

# Identification of a Novel Gene, *GASC1*, within an Amplicon at 9p23–24 Frequently Detected in Esophageal Cancer Cell Lines<sup>1</sup>

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## Abstract

In a recent study, we identified frequent amplification of DNA copy number at chromosome 9p23–24 in cell lines derived from esophageal squamous cell carcinomas (ESCs), using comparative genomic hybridization. Because amplified regions often harbor oncogenes and/or other tumor-associated genes, and because 9p23–24 amplification had been reported in various other types of cancers, we used fluorescence *in situ* hybridization and Southern blot analysis to map the 9p23–24 amplicon. We then screened target genes/transcripts present within this amplicon by Northern blotting. With this strategy, we successfully cloned a novel gene, designated *gene amplified in squamous cell carcinoma 1 (GASC1)*, that was amplified and overexpressed in several ESC cell lines. The deduced amino acid sequence of *GASC1* contains two PHD-finger motifs and a PX domain. PHD-finger motifs are found in nuclear proteins that participate in chromatin-mediated transcriptional regulation and are present in a number of proto-oncogenes. Our findings suggest that overexpressed *GASC1* may play an important role in the development and/or progression of various types of cancer including ESC.

## Introduction

Amplification of genetic material is often observed in tumor cells; it represents one of the mechanisms leading to the activation of proto-oncogenes that can contribute to tumor progression (1). Identification and characterization of genes present in amplified regions can provide important insights into the pathogenesis of cancer.

Esophageal carcinoma is the sixth most frequent cause of cancer deaths throughout the world (2). Of the two major histopathological types of tumor arising in this tissue, ESC<sup>3</sup> and adenocarcinoma, ESC is the more frequent type in Japan as elsewhere (Annual Statistical Report of National Health Conditions, Japan Health and Welfare Statistics Association, 1999). Some of the genetic alterations associated with development, progression and/or metastasis of ESC have been identified, including amplifications of *MYC*, *EGFR*, and *CCND1* (3, 4). However, recent CGH studies have identified at least 10 additional regions of amplification in ESCs (5–7), although as yet no

ESC-associated genes have been identified at those newly detected chromosomal sites. Recently, we investigated 29 ESC cell lines for aberrations in DNA copy number and detected several novel amplified regions, among them 9p23–24 (8).

Amplification at 9p23–24 is of particular interest because genomic alterations in this region have been implicated in various malignancies including non-small cell lung cancers, carcinomas of liver, ovary, uterine cervix, and breast, as well as osteosarcomas and mediastinal thymic B-cell lymphomas (9). The combined evidence strongly suggests that the 9p23–24 region may harbor one or more genes that become oncogenic in an amplified state, regardless of the type of tissue involved.

To identify putative tumor-associated genes within the 9p23–24 amplicon, we performed molecular cytogenetic characterization of the region CGH had identified as amplified in several ESC cell lines. We identified a novel gene, designated *GASC1* (*gene amplified in squamous cell carcinoma 1*), that encodes a protein containing PHD and PX domains. Its amplification and consequent overexpression in some of the 29 ESC cell lines examined suggest that this gene might be involved in the progression of a proportion of primary esophageal cancers.

## Materials and Methods

**ESC Cell Lines and Preparation of Metaphase Slides.** The 29 ESC cell lines examined (KYSE series) had been established from surgically resected tumors (10). Copy number aberrations among all of these lines were reported elsewhere (8). Metaphase chromosome slides were prepared and used in FISH experiments in the manner described previously (6).

**FISH Analysis using YACs and a PAC as Probes.** The locations of YACs within the region of interest were compiled from information archived by the Whitehead Institute/MIT Genome Center<sup>4</sup> and by Resources for Human Molecular Cytogenetics.<sup>5</sup> YAC clones in the vicinity of 9p23–24 were then isolated from the Center d'Etude du Polymorphisme Humain YAC library, and FISH probes for these YACs were generated by *Alu*-PCR, as described elsewhere (6). A PAC clone containing a known gene, *JAK2*, mapped in 9p24, kindly given to us by Dr. Peter Marynen (University of Leuven, Belgium), was also used as a probe. Probes were labeled by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim, Tokyo, Japan). Chromosomal *in situ* suppression hybridization and fluorescent detection of hybridization signals were carried out as described previously (6). After washing, slides were counterstained with DAPI and mounted in antifade 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 EST clones in the 9p23–24 region, chosen from the Whitehead Institute for Genomic Research Database,

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<sup>3</sup> The abbreviations used are: ESC, esophageal squamous cell carcinoma; CGH, comparative genomic hybridization; *GASC1*, gene amplified in squamous cell carcinoma 1; YAC, yeast artificial chromosome; PAC, P1-artificial chromosome; FISH, fluorescence *in situ* hybridization; *JAK2*, Janus kinase 2; DAPI, 4',6'-diamidino-2-phenylindole; HSR, homogeneously staining region.

