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Identification and Characterization of a 19q12 Amplicon in Esophageal Adenocarcinomas Reveals *Cyclin E* as the Best Candidate Gene for this Amplicon[1]

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ABSTRACT

Genomic DNA amplification in tumors is frequently associated with an increased gene copy number of oncogenes or other cancer-related genes. We have used a two-dimensional whole-genome scanning technique to identify gene amplification events in esophageal adenocarcinomas. A multicopy genomic fragment from a tumor two-dimensional gel was cloned, and genomic amplification encompassing this fragment was confirmed by Southern blot analysis. The corresponding DNA sequence was matched by BLAST to a BAC contig, which allowed the use of electronic-PCR to localize this amplicon to 19q12. Sequence tagged site-amplification mapping, an approach recently implemented in our laboratory (Lin, L. *et al.*, Cancer Res., *60:* 1341–1347, 2000), was used to characterize the amplicon. Genomic DNA from 65 esophageal and 11 gastric cardia adenocarcinomas were investigated for 19q12 amplification using quantitative PCR at 11 sequence tagged site markers neighboring the cloned fragment. The amplicon was narrowed from >8 cM to a minimal critical region spanning <0.8 cM, between D19S919 and D19S882. This region includes the *cyclin E* gene. Fourteen expressed sequence tags (ESTs) covering the minimal region were then assayed for potential gene overexpression using quantitative reverse transcription-PCR. Seven of the selected ESTs were found to be both amplified and overexpressed. Among these seven ESTs, *cyclin E* showed the highest frequency of gene amplification and overexpression in the tumors examined, which allowed us to finalize the core-amplified region to <300 kb. These results indicate that *cyclin E* is the likely target gene selected by the amplification event at 19q12. The fact that *cyclin E* overexpression was found only in the amplified tumors examined indicates that gene amplification underlies the *cyclin E* gene overexpression. Our study represents the first extensive analysis of the 19q12 amplicon, and is the first to physically map the core-amplified domain to a region of <300 kb that includes *cyclin E*. Amplification of 19q12 was found neither in the 28 esophageal squamous cancers nor in the 39 lung adenocarcinomas examined but was observed in 13.8% of esophageal and 9.1% of gastric cardia adenocarcinomas.

INTRODUCTION

A striking increase in the incidence of esophageal adenocarcinoma has been observed in the United States over the past 2 decades (1), yet the 5-year survival rate remains unchanged and <10% (2). Chronic gastroesophageal reflux is the major risk factor for esophageal adenocarcinoma, which can result in the replacement of the normal squamous mucosa with a metaplastic, intestinal-like columnar epithelia, designated Barrett's esophagus (3). Esophageal adenocarcinomas are reported to occur at a higher rate among patients with preexisting Barrett's esophagus as compared with those without, and the malignancy is often associated with Barrett's epithelia (4).

The genetic alterations underlying development and/or progression of esophageal adenocarcinoma remain poorly understood. Gene amplification and overexpression of the oncogenes *erbB2*, *EGFR*, and K-*ras* have been reported in this type of tumor[3] (5, 6). A recent study using CGH[4] by van Dekken *et al.* (7) reported amplification at 8q23–24.1, 15q25, 17q12–21, and 19q13.1 in a series of 28 Barrett's adenocarcinomas. The candidate genes for these amplicons were suggested as *myc* for 8q23–24.1; *IGF1R* for 15q25; *erbB2* for 17q12–21; and *TGFβ1*, *BCL3*, and *AKT2* for 19q13.1. Another CGH study, analyzing 58 primary gastric cancers, also demonstrated genomic amplification at multiple chromosomal locations including 6p21, 7q31, 8p22–23, 8q23–24, 11q13, 12p12–13, 17q21, 19q12–13, and 20q13 (8). We have recently identified and characterized the 8p22–23 amplicon that was detected in 12.1% of 66 esophageal adenocarcinomas (9, 10). Two genes, the lysosomal protease *cathepsin B* and the zinc finger transcription factor *GATA-4*, were localized within the minimal critical region and are likely candidate genes for the 8p22–23 amplicon in esophageal adenocarcinomas (9, 10).

In the present study, a potentially amplified *Not*I/*Dpn*II DNA fragment was identified and cloned from an esophageal adenocarcinoma using the two-dimensional gel RLGS technique. The amplification was confirmed and then localized to 19q12. STS-amplification mapping was applied to characterize the amplicon using the QG-PCR assay with 11 STS markers neighboring the location of the two-dimensional fragment and spanning >8 cM in 19q12. Genomic DNA from 65 normal-esophageal adenocarcinoma pairs and 11 normal-gastric cardia carcinoma pairs were analyzed. The core amplified region was determined to be localized between D19S919 and D19S882, a region of <300 kb that includes *cyclin E*. *Cyclin E* is a G_1 cyclin that regulates cell entry into the S phase (11, 12). Amplification and overexpression of *cyclin E* have been observed in ovarian and gastric carcinomas (13–16). These results strongly support the theory that *cyclin E* is the likely target gene selected by the 19q12 amplicon.

MATERIALS AND METHODS

Tumor Tissue Collection and DNA/RNA Isolations. Sixty-nine esophageal adenocarcinomas and the corresponding normal esophageal or gastric mucosa, 20 Barrett's metaplasia specimens, as well as the esophageal squamous cell carcinomas and lung adenocarcinomas were obtained after informed consent from patients undergoing esophagectomy and pulmonary resection for cancer at the University of Michigan Medical Center from 1992 to 1999. Patients receiving treatment with chemotherapy and/or radiotherapy prior to surgery were excluded from the present study. A small portion of each tissue

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[3] D. G. Beer *et al*., unpublished data.

