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• *ESOPHAGEAL CANCER* •

Copy number changes of target genes in chromosome 3q25.3-qter of esophageal squamous cell carcinoma: *TP63* is amplified in early carcinogenesis but down-regulated as disease progressed

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Abstract

AIM: By using comparative genomic hybridization, gain of 3q was found in 45-86% cases of esophageal squamous cell carcinoma (EC-SCC). Chromosome 3q25.3-qter is the minimal common region with several oncogenes found within this region. However, amplification patterns of these genes in EC-SCC have never been reported. The possible association of copy number changes of these genes with pathologic characteristics is still not clear.

METHODS: Real-time quantitative PCR (Q-PCR) was performed to analyze the copy number changes of 13 candidate genes within this region in 60 primary tumors of EC-SCC, and possible association of copy number changes with pathologic characteristics was analyzed by statistics. Immunohistochemistry (IHC) study was also performed on another set of 111 primary tumors of EC-SCC to verify the association between *TP63* expression change and lymph node metastasis status.

RESULTS: The average copy numbers (±SE) per haploid genome of individual genes in 60 samples were (from centromere to telomere): *SSR3*: 4.19 (±0.69); *CCNL1*: 5.24 (±0.67); *SMC4L1*: 2.01 (±0.16); *EVI1*: 2.02 (±0.12); *hTERC*: 5.28 (±0.54); *SKIL*: 2.71 (±0.14); *EIF5A2*: 1.95 (±0.12); *ECT2*: 9.18 (±1.68); *PIK3CA*: 8.13 (±1.17); *EIF4G1*: 1.07 (±0.05); *SST*: 3.07 (±0.25); *TP63*: 2.51 (±0.22); *TFRC*: 2.42 (±0.19). Four clusters of amplification were found: *SSR3* and *CCLN1* at 3q25.31; *hTERC* and *SKIL* at 3q26.2; *ECT2* and *PIK3CA* at 3q26.31-q26.32; and *SST*, *TP63* and *TFRC* at 3q27.3-q29. Patients with lymph node metastasis had significantly lower copy number of *TP63* in the primary tumor than those without lymph node metastasis. IHC study on tissue arrays also showed that patients with lymph node metastasis have significantly lower *TP63* staining score in the primary tumor than those without lymph node metastasis.

CONCLUSION: This study showed that different amplification patterns were seen among different genes within 3q25.3-qter in EC-SCC, and several novel candidate oncogenes (*SSR3*, *SMC4L1*, *ECT2*, and *SST*) were identified. *TP63* is amplified in early stage of EC-SCC carcinogenesis but down-regulated in advanced stage of disease.

Key words: Chromosomal aberration; Comparative genomic hybridization; Esophageal neoplasm; Immunohistochemistry; Quantitative real-time PCR; Tissue array; Tumor protein 63

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INTRODUCTION

Esophageal cancer (EC) is one of the most lethal cancers in the world[1] as well as in Taiwan (http://www.doh.gov.tw/dohenglish/Upload/Statistics/S02/9110-eng.xls). In most Asian countries, squamous cell carcinoma (EC-SCC) is the most frequent histological subtype of EC. By using modern molecular cytogenetic study such as comparative genomic hybridization (CGH)[2] and spectral karyotyping (SKY)[3], several recurrent chromosomal aberrations of EC-SCC were identified. Among them, chromosome 3q is especially important, because 45-86% cases of EC-SCC were found to have amplification in this region, with minimal overlapping region over 3q25.3-qter[4-8]. Gain of chromosome 3q was also reported in many other tumors, such as cancer of the lung[9], ovary[10], and cervix[11,12], and head and neck squamous cell carcinoma (HNSCC)[13,14].

The size of 3q25.3-qter is about 40 MB, containing about 367 known genes within this region, not to mention the unknown ones. Several candidate oncogenes were located in this chromosomal region, such as cyclin L1 (*CCNL1*), at 3q25; human telomerase RNA component (*hTERC*), at 3q26; phosphoinositide-3-kinase, catalytic, alpha polypeptide (*PIK3CA*), at 3q26; Ski-like protein (*SKIL*), at 3q26; ecotropic viral integration site 1 (*EVI1*), at 3q26; eukaryotic translation initiation factor 5A2 (*EIF5A2*), at 3q26; eukaryotic translation initiation factor 4gamma1 (*EIF4G1*), at 3q27; tumor protein 63 (*TP63*), at 3q28; and transferrin receptor (*TFRC*), at 3q29 (GeneCards™, http://bioinformatics.weizmann.ac.il/cards/). However, amplification patterns of these genes in EC-SCC have never been reported.

