

## Nonrandom Chromosomal Imbalances in Esophageal Squamous Cell Carcinoma Cell Lines: Possible Involvement of the *ATF3* and *CENPF* Genes in the 1q32 Amplicon

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Using comparative genomic hybridization (CGH), we investigated copy number aberrations in 29 esophageal squamous cell carcinoma (ESC) cell lines. All lines displayed numerous chromosome imbalances. The most frequent losses were observed on chromosome 18q (65.5%), Xp (48.3%), 3p (44.8%), 4q (44.8%), 8p (41.4%), 11q23–25 (34.5%) and 4p (27.6%), whereas the most common copy number gains were noted at 8q (86.2%), 3q (82.8%), 5p (69%), 7p (69%), 20q (65.5%), 9q (55.2%), 11q (55.2%), 1q (48.3%), Xq (44.8%) and 18p (37.9%). High-level gains (HLGs) were detected at 3q26 (9 cases), 8q23 (6 cases), 5p14–15 (6 cases), 18p11.2–11.3 (6 cases), 3q27–28 (5 cases), 5p13 (3 cases), 7p14–15 (3 cases), 20q12–13 (3 cases), 11q13 (3 cases), 14q21 (2 cases), 20p11.2 (2 cases), 13q32 (2 case), and 1q32 (1 case). Among them, HLGs of 1q32 have been reported in other types of cancer, including glioblastoma and breast cancers. We successfully narrowed down the smallest common amplicon involving 1q-gain to the genomic segment between D1S414 and D1S2860 by fluorescence *in situ* hybridization (FISH). Southern and northern blot analysis clearly demonstrated that *ATF3*, human activating transcription factor-3 and *CENPF*, centromere protein F, mapped within this region, were significantly amplified and over-expressed in 1q32 amplicon.

**Key words:** Esophageal squamous cell carcinoma (ESC) — Comparative genomic hybridization (CGH) — Amplicon map — Human activating transcription factor 3 (*ATF3*) — Centromere protein F (*CENPF*)

Esophageal carcinoma is the sixth most frequent cause of cancer death in the world.<sup>1)</sup> In Japan, it ranked eighth among cancer-related causes of death in 1997.<sup>2)</sup> There are two different histopathological types of esophageal carcinoma, squamous cell carcinoma and adenocarcinoma. Esophageal squamous cell carcinoma (ESC) occurs more frequently in Japan as well as the developing countries, with marked regional variations in incidence<sup>3)</sup>; mortality rates are in accordance with the incident rates due to the relatively late detection and the poor efficiency of therapy.<sup>1)</sup> Based on epidemiological and clinical studies, risk factors for primary ESC include tobacco smoking, alcohol consumption, certain dietary habits and esophageal reflux.<sup>4,5)</sup>

Recently, evidence has been accumulated suggesting that multistep genetic alterations in nucleotide level and chromosome level may underlie carcinogenesis.<sup>6,7)</sup> Using classical cytogenetic methods, many chromosomal aberrations have been identified in human cancer. This informa-

tion has facilitated identification of a number of important genes associated with tumorigenesis.<sup>8)</sup> Some of the genetic alterations associated with development and/or progression of ESC have been identified, including amplifications of *MYC*, *EGFR* and *CCND1*.<sup>9,10)</sup> With respect to tumor suppressor genes, loss of heterozygosity (LOH) studies in ESC have indicated possible involvement of the *TP53* (17p13), *RBI* (13q14), *APC* (5q12), *MCC* (5q12), *CDKN2A* (9p21–22), and *DCC* (18q23).<sup>11)</sup> Furthermore, putative tumor suppressors involved in the development or progression of ESC are also thought to lie on 3p, 3q, 10p, 17q, 19q and 21q.<sup>11,12)</sup> However, few of them have been demonstrated to be associated with phenotype, including prognosis in ESC. Therefore, novel genes associated with the progression of ESC still need to be identified.

Comparative genomic hybridization (CGH) is a powerful molecular cytogenetic tool that allows comprehensive analysis of the entire genome and has now become the most popular genome scanning technique.<sup>13–15)</sup>

In this study, we investigated chromosome copy number aberrations in 29 ESC cell lines using CGH analysis and

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identified novel chromosomal regions that may be relevant for development and/or progression of ESC.