[4] The abbreviations used are: CGH, comparative genomic hybridization; RLGS, restriction landmark genomic scanning; STS, sequence tagged site; EST, expressed sequence tag; QG-PCR, quantitative genomic-PCR; E-PCR, electronic PCR; RT-PCR, reverse transcription-PCR; Ts/c and Ns/c, the intensity ratio of tumor (Ts/c) or normal (Ns/c) sample *versus* GAPDH control from QG-PCR; NCBI, the National Center for Biotechnology Information; WICGR, Whitehead Institute Center for Genome Research; SHGC, Stanford Human Genome Center; LLNL, Lawrence Livermore National Laboratory; RH, radiation hybrid; BLAST, Basic Local Alignment Search Tool; BAC, bacterial artificial chromosome; CR, centi-ray.

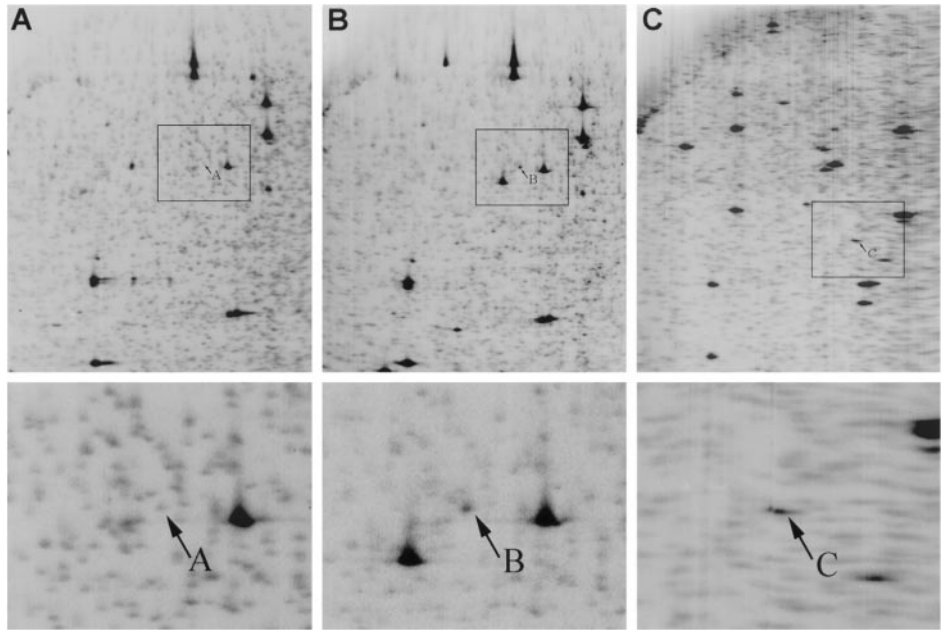

Fig. 1. Two-dimensional RLGS analysis of DNA from the normal and tumor tissues of patient F12. Two-dimensional PAGE was used to separate the *Not*I/*Hin*fI DNA fragments of normal tissue from patient F12 (*A*) and esophageal adenocarcinoma from the same patient (*B*). *C*, *Not*I/*Dpn*II fragments from the same patient. *Not*I/*Dpn*II digestion was performed for cloning purposes. Comparison of the relative intensities of the DNA fragments *A* (*arrow* normal) and *B* (tumor) indicates an increased DNA amount presented in the tumor genome, which suggests potential genomic amplification. Spot *C* (*arrow*), a *Not*I/*Dpn*II fragment, represents the cloned fragment.

specimen was embedded in OCT compound (Miles Scientific, Naperville, IL), and the remainder of the tissue was immediately frozen in liquid nitrogen. All of the samples were stored at −70°C. High-molecular-weight DNA was isolated as described previously (17). DNA was dissolved in TE buffer [10 mM Tris, 1 mM EDTA (pH 8.0)] and stored at −20°C. Total RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD). Agarose gel electrophoresis and the A260:280 nm ratio were used to assess RNA quality. The RNA samples were stored at −70°C until use.

Two-Dimensional Gel Electrophoresis for RLGS. High- molecular-weight DNA from normal and tumor samples were subjected to two-dimensional gel electrophoresis as described previously (18). In brief, DNA samples were digested using the restriction enzyme *Not*I (New England BioLabs Inc., Beverly, MA) followed by labeling *Not*I ends with [α-^{32}P]dCTP and [α-^{32}P]dGTP (NEN Life Science Products, Boston, MA). A second enzyme, *Eco*RV, was used to digest the DNA fragments prior to electrophoresis in a 0.9% disc-agarose gel (one-dimensional). After first-dimensional separation, additional digestion was carried out within the gel using *Hin*fI enzyme (or *Dpn*II for cloning purposes). The disc-agarose gel was then placed on the top of 5.25% polyacrylamide gel and subjected to electrophoresis. The resulting gels were dried and autoradiographed using PhosphorImage screens (Molecular Dynamics, Sunnyvale, CA). Computer analysis of potential amplification events was performed using previously developed software (18). For cloning, preparative gels were run, and the selected amplified DNA fragments were cut out for subsequent cloning as described previously (9).

Isolation and Cloning of the Two-Dimensional DNA Fragments. The gel pieces containing the amplified DNA fragments were isolated, and the gel was electrophoresed onto a DEAE membrane (Schleicher & Schuell, Keene, NH). The DNA was then eluted out and purified as described previously (9) and was dissolved in 7 μl of distilled water. A pBC vector (Stratagene, La Jolla, CA) was cleaved with the enzymes *Not*I and *Bam*HI (*Dpn*II compatible end), and was gel-purified using SeaKem GTG agarose (FMC BioProducts, Rockland, ME). After the incubation of 7 μl of two-dimensional DNA, 1 μl of vector (1 ng/μl), and 1 μl of 10× ligation buffer (Boehringer Mannheim, Indianapolis, IN) at room temperature for 10 min and at 65°C for 7 min, 1 μl T4 ligase (5 u/μl; Boehringer Mannheim, Indianapolis, IN) was added, and the ligation reaction was carried at 16°C for 25 h. The ligation mixture was purified using a standard phenol-chloroform extraction protocol (19). The XL1 Blue competent cells were then transformed with the purified vector two-dimensional DNA via electroporation using E Coli Pulser (Bio-Rad Laboratories, Hercules, CA). Individual colonies were used for DNA isolation (mini-preps) via the alkaline lysis method as described by Sambrook *et al.* (19).

