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# Gene amplification profiling of esophageal squamous cell carcinomas by DNA array CGH

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**Abstract**

Gene amplification is one of the basic mechanisms that lead to overexpression of oncogenes. DNA array comparative genomic hybridization (CGH) has great potential for comprehensive analysis of both a relative gene-copy number and altered chromosomal regions in cancers, which enables us to identify new amplified genes and unstable chromosomal loci. We examined the amplification status in 32 esophageal squamous cell carcinomas (ESCCs) and 13 ESCC cell lines on 51 frequently amplified loci in a variety of cancers by both DNA array CGH and Southern blot analyses. The 1p34 locus containing *MYCL1*, 2p24 (*MYCN*), 7p12 (*EGFR*), and 12q14 (*MDM2*) were amplified in one of the 32 cases (3%), and the 17q12 locus (*ERBB2*) and 8p11 (*FGFR1*) in two of the 32 cases (6%), while only the 11q13 locus (*Cyclin D1*, *FGF4*, and *EMS1*) was frequently amplified (28%, 9/32), demonstrating this locus to be a major target in ESCCs. One locus, 8q24 (c-*MYC*) was found to be amplified only in the cell lines. Eight out of 51 loci (15.7%) were found to be amplified in at least one of the 32 primary ESCCs or the 13 ESCC cell lines, suggesting that chromosomal loci frequently amplified in a type of human cancer may also be amplified in other types of cancers. This paper is the first report of an application of DNA array CGH to ESCCs. 

*Keywords:* Amplification; Esophageal cancer; Array CGH; *MYCL1*; *MYCN*; *EGFR*; *FGFR1*; *MDM2*; *ERBB2*; *Cyclin D1*

Gene amplification has been demonstrated in different types of human cancers, and can be considered to be one of the typical end results of genomic instability in cancer. Study of drug-selected intrachromosomal gene amplification reveals that site-specific breaks triggered by fragile site inducers account for the early features of intrachromosomal amplification [1] and that hypoxia is a potent fragile site inducer which facilitates the fusion of double minute chromosomes and their targeted reintegration into chromosomal fragile sites, generating homogeneous staining regions [2]. Thus, it is possible that the limited genomic DNA region, in which a double-strand break may frequently be induced for gene amplification and translocation can be shared or that translocated regions are unstable, inducing gene amplification. DNA array CGH has great potential for comprehensive analysis of a relative gene-copy number in cancers [3,4], which enables us to identify new amplified genes and unstable chromosomal loci. In East Asian countries including Japan and China, and in some parts of Europe, esophageal carcinoma consists mainly of squamous cell carcinomas located mostly in the thoracic esophagus, while adenocarcinoma in the distal part of the esophagus has increasingly become the major pathological type found in Europe and North America. Thus, to identify unstable chromosomal regions, esophageal carcinoma is a good target for genetic analysis compared with carcinomas classified into multiple pathological types such as a gastric carcinoma.

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## Materials and methods

*Genomic DNA preparation from tumor tissues and cell lines*. A total of 32 primary esophageal carcinoma tissues and their adjacent normal tissues were obtained at the National Cancer Center Hospital, Tokyo. All of the specimens were frozen immediately in liquid nitrogen and stored at −80 °C until use. Thirteen esophageal cancer cell lines, TE1–TE13 were maintained in RPMI1640 supplemented with 10% fetal calf serum, 0.15% sodium bicarbonate, 2 mM L-glutamine, and penicillin–streptomycin. Genomic DNA was prepared from culture cells and tissues using a standard method [5].

*DNA array CGH*. Genome copy number was assessed using a commercial array (AmpliOnc 1 Array, Vysis, IL) according to manufacturer's protocol. The array contains 51 gene loci composed of 59 BAC clones which have been reported to be amplified in various human cancers (list available from the manufacturer's web site, http://www.vysis.com/). Briefly, DNA samples isolated from normal human lymphocytes (reference DNA) and tumor samples (test DNA) were labeled by nick translation with fluorophore-labeled dUTPs. The DNA probes (0.5 μg) were mixed with 20 μg of unlabeled Cot-1 DNA and were hybridized to the genomic array, which was then counterstained with DAPI and analyzed by the fluorescent image capturing system, GenoSensor (Vysis, IL).

