Gastric Cancers Overexpress DARPP-32 and a Novel Isoform, t-DARPP[1]

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ABSTRACT

Gastric carcinoma is the second most common cause of cancer-related death worldwide. Recently, we have demonstrated that expressed sequence tag AA552509 was frequently amplified and the most consistently overexpressed target at 17q in gastric cancers. Herein, we report that DARPP-32 (dopamine and cAMP-regulated phosphoprotein of M_r 32,000) is the target gene for overexpression of expressed sequence tag AA552509. In addition, we have identified full-length cDNA of DARPP-32 (GenBank accession number AF464196) with 467 bp of additional untranslated mRNA nucleotides upstream of the previously known translation start site in exon 1. Additionally, we have discovered a novel truncated isoform of DARPP-32 that we named t-DARPP (GenBank accession number AY070271), which is also overexpressed in gastric cancers. Using quantitative real-time reverse transcription-PCR, Western blots, and staining of tumor tissue arrays, the two DARPP mRNA transcripts and proteins were overexpressed in gastric cancer cells and exhibited abundant protein overexpression in neoplastic but not normal gastric epithelial cells. DARPP-32 is the only known protein that acts as a protein phosphatase 1 inhibitor or a protein kinase A inhibitor. The novel truncated isoform, t-DARPP, lacks the phosphorylation site related to protein phosphatase 1 inhibition but maintains the phosphorylation site with the protein kinase A inhibitory effect. Our results reveal for the first time the presence of these signaling molecules in human cancer and suggest that they may be important for gastric tumorigenesis.

INTRODUCTION

GC[3] remains a leading cause of cancer-related death worldwide. Moreover, gastroesophageal junction and cardia adenocarcinomas have the most rapidly rising incidence of all visceral malignancies in the industrialized Western world, for reasons that are unclear. However, the critically important genes involved in the development of gastric tumorigenesis remain largely undefined (1, 2). Indeed, no gene has been shown to be consistently and specifically activated in GCs. Several studies have shown that 17q DNA amplifications are frequent in gastric cancers (3–5), but until recently, the targets of amplification have been unclear.

A comprehensive analysis of copy number and expression levels of 636 chromosome 17-specific genes in gastric cancer by using a custom-made chromosome 17-specific cDNA microarray revealed increased copy numbers of 18 genes and ESTs such as *ERBB2, TOP2A, GRB7,* and EST AA552509 that mapped to the 17q12-q21 regions (6). Gene expression analyses on an identical cDNA microarray showed overexpression of only three of the commonly amplified targets (*ERBB2, TOP2A,* and EST AA552509). EST AA552509 was

frequently amplified (45% of studied samples) and was the most consistently overexpressed target (82% of studied samples) in gastric cancers as compared with normal gastric epithelial tissues (6).

Herein, we have characterized EST AA552509 and demonstrated that DARPP-32 (dopamine and cAMP-regulated phosphoprotein of M_r 32,000) and a novel isoform (t-DARPP) are critically altered targets at 17q in gastric cancer.

MATERIALS AND METHODS

Plasmid Transfer. The expression plasmid for FLAG-DARPP-32 was generated by PCR amplification of the full-length DARPP-32 cDNA and cloned in-frame into a modified pcDNA3.1Zeo (Invitrogen) plasmid containing an oligonucleotide corresponding to the FLAG peptide (DYKDDDDK) inserted into the HinDIII and EcoRI sites. The expression plasmid for t-DARPP was generated by PCR amplification of the full-length (including the 5′-untranslated region) t-DARPP cDNA and cloning into the HinDIII and XhoI sites of pcDNA3.1Zeo. AGS cells were transfected using Lipofectamine (Invitrogen) and selected for zeocin resistance. Individual clones were screened by Western blot for expression of the DARPP proteins.

Western Blot. Western blot analysis of DARRP proteins was performed by disrupting cells (either cell lines, primary tissue, or xenografts) directly in SDS sample buffer and boiling for 5 min. DNA was sheared by passage through a 25-gauge needle. Aliquots of lysates were electrophoresed through a 12.5% SDS-PAGE and electroblotted to nitrocellulose. DARPP proteins were detected using a rabbit polyclonal antibody corresponding to residues in the common COOH terminus of DARPP-32 and t-DARPP (H-62; Santa Cruz Biotechnology, Santa Cruz, CA).