E-PCR. The cloned fragment was sequenced by University of Michigan Sequencing Core facility. The sequences were analyzed by BLAST for similarity matches in the databases. Resulting sequences can be then used as templates to perform the E-PCR (on the NCBI web site) analysis. Chromosomal location of the template was determined based on matches to a previously analyzed group of sequences in the databases.

Southern Blot Analysis. DNA from three normal-tumor tissue pairs, including the tumor from which the two-dimensional fragment was isolated, was cleaved by *Eco*RI and size-fractionated following the standard protocol for Southern blot analysis (19). The cloned DNA sequence was used as the probe and hybridized to the membrane containing the three pairs of DNA samples, using the hybridization and washing conditions provided by the manufacturer (NEN Life Science Products).

STS-Amplification Mapping Using QG-PCR. The amplified fragment was used as the anchor to select neighboring STS markers by searching STS databases as described previously (10). Briefly, PCR primers for each STS fragment were designed to ensure that the melting temperature (*Tm*) of the STS PCR fragments matched the *Tm* of the internal control (*GAPDH*), which was coamplified in each PCR reaction. Genomic DNA from tumor and normal tissues was quantified to have equity of starting materials. The forward primers of the control and test fragments were end-labeled with [^{32}P-γ]ATP (NEN Life Science Products) using T4 polynucleotide kinase (New England BioLabs). PCR was conducted with a 50-ng template in 25 μl of total reaction volume using *Taq* polymerase (Promega, Madison, WI). The PCR products were then resolved on 8% denaturing polyacrylamide gels. The signal ratios (Ts/c:Ns/c) for both the tumor (Ts/c, tumor STS fragment/tumor *GAPDH* fragment) and normal DNA samples (Ns/c, normal STS fragment/normal GAPDH fragment) were determined using ImageQuant software (Molecular Dynamics).

Positional Candidate Analysis Using Quantitative RT-PCR. Several databases (NCBI,[5] WICGR,[6] SHGC[7]) were searched to select available ESTs or known genes within the defined minimal critical region of the 19q12 amplicon. All of the total RNA samples used were treated with DNase I (Promega) prior to performing reverse transcription. Two μg of total RNA were reverse transcribed using reverse transcriptase (Life Technologies) and primed by both $(dT)_{18}$ and random hexamers in a total 40 μl of reaction volume. Two μl of the cDNA were then subjected to RT-PCR with *GAPDH* coamplified as the internal control. The PCR products were resolved on 8% PAGE gels, and gel data analyses were performed using ImageQuant software as in QG-PCR analysis.

Immunohistochemical Analysis. Frozen specimens were sectioned at 5 μm, placed on 0.1% poly-L-lysine-coated slides, and fixed in 100% acetone at

[5] Internet address for NCBI: http://www.ncbi.nlm.nih.gov.
[6] Internet address for WICGR: http://www-genome.wi.mit.edu.
[7] Internet address for SHGC: http:www-shgc.stanford.edu.

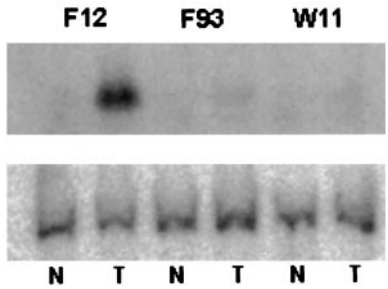

Fig. 2. Southern blot analysis of DNA samples from three paired normal-adenocarcinomas. The cloned two-dimensional DNA fragment was ^{32}P-labeled and hybridized to the Southern blot membrane, which contained the DNA from normal-tumor pairs of patients F12, F93, and W11. DNA amplification was confirmed in tumor F12, which demonstrated a 6.5-fold increase as compared with its normal tissue counterpart. The *lower panel* is a control to show equal DNA loading.

Table 1 *An outline of the resulting STS DNA fragments and two-dimensional fragment in BAC clone AC007786 retrieved by E-PCR analysis*

Name	GenBank accession no.	Location in contig (bp)	Genetic map
SHGC-80721	G54241	19280–19574	NA[a]
SHGC-130179	G59466	31591–31791	NA
UTR-9734	G13234	107360–107538	NA
F12 two-dimensional fragment	Not applied	112467–112870	
HS269XG5 (D19S409)	Z23912	139732–139902	50.0 cM
HSA084XF1 (D19S932)	Z51953	186844–186987	49.4 cM
HSA224WC9 (D19S875)	Z52589	206471–206583	49.4 cM

[a] NA, not available.

−20°C for 10 min. Endogenous peroxidase activity was quenched with three changes of 1.2% hydrogen peroxide for 30 min each. Nonspecific binding was blocked using a 1:20 dilution of rabbit serum in PBS-1% BSA. The cyclin E protein was detected using the anti-cyclin E antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution in PBS-1% BSA. A section of each tissue was also incubated without the primary antibody as a negative control. Immunoreactivity was detected using the Vectastain avidin/biotin complex kit (Vector Laboratories, Burlingame, CA) with 3,3′-diaminobenzadine as a substrate. The slides were lightly counterstained with Harris-modified hematoxylin and permanently mounted as described previously (20).

RESULTS

Two-Dimensional Gel Analysis of Esophageal Adenocarcinomas. DNA samples from 44 primary esophageal adenocarcinomas were analyzed using the two-dimensional gel RLGS technique. Cleavage sites of the rare cutting restriction enzyme *Not*I were labeled with [α-^{32}P]dCTP and [α-^{32}P]dGTP and served as the landmarks to scan the whole genome of tumor samples and associated normal tissues. Approximately 3,000 restriction fragments were resolved in each two-dimensional gel (21). Comparison of normal and tumor two-dimensional patterns revealed a *Not*I/*Hin*fI fragment that was 3100 bp in size in the first-dimension separation and 400 bp in the second-dimension, which shows higher intensity in two tumors (F12 and B81) relative to normal tissue controls (Fig. 1, *A* and *B*). Two-dimensional gels were digested with *Not*I/*Dpn*II restriction enzymes for cloning purposes (Fig. 1*C*). Most of the very intensive spots visible in the two-dimensional gels represent ribosomal DNA, which are present in multiple copies. Changes in the patterns of both of these two-dimensional fragments and other CpG island-containing fragments, attributable to alterations of DNA methylation, are often observed during tumor development (22, 23).