*Southern blot analysis*. The extracted DNA was digested with *Eco*RI, and 1 μg of the digested DNA was fractionated on 1% agarose gel and transferred to Hybond-$N^+$ (Amersham). Hybridization was carried out in 50% formamide, 5× standard saline citrate (SSC) (1× SSC: 150 mM NaCl, 15 mM sodium citrate), 5× Denhardt's reagent, 5 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA at 42 °C for 14–16 h. The cDNA fragments for the probes were prepared by RT-PCR. All cDNA probes were labeled with [α-$^{32}$P]dCTP. The filter was washed twice in 0.1× SSC and 0.1% SDS at room temperature and twice at 65 °C, and exposed to Kodak XAR film at −70 °C. The hybridization intensity in each sample was quantified by Fujix BAS2000 bioimaging analyzer (Fuji Photo Film, Tokyo, Japan). The criteria for gene amplification used in this study was that the intensity of the hybridization signal was elevated more than 3-fold compared with a normal one.

## Results and discussion

Presently, 256 genes are recognized as oncogenes in Locus Link, a public database. However, only a limited

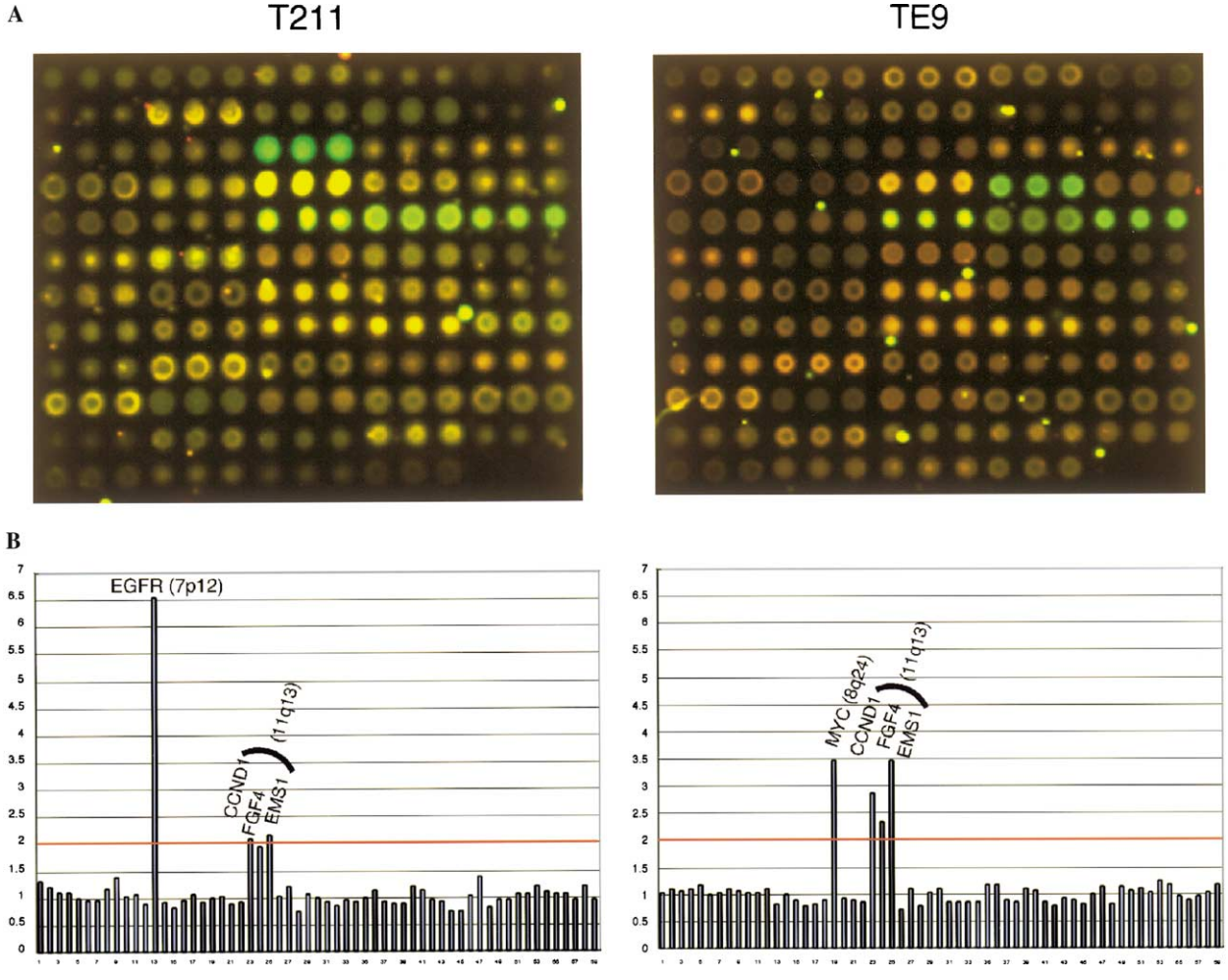


Fig. 1. Representative results of DNA array CGH on primary ESCCs and ESCC cell lines. (A) Analysis of DNA copy-number variation in ESCCs (green) compared with normal male genomic DNA (red). Left, a primary tumor T211. Right, TE9 cell line. (B) Fluorescence ratios on the 51 loci composed of 59 BAC clones. In T211, the 7p12 locus containing *EGFR* and the 11q13 locus containing *CCND1*, *FGF4*, and *EMS1* were found to be amplified, and the 8q24 containing *MYC* and the 11q13 were found to be amplified in TE9.

number of genes have been found to be activated by genetic alterations including amplification and mutation. To examine the amplification status in esophageal squamous cell carcinomas (ESCCs) on the 51 amplified loci reported previously in each type of cancer (see Materials and methods), we analyzed 32 primary ESCCs and 13 ESCC cell lines by DNA array CGH. Representative results of the array CGH on the primary ESCC and the ESCC cell line are shown in Fig. 1. The criteria for gene amplification by DNA array CGH was that the intensity