

A Novel Amplicon at 9p23–24 in Squamous Cell Carcinoma of the Esophagus That Lies Proximal to *GASCI* and Harbors *NFIB*

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The non-random amplification of DNA at 9p23–24 observed in various types of human cancers, including esophageal squamous cell carcinomas (ESCs), may reflect the locations of important tumor-associated genes. Our previous studies using ESC cell lines defined an amplicon in this region and identified a novel gene, *GASCI*, as a target of the amplification. Since different regions within the same chromosome arm are often involved in amplification in a syntenic or non-syntenic manner, we characterized the amplicon at 9p23–24 in 35 ESC cell lines (29 KYSE series and 6 YES series), and examined possible involvement of non-syntenic amplifications at 9p23–24 in 32 primary ESCs. Our results clearly indicated that two target regions for DNA amplification exist at 9p23–24; the major amplicon contains *GASCI*, and the minor one harbors a transcription factor, *NFIB*, centromeric to the *GASCI* locus.

Key words: Esophageal squamous cell carcinoma — Amplification — 9p23–24 — *NFIB* — *GASCI*

Amplifications of chromosomal DNA, with consequent up-regulation of specific genes within the amplicons, play an important role in tumorigenesis of human neoplasia.¹⁾ Thus, investigating regions of amplification and exploring genes within them can provide valuable information for a better understanding of carcinogenesis. Using comparative genomic hybridization (CGH), a molecular cytogenetic technique, investigators are now able to screen DNA samples for aberrations in copy number that include amplification at the level of whole chromosomes.²⁾ Cumulative data obtained by CGH in various types of tumor have revealed novel amplifications in specific regions of certain chromosomes.³⁾ Subsequent efforts to explore these amplicons for target genes have identified several cancer-associated genes, including *PIK3CA* and *hTR* amplified at 3q26 in non-small cell lung carcinomas⁴⁾; *MASLI* at 8p23.1 in malignant fibrous histiocytomas⁵⁾; *PS6K* at 17q23 in breast cancers⁶⁾; and *AIB1*,⁷⁾ *BTAK*,⁸⁾ *DcR3*,⁹⁾ and *ZNF217*¹⁰⁾ at 20q in tumors of the breast, ovary or colon. Furthermore, detailed analyses of genomic structures and sequences of such amplified regions have revealed that amplicons frequently involve non-syntenic as well as syntenic DNA from the same chromosomal region and that they can harbor multiple genes likely to be associated with tumorigenesis.¹¹⁾

Amplification at 9p has been implicated in non-small cell lung cancers and in carcinomas of the liver, ovary,

uterine cervix, and breast.³⁾ Using CGH, we detected in earlier studies a region of amplification at 9p23–24 in cell lines derived from esophageal squamous cell carcinomas.¹²⁾ From that amplicon we isolated a novel gene, *GASCI* (gene amplified in squamous cell carcinoma 1) that could be associated with tumorigenesis.¹³⁾ However, the possibility that DNA amplifications non-syntenic to *GASCI* might also occur in tumors bearing high-level gains (HLGs) at 9p was not examined.

In the present study we performed molecular cytogenetic characterization of the amplicon at 9p23–24 in 35 esophageal squamous cell carcinoma (ESC) cell lines including the 29 (KYSE series) used in the previous study and six lines of the YES series; one of the latter had shown a HLG indicative of amplification at 9p by CGH analysis.¹⁴⁾ We also examined possible involvement of non-syntenic amplifications at 9p23–24 in primary ESCs.

MATERIALS AND METHODS

ESC cell lines and primary tumor samples The 35 ESC cell lines examined consisted of the 29 lines of the KYSE series¹⁵⁾ and lines 1–6 of the YES series.¹⁴⁾ Data from CGH analyses in all lines of these two series have been reported elsewhere.^{12,14)} Primary tumor samples were obtained from 32 patients at the time of surgery for ESC in the Kyoto University Hospital. Informed consent was obtained, in the formal style approved by the Ethics Committee of the Kyoto University Hospital, before the present study began.

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Fluorescence *in situ* hybridization (FISH) analysis using YAC probes Metaphase-chromosome slides were prepared and employed in FISH experiments in the manner described previously.^{16,17} The locations of YACs within the region of interest were compiled from information archived by the Whitehead Institute/MIT Genome Center (<http://www-genome.Wi.Mit.Edu/>) and by Resources for Human Molecular Cytogenetics (<http://bioserver.uniba.it/fish/rocchi/welcome.html>). YAC clones in the vicinity of 9p23–24 were then isolated from the Centre d'Etude du Polymorphisme Humain (CEPH) YAC library, and FISH probes for these YACs were generated by *Alu*-PCR, as described elsewhere.¹⁷ Probes were labeled by nick-translation with biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim, Tokyo). Chromo-

somal *in situ* suppression hybridization (CISS) and fluorescent detection of hybridization signals were carried out as previously described.¹⁷ After washing, slides were counter-stained with 4',6'-diamidino-2-phenylindole (DAPI) and mounted in anti-fade solution. Images were recorded with a cooled, charge-coupled device (KAF1400; Photometrics, Tucson, AZ) and processed using IPLab Spectrum software (Signal Analytics Corp., Vienna, VA). The copy number and molecular organization of the region of interest were assessed according to the hybridization patterns observed on both metaphase and interphase chromosomes.