## MATERIALS AND METHODS

**Tumor samples** All of 29 ESC cell lines (KYSE series) were established from surgically resected esophageal cancer.<sup>16</sup> The cell lines were maintained in RPMI1640 supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin. High-molecular-weight DNAs were isolated from all cell lines using standard procedures. RNAs were also isolated from all cell lines using Trizol (Life-Technologies, Gaithersburg, MD).

**Slide preparation** Metaphase chromosome spreads were prepared from peripheral blood leukocytes from healthy donors or cell lines. Blood cells were cultured for 72–84 h in RPMI1640 containing 15% fetal bovine serum, penicillin-streptomycin (100  $\mu\text{g}/\text{ml}$ ), glutamine, and phytohemagglutinin (PHA 5  $\mu\text{g}/\text{ml}$ ). Both blood cells and cell lines were harvested by arresting with Colcemid (0.05 mg/ml) for 1 h, followed by hypotonic treatment in KCl (0.54%) for 20 min on ice and fixation in cold methanol:acetic acid (3:1).

**CGH** CGH was performed using directly fluorochrome-conjugated DNA as described by Kallioniemi *et al.*,<sup>13</sup> with

minor modification.<sup>17, 18</sup> Briefly, tumor DNAs and normal DNAs were labeled with Spectrum Green-dUTP (Vysis, Chicago, IL) and Texas Red-dUTP (DuPont, Boston, MA) by nick translation, respectively. Labeled tumor and normal DNAs (400 ng each), together with 10  $\mu\text{g}$  of Cot-1 DNA (Gibco BRL, Gaithersburg, MD) in a 10- $\mu\text{l}$  hybridization solution (50% formamide, 10% dextran sulfate and 2 $\times$  standard sodium citrate (SSC)), were denatured at 72°C for 5 min and hybridized to normal male metaphase chromosome at 37°C for 48 h. The slides were washed in 2 $\times$  SSC/0.3% NP-40 at 72°C for 2 min and 0.1 $\times$  SSC/0.1% NP-40 at room temperature for 5 min. Air-dried slides were counterstained with 0.1  $\mu\text{g}/\text{ml}$  of 4', 6'-diamidino-2-phenylindole (DAPI).

**Digital image analysis** Three single-color images (DAPI, Spectrum Green, and Texas Red fluorescence) were collected from each hybridization in 6–10 metaphase cells using an epifluorescence microscope (Olympus, Tokyo) and a cooled charge-coupled device (CCD) camera (Photometrics, Tucson, AZ) and analyzed by using a digital image analysis system (Vysis/Quip CGH software). Target regions were determined according to the green-to-red profiles of fluorescence intensity and information obtained by visual inspection of the digital images. Chromosome regions where the mean ratio fell below 0.8 were consid-