of the hybridization signal was elevated more than 2-fold compared with the normal one. By DNA array CGH analysis, the 1p34 locus containing *MYCL1*, 2p24 (*MYCN*), 3q26 (*TERC* and *PIK3CA*), 7p12 (*EGFR*), 8p11 (*FGFR1*), 11q13 (*Cyclin D1*, *FGF4*, and *EMS1*), 12q14 (*MDM2*), and 17q12 (*ERBB2*) were found to be the candidates amplified in the primary ESCCs.

Next, we checked the gene amplification in these eight loci with a corresponding case by Southern blot analysis. All the data are shown in Fig. 2. By Southern blot analysis, seven out of the eight loci were confirmed to be amplified in at least one of the 32 primary ESCCs, while only one locus, 3q26 (*TERC* and *PIK3CA*) was unable to demonstrate high copy-number amplification. The ratio of the copy number change on this locus identified by DNA array CGH was lower than that on the other seven loci, a fact that may be explained not by gene amplification but by chromosomal aneuploidy.

Therefore, we concluded that seven out of 51 loci (13.7%) were amplified in at least one of the 32 primary ESCCs. The 1p34 locus containing *MYCL1*, 2p24 (*MYCN*), 7p12 (*EGFR*), and 12q14 (*MDM2*) were amplified in one of the 32 cases (3%), and the 17q12 locus (*ERBB2*) and 8p11 (*FGFR1*) in two of the 32 cases (6%), while only the 11q13 locus (*Cyclin D1*, *FGF4*, and *EMS1*) was frequently amplified (28%, 9/32). One locus, 8q24 (c-*MYC*) was also found to be amplified only in the ESCC cell lines but not in the primary ESCCs by Southern blot analysis (data not shown).

Two oncogenes, *EGFR* and *Cyclin D1*, have already been identified as amplification targets on the primary ESCCs [6–9], correponding to this study, while six other oncogenes, *MYCL1*, *MYCN*, *EGFR*, *FGFR1*, *MDM2*, and *ERBB2* have not been reported to be amplified. Recently, in four of the 42 primary ESCCs (9.5%), the 11q22 locus containing *cIAP1* and *MMP1*, *7*, *8*, and *10* genes has been reported to be amplified [10]. Taken together, the 11q13 locus containing *Cyclin D1*, *FGF4*, and *EMS1* genes was still a most frequently amplified locus, demonstrating this locus to be a major target in ESCC development or progression.