Southern- and northern-blot analyses Eight IMAGE expressed-sequence tag (EST) clones in the 9p23–24 region, chosen from the Whitehead Institute for Genomic

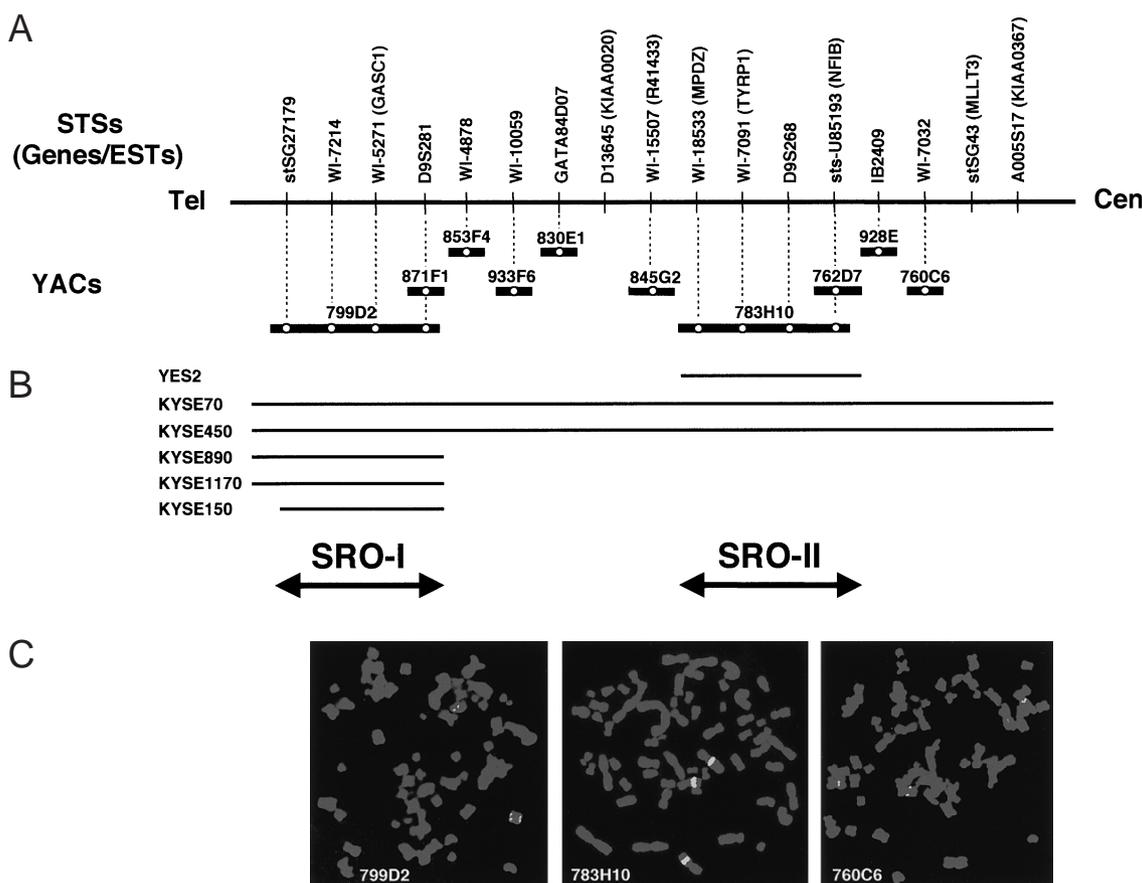


Fig. 1. (A) Map of the 9p23–24 amplicon surrounding the *NFIB* gene. The genes/ESTs above the chromosome are probes used for Southern blotting; the YACs used for FISH are represented below the chromosome by horizontal bars; circles within the bars denote the anchor points of markers on the respective YAC clones. Relative sizes of the YACs and the spacing of the markers are not drawn to scale. (B) The extent of two 9p23–24 amplicons in each of six ESC cell lines, as determined by Southern-blot analysis. The smallest region of overlap (SRO) was determined by FISH together with the Southern-blotting results. SRO-I contains *GASC1*¹³ and SRO-II contains *NFIB*. Details of SRO-I have been described elsewhere.¹³ (C) Typical result of FISH analysis in YES-2. Middle panel: the image shows HSRs on three marker chromosomes with YAC783H10. YAC799D2 (containing the *GASC1* gene; left panel) and YAC760C6 (right panel), both outside the amplified region, show no amplifications.