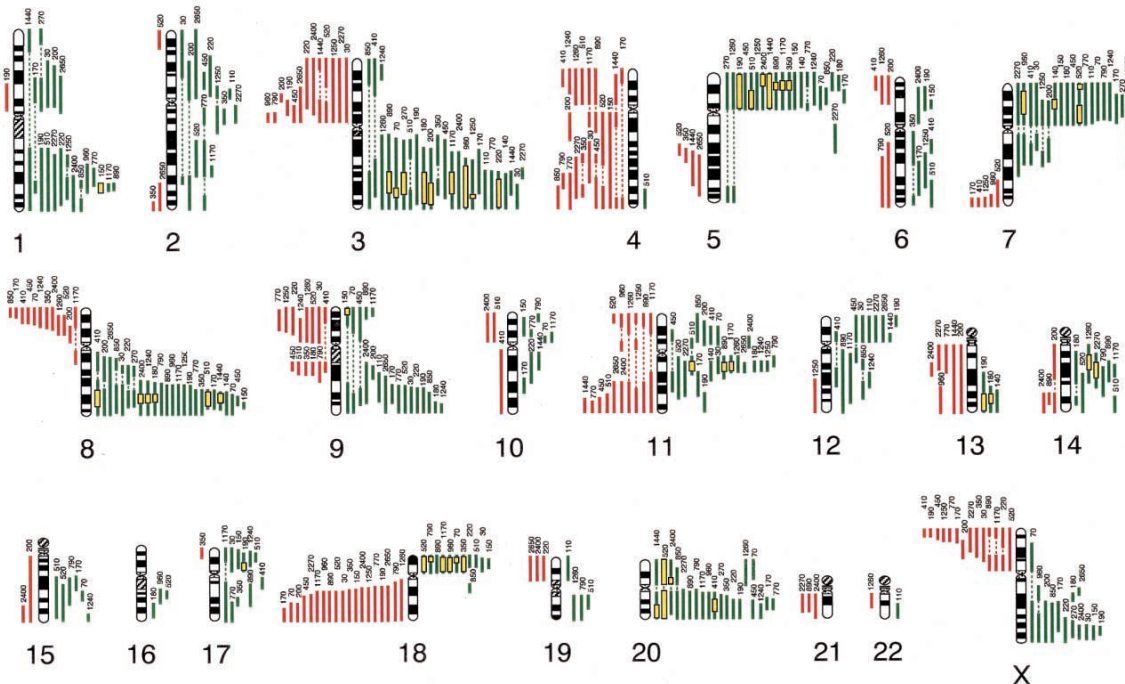


Fig. 1. Summary of genomic imbalances in 29 ESC cell lines detected by CGH. The 22 autosomes and X chromosome are represented by ideograms showing G-banding patterns. As judged by the computerized green-to-red profiles (see "Materials and Methods"), vertical lines on the left of each chromosome ideogram show losses of genomic material in cell lines, whereas those on the right correspond to copy number gains. High-level gains (HLGs) are represented as rectangles.

Table I. Minimal Overlapping Regions of Common DNA Copy Number Changes in ESC Cell Lines

DNA copy number	Chromosome regions	Frequency (%)	Putative target genes	
Gains	8q21–24	86.2 (25/29)	<i>MYC</i>	
	3q26	82.8 (24/29)	<i>PIK3CA</i>	
	5p14–15	69.0 (20/29)		
	20q12–13	65.5 (19/29)		
	7p14–15	62.1 (18/29)		
	1q32	48.3 (14/29)	<i>GAC1</i>	
	11q13	44.8 (13/29)	<i>CCND1</i> , <i>BCL1</i> , <i>INT2</i>	
	Xq	44.8 (13/29)		
	18p	37.9 (11/29)		
	9q34	37.9 (11/29)	<i>ABL</i>	
	14q	27.6 (8/29)		
	2q	27.6 (8/29)		
	High level gains (Amplifications)	3q26	31.0 (9/29)	<i>PIK3CA</i>
		8q23–24	20.7 (6/29)	<i>MYC</i>
5p14–15		20.7 (6/29)		
18p11.2–11.3		20.7 (6/29)	<i>YES1</i>	
3q27–28		17.2 (5/29)		
5p13		10.3 (3/29)		
20q12–13		10.3 (3/29)		
7p14–15		10.3 (3/29)		
11q13		10.3 (3/29)	<i>CCND1</i> , <i>BCL1</i> , <i>INT2</i>	
14q21		6.9 (2/29)		
20p11.2		6.9 (2/29)		
13q32		6.9 (2/29)		
1q32		3.4 (1/29)	<i>GAC1</i>	
7p22		3.4 (1/29)		
9p23–24		3.4 (1/29)		
9q34		3.4 (1/29)	<i>ABL</i>	
17p11.2	3.4 (1/29)			
20p12–13	3.4 (1/29)			
20q11.2	3.4 (1/29)			
Losses	18q22–23	65.5 (19/29)		
	18q12	51.7 (15/29)	<i>DCC</i> , <i>DPC4</i>	
	Xp22.2–22.3	44.8 (13/29)		
	3p	44.8 (13/29)		
	8p22–23	37.9 (11/29)		
	11q23–25	34.5 (10/29)		
	4p15–16	27.6 (8/29)		
4q33–34	24.1 (7/29)			

ered to reflect losses of DNA (under-represented), whereas regions where the mean ratio exceeded 1.2 were considered gained (over-represented) in the tumor genome. The overrepresentations were considered to be high-level gains (HLGs) when the fluorescence ratio exceeded 1.5.<sup>18,19)</sup> Heterochromatic regions near the centromeres and the entire Y chromosome were excluded from the analysis.