Sequencing. High molecular weight genomic DNA was extracted from 40 gastric cancer samples according to classic organic methods for sequence analyses of the DARPP-32 exon 2 and exon 4 that contain the Thr^{34} and Thr^{75} phosphorylation sites, respectively. Each DNA region was amplified using PCR with primer pairs in adjacent introns (sequences are available on request) obtained from GeneLink (Hawthorne, NY). PCR amplifications were performed for 35 cycles in a standard fashion. The DNA products were electrophoresed in 2% agarose gel, and the bands were purified using a QIAquick Gel Extraction kit (Qiagen) as per the manufacturer's instructions. The products were sequenced using the forward amplimer with the Thermo Sequence Radiolabeled Terminator Cycle Sequencing Kit (USB Corp., Cleveland, OH) according to manufacturer's instructions. After performing the sequencing reaction, samples were heated to 80°C for 5 min and loaded on a 6% denaturing polyacrylamide gel for electrophoresis and autoradiography.

Quantitative Real-time PCR. For quantitative real-time PCR, 50 primary and xenografted gastric cancers and 13 normal gastric epithelial samples were collected. All tumors and normal gastric mucosal epithelial tissues were verified by our histopathologist (H. F. F. and C. A. M.). mRNA was isolated using the RNeasy kit (Qiagen, Gmbh, Hilden, Germany). Single-stranded cDNA was synthesized using the Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA). Quantitative PCR was performed using iCycler (Bio-Rad, Hercules, CA), and threshold cycle number was determined using iCycler software version 2.3. Reactions were performed in triplicates, and threshold cycle numbers were averaged. The results for DARPP-32 and t-DARPP were normalized to B-amyloid precursor protein, which had minimal variation in all normal and neoplastic gastric samples that we tested. Overexpression fold was calculated as previously (7). Overexpression fold was calculated according to the formula $2^{(Rt–Et)}/2^{(Rn–En)}$, where Rt is the threshold cycle number for the reference gene observed in the tumor, Et is the threshold cycle number for the experimental gene observed in the tumor, Rn is the threshold cycle number for
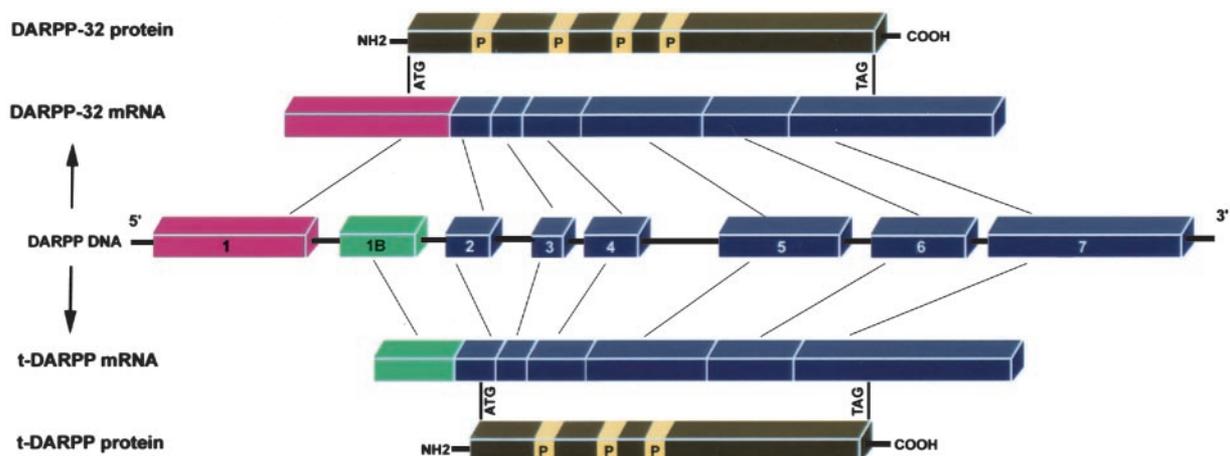
Fig. 1. The genomic structure of DARPP-32 and t-DARPP. The mRNA of DARPP-32, including the untranslated 3′ and 5′ ends, is 1983 bp, and the mRNA of t-DARPP is 1502 bp. DARPP-32 and t-DARPP share an identical sequence from exon 2 to the 3′ end. Exon 1 of t-DARPP is spliced from the intron 1 of DARPP-32. DARPP-32 encodes a protein of 204 amino acids, whereas t-DARPP encodes a 168-amino acid protein. DARPP-32 contains four phosphorylation sites at Thr^{34}, Thr^{75}, Ser^{102}, and Ser^{137}, whereas t-DARPP lacks the Thr^{34} phosphorylation site of DARPP-32.