Fluorescence ratios between cancers and normal tissues are inevitably under-estimated by saturation of signals from the amplified locus in the scanning process compared with those estimated by Southern blot

Table 1

| Name | Ratio | *FGR* | *MYCL1* | *NRAS* | *LAMC2* | *MYCN* | *RAF1* | *TERC* | *PIK3CA* | *EGFR* | *PGY1* | *FGFR1* | *MOS* | *MYC* | *CCND1* | *FGF4/FGF3* | *EMSI* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | | 1p36 | 1p34 | 1p13 | 1q25 | 2p24 | 3p25 | 3q26 | 3q26 | 7p12 | 7q21 | 8p11 | 8q11 | 8q24 | 11q13 | 11q13 | 11q13 |
| Primary ($N = 32$) | >2 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 2 | 0 | 0 | 9 | 7 | 9 |
| | 1.5–2 | 1 | 1 | 2 | 1 | 0 | 0 | 2 | 1 | 1 | 1 | 2 | 0 | 0 | 0 | 2 | 0 |
| Cell line ($N = 12$) | >2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 3 | 5 | 5 | 5 |
| | 1.5–2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |

| Name | Ratio | *GARP* | *PAK1* | *CCND2* | *KRAS2* | *MDM2* | *ERBB2* | *RPS6KB1* | *JUNB* | *CCNE1* | *AIB1* | *STK15* | *MYBL2* | *PTPN1* | *ZNF217* | *BCR* | *AR3'* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | | 11q13 | 11q13 | 12p13 | 12p12 | 12q14 | 17q12 | 17q23 | 19p13 | 19p13 | 20q12 | 20q13 | 20q13 | 20q13 | 20q13 | 22q 11 | Xq11 |
| Primary ($N = 32$) | >2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1.5–2 | 1 | 5 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Cell line ($N = 12$) | >2 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1.5–2 | 0 | 7 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 5 | 3 | 2 | 6 | 5 | 1 | 0 |

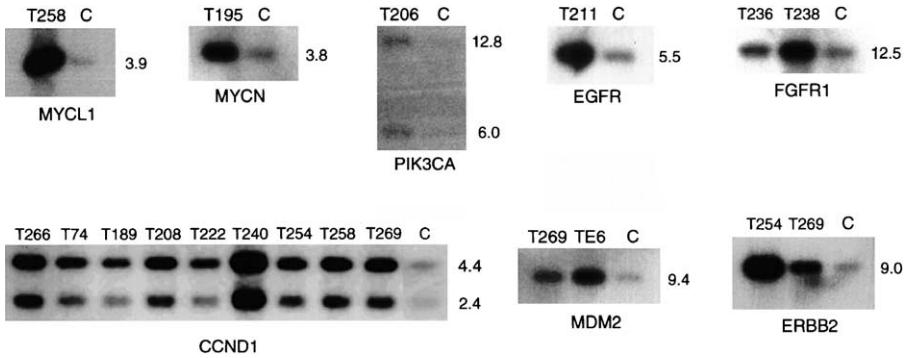


Fig. 2. Southern blot analysis of DNA from primary ESCCs. *Eco*RI digested DNA was hybridized with *MYCL1*, *MYCN*, *PIK3CA*, *EGFR*, *FGFR1*, *CCND1*, *MDM2*, and *ERBB2* probes. Probes (lower), and sample number (upper). The approximate sizes in kilobase of the represented bands are shown at the right side. As a control for the equal loading, transfer, and hybridization of the DNA samples, the filters were stripped and re-hybridized with a probe for *HOX2I*, which is not amplified in all the ESCCs examined. The intensities of the *HOX2I* hybridizing fragments were used as references for normalization of the results. The criterion for gene amplification used in this study was that the intensity of the hybridization signal was elevated more than 3-fold compared with the normal control, C.

analysis, which is a more faithful method. Therefore, some chromosomal loci, whose fluorescence ratios between a cancer tissue and a normal tissue were elevated 1.5–2-fold, are possibly identified as an amplified locus, or at least as a candidate. The candidates of amplified loci and genes identified by the DNA array CGH in both the primary ESCCs and the ESCC cell lines are summarized in Table 1. This gene list could be helpful for identifying new amplified genes other than genes confirmed in this study.

A double-strand break in the limited genomic DNA regions such as chromosomal fragile sites has been reported to trigger gene amplification. Therefore, chromosomal loci frequently amplified in a type of human cancer may also be amplified in other types of cancers, which was supported by our data that at least eight (15.7%) out of the 51 loci examined were amplified in at least one of the 32 primary ESCCs or the 13 cell lines.

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