the frequency was 70 % (Fig. 4a, b). We further found that *ALG3* at 3q27.1 was significantly overexpressed in tumor tissues comparing with paracancerous normal tissues (Fig. 4c). The mRNA expression of *ALG3* in cell lines with amplification was higher than those without amplification (Fig. 4d, e). FISH assay showed that *ALG3* was amplified in 4 ESCCs ($n = 8$, Fig. 4f). And high

expression of *ALG3* was significantly associated with lymph node metastasis ($P = 0.038$; $n = 33$; Table 3).

Discussion

Genomic aberrations can contribute to the carcinogenesis and tumor progression. Earlier reports have identified multiple abnormal regions in ESCC, including amplifications at 1p34, 3q, 5p, 7p12, 8q, 11q13, 12p, 17q12, 22q as well as deletions at 2q, 3p, 4q, 5q13-q21, 9p21.3 and 13q [1, 3, 6, 13]. In the present study, we found 34 gains and 16 losses in ESCC with frequency over 50 %, especially six

Fig. 3 Target screening of 11q13.2 amplification region. **a** Frequency plot of copy number changes at chromosome 11. *Right of 0*: amplification; *left of 0*: homozygous deletion. **b** Typical amplification status of 11q13.2 in four ESCC cases. **c** The relative mRNA expression level of CHKA, GAL, KIAA1394, LRP5 and PTPRCAP. **d** FISH assay on the GAL amplification (RP11-569N5). **e** FISH assay on the KIAA1394 amplification (RP11-389P13)



high-level amplifications at 7p11.2, 8p12, 8q24.21, 11q13.2-q13.3, 12p11.21, 12q12 and four homozygous deletions at 2q22.1, 8p23.1-p21.2, 9p21.3 and 14q11.2 were identified. EGFR (7p11.2) was amplified and overexpressed in ESCC, and copy number increase of EGFR led to protein overexpression [14]. MYC (8q24.21) was frequently amplified, and its copy number increase was associated with worse overall survival of ESCC [4]. CCND1 at 11q13.3 was another common amplification gene, and both copy number increase and overexpression

of CCND1 were significantly linked with lymph node metastasis [15]. And elevated level of CCND1 DNA in plasma was significantly associated with poor prognosis [16]. However, target genes of other amplifications were largely unknown.

Amplification of 11q13 is a common genomic event in cancers, including esophageal, breast, gastric, oral and liver cancer [15, 17–21]. The 11q was gained in 75 % of gastric adenocarcinoma, and the two minimal amplification regions harbored two candidate target genes CCND1 and

Fig. 4 Overexpression of ALG3 at 3q27.1 in ESCC. **a** Typical amplification status of 3q27.1 in six ESCC cases. **b** Frequency plot of copy number changes at 3q. *Right of 0*: amplification; *left of 0*: homozygous deletion. **c** Relative mRNA expression of ALG3 in paired ESCC samples. **d** Logratio of ALG3 in four ESCC cell lines. **e** Relative mRNA expression of ALG3 in four ESCC cell lines. **f** FISH assay on the ALG3 amplification (RP11-631P04)