**Fluorescence *in situ* hybridization (FISH)** Since we detected novel HLGs involved in the 1q32 region, we carried out FISH using yeast artificial chromosomes (YACs) and bacterial artificial chromosome (BAC) clones. Twenty-two YACs (804G1, 753H7, 877B3, 823E5, 812D11, 950C7, 810G4, 622F2, 914G4, 952G5, 851D9, 958E1, 750E4, 956E4, 945F7, 873A8, 762A6, 890H10, 956E6, 960E7, 905H5, 765H10), archived by the Whitehead Institute Genome Center (<http://www.genome.wi.mit.edu/>) and BAC (4p17) containing the known *GAC1* gene were used as probes. Each of the human genomic DNAs cloned by YAC was amplified by *Alu*-PCR as previously reported.<sup>17)</sup> All DNA probes were labeled with biotin (BIO)-16-dUTP by nick translation (Boehringer Mannheim, Tokyo) denatured and hybridized to the denatured tumor chromosome slides. Fluorescence signals specific for BIO-labeled probes were detected with fluorescein isothiocyanate (FITC)-avidin (Boehringer Mannheim). We examined the number of FISH signals specific to each probe in ten to fifteen nuclei of each cell line. Hybridizations to normal lymphocyte nuclei were performed as controls to ascertain that the probes recognize a single-copy target.

**Southern and northern blot hybridization** Since our CGH analysis showed distinct HLGs at 1q32, we performed Southern and northern blot analyses to investigate the amplification and overexpression status of candidate genes mapped in this region. cDNA of 0.6 kb *ATF3*, kindly provided by Prof. Sigetaka Kitajima, and cDNA of 3.3 kb, *CENPF* (clone ID: 1739010), purchased from Genome Systems (St. Louis, MO), were used as probes. In Southern blot analysis, 5 µg of genomic DNA from each cell line was digested with *EcoRI*, electrophoresed in a 0.8% agarose gel, and then transferred to nylon membrane (BIODYNE B, PALL, Tokyo). On the other hand, in northern blot analysis, 10 µg of total RNA extracted from each cell line was electrophoresed in 1.0% agarose/0.67 M formaldehyde gel, and then transferred to a positively charged nylon membrane (Hybond N<sup>+</sup>, Amersham Pharmacia Biotech, Tokyo). Probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using a random priming method (Megaprime, Amersham Pharmacia Biotech) and hybridized to the pre-hybridized membranes. All membranes were washed in the final solution of 0.1× SSC/0.1% sodium dodecyl sulfate (SDS). Autoradiograms were developed after 48–84 h exposure at –70°C.

**RESULTS**

**Overview of genomic changes in ESC cell lines** An overview of the genetic changes in 29 ESC cell lines is shown in Fig. 1. All of the lines (100%) showed copy number aberrations, and gains predominated over losses in a ratio of 2:1. The average number of aberrations per line

was 12.5 (range, 6–17); the average numbers of gains and losses were 11.3 (range, 6–15) and 5.6 (range, 0–13), respectively. No specific relationship between copy number alterations and the staging of the original tumors was identified in this study (data not shown).

**Common regions involved in DNA copy number aberrations** As shown in Table I, the minimal common regions of losses that occurred most frequently were at 18q22–23 (19/29; 65.5%), 18q12 (15/29; 51.7%), Xp22.2–22.3 (13/29; 44.8%), 8p22–23 (11/29; 37.9%), 11q23–25 (10/29; 34.5%), 3p14 (9/29; 31%), 4p15–16 (8/29; 27.6%), 3p22–23 (7/29; 24.1%), 3p24–26 (7/29; 24.1%) and 4q33–34 (7/29; 24.1%).