A recently introduced method, real-time quantitative PCR (Q-PCR), could be used to accurately evaluate copy numbers of genes with only minimal amounts of tumor materials with high-throughput capacity[15-17]. In previous study, the accuracy of assessing copy number changes by Q-PCR in comparison with fluorescence *in situ* hybridization (FISH) had been demonstrated[18]. In this study, Q-PCR was used to study the copy number changes of the afore mentioned nine candidate oncogenes, together with four genes [signal sequence receptor gamma (*SSR3*) at 3q25.31, structural maintenance of chromosomes 4-like 1 (*SMC4L1*) at 3q26.1, epithelial cell transforming sequence 2 oncogene (*ECT2*) at 3q26.31, and somatostatin (*SST*) at 3q27.3] that were not down-regulated in our preliminary expression profile study, within chromosome 3q25.3-qter region in 60 primary tumors of EC-SCC, and the possible associations of these changes with disease progression were analyzed. Immunohistochemistry (IHC) was also used to verify the association of expression change of *TP63* with lymph node metastasis status on another 111 cases of EC-SCC.

MATERIALS AND METHODS

Primary tumors and cell lines of EC-SCC

From 1995 to 1997, 60 ethic Chinese patients with EC-SCC were enrolled for Q-PCR study. All patients underwent primary surgical resection without neoadjuvant chemotherapy or radiotherapy. Only patients with written informed consent were included. Pathological evaluation of depth of tumor invasion, tumor differentiation and lymph node metastasis were done by one of our pathologists (Jung-Ta Chen), and staging and grading of tumor were defined according to the Cancer Staging Manual (5th edition; American Joint Committee on Cancer). Portions of tumor from the paraffin-embedded primary tumor samples, of which at least 70% were tumor cells, were identified under the microscope by one of our pathologists (Jung-Ta Chen) and were cut out for study. The procedure of DNA extraction was modified from a previously described protocol[19]. CGH analysis of part of these patients had been previously reported[8]. DNA extracted from five volunteer donor lymphocytes was used as control. EC-SCC cell lines CE 48T/VGH, CE 81T/VGH, TE6 and TE9, which have been previously characterized by molecular cytogenetics[18], were used to validate the accuracy of Q-PCR.

Fluorescence in situ hybridization (FISH)

FISH was performed using methods as previously suggested[20]. The search for FISH probes covering the 13 genes was done by browsing Ensembl Genome Browser http://www.ensembl.org/ and UCSC Genome Browser http://genome.ucsc.edu for candidate bacterial artificial chromosome (BAC) clones. The resulting clones were then obtained from RPCI-11 BAC library (Table 1)[21]. Their identities were verified by FISH-mapping onto normal lymphocyte metaphases. For each cell line, FISH signals were counted in 10 metaphases, and FISH signals per haploid genome were calculated by using average FISH signals per cell ×23/ average number of chromosomes per cell[18].

Real-time quantitative PCR (Q-PCR)

All primers were designed with Primer Express 3.0 software (Applied Biosystems Foster City, CA) using default parameters, with modified minimum amplicon length requirements (85 bp). An additional requirement consisted of a maximum GC content of 40% for the five last 3' end nucleotides. The sequences of the primers are listed in Table 1. PCR reactions were performed as previously described[18]. DNA content was normalized to that of long interspersed elements (*LINE1*), a repetitive element for which copy numbers per haploid genome are similar both in normal or neoplastic cells[17]. Copy number changes per haploid genome were calculated by using the formula $2^{(Nt-Nline)-(Tt-Tline)}$, where Nt is the threshold cycle number observed for an experimental primer in the normal DNA sample, Nline is the threshold cycle number observed for a *LINE1* primer in the normal DNA sample, and Tt and Tline are the threshold cycle numbers observed for the experimental primer and *LINE1* primer in test DNA sample, respectively[17]. For normal cell the copy number of a gene per haploid genome should be one. PCR for each primer set were performed in triplicate, and calculated copy number changes per haploid genome were averaged.