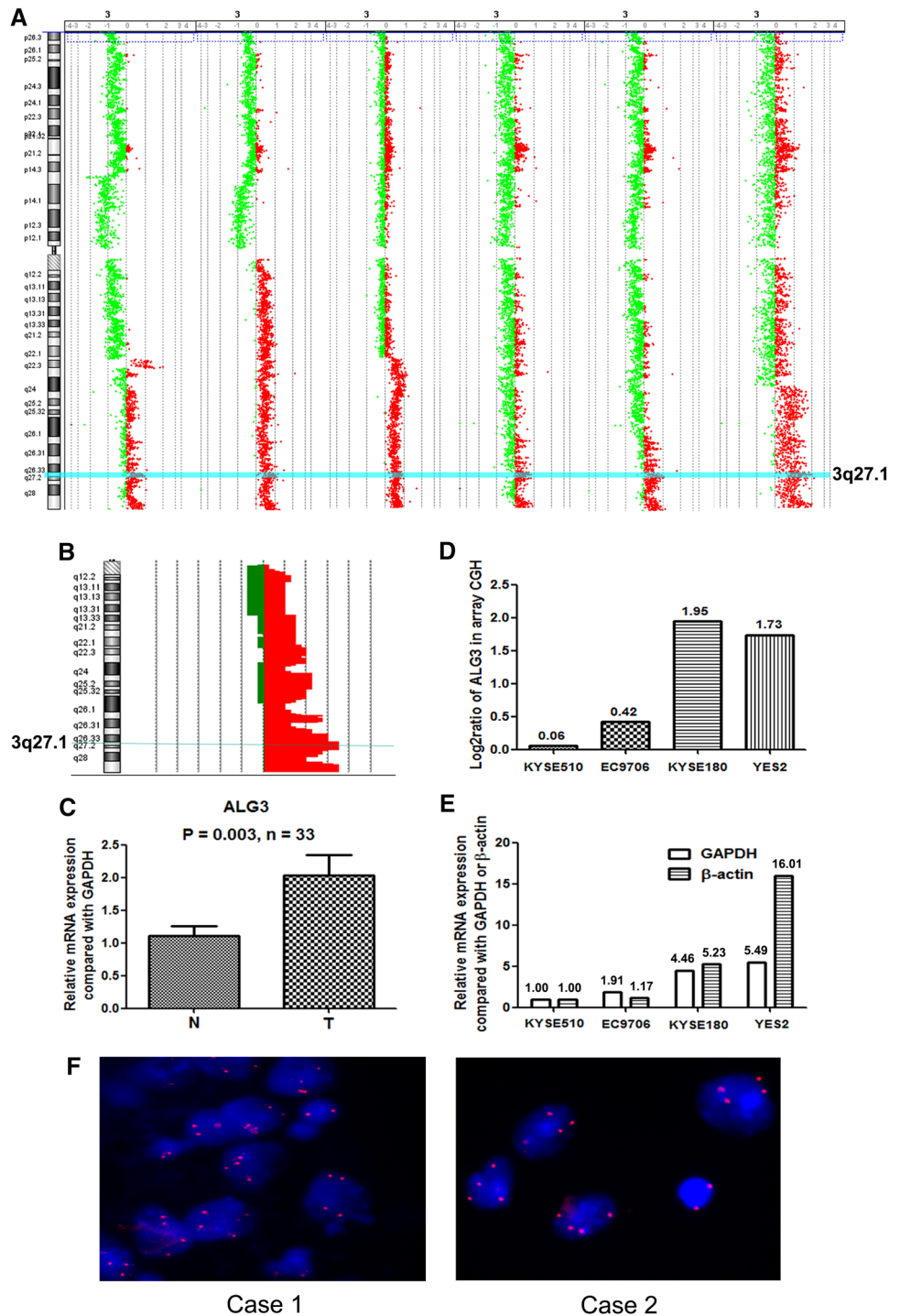


Table 3 ALG3 mRNA expression in lymph node metastatic cases

ALG3 overexpression	N1	N0	P value*
Positive	14	4	0.038
Negative	6	9	

* Fisher’s exact test

N1 positive lymph node metastasis; N0 no lymph node metastasis

ORAOV1 [17]. In oral squamous cell carcinoma (OSCC), gain of 11q22.1-q22.2 and loss of 11q23-q25 was associated with loco-regional recurrence and shorter survival [18]. ORAOV1 at 11q13.3 was amplified in OSCC, and its expression level in OSCC cell lines with 11q13 amplification was higher than those without amplification [19]. 11q13 was also a common amplification region in liver

cancer, and knockdown of CCND1 or FGF19 located in this region can significantly inhibit clonal growth and tumorigenicity of hepatocellular carcinoma cells [20]. In ESCC, 11q13 was frequently amplified, in which CTTN promoted tumor metastasis by anoikis resistance via activation of the phosphatidylinositol 3-kinase/Akt pathway [22]. Our present study screened 37 genes at 11q13.2 and found that five genes including CHKA, GAL, KIAA1394, LRP5 and PTPRCAP were significantly overexpressed in ESCC. It has been reported that CHKA was overexpressed in 35 % of hepatocellular carcinoma, and its overexpression was significantly correlated with advanced tumor stage and poor prognosis [23]. In breast cancer cell lines, CHKA interacted with EGFR kinase domain and was phosphorylated in a c-Src-dependent manner, and finally promoted EGF-dependent cell proliferation [24]. GAL was overexpressed in ESCC, and GAL together with its receptor GalR1 could reduce caspase-8-dependent apoptosis via upregulation of the caspase-8 inhibitor FLIP(L) and induce chemoresistance [12, 25]. Amplification and overexpression of LRP5 were detected in sarcoma lines, and together with overexpression of co-receptor LRP6 were one of the mechanisms of activating Wnt signaling [26]. However, the biological function about the genes KIAA1394 and PTPRCAP in tumorigenesis was limited. Therefore, our data may help to identify novel candidate amplification target genes in ESCC.

Amplification of 3q is also a common genomic feature in cervical, lung and esophageal cancer [27–29]. In lung squamous cell carcinoma, EPHB3 (3q27.1), MASP1 (3q27.3) and SST (3q27.3) were amplified at frequency of 18, 18 and 14 %, respectively [27]. Overexpression of FXR1, CLAPM1, EIF4G and TP63 located at 3q26-q27 was also reported, especially that copy number increase and overexpression of TP63 were an indicator of prolonged survival [28]. Choi et al. reported that gain of 3q27.1 especially ALG3 at 3q27.1 was correlated with the cervical carcinogenesis [30], so we conducted the study to explore whether ALG3 was the novel target of 3q27.1 amplification in ESCC. Our study revealed that ALG3 at the amplification region 3q27.1 was significantly overexpressed and associated with lymph node metastasis. Therefore, ALG3 may be the novel important gene for 3q27.1 amplification, but the role of it in the tumorigenesis was still needed to be clarified.

In summary, our study identified multiple DNA copy number-changed chromosome regions and candidate targets of amplification regions 11q13.2 and 3q27.1. These findings provide important insights into molecular alterations occurred in ESCC. Further study should be addressed to explore the possible tumorigenic roles of these candidate genes.

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Conflict of interest We declare that we have no conflict of interest.

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