Southern Blot Analysis for Genomic Amplification. High-molecular-weight DNA from three normal-tumor pairs of esophageal adenocarcinomas, including patient F12, were digested by restriction enzyme *Eco*RI prior to Southern blot analysis. The cloned two-dimensional fragment was labeled with [α-^{32}P]dCTP as the probe and was hybridized to a membrane containing the paired normal-tumor DNAs. As shown in Fig. 2, increased DNA copy number of the two-dimensional fragment was detected in tumor F12 but not in another two pairs of normal-tumor DNA, F93 and W11.

Chromosomal Localization of Amplified Two-Dimensional Fragment. The amplified fragment was sequenced, and a BLAST search revealed that the 396-bp sequence was matched within a BAC clone, AC007786. AC007786 consists of more than 229 kb of completed sequences and was mapped to 19q12 between the STS marker D19S222 and the gene *UQCRFS1* (ubiquinol-cytochrome C reductase iron-sulfur subunit), a chromosomal interval of 2–3 cM centromeric to the *UQCRFS1* gene. AC007786 was then used as the template and submitted for an E-PCR analysis. Table 1 shows the outline of the six STS markers as well as the amplified two-dimensional sequence that localized within this BAC clone. This allowed not only the chromosomal location of the amplicon in question to be confirmed but the neighboring physical map to be determined as well. This analysis also revealed that the amplified two-dimensional *Not*I/*Dpn*II fragment was actually a part of the 5′ end of the *UQCRFS1* gene and was 27 kb away from the STS marker D19S409.

Characterization of the 19q12 Amplicon and Narrowing of the Minimal Region by STS-Amplification Mapping. To determine the size and frequency of the 19q12 amplicon in esophageal adenocarcinomas and, most importantly, the minimal critical region, 11 STS markers spanning >8 cM were selected in proximity to the location of the two-dimensional fragment in 19q12 based on a search of NCBI, WICGR, and SHGC databases. The QG-PCR assay was applied to the DNA from 65 paired normal tissue-esophageal adenocarcinomas and 11 paired normal-gastric cardia adenocarcinomas using these 11 STS markers. The house-keeping gene *GAPDH* was PCR-coamplified as an internal control. Increased DNA dosage in the tumors containing the 19q12 amplicon was observed (Fig. 3). The 19q12 amplicon spans more than 8 cM in tumor genome and occurred in 13.8% (9 of 65) of esophageal and in 9.1% (1 of 11) of gastric cardia adenocarcinomas.

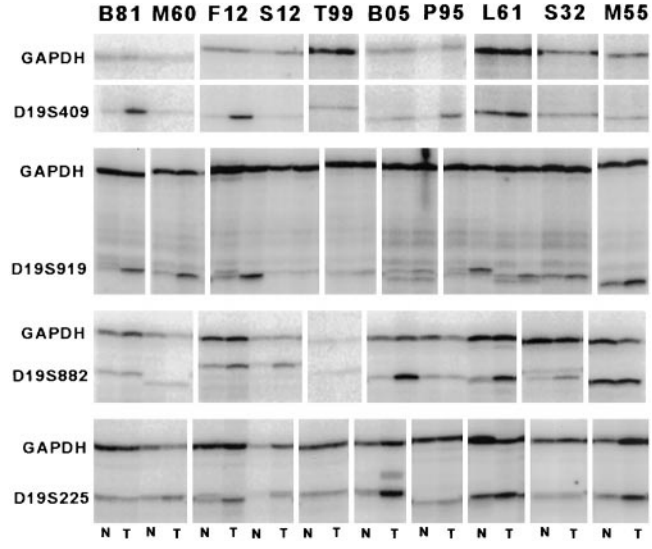

Fig. 3. The QG-PCR assay was applied to characterize the 19q12 amplicon and to map the minimal critical region in 76 esophageal and gastric cardia adenocarcinomas. The housekeeping gene *GAPDH* was coamplified in the PCR reactions along with the STS markers. The STS markers selected were those neighboring the two-dimensional fragment on 19q12 as determined from the physical maps and STS databases. The DNA from the 10 of 76 tumors that were amplified at 19q12 is presented here. As shown, genomic amplification in tumors B81, M60, and P95 is observed for markers D19S409 and D19S919 but not for the markers D19S882 and D19S225. Amplification is observed at marker D19S882 in tumors S12, T99, B05, and S32, and also at D19S225 in tumor B05 but not at markers D19S409 or D19S919. Amplification in tumor M55 is observed at all of the loci except D19S409 as shown. D19S409 is a STS marker tightly linked to the cloned two-dimensional fragment that is 27 kb away (Table 1).