Construction of tissue arrays

Tissue arrays of another 120 EC-SCC tumor samples were constructed using method as previously described[22]. Briefly, the H&E-stained slides of selected tumor samples were examined under a light microscope. The areas of interest were circled with a color pen, and a 16-gauge bone marrow

Table 1 Covering BAC clones and position of 13 genes and sequence of Q-PCR primers of *LINE1* and 13 genes over 3q25.3-qter

Genes	BAC clones	Position	Forward primer	Reverse primer
LINE1	-	-	CCGCTCAACTACATGGAAACTG	GCGTCCCAGAGATTCTGGTATG
SSR3	304C15	3q25.31	GCCCAGGCATATGAGAGTTGTC	CCAACATGGCAGGGTCAAGT
CCNL1	6F2	3q25.31	*TCATGGCAGTCAACCAACAT*	CCATTGTAAGGGCTTTTGGA
SMC4L1	227J5	3q26.1	GGCAAAGTCCTAAGCAAGGTTGT	TCAACTGGCAAGCTAAGTGGAA
EVI1	141C22	3q26.2	CATGCATGCTGATTGCAGAAC	CCACCTGCCGCAAAATGGT
hTERC	40O08	3q26.2	CGTAGGCGCCGTGCTTT	TTTTCCGCCCGCTGAA
SKIL	543D10	3q26.2	GCTCGGCATTCCCAAGAAA	CCCCTTCCAACACAGTCTGAGT
EIF5A2	110O7	3q26.2	GGCTTCCAGCACTTACCCTATG	GACCATGCTTTCCAGTTTTGG
ECT2	453J16	3q26.31	CCTAACAGCAATCGCAAACG	CTGTCTCTCTTGAAAGCTGAGCAA
PIK3CA	245C23	3q26.32	GGAGGATGCCCAATTTGATG	AACAGTCCATTGGCAGTTGAGA
EIF4G1	481O2	3q27.1	CCGGTTCCAGAATCTGAGTTTT	GATTCGGAGGGGCAAGCTG
SST	211G3	3q27.3	GATGCCCTGGAACCTGAAGA	GCCGGGTTTGAGTTAGCAGAT
TP63	313I6	3q28	CCTCGTCCACCAGTCCCTAT	GGAAGGACACGTCGAAACTG
TFRC	313F11	3q29	CAAAGTGGCACGAGCAGCTG	GCTCTAGAATGAACGGTGGAAG

LINE1: long interspersed elements; *SSR3*: signal sequence receptor gamma; *CCNL1*: cyclin L1; *SMC4L1*:structural maintenance of chromosomes 4-like 1; *EVI1*: ecotropic viral integration site 1; *hTERC*: human telomerase RNA component; *SKIL*: Ski-like protein; *EIF5A2*: eukaryotic translation initiation factor 5A2; *ECT2*: epithelial cell transforming sequence 2 oncogene; *PIK3CA*: phosphoinositide-3-kinase, catalytic, alpha polypeptide; *EIF4G1*: eukaryotic translation initiation factor 4gamma1; *SST*: somatostatin; *TP63*:tumor protein 63; *TFRC*: transferrin receptor. BAC clones, clones of bacterial artificial chromosomes covering the genes obtained from RPCI-11 library.

biopsy trephine apparatus was used to punch at the circled areas, extracting a tissue cylinder with 2.0 mm diameter. At least three cylindrical core biopsies were taken from different sites of each tumor. About 40 cylinders (8×5) were carefully transferred with forceps to a recipient metal paraffin block box. The recipient box was then covered with a plastic cassette and then liquid wax was gently poured into the box until it was full. The box was then put on a hot plate for 1 min to homogenize the wax, after which the box was removed from the hot plate and cooled to room temperature slowly. Four-micrometer sections were cut and mounted on silane-coated slides.

Immunohistochemistry (IHC) for TP63

Immunostaining for *TP63* was performed on tissue array slides using a mouse anti-human *TP63* monoclonal antibody clone: 7JUL (1∶30; Novocastra, New Castle Upon Tyne, UK), which recognized all *TP63* isoforms, and high temperature antigen unmasking technique (autoclave in pH 8.0 EDTA buffer) as previously described[23]. The bound antibodies were detected using the DAKO Envision system. Positive control (non-neoplastic tonsil tissue) and negative control (replacement of primary antibody by PBS) were included. The slides were independently reviewed by two of the authors (Chin-Chen Pan, Paul Chih-Hsueh Chen) who were blinded to the clinicopathologic data. *TP63* immunoreactivity was semi-quantified using a combined intensity and percentage of positive scoring method. Intense nuclear staining was scored as 2, weak as 1, and negative as 0 (Figure 1)[24]. The percentage of cells with each intensity score was estimated. A *TP63* staining score was defined as the sum of the percentage of positive cells with each intensity level multiplied by the intensity score [e.g., a case with 30% intense staining and 40% weak staining would be scored as 100 (30×2+40×1)][24].