Research Database, were purchased from Incyte Genomics Inc. (St. Louis, MO) for use as probes for Southern and northern blotting. For Southern-blot analysis, 10- μ g aliquots of *Eco*RI-digested DNA extracted from each cell line or from normal lymphocytes were electrophoresed in 0.8% agarose gels and transferred to nylon membranes (BIODYNE B, Nihon Pall, Tokyo). For dot-blot analysis, 2 μ g of DNA from each primary tumor, each cell line or normal lymphocytes was denatured with 0.4 N NaOH, and then transferred to nylon membranes (BIODYNE B, Nihon Pall). For northern blotting, 20 μ g of total RNA extracted from each cell line was size-fractionated in 1.0% agarose/0.67 M formaldehyde gel, and transferred onto a positively charged nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Tokyo). Membranes were hybridized with [α^{32} P]dCTP-labeled cDNA probes prepared from EST clones under appropriate conditions, washed, and then exposed to Kodak X-OMAT film as described elsewhere.¹³⁾

RESULTS

Definition of the 9p23–24 amplicon in YES-2 by FISH

Among six cell lines of the YES series (YES1–6), YES-2 had shown HLG indicative of amplification at 9p in a previous CGH study.¹⁴⁾ Thus, we attempted to define the 9p amplicon in this cell line by FISH, using ten YACs as probes (Fig. 1A). FISH signals generated with YACs 783H10 and 762D7 exhibited strong signals as homogeneously staining regions (HSRs) on three different marker chromosomes (Fig. 1C, middle panel). On the other hand, the number of FISH signals from YACs on either side, 845G2 and 928E7, ranged from four to nine per nucleus. More distal YACs 830E1 and 799D2, the latter harboring *GASC1*, as well as more proximal 760C6, showed only two or three twin-spot signals.

Next, we performed FISH in five KYSE cell lines (KYSE70, 150, 450, 890 and 1170), that had shown copy-number gains on 9p in our previous studies.¹²⁾ The number of FISH signals achieved with YACs 845G2, 783H10, 762D7, 928E and 760C6, all mapping between WI-15507 and WI-7032, ranged from six to nine in KYSE70 and 450. These results were consistent with our previous FISH results,¹³⁾ and copy-number abnormalities in KYSE70 and 450 cell lines extended beyond the region amplified in YES-2. Therefore two distinct amplicons appeared to exist at 9p23–24 in ESCs; the distal amplicon harbors *GASC1* (Fig. 1B) and the proximal one lies within the region covered by YACs 783H10 and 762D7, which are located 10 cM away from *GASC1* according to GeneMap'99 (<http://www.ncbi.nlm.nih.gov/genemap/>).

Molecular definition of the 9p23–24 amplicon and analysis of transcripts Southern-blot analyses of the six YES-series cell lines with cDNA probes representing

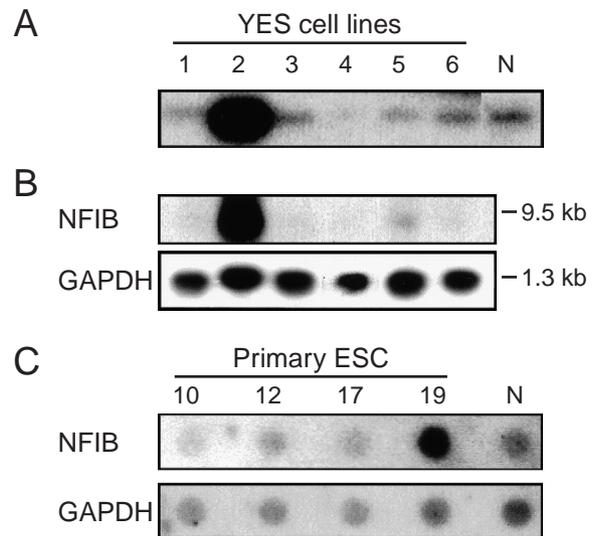


Fig. 2. Amplification of *NFIB* in ESC cell lines and primary tumors, and expression status in cell lines. (A) Representative Southern blot using *NFIB* cDNA as the probe. DNAs of six ESC cell lines (YES1–6) and peripheral blood lymphocytes of a healthy donor (N) were digested with *Eco*RI, electrophoresed, transferred onto nylon membranes, and hybridized with *NFIB* cDNA. *NFIB* was amplified more than 20-fold in YES-2 compared with control DNA. (B) A northern blot containing total RNAs from the six YES cell lines was hybridized with an [α^{32} P]dCTP-labeled *NFIB* cDNA clone and a control probe (*GAPDH*). mRNA is over-expressed in the cell line (YES-2) that had shown *NFIB* amplification. (C) Representative dot blot using *NFIB* cDNA and a control probe (*GAPDH*) in primary ESC samples. Only tumor No. 19 showed an amplified signal for *NFIB*.