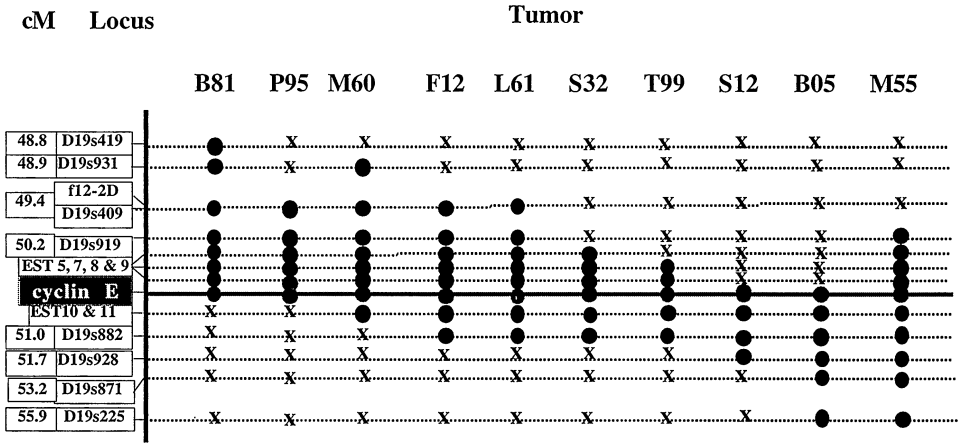

Fig. 4. Diagrammatic representation of the 19q12 amplicon in esophageal adenocarcinomas as determined using both the QG-PCR and quantitative RT-PCR analyses shown in Figs. 3 and 5. ●, genomic amplification detected in the individual tumors at the markers tested; **X**, DNA amplification was not detected at the loci applied. The map order is based on the DNA amplification analysis of 76 normal-tumor pairs of esophageal and gastric cardia adenocarcinomas but closely follows the published genetic, RH, and expression maps from the NCBI, WICGR, and SHGC databases. As shown, the minimal amplified region is confined between D19S919 and D19S882, which spans about 0.8 cM. The core amplified domain was determined by examining the overexpression status of the 14 ESTs selected within the minimal region, which was located between EST 9 and EST 10 (<300 kb in size). *Cyclin E* shows the highest frequency of gene amplification and overexpression and resides within this <300-kb core domain.

After quantification, a 6.5-fold increase was observed in tumor F12 using Southern hybridization analysis (Fig. 2), and an 8.6-fold increase was present in tumor B81 using the QG-PCR assay (Fig. 3). The minimal critical region was mapped between the markers D19S919 and D19S882, telomeric to the *UQCRFS1* gene and was ~0.8 cM in size based on the physical maps from WIGRC and SHGC (Fig. 4). *Cyclin E*, a G_1 cyclin that regulates cell entry into the S phase, is located within this region.

Localization of the Core Amplified Domain by Analyzing Amplification and Overexpression Mapping Patterns of ESTs within the Minimal Critical Region. The fact that the 19q12 amplicon was narrowed to a minimal region of less than 1 cM facilitates the approach of positional candidate analysis. Fourteen ESTs within the minimal region, including *cyclin E*, were selected from the database for expression analysis using quantitative RT-PCR (Fig. 5). Quantitative RT-PCR was applied to 7 (of 10 amplified) tumors containing the 19q12 amplicon and 5 tumors without 19q12 amplification. EST 9 (sts-N21279) showed elevated expression in tumors P95, B81, F12, and L61, but not in tumors M60 and S12, relative to their corresponding normal tissue RNA. Tumors P95 and B81 did not show increased expression at EST 11 (sts-Z41049); however, tumors M60 and S12 demonstrated a 6- to 20-fold increased expression of this EST as compared with their normal tissue mRNA (Fig. 5). Tumor P15 was not observed to be amplified and was used as a negative control for the expression assay (Fig. 5). The expression levels between normal and tumor cDNA from patient P15 are thus similar at ESTs 9 and 11. The mRNA from one Barrett's tissue, P95, showed overexpression of EST 9 and cyclin E, but not EST 11 (Fig. 5). QG-PCR for assessment of DNA amplification was then applied to all of the 76 esophageal and gastric cardia adenocarcinomas using those EST fragments which showed overexpression in the tumors to determine the frequency of the genomic amplification of these ESTs. Seven ESTs of the 14 selected within the minimal region were observed to be both amplified and overexpressed in the tumors (Fig. 5; Table 2). A high resolution map representing the characteristics of tumor RNA overexpression and DNA amplification within the minimal critical region was established, and the core amplified domain of the 19q12 amplicon was localized between ESTs sts-W74757 (EST 9, 179.90 cR in GB4 RH GeneMap'99) and sts-R63092 (EST 10, 180.67 cR; Fig. 4).

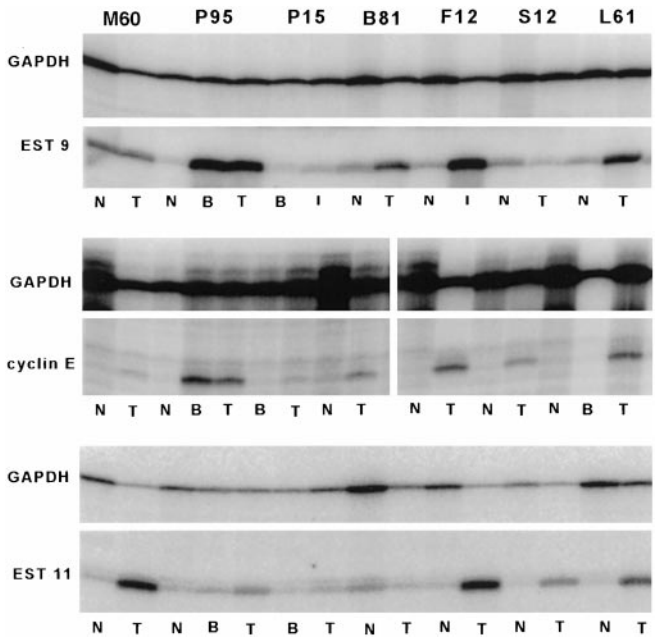

Fig. 5. Quantitative RT-PCR analysis applied to 14 ESTs within the minimal critical region of the 19q12 amplicon. *GAPDH* was coamplified as the internal control in each reaction. As shown, the mRNA level is elevated for EST 9 (sts-N21279) in tumors P95, B81, F12, and L61; however, a similar level is observed between normal tissue and tumors in M60 and S12 RNA. The mRNA from tumors P95 and B81 shows no increase in EST 11 (sts-Z41049), but tumors M60 and S12 demonstrate in 6- to 20-fold increased expression as compared with their normal RNA for this EST. The mRNA from one Barrett's mucosa from patient P95 shows overexpression of EST 9 and *cyclin E*, but not EST 11. *N*, mRNA from normal esophagus; *B*, mRNA from Barrett's mucosa; *T*, mRNA from tumor esophageal RNA.