Statistical analysis

Statistical comparisons were performed using SPSS 12.0 software. Pearson coefficient of correlation was used to determine the correlation between the copy number changes assessed by FISH and Q-PCR. Student's *t*-test was used to test the association between the results of Q-PCR or the *TP63* staining score and lymph node metastasis or differentiation status. Kruskal-Wallis test was used to test the association between the results of Q-PCR or the *TP63* staining score and primary tumor extent.

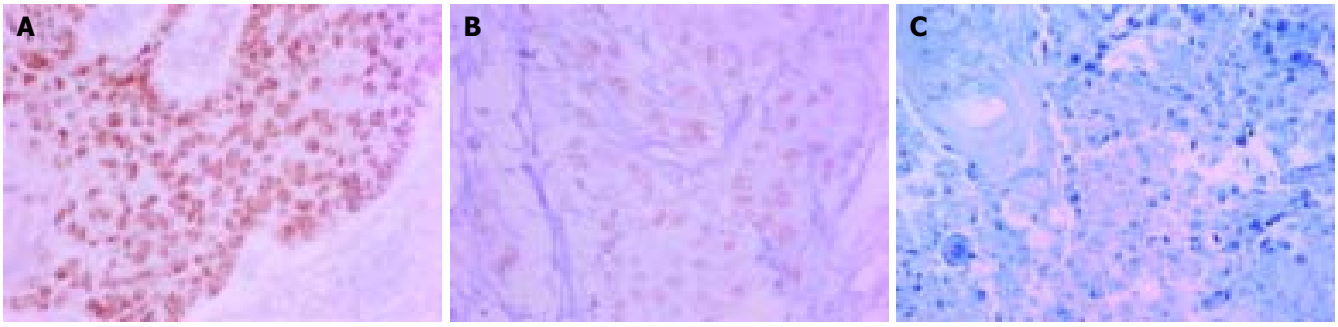

Figure 1 Immunoreactivity for *TP63*. A: Intense nuclear *TP63* staining; B: Weak nuclear *TP63* staining; C: Negative staining. (Original magnification: ×400).

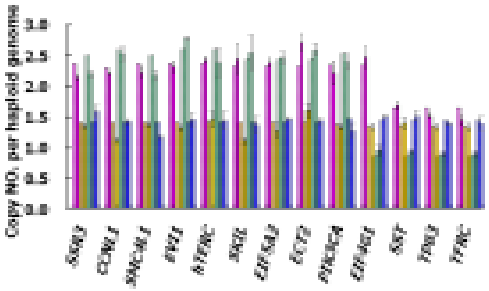

Figure 2 Comparison of copy numbers assessed by FISH (stripped bar) and Q-PCR (±SD) (hollow bar) of 13 genes in 4 EC-SCC cell lines [CE 48T/VGH (pink), CE 81T/VGH (yellow), TE6 (green), TE9 (blue)]. Pearson coefficients of correlation for comparing FISH and Q-PCR of 13 genes in 4 cell lines were all >0.9.

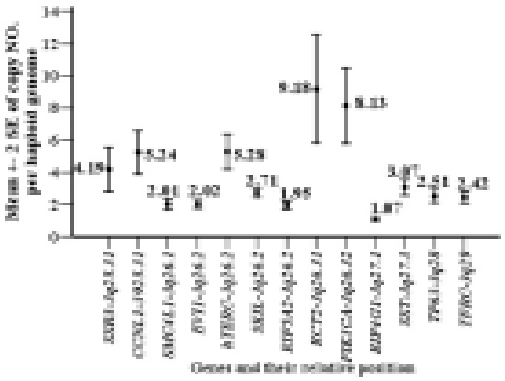

Figure 3 Average copy number changes (indicated by the circles with number listed beside them; crossbar represented 2×standard error) of 13 genes in association of their relative position on chromosome 3q. Four clusters of amplifications could be found: *SSR3* and *CCLN1* at 3q25.31; *hTERC* and *SKIL* at 3q26.2; *ECT2* and *PIK3CA* at 3q26.31-q26.32; and *SST*, *TP63* and *TFRC* at 3q27.3-q29.