<sup>4</sup> Internet address: <http://www-genome.Wi.Mit.Edu/>.

<sup>5</sup> Internet address: <http://bioserver.uniba.it/fish/rocchi/welcome.html>.

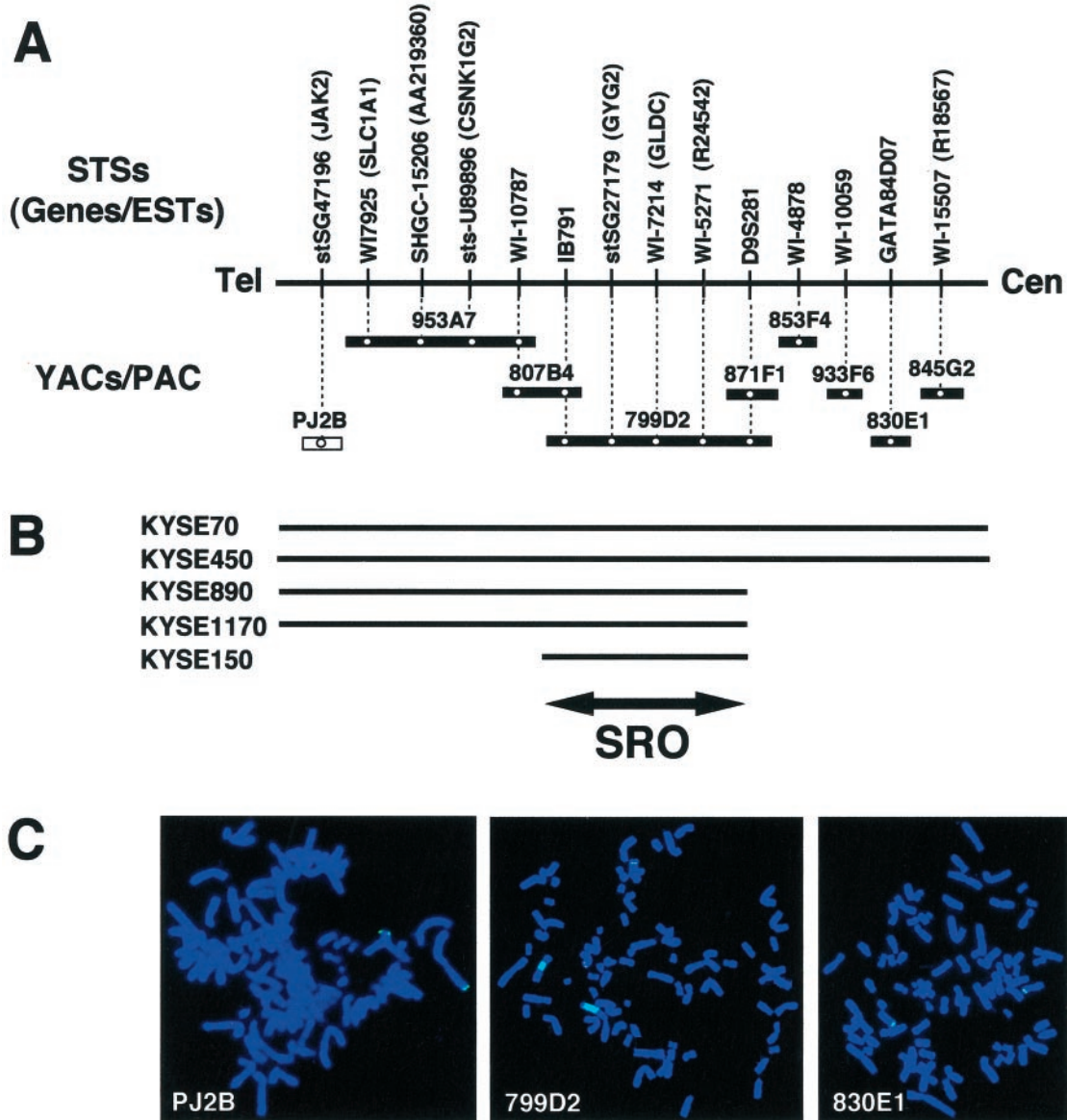


Fig. 1. A, map of the 9p23-24 amplicon surrounding the novel gene represented by IMAGE clone 131865 (R24542). The genes/expressed sequence tags above the chromosome are probes used for Southern blotting; the YACs and one PAC used for FISH are represented below the chromosome by horizontal black and open bars, respectively; circles within the bars denote the anchor points of markers on the respective YAC or PAC clones. Relative sizes of the YACs and PAC and the spacing of the markers are not drawn to scale. B, the extent of the 9p23 amplicon in each of five ESC cell lines, as determined by Southern blot analysis. The smallest region of overlap (SRO) was determined by FISH together with the Southern blot results. C, typical result of FISH analysis in KYSE-150. The images show HSRs on two marker chromosomes with YAC 799D2; PAC PJ2B (containing the *JAK2* gene) and YAC 830E1, both outside the amplified region, show no amplifications.

were purchased from Research Genetics (Huntsville, AL) and used as probes for Southern and Northern blotting. Tumor DNAs were extracted from cultured ESC cell lines by standard methods. For Southern blot analysis, 10- $\mu$ 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, Japan). For Northern blotting, 20  $\mu$ g of total RNA extracted from each cell line were electrophoresed in 1.0% agarose/0.67 M formaldehyde gel and transferred onto a positively charged nylon membrane (Hybond-N<sup>+</sup>; Amersham Pharmacia Biotech, Tokyo, Japan). Each membrane was hybridized with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled EST probes under appropriate conditions, washed, and then exposed to Kodak X-OMAT film as described elsewhere (6).