The minimal regions involving copy number gains were identified at 8q21–24 (25/29; 86.2%), 3q26 (24/29; 82.8%), 5p14–15 (20/29; 69%), 20q12–13 (19/29; 65.5%), 7p14–15 (18/29; 62.1%), 1q32 (14/29; 48.3%), 11q13 (13/29; 44.8%) and 9q34 (11/29; 37.9%). The smallest regions of HLGs were seen at 3q26 (9 cases), 8q23 (6 cases), 5p14–15 (6 cases), 18p11.2–11.3 (6 cases), 3q27–28 (5 cases), 5p13 (3 cases), 20q12–13 (3 cases), 7p14–15 (3 cases), 11q13 (3 cases), 14q21 (2 cases), 20p11.2 (2 cases) and 13q32 (2 cases). Additional HLGs were detected in one case each at 1q32, 7p22,

9p23–24, 9q34, 17p11.2, 20p12–13 and 20q11.2. The minimal overlapping regions and the putative amplified genes are summarized in Table I.

**Definition of smallest region of the 1q32 amplicon by FISH** In order to determine the smallest common region amplified among cell lines that exhibited gains involving 1q32 in CGH analysis, FISH analysis was performed using those fourteen cell lines with 22 YACs mapped within 1q32 as probes. As shown in Fig. 2, one marker chromosome exhibited a homogeneous staining region (HSR) signal for YAC from D1S414 (YAC 662F2) to D1S2860 (YAC 762A6) in KYSE150. Five to six FISH signals were found in six cell lines (KYSE510, 770, 1250, 1440, 2270 and 2400), while three to four FISH signals were found in the other cell lines (KYSE170, 190, 590, 850, 890, 960, and 1170) at the same region.

To determine whether the *GAC1* gene, which has been identified from the amplified region at 1q32 in a case of glioblastoma,<sup>20</sup> is involved in the present 1q32 amplicon, we also carried out FISH analysis using *GAC1* gene-specific BAC (4p17) as a probe. We found that this gene was mapped proximal to this amplicon of KYSE150 (Fig. 2), and no increase in its probe signal was observed.