The *Cyclin E* Gene Is the Likely Target Selected by the Amplification Event at 19q12. The core amplified domain was estimated to span ~100–300 kb based on the genetic and RH maps, which is in agreement with the analysis of sequenced contigs of band 19q12 (from the database of LLNL[8]). A BAC clone, AC008798, containing >137 kb of sequenced DNA (updated as of February 23, 2000), includes the *cyclin E* sequence but not any of the other neighboring ESTs analyzed (Table 3). The sequences of ESTs 10, 11, 12, and 14 were matched to the clone AC008507, containing >213 kb of 11 ordered pieces of sequences and adjacent telomerically to AC008798. ESTs 3, 7, 8, and 9 were found to be contained in AC010513, which is centromeric to AC008798. ESTs 4 and 5 were contained in AC010505 (Table 3), centromeric to AC010513. AC007786 which contains the amplified two-dimensional *Not*I/*Dpn*II fragment is ~2 cM centromeric to AC010505 (Table 3). Dissection or physical mapping of the minimal critical region with these ESTs in addition to the genomic amplification mapping data clearly indicates that *cyclin E* is located between ESTs 7, 8, 9 and ESTs 10, 11 (Fig. 4; Table 3), rather than between EST 5 and EST 7 as shown in GeneMap'99. Moreover, an unknown EST, sts-X95406, which is mapped between EST 9 and EST 10 in GeneMap'99, shows homology to the *cyclin E* gene. Furthermore, among these seven amplified and overexpressed ESTs,

[8] Internet address for LLNL: http://www-bio.llnl.gov.

Table 2 *Candidate search results of the 14 ESTs[a] located within the minimal critical region of the 19q12 amplicon position*

Quantitative RT-PCR analysis was performed using these selected ESTs. Their expression status in esophageal and gastric cardia adenocarcinomas is listed.

EST no. in this study	EST name	GenBank accession no.	GB4 RH Map (cR)	Overexpression[b]
EST 13[c]	sts-H99213	H99213	174.40	Not detected
EST 1[c]	SGC35855	R59989	175.34	Not detected
EST 4 (LCN2)	stSG44980	N27931	179.72	+[d]
EST 5	WI-11914	R27320	179.72	+
EST 6 (CCNE1)	stSG16104	T54121	179.72	+
EST 7	stSG22464	N20512	179.72	+
EST 8	stSG25791	AA062869	179.90	Not detected
EST 9	sts-N21279	N21279	180.63	+
EST 10	sts-R63092	R63092	180.67	+
EST 11	sts-Z41049	Z41049	181.52	+
EST 12	stSG30380	AA164546	181.52	−
EST 2[c]	SHGC-33351	T71014	1099 (G3)	Not detected
EST 3[c] (D19S1092)	SHGC-11607	T56452	1122 (G3)	−
EST 14[c] (D19S856)	SHGC-3166	G07557	NA	−

[a] The selected ESTs are located between (telomeric to) D19S919 and (centromeric to) D19S882, which spans ~7 cR (about 2 Mb according to the calculation) in GeneMap'99 GB4 RH Map.

[b] The ratio of T(s/c):N(s/c) >2.5 was defined as gene overexpression.

[c] EST 13 and 1 are centromeric to D19S919, and ESTs 2, 3, and 14 are telomeric to D19S919 in a consecutive order selected from Stanford Chromosome 19 RH Map Data v2.0 (G3 RH Map).

[d] +, overexpressed; −, not overexpressed; NA, not available.

ESTs 4, 5, and 7 were found to be amplified in 7 of 10 tumors containing the 19q12 amplicon. ESTs 9, 10, and 11 were found to be amplified in 8 of 10 tumors containing the 19q12 amplification. None of the 13 ESTs (excluding EST 6, which represents *cyclin E*) were seen to have increased gene copy number without *cyclin E* amplification. Therefore, *cyclin E* demonstrates the highest frequency of both gene amplification and mRNA overexpression (Fig. 4).

An immunohistochemical study of *cyclin E* protein expression was applied to 30 esophageal adenocarcinomas, which demonstrated abundant nuclear staining in all of the 10 tumors possessing the 19q12 amplicon (Fig. 6, *A–D*), but not in any tumors without the 19q12 amplification. The increased staining was also observed in a region of Barrett's mucosa from patient P95 that represents either high-grade dysplasia or early adenocarcinoma *in situ* (Fig. 6*B*). This was consistent with the overexpression of *cyclin E* mRNA observed in the Barrett's mucosa from the same patient (Fig. 5). To determine whether increased DNA dosage might underlie the overexpression of *cyclin E* in these dysplastic Barrett's cells, the region of high *cyclin E* nuclear staining from the Barrett's mucosa was microdissected, and the DNA was isolated. *Cyclin E* amplification is observed in the microdissected DNA from this dysplastic Barrett's mucosa (Fig. 6*E*).

We examined the correlation of *cyclin E* amplification and overexpression with tumor stage and size. The analysis indicated that the size of the tumors with *cyclin E* gene amplification (4.34 cm in stage II, 5.75 cm in stage III, and 5.00 cm in stage IV) was not appreciably different from that of nonamplified tumors (4.31 cm in stage II, 5.39 cm in stage III, and 5.23 cm in stage IV). However, *cyclin E* amplification might likely be a relatively early event because it occurred in one dysplastic Barrett's mucosa and in 50% (5 of 10) of stage II esophageal adenocarcinomas. We were unable to correlate patient prognosis with *cyclin E* amplification in this study because of the small number of tumors amplified and the extremely low (<10–15%) 5-year survival for patients with this type of malignancy.