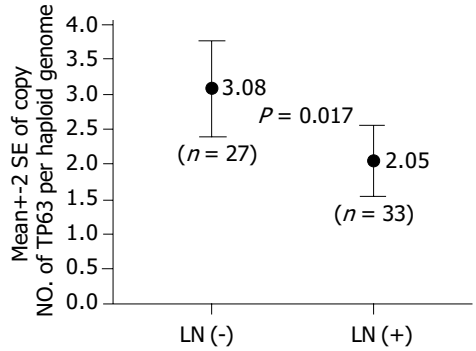

Figure 4 In Q-PCR analysis of 60 EC-SCC tumors, patients with lymph node metastasis [LN (+)] have significantly lower copy number of *TP63* in the primary tumor than those without lymph node metastasis [LN (-)] (2.05 *vs* 3.08; *P* = 0.017).

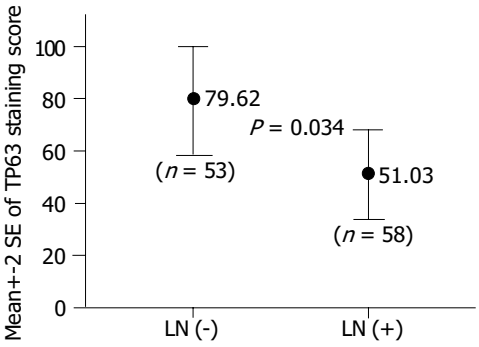

Figure 5 In IHC study of 111 EC-SCC tumors, patients with lymph node metastasis [LN (+)] have significantly lower *TP63* staining score in the primary tumor than those without lymph node metastasis [LN (-)] (51.03 *vs* 79.62; *P* = 0.034).

RESULTS

Evaluation of Q-PCR accuracy

The accuracy of the assay was tested on 4 EC-SCC cell lines. In Figure 2, copy number changes of 13 genes on these 4 EC-SCC cell lines, assessed by both FISH and Q-PCR, were quite compatible. The Pearson coefficients of correlation for comparing FISH and Q-PCR of 13 genes in 4 cell lines were all >0.9.

Assessment of copy number changes of candidate genes in primary tumors by Q-PCR

Copy number changes of 13 candidate genes were assessed by Q-PCR in 60 primary EC-SCC tumors. The average copy numbers (±SE) per haploid genome of individual genes in 60 samples were (from centromere to telomere): *SSR3*: 4.19 (±0.69); *CCNL1*: 5.24 (±0.67); *SMC4L1*: 2.01 (±0.16); *EVI1*: 2.02 (±0.12); *hTERC*: 5.28 (±0.54); *SKIL*: 2.71 (±0.14); *EIF5A2*: 1.95 (±0.12); *ECT2*: 9.18 (±1.68); *PIK3CA*: 8.13 (±1.17); *EIF4G1*: 1.07 (±0.05); *SST*: 3.07 (±0.25); *TP63*: 2.51 (±0.22); *TFRC*: 2.42 (±0.19). Four clusters of amplification could be found: *SSR3* and *CCLN1* at 3q25.31; *hTERC* and *SKIL* at 3q26.2; *ECT2* and *PIK3CA* at 3q26.31-q26.32; and *SST*, *TP63* and *TFRC* at 3q27.3-q29 (Figure 3).

Association of copy number changes of candidate genes in primary tumors with disease staging or differentiation

The association between copy number changes of 13 genes and primary tumor extent, lymph node metastasis or tumor differentiation was analyzed. Patients with lymph node metastasis have significantly lower copy number of *TP63* in the primary tumor than those without lymph node metastasis (2.05 *vs* 3.08; *P* = 0.017) (Figure 4). There were no significant association between copy number changes of the other 12 genes and lymph node metastasis. Also the copy number changes of all 13 genes had no statistical association with either primary tumor extent or tumor differentiation.

Association of TP63 staining score in primary tumors with disease staging or differentiation

Immunostaining of *TP63* could be evaluated in 111 cases. The association between *TP63* staining score and primary tumor extent, lymph node metastasis or tumor differentiation was analyzed. As in the study of Q-PCR, patients with lymph node metastasis have significantly lower *TP63* staining score in the primary tumor than those without lymph node metastasis (51.03 *vs* 79.62; *P* = 0.034) (Figure 5). There was no statistical association between *TP63* staining score and either primary tumor extent or tumor differentiation.