**Southern and northern blot analyses** Since significant

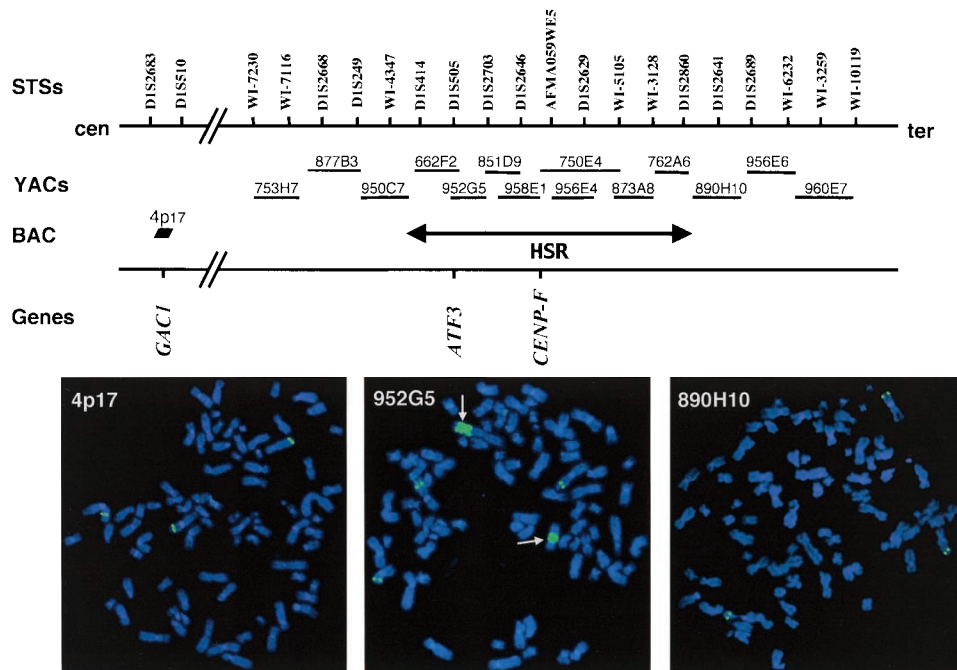


Fig. 2. Amplicon map of 1q32 in ESC cell lines between WI-7230 and WI-10119. The upper scale shows the ordering of STS markers mapped on this region (<http://www.ncbi.nlm.nih.gov/genemap99/> and <http://www.genome.wi.mit.edu/>). BAC and YACs anchored to the markers are represented as rectangles and bars, respectively. Based on FISH analysis using BAC and YAC clones, a homogeneous staining region (HSR, closed arrow) is indicated in relation to mapped genetic markers between D1S414 (766F2) and (762A6). Typical FISH images show HSRs (arrows) with 952G5 YACs clones, while BAC 4p17 which contains *GAC1* gene and other YACs localized out of the amplified region did not show any amplification.

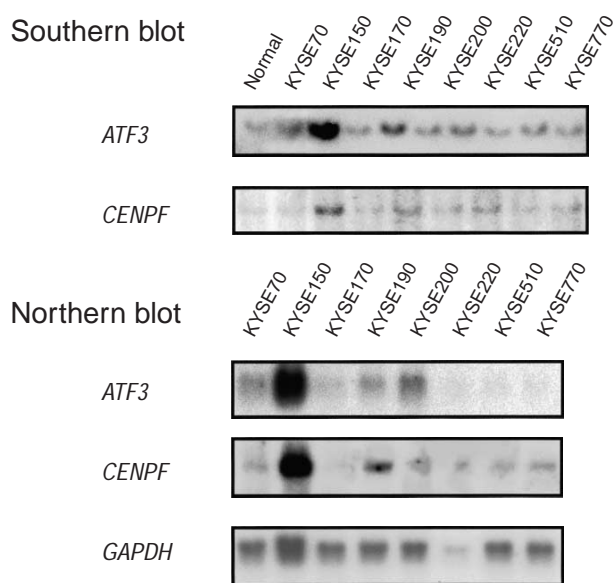


Fig. 3. Southern and northern blot analyses of the *ATF3* and *CENPF* genes in nine ESC cell lines. KYSE70 and 200 cell lines showed normal copy number in CGH analysis, whereas the other 6 cell lines showed a gain pattern; KYSE150 cell line demonstrated HSR at this region by FISH analysis (see Fig. 2). Note that overexpression of *ATF3* and *CENPF* is clearly observed in KYSE150 cell line.

amplification was identified at 1q32 in one line (KYSE150) and *GAC1*, a known target gene within the 1q32 amplicon in glioma,<sup>20</sup> was not involved in our amplicon, this amplicon might harbor other tumor-associated gene(s). To ascertain whether other candidate genes mapped in this region, such as *ATF3* and *CENPF*, are involved in this amplicon, we performed Southern and northern blot analyses with fourteen cell lines, which show copy number gain at 1q32 in CGH analysis, and 2 cell lines (KYSE70, 200), which show normal copy number, as controls. Amplification and overexpression of *ATF3* and *CENPF* were clearly observed in one line; KYSE150. Representative Southern and northern analyses are shown in Fig. 3.

## DISCUSSION

In our CGH analysis of 29 ESC cell lines, frequent losses were observed on 18q, 3p, 4q, 4p and 11q. Common gains were detected at 3q, 5p, 7p, 8q, 9q, 11q13, 14q, 18p, 20q and Xq. These results are concordant with recent CGH analyses in both primary tumors and cell lines of ESC,<sup>18, 21</sup> except for more frequent losses involving 8p and gains involving 18p and 1q in the present study. Therefore these regions might contain important gene(s) associated

with tumorigenesis in ESC. Loss of the short arm of chromosome 8 is the third most common loss among solid tumors.<sup>8</sup> In particular, 8p21.3–22 contains *DLC1* (dynein light-chain gene 1) and *PRLTS* (platelet-derived growth factor (PDGF)-receptor- $\beta$ -like tumor suppressor). Recently, *DLC1* was found to be frequently deleted in liver cancer<sup>22</sup> and *PRLTS* is well known to be altered in liver, colorectal, and non-small cell lung cancer.<sup>23</sup>