MPDZ (multiple PDZ domain protein), *TYRP1* (tyrosinase-related protein 1) and *NFIB* (nuclear factor I/B), all located on YAC783H10, showed strong amplification in the YES-2 cell line (Fig. 2A). By contrast, probes for other genes or transcripts from the region surrounding YAC783H10: *MLLT3* (myeloid/lymphoid or mixed-lineage leukemia trithorax *Drosophila* homolog; translocated to 3); *KIAA0020*; *KIAA0367*; and cDNA 30354 (R41433), revealed no amplification in YES-2. Based on comparison of the hybridization signals of amplified DNA versus normal DNA, a rough estimation revealed >20-fold amplifications of the first three probes (*MPDZ*, *TYRP1* and *NFIB*) in YES-2. However, of those three genes only *NFIB* showed over-expression (Fig. 2B). In 29 KYSE cell lines, on the other hand, *NFIB* was not over-expressed in KYSE70 and 450, even though an increase in copy-number of this gene was observed in these two cell lines (data not shown).

Since *NFIB* seems to be one of the target genes in the proximal amplicon at 9p23–24, we examined 32 primary

ESC tumors to determine whether *NFIB* amplification had occurred in any of them. By dot-blot analysis we detected amplification of *NFIB* in one tumor, but *GASC1* did not appear to be amplified in the same neoplasm (Fig. 2C).

DISCUSSION

Amplification of 9p, particularly the portion distal to the *CDKN2A* gene at 9p21, has been reported in breast cancers, ESCs, carcinomas of lung or ovary, high-grade astrocytomas, and glioblastomas.^{18–22} A CGH analysis of primary human ovarian carcinomas revealed that 9p21-ter was one of the most common regions of copy-number increases; one case in every nine showed specific 9p24 amplification; furthermore, gains at 9p21-ter tended to be more common in advanced-stage tumors.²⁰ In a previous study we detected frequent amplification of DNA copy-number at chromosome 9p23–24 in ESC cell lines, and successfully cloned a novel gene, *GASC1*, that was amplified and over-expressed in several ESC cell lines of the KYSE series.¹³

Cumulative CGH data have revealed that amplification in tumor cells is complex; i.e., several different regions along the same chromosome arm (e.g. 8q, 17q and 20q) are sometimes involved in amplifications, leading to co-activation of multiple genes.^{11, 23, 24} In fact, studies in breast cancer have revealed syntenic or non-syntenic amplifications involving at least three regions of 20q (q11, q12, and q13.2), and several candidate genes including *AIB1*, *AIB3*, *AIB4*, *ZNF217*, *DcR3*, *CAS* and *BTAK* have been defined in those regions.^{8–11, 25}

Complex genetic alterations have also been described in chromosome 9p. In G5, a human cell line derived from a high-grade astrocytoma, two distinct genetic loci involved in amplifications at 9p have been revealed by CGH and FISH analyses.¹⁹ In addition, using a large set of microsatellite loci, An *et al.* identified two distinct regions of allelic imbalances at 9p23–24 in 80 primary breast can-

cers.²⁶ The more distal of those regions, containing D9S281 and D9S286 (an approximately 4-cM interval), overlaps with the amplicon involving *GASC1* that we reported previously in ESC cell lines¹³; the proximal amplicon harboring D9S1808 and D9S268 overlaps with the novel amplicon involving *NFIB* reported here. These findings suggest that 9p may indeed have at least two target regions of amplification. No structural or functional background has been put forward so far to account for amplification in specific regions of certain chromosomes.

By screening several positional-candidate genes around the novel 9p23–24 amplicon proximal to *GASC1*, we identified *NFIB* as a likely target. *NFIB* is a member of the human nuclear factor I (*NFI*) gene family, whose products were initially identified as host-encoded proteins required for the efficient initiation of adenovirus replication *in vitro*,²⁷ and were subsequently shown to be necessary for expression of many cellular genes.^{28–31} Genetic alterations of *NFIB* have been observed in some tumors; for example, Geurts *et al.* identified *NFIB* as a recurrent translocation partner of *HMGIC* in two pleomorphic adenomas.³² Moreover, over-expression of avian NFI proteins induces a morphological change in chick embryo fibroblasts, increasing cell adherence and flattening of the cell monolayer.³³ Those findings, along with ours, suggest that amplification and subsequent over-expression of *NFIB* might be associated with the development and progression of ESC, although the relevance of this gene to esophageal carcinogenesis remains to be determined.

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