DISCUSSION

In this study, the pattern of copy number changes of 13 potential target genes located on 3q25.3-qter in EC-SCC and the association of these changes with disease progression were analyzed. In general, all genes except *FIE4G1* had increased copy number. However, four clusters of genes with higher amplification were found: *SSR3* and *CCNL1* at 3q25.31; *hTERC* and *SKIL* at 3q26.2; *ECT2* and *PIK3CA* at 3q26.31-q26.32; and *SST*, *TP63* and *TFRC* at 3q27.3-q29, with highest peaks over *ECT2* and *PIK3CA* (Figure 3). In previous study, it was demonstrated that copy number of *PIK3CA* was significantly higher than that of *TP63* in EC-SCC[18]. Therefore, in EC-SCC, 3q26.31-q26.32 might be the mostly amplified area within this region.

CCNL1[25], *hTERC*[26-28], *SKIL*[29], *PIK3CA*[30-32], *TP63*[33-37] and *TFRC*[38] were reported as potential oncogenes in different diseases . However, *SSR3*, *SMC4L1*, *ECT2* and *SST* have never been previously identified as oncogenes. *SSR3* is one of the 4 members of translocon-associated protein (TRAP) over endoplasmic reticulum (ER) membrane. TRAP is responsible for the passage of peptide through ER membrane[39]. *SMC4L1* is one of the members of structural maintenance of chromosomes (SMC) proteins. The eukaryotic SMC proteins could form heterodimers, which may be involved in chromosome condensation, sister chromatid cohesion, and DNA recombination[40]. *ECT2* was identified as an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis[41]. Recent studies have showed that N-terminal truncation of *ECT2* may render it as an oncogenic protein, which may cause malignant transformation of cell[42]. *SST* (somatostatin) and its analogues have been used in the treatment of endocrine tumor. Whether these genes play roles in carcinogenesis of EC-SCC deserve further investigation.

It is very interesting to find that the copy number changes as well as expression of *TP63* decrease in more advanced stage of disease in EC-SCC. Heselmeyer *et al.* reported that gain of chromosome 3q could be found in the early dysplasia lesion as well as in the invasive carcinoma of cervical cancer, but at reduced frequency in advanced stage of disease[11,12]. However, this phenomenon has never been reported in EC-SCC.

TP63 gene, one of *TP53* gene family, is a well-known oncogene within this region. Overexpression of *TP63* was found in most squamous cell carcinoma[33-37]. In a IHC study of EC-SCC, *TP63* protein was found highly expressed in carcinoma (50/51), dysplasia (10/11), and even in histologically normal epithelia of esophagus adjacent to the cancerous tissues (45/47)[35]. On the contrary, in the study of *TP63* expression in urothelial carcinoma, Koga *et al.* reported that lower *TP63* expression was significantly associated with higher Tumor-Node- Metastasis (TNM) stage ($P = 0.0004$) and lymph-node metastasis ($P = 0.013$). It was found that cancer cells with lower *TP63* staining had higher chance of lower membranous *β-catenin* expression, which plays a role in cell-cell adherent junctions, and cancer invasion and metastasis could be promoted by reduced membranous *β-catenin* expression[24]. In a similar study of upper urinary tract urothelial carcinoma, Zigeuner *et al.* found that decreased *TP63* immunoreactivity was significantly associated with advanced tumor stages and poor prognosis. They also found that cases with decreased *TP63* immunoreactivity had higher chance of *TP53* overexpression in comparison with cases with normal *TP63* immunoreactivity.

By combining results of this study with other reports, it is very likely that amplification of genes located on 3q25.3-qter may occur in quite early stage in the carcinogenesis of EC-SCC. But some of the genes, such as *TP63*, may be down-regulated as disease progressed. One possible explanation is that the alterations of genes are no longer necessary for maintenance of cancer cells survival. The other possibility is that down-regulation of these genes may accompany alterations of other genes (such as *β-catenin* or *TP53*), which may render cancer cell more invasive or malignant.

In conclusion, this study demonstrated different amplification patterns of different genes within 3q25.3-qter in EC-SCC, with highest amplification over 3q26.31-q26.32. *SSR3*, *SMC4L1*, *ECT2* and *SST* were identified as novel candidate oncogenes within this region. *TP63* is amplified in early stage of EC-SCC carcinogenesis but down-regulated in advanced stage of disease.

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