The long arm of chromosome 18, showing the highest incidence of losses (65.5%) in our study, is frequently lost in several types of malignancy. Several candidate tumor suppressor genes such as *DCC*, *SMAD2*, and *SMAD4*<sup>24</sup> have been isolated and mapped to this chromosome arm. Nevertheless, infrequent mutations of *SMAD4* have been reported in ESC,<sup>25</sup> and allelic losses of *DCC* do not appear to be common in ESC.<sup>26</sup>

Gains of 8q24, the most frequent gains in solid tumors,<sup>8</sup> were also the most frequent aberration in the present study. This was expected because the 8q24 region contains *MYC*, which is well known to be amplified in various types of tumor including ESC.<sup>18</sup> Copy number gains at 3q, especially 3q24–28, were the second most frequently detected in this study with the highest frequency of HLGs at 3q24–26 (9/29; 31%). The amplification at 3q has been detected in ESC<sup>18</sup> and other types of tumor such as ovarian cancer,<sup>27</sup> and squamous cell carcinoma (SCC) of the lung,<sup>28</sup> and head and neck including oral cavity.<sup>29</sup> The possible candidate genes involved in tumor development include the genes for ribosomal protein L22 (*RPL22*), butyrylcholinesterase (*BCHE*), glucose transporter 2 (*SLC2A2*), transferrin receptor (*TFRC*), thrombopoietin (*THPO*) and the phosphatidylinositol-3 kinase catalytic  $\alpha$  polypeptide (*PIK3CA*). The combined amplification and expression of these genes in SCC in several organs raise the possibility that several amplified and functionally important genes at 3q26 may be involved in the pathogenesis of SCC, especially lung cancer.<sup>28</sup>

Our CGH results for 29 ESC cell lines also showed frequent gains and HLGs at 18p (11/29; 37.9% and 6/29; 20.7%, respectively). HLGs at this chromosome site were frequently observed in several types of malignancy, such as malignant fibrous histiocytoma,<sup>30</sup> ovarian cancer,<sup>27</sup> and non small-cell lung cancer.<sup>31</sup> Together with previous reports, our results strongly suggest that there might be important gene(s) responsible for tumor progression regardless of the specific type of tumors.

Copy number gain at 1q32, firstly identified in ESC in this study, is the second most common gain in solid tumors.<sup>8</sup> Recently, 1q32 gains have been reported to be amplified in various types of tumors, including lung cancer,<sup>31–33</sup> ovarian cancer,<sup>34</sup> brain tumor,<sup>20, 35</sup> breast cancer,<sup>36, 37</sup> and osteosarcoma.<sup>38, 39</sup> Based on previous reports and the results of the present study, candidate genes located in this amplified region are *GAC1*, *ATF3*



and *CENPF*. *GAC1*, which encodes a protein belonging to the leucine-rich repeat (LRR) superfamily, is reported to be amplified in malignant glioma.<sup>20)</sup> According to our FISH analysis, however, we found that *GAC1* is not involved in 1q32 amplicon found in ESC cell lines, suggesting that the amplicon harbors another tumor-associated gene, whose overexpression may play an important role in the progression of ESC.

On the other hand, our Southern and northern blot analyses clearly showed the amplification and overexpression of *ATF3* and *CENPF* in a cell line, KYSE150, that exhibited high-level gain at 1q32. Therefore, these genes may play an important role in tumor development and/or progression in ESC. Interestingly, TI-241, a mouse *ATF3* homologue, was recently reported to regulate the expression of various genes as a transcription factor in the complex process of metastasis in B16 murine melanoma subline.<sup>40)</sup> *CENPF*, a newly characterized cell cycle-associated nuclear antigen that is expressed in small amounts in G0/G1 cells and accumulates in the nuclear matrix during S phase with maximal expression in G2/M cells, was investigated as a marker for cell proliferation in several

human malignancies.<sup>41)</sup> Those data support the idea that these genes may be preferentially up-regulated by an amplification mechanism among the numerous genes localized on 1q32. However, some other known genes or unknown transcripts, which were not tested in this study but are located within this amplicon, may also be activated via amplification. To determine the significance of the amplification at 1q32 in the progression of ESC and to identify the real target gene(s) in this amplicon, preparation of a more detailed amplicon map and analyses of transcripts within this region are warranted.

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