To evaluate expression patterns in different tissues, a multiple-tissue Northern blot (MTN-Human 12 lanes; Clontech, Palo Alto, CA) was hybridized with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled IMAGE cDNA clone 131865 (GenBank accession number: R24542).

**cDNA Library Screening and DNA Sequencing.** Two cDNA libraries were constructed from RNA of a gastric cancer cell line (HSC39), one by the

oligo-capping method (11) and the other with the ZAP-cDNA Gigapack III Gold cloning kit (Stratagene, La Jolla, CA). Each library was screened with IMAGE clone 131865 as the probe. Six overlapping cDNA clones were isolated; their DNA sequences were determined using a 377 ABI auto-sequencer (PE Biosystems, Foster City, CA).

To confirm the sequences of the isolated clones, we carried out reverse transcription-PCR analysis using cell line-derived RNAs as templates and two primer pairs generated on the basis of sequences determined from the clones isolated by screening with clone 131865. These primers were: W1f, 5'-CGGGTTAAGAGGGTCTCTG-3' and W1r, 5'-GGATGTCCCTTCA-TCTTCTC-3' (nucleotides 2592-3382 of the assembled cDNA); and W2f, 5'-AATACCTTGCATACATGGAGTC-3' and W2r, 5'-CTTCTCAAC-CACATCCAAGAC-3' (nucleotides 238-639 of the assembled cDNA).

**Results**

**Definition of the 9p23-24 Amplicon by FISH.** In our previous CGH analysis, a gain of copy number on 9p had been detected in 5 of