Amplification of *BCL3*, *TGFβ1*, and the STS Markers Closely Linked to *AKT2* Was Not Detected in This Series of 76 Esophageal and Gastric Adenocarcinomas. 19q13.1 was reported to be amplified in 28 esophageal adenocarcinomas by a previous study using a CGH approach (7). *AKT2*, *BCL3*, and *TGFβ1* were proposed as the potential candidate genes for the 19q13.1 amplicon. To determine whether the 19q13.1 amplicon is an extension from 19q12 or whether two individual amplicons exist at chromosome 19q, the QG-PCR assay was used to analyze the genes *BCL3* and *TGFβ1*, and two STS fragments (D19S421 and D19S420) that are closely linked to *AKT2*, as well as four other STS DNA markers spanning the 19q13.1 band. Genomic amplification was not detected in these two genes nor in the STS markers closely linked to *AKT2*. Because none of the markers and genes selected from the 19q13.1 region were found to be amplified (data not shown), this indicates that the 19q12 amplicon is not an extension of the previously reported 19q13.1 amplicon and is the only amplicon in the 19q centromeric region in this series of 76 esophageal and gastric cardia adenocarcinomas.

The 19q12 Amplicon Was Not Observed in Esophageal Squamous Cell Carcinomas nor in Lung Adenocarcinomas. *Cyclin E* amplicon was observed in 13.8% of 65 paired normal-tumors of esophageal adenocarcinoma and in 9.1% of 11 gastric cardia adenocarcinomas. Using the QG-PCR assay for analyzing genomic amplification at 19q12, increased DNA copy number, however, was detected in neither 28 paired normal-esophageal squamous cell carcinomas nor in 39 paired normal-lung adenocarcinomas. The results suggest a potential tissue specificity of the 19q12 amplicon in the development of esophageal adenocarcinoma.

DISCUSSION

Our two-dimensional genome scanning approach identified the amplification of a *Not*I fragment that was mapped to 19q12. This led to the characterization of a 19q12 amplicon that appears to play a role in the development and/or progression of esophageal and gastric cardia adenocarcinomas. The STS-amplification mapping approach using a QG-PCR assay allowed the minimal critical region of the 19q12 amplicon to be localized between D19S919 and D19S882. Fourteen ESTs selected within the minimal region were analyzed using quantitative RT-PCR to determine the expression patterns in the tumors containing the 19q12 amplicon. *Cyclin E* demonstrated the highest frequency of gene amplification and overexpression among the tumors examined and was localized within the <300-kb core amplified domain of the 19q12 amplicon. *Cyclin E* amplification and overexpression might be a relatively early event, inasmuch as amplification and overexpression were both detected in dysplastic Barrett's mucosa and in 50% (5 of 10) of stage II tumors containing this amplicon. The present study is the first to fully characterize the 19q12 amplicon. It is also the first to physically map the core amplified domain, narrowing it to a region <300 kb that includes *cyclin E*.

Genomic amplification is an event that may selectively increase the dosage of a gene. Coamplification of neighboring genes may also

Table 3 *Determination of the core amplified domain by analyzing the sequenced BAC or cosmid contigs from the LLNL and JGI databases using BLAST 2 SEQUENCES alignment and E-PCR in NCBI*

Contig order	EST[a] matched	STS matched	Note
AC007786	None	D19S409	Two-dimensional fragment
AC007759	None	NA[b]	
AC006134	None	NA	
AC011474	EST 2	SHGC-101991	
AC010505	EST 4 and 5	SHGC-141601	
AC010512	None	N/A	
AC010644	None	G25801	
AC010513	EST 3, 7, 8, 9	EST 3 = D19S1092	
AC008989	None	NA	
AC008798	*Cyclin E* (EST 6)	NA	
AC008507	EST 10, 11, 12, 14	D19S590 WI-30870	

[a] Only for the ESTs selected within the minimal critical region in the present study.

[b] NA, not available from E-PCR analysis at the time this article was written in February 2000.