the reference gene observed in the normal sample, and En is the threshold cycle number for the experimental gene observed in the normal sample. Rn and En values were an average value for the 13 normal samples that were analyzed. The primers used for RT-PCR were obtained from GeneLink, and their sequences are available on request.

IHC. Tissue microarrays containing 0.6-mm cores from 92 different formalin-fixed paraffin-embedded gastric cancer specimens were constructed using a tissue microarrayer (Beecheer Instruments, Silver Spring, MD). Most of the cases had two to three cores of cancer for IHC on the tissue microarrays and on whole tissue sections of completely normal stomach. The avidin-biotin immunoperoxidase method was performed after slides had been placed in citrate buffer and treated with microwave heat for 20 min; the rabbit anti-DARPP antibody (H-62; 1:200 dilution; Santa Cruz Biotechnology) was applied for IHC at room temperature (the antibody had been raised against a recombinant protein corresponding to amino acids 134–195 at the COOH terminus). Immunoreactivity was scored as follows: 1, absent/weak; or 2, moderate/strong in the majority of neoplastic cells.

RESULTS AND DISCUSSION

Our gene expression analyses using a custom-made chromosome 17-specific cDNA microarray identified EST AA552509 as the most consistently overexpressed target in gastric cancers as compared with normal gastric epithelial tissues (6). Sequence assembly of the spliced ESTs nearest to EST AA552509 and guided by the Ensembl assembly and annotation of the human genome sequence[4] determined that this EST corresponded to the 3′-untranslated region of the DARPP-32 gene. By analyses of the nearby ESTs, we detected two ESTs (BF725600 and BF724182) that overlap with the 5′ end of DARPP-32 exon 1. The sequence analysis identified 467 bp of additional untranslated mRNA nucleotides of DARPP-32 upstream of the previously known translation start site in exon 1. Further cloning and sequencing verified the full-length DARPP-32 cDNA sequence (submitted to the GenBank/EMBL database with accession number AF464196). The overexpression of full-length DARPP-32 mRNA in gastric cancers with minimal expression in normal gastric epithelial tissues was confirmed by cloning and sequencing of RT-PCR products that were obtained with specific primers nearest the 5′ and 3′ ends.

The fact that many genes encode multiple protein isoforms (8, 9) triggered us to investigate the possibility that DARPP-32 may have alternative isoforms. The 5′ extension of the cDNA of DARPP-32 was

performed by RACE (Smart RACE system; Clontech) using a gene-specific primer nearest to the 3′ end of DARPP-32 mRNA. Cloning and sequencing of the RACE-amplified cDNA fragments identified a unique sequence that used an alternative first exon located within intron 1 of DARPP-32 to form the first exon for this novel molecule, which we have termed t-DARPP (Fig. 1). The nucleotide and protein sequence of t-DARPP has been deposited in the GenBank/EMBL database with accession number AY070271. Sequence analyses of PCR products obtained after RT-PCR of gastric tumor mRNA with a t-DARPP-specific 5′ primer and a common 3′ primer verified that t-DARPP shares an identical sequence with DARPP-32 from exon 2 to exon 7 but has a unique sequence for its first exon and an alternative translational start in exon 2 of the original DARPP-32 gene (Fig. 1).

To quantify the transcript expression of DARPP-32 and t-DARPP in gastric cancers as compared with normal gastric epithelium, quantitative real-time RT-PCR was performed on 50 primary and xenografted gastric cancers using iCycler (Bio-Rad). The real-time RT-PCR analyses of 50 GCs that included all stages of development [tumor-node-metastasis (TNM) stages 1 to IIIa], histopathology (well differentiated to poorly differentiated, intestinal type, and diffuse type), and location (gastroesophageal junction to antrum) showed consistently high levels of both DARPP-32 and t-DARPP transcript expression, irrespective of the carcinoma subtype, in >80% of gastric cancer samples with minimal expression in normal gastric epithelial tissues (Fig. 2). Moreover, more than half of the tumors exhibited >20-fold higher mRNA expression levels of DARPP-32 and t-DARPP.