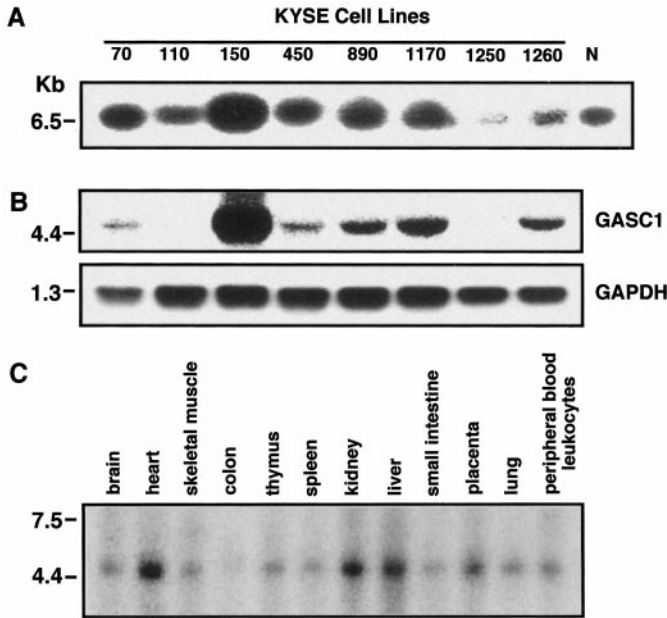


Fig. 2. Amplification and overexpression of GAS1 in ESC cell lines. A, representative Southern blot using IMAGE clone 131865 as the probe. Eight tumor DNAs and DNA from peripheral blood lymphocytes of a healthy donor (N) were digested with *EcoRI*, electrophoresed, transferred onto nylon membranes, and hybridized with this partial cDNA clone. B, Northern blot containing total RNAs from various ESC cell lines was hybridized with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled IMAGE clone 131865 and a control probe (*GAPDH*, glyceraldehyde-3-phosphate dehydrogenase). Note that mRNA is overexpressed in the five ESC cell lines that showed *GAS1* gene amplification (KYSE-70, -150, -450, -890, and -1170) as well as in one cell line that did not (KYSE-1260). C, expression of the novel gene in normal human tissues. Membranes containing poly(A)<sup>+</sup> RNA derived from 12 different tissues were hybridized to [ $\alpha$ -<sup>32</sup>P]dCTP-labeled IMAGE clone 131865.

(R18567), revealed no amplifications in KYSE150 (Fig. 1B). On the basis of a comparison of the hybridization signals of amplified DNA versus normal DNA, a rough estimation revealed more than 12-fold amplifications of the first three probes (*GYG2*, *GLDC*, and IMAGE clone 131865) in KYSE150 and about 3- to 6-fold amplifications in the other four cell lines (e.g., Fig. 2A). On the other hand, their hybridization signals decreased in KYSE1250 and 1260 as compared with normal diploid cells, suggesting that losses of 9p had occurred in those two cell lines. Indeed, we confirmed that only two signals specific for YAC 799D2 could be detected in these cell lines, which bear near-tetraploid and -triploid chromosome backgrounds, respectively.

Although the results of Southern blotting prompted us to analyze the expression levels of three transcripts, only IMAGE clone 131865 showed overexpression in cell lines that had exhibited amplification at 9p23-24 (Fig. 2B). In addition, this clone showed an amplification pattern in 2 of 46 (4.3%) of primary ESC samples by Southern and dot blot analysis (data not shown). This result suggested that IMAGE clone 131865 represented part of a candidate target gene, which we named *GAS1*, within this amplicon. A multiple-tissue Northern blot hybridized with IMAGE clone 131865 showed ubiquitous expression of a single 4.5 kb-transcript (Fig. 2C).

**Isolation of Full-Length cDNA of the Gene Represented by Clone 131865.** To obtain the complete sequence, we screened two different cDNA libraries using IMAGE clone 131865 as a probe. The alignment of cDNA sequences from six overlapping clones isolated in this manner revealed a 4235-bp transcript. Because the size corresponded well with that indicated by Northern blots, our cDNA was considered to be full-length. Nucleotide sequence analysis revealed that translation was considered to be initiated at nucleotide 146, because there the consensus sequence for initiation of translation (Kozak's rule) is well conserved. Two AATAA polyadenylation sig-

the 29 ESC cell lines examined (17.2%); high-level amplification was present in one of those (KYSE150; Ref. 8). On the basis of this CGH result, we performed FISH analysis in KYSE150 using eight YACs and one PAC as a probe to generate a map of the 9p23-24 amplicon (Fig. 1, A and B). YACs 799D2 and 807B4 produced strong signals as HSRs on two marker chromosomes (Fig. 1C). By contrast, the number of FISH signals with YACs 953A7, 871F1, 853F4, and 933F6 ranged from four to nine, but with PAC PJ2B and YAC 830E1 only two to three. To define the smallest common region of amplification at 9p23-24, FISH analyses were performed on the other four cell lines (KYSE70, 450, 890, and 1170) that had shown copy number gains on 9p in our CGH analysis (8). Hybridization signals with YACs 799D2 and 807B4 were detected as small but distinct HSRs on a marker chromosome in both KYSE890 and 1170 (data not shown), whereas in KYSE70 and 450 the number of signals ranged from six to nine. However, none of these cell lines was useful for narrowing the size of the amplicon defined in KYSE150, because the copy number abnormalities in all four cases extended beyond the region amplified in KYSE150. Thus, the target gene(s) in the 9p23-24 amplicon seemed to lie within the relatively narrow region covered by YACs 799D2 and 807B4.