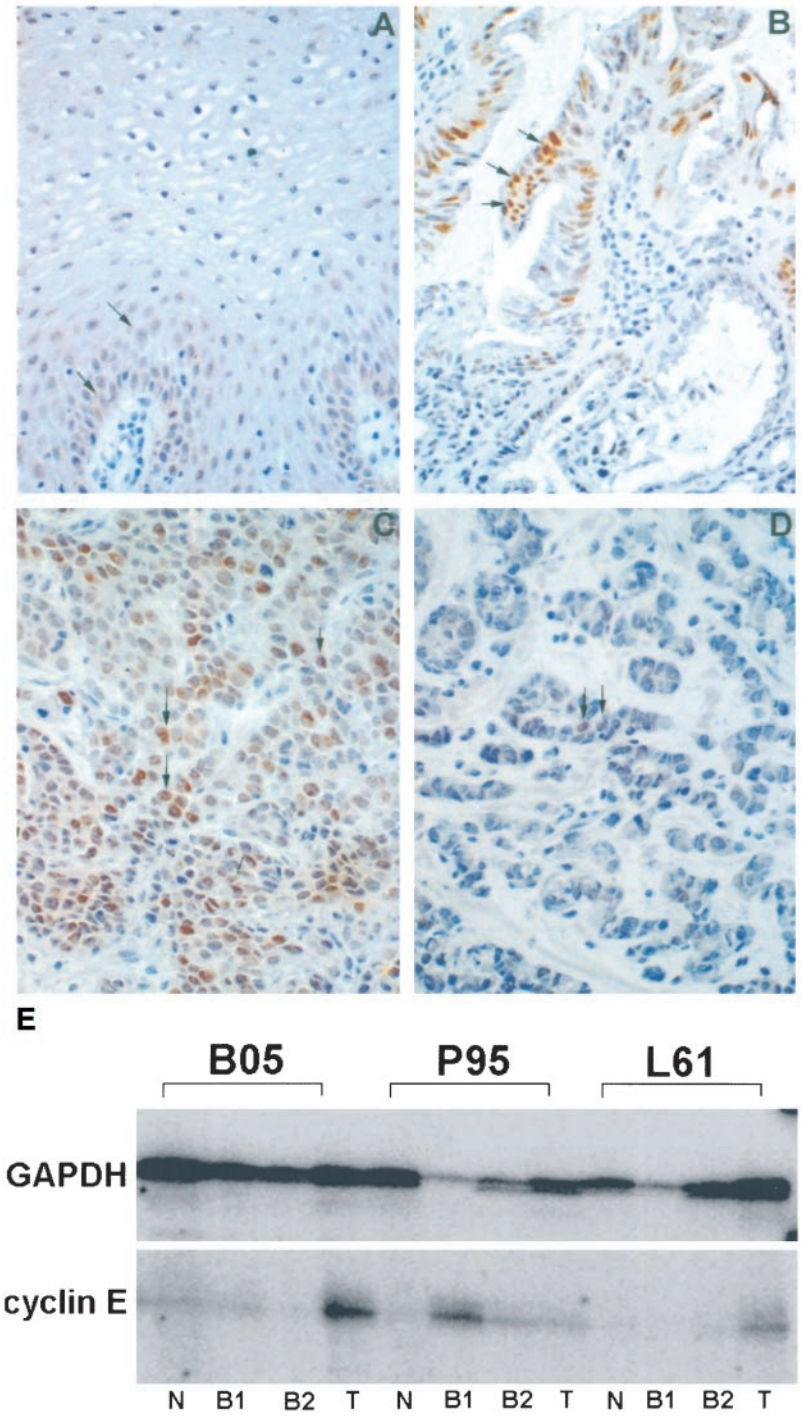

Fig. 6. Immunohistochemical analysis of cyclin E protein expression in human normal esophagus, dysplastic Barrett's tissue, and esophageal adenocarcinomas with or without cyclin E amplification. *A*, normal esophageal epithelia from patient M60; *arrows*, cyclin E expression in the basal area. Cyclin E expression in the upper layers of differentiated cells was not detected. *B*, increased nuclear staining of cyclin E is observed in an area of dysplastic cells in Barrett's mucosa from patient P95 (*e.g.*, arrows). Increased mRNA was also detected in this Barrett's tissue (Fig. 5). *C*, increased nuclear staining of cyclin E (*arrows*) is observed in the tumor from patient M60 which contains the 19q12 amplicon. *D*, cyclin E expression is occasionally seen in the nuclei of tumor cells not containing the 19q12 amplicon as shown in this tumor from patient B42. *E*, QG-PCR analysis was applied to the DNA samples from Barrett's mucosa of patients B05, P95, and L61. The DNA was either microdissected directly from the tissue slides of Barrett's mucosa *(Lanes B1*) or from the whole Barrett's tissues *(Lane B2*). *Cyclin E* amplification is observed in the DNA from the dysplastic Barrett's mucosa of patient P95 (DNA in *Lane B1* of P95 was extracted from the section shown in Fig. *6B*). However, amplification is not observed in Barrett's samples B05 and L61. In Barrett's sample P95, the level of *cyclin E* amplification is lower in the DNA of *Lane B2* as compared with the DNA in the *Lane B1*. This may be because DNA isolated from the whole Barrett's tissue was mixed with many nonamplified cells.

occur because of the fact that amplification can involve a large chromosomal segment (reviewed in Ref. 24). Although programmed gene amplification is observed as a part of a normal developmental progress in lower organisms (25, 26), unscheduled gene amplification in mammals is predominantly observed after exposure to cytotoxic drugs (27) and during tumorigenesis (28, 29). In tumors, genes considered to be the target of the amplification are frequently dominant-acting oncogenes. Identification of amplicons in a tumor genome, therefore, provides a means to discover dominant-acting oncogenes or tumor-related genes. The two-dimensional RLGS protocol is an effective method to achieve this goal as shown in this and other studies (9, 10, 18). The STS-amplification mapping approach that we have implemented efficiently reduces the size of the minimal critical region, thus narrowing the choice of potential candidate genes (10).

Cyclin E is a regulatory subunit of the *cdk2-E complex*, which acts during later G_1 phase into the S phase to ensure cells entry into the S phase (11, 12). Amplification of the *cyclin E* gene underlies the observed up-regulated *cyclin E* mRNA and protein levels in the tumors containing the *cyclin E* amplicon as shown in the present study. Interestingly, the presence of *cyclin E* is required for amplification of another gene, *chorion*, during the *Drosophila* oogenesis (30). *Cyclin E-cdk2* activity is necessary for the growth anchorage-dependence of nontransformed cells (31), and the cytoplasmic displacement of the *cyclin E-cdk2* complex, along with the *cdk* inhibitors, is be-

lieved to contribute to the growth anchorage-independence of transformed cells (32). Anchorage-independence of transformed cells is thought to contribute to tumor invasiveness and metastasis (33). Deregulated *cyclin E* may also be a factor contributing to genomic instability in tumor genomes (34, 35). *Cyclin E* can function like *cyclin D1* in the setting of *cyclin D1* deficiency (36), and deregulated *cyclin D1* was proposed to act like an oncogene and is found to be amplified and overexpressed in many human tumors (37, 38 and reviewed in Ref. 39). *Cyclin E* amplification is observed in ovarian (12.5–21%), colon (9.4%), and gastric (15.6%) carcinomas (13–16). Expression of the cyclin E protein was reported in dysplastic Barrett's mucosa as well as in adenocarcinomas, and increased nuclear staining was associated with regeneration and proliferation of adjacent metaplastic epithelium of erosions or ulcerations in Barrett's (40). Overexpression of the *cyclin E* mRNA and protein was also observed in chemical-induced rat esophageal tumors (41). Taken together, the previous existing evidence and our present results indicate that *cyclin E* is the best candidate selected by the 19q12 amplification event.

Genomic amplification in tumors does not usually contain only the core domain but may extend several hundred kilobases to several megabases flanking the selected gene(s) (42, 43). One potential hypothesis is that the additional coamplified and cooverexpressed gene(s) could be a factor that influences the tumor phenotype and/or clinical outcome. This may explain why patients with the same amplicon in their tumors demonstrate a different clinical prognosis (44). In the present study, at least six other ESTs were shown to be coamplified along with *cyclin E*. Whether the genes coamplified in a given amplicon are just physically linked by sharing the same origin, or whether there is any functional correlation between the coamplified genes in the same amplicon is unclear and will require further study.

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