**Molecular Definition of the 9p23-24 Amplicon and Analysis of Transcripts.** Southern blot analysis of all 29 ESC cell lines with cDNA probes representing glycogenin 2 (*GYG2*), glycine dehydrogenase (*GLDC*), and IMAGE clone 131865 (R24542) located on YAC 799D2 showed amplification patterns in all five lines that had exhibited copy number gains at 9p in CGH and FISH experiments. By contrast, probes for other genes in the region, *JAK2*, a member of the solute-carrier family (*SLC1A1*), casein kinase 1  $\gamma$  2 (*CSNK1G2*), IMAGE clone 650495 (AA219360), and IMAGE clone 30354

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1  M E V A E V E S P L N P S C K I M T F R P S M E E F R E F N
31  K Y L A Y M E S K G A H R A G L A K V I P P K E W K P R Q C
61  Y D D I D N L L I P A P I Q Q M V T G Q S G L F T Q Y N I Q
91  K K A M T V K E F R Q L A N S G K Y C T P R Y L D Y E D L E
121 R K Y W K N L T F V A P I Y G A D I N G S I Y D G E D E W
151 N I A R I N T V L D V V E E E C G I S I E G V N T P Y L Y F
181 G M W K T T F A W H T E D M D L Y S I N Y L H F G E P K S W
211 Y A I P P E H G K R L E R L A Q G F F P S S Q G C D A F L
241 R H K M T L I S P S V L K K Y G I P F D K I T Q E A G E F M
271 I T F P Y G Y H A G F N H G F N K A E S T N F A T V R W I D
301 Y G K V A K L C T C R K D M V K I S M D I F V R K F Q P D R
331 Y Q L W K Q G K D I Y T I D H T K P T P A S T P E V K A W L
361 Q R R R K V R K A S R S F Q C A R S T S K R P K A D E E E E
391 V S D E V D G A E V P N P D S V T D D L K V S E K S E A A V
421 K L R N T E A S S E E E S A S A R M Q V E Q N L S D H I K L
451 S G N S C L S T S V T E D I K T E D D K A Y A R S V S P S I
481 S S E A D D S I P L S T G Y E K P E K S D P S E L S W P K S
511 P E S C S S V A E S N G V L T E G E E S D V E S H G N G L E
541 P G E I P A V P S G E R N S F K V P S I A E G E N K T S K S
571 W R H P L S R P P A R S P M T L V K Q Q A P S D E L P E V
601 L S I E E E V E E T E S W A K P L I H L W Q T K S P N F A A
631 E Q E Y N A T V A R M K P H C A I C T L L M P Y H K P D S S
661 N E E N D A R W E T K L D E V V T S E G K T K P L I P E M C
691 F I Y S E E N I E Y S P P N A F L E E D G T S L L I S C A K
721 C C V R V H A S C Y G I P S H E I C D G W L C A R C K R N A
751 W T A E C C L C N L R G G A L K Q T K N L R W A H V M C A V
781 A V P E V R F T N V P E R T Q I D V G R I P L Q R L K L K C
811 I F C R H R V K R V S G A C I Q C S Y G R C P A S F H V T C
841 A H A G V L M E P D D W P Y V V N I T C F R H K V N P N V
871 K S K A C E K V I S V G Q T V I T K H R N T R Y Y S C R V M
901 A V T S Q T F Y E V M F D D G S F S R D T F P E D I V S R D
931 C L K L G P P A E G E V V Q V K W P D G K L Y G A K Y F G S
961 N I A H M Y Q V E F E D G S Q I A M K R E D I Y T L D E E L
991 P K R V K A R F S T A S D M R F E D T F Y G A D I I Q G E R
1021 K R Q R V L S S R F K N E Y V A D P V Y R T F L K S S F Q K
1051 K C Q K R Q
    
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Fig. 3. Predicted amino acid sequence of GAS1. Two PHD-finger motifs are underlined, and a PX domain is doubly underlined. A putative nuclear localization signal is indicated in bold type.



nals were found at the 3' end, followed by a poly(A) stretch. Therefore, the predicted protein sequence is likely to contain 1056 amino acids (Fig. 3). Interestingly, nucleotides 10–3140 of *GASC1* showed striking homology to part of the cDNA sequence of KIAA0780 (GenBank accession no. AB018323). However, reverse transcription-PCR designed to cover nucleotides 2592–3382 of *GASC1*, using tumor and normal RNAs as templates, yielded a single-band product of the expected size, confirming that our sequence was correct. In addition, a probe generated from nucleotides 238–639 hybridized to YAC 799D2, which contains IMAGE clone 131865 (R24542), and also showed amplification signals on Southern blots with all five relevant tumor lines.

Analysis of the predicted amino acid sequences suggested that the gene product would contain two PHD fingers and a PX domain (Fig. 3). Computer prediction of its subcellular localization, using the PSORT II program, indicated that *GASC1* would probably localize to the nucleus; one typical bipartite nuclear location signal occurs at residues 979–996.

## Discussion

Genetic alterations in 9p have been seen in a wide range of human cancers, including ESC. Earlier molecular genetic studies of ESCs had focused on 9p21–22, in particular the region encompassing the *MTS1* (*P16/CDKN2A*) gene, which encodes an inhibitor of cyclin-dependent kinase 4/6 that negatively regulates G<sub>1</sub>-S transition of proliferating cells (12). Recent studies using CGH and FISH have revealed, however, that amplification of DNA on 9p, particularly at 9p23–24, frequently occurs in ESCs as well as other types of tumor (8, 13–17). For example, 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 among nine showed specific 9p24 amplification, and furthermore, gains at 9p21–ter tended to be more common in advanced-stage tumors (13). Amplifications of 9p23–24 have been observed also in breast cancers, lung carcinomas, high-grade astrocytomas, and glioblastomas (14–17). A breast cancer cell line, COLO 824, shows an ~10-fold increase in DNA copy number at 9p23–24, far distal to *P16/CDKN2A* (17). In addition, coexistence of a constitutional duplication of 9p23–24 and a germ-line mutation of *BRCA2* has been reported in three brothers with breast cancer (18). The combined evidence indicates that 9p23–24 is likely to harbor at least one tumor-associated gene that may be involved in multiple types of neoplasm. On the basis of that hypothesis, in the study reported here we attempted to define the smallest 9p23–24 amplicon among our ESC cell lines and identified a novel candidate gene, *GASC1*, that was amplified and overexpressed in 5 of the 29 cell lines examined.

*GASC1* contains one PX domain and two PHD fingers. PX domains are present in a diverse set of proteins and may participate in protein-protein interactions, although the function of this motif has not yet been well defined (19). The PHD finger, a zinc finger-like sequence, is widely found in nuclear proteins involved in chromatin-mediated transcriptional regulation, such as the *Drosophila trl* and *pcl* gene products (20). Several PHD-finger proteins have been identified recently, including the transcriptional coactivators transcriptional intermediary factor 1, the putative chromatin-associated acetylase *MOZ* (monocytic leukemia zinc-finger protein), and the dermatomyositis-specific autoantigen *Mi2* (21–23). The transcriptional intermediary factor 1 family of proteins ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) appears to play important roles in cell differentiation, oncogenesis, and signal transduction (21). *Mi2*, on the other hand, is found in a complex that possesses histone deacetylase and nucleosome-remodeling activities and is involved in chromatin

reorganization; the PHD fingers in *Mi2* appear to be required for direct interaction with histone deacetylase (23).

The PHD motif is also present in several potential proto-oncogenes. *HRX/ALL1/MLL* (*HRX*, human trithorax; *ALL*, acute lymphoblastic leukemia; *MLL*, mixed lineage leukemia), a human homologue of *trx*, is frequently altered in acute lymphocytic leukemias in children (24). Amplification of *MLL2*, another human homologue of *trx*, has been observed in tumor cell lines derived from a variety of solid tissues (25). *PLU-1* is expressed consistently in breast cancers, although its expression is highly restricted in normal adult tissues (26). Mutations within the PHD finger of the *AIRE* gene have been found in DNA from patients with an autoimmune disease known as polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), who develop squamous cell carcinoma in the oral cavity (27). The *MOZ* gene was found fused with the *CBP* gene [t(8;16)(p11;p14)] in a case of acute myeloid leukemia (22), and fusion of *Tif1* to the *RET* receptor tyrosine kinase gene has been reported in cases of pediatric papillary thyroid carcinoma (28).

We conclude that *GASC1*, which contains both of these potentially “oncogenic” motifs, may play an important role in the carcinogenesis or progression of multiple tumors, although its function has not yet been defined. However, involvement of the *GASC1* protein in some basic biological process is likely, a view supported by its ubiquitous expression in normal tissues. Its predicted amino acid structure and our other findings strongly suggest that up-regulation of the *GASC1* transcript may be related to development and/or progression in various types of tumors including ESC.

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## Identification of a Novel Gene, *GASC1*, within an Amplicon at 9p23–24 Frequently Detected in Esophageal Cancer Cell Lines

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