We next sought to evaluate protein expression levels for the two DARPP isoforms in gastric cancers. DARPP-32 encodes a 204-amino acid protein, whereas t-DARPP contains an open reading frame that is predicted to encode a protein of 168 amino acids. To determine whether the t-DARPP mRNA could in fact be translated and generate a protein of the predicted size, AGS gastric cancer epithelial cells, which do not express either isoform of DARPP, were stably transfected with expression plasmids encoding either epitope-tagged DARPP-32 or untagged t-DARPP (9). Western blot analyses of these transfected cell lines with a polyclonal antibody specific for the common COOH terminus of DARPP (H-62; Santa Cruz Biotechnology) confirmed the expression of both DARPP proteins of the predicted molecular weights, DARPP-32 at M_r 32,000 and t-DARPP at

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[4] http://www.ensembl.org/.

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A) Multiplex RT PCR on a gel
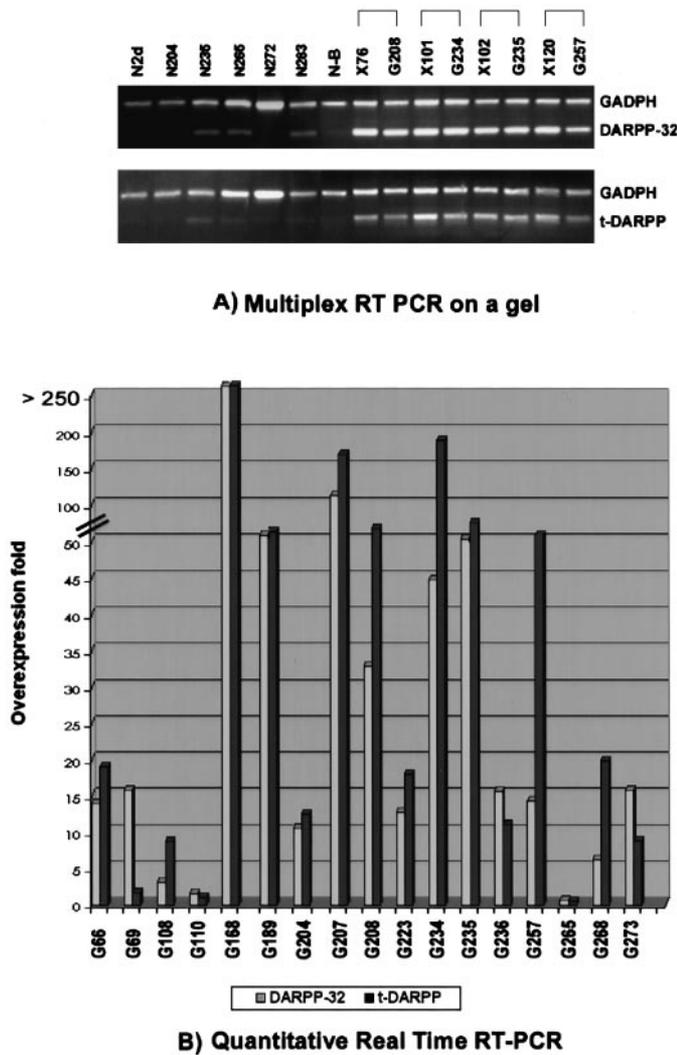

B) Quantitative Real Time RT-PCR

Fig. 2. RT-PCR expression analyses. *A,* a gel image for expression analyses of DARPP-32, t-DARPP, and glyceraldehyde-3-phosphate dehydrogenase at 30 cycles by multiplex RT-PCR. Seven different samples of normal gastric epithelium (*N*), four primary gastric cancers (*G*), and their corresponding xenografts (*X*) are shown. A primary tumor and its corresponding xenograft are in adjacent lanes. *B,* quantitation of expression of DARPP-32 and t-DARPP was performed in 50 gastric cancer samples using iCycler (Bio-Rad). Seventeen representative gastric cancer samples are shown in this figure. The expression of DARPP-32 and t-DARPP was compared with B-amyloid precursor protein gene, which had minimal variation in all normal and neoplastic gastric samples that we tested. More than half of the tumors had at least 20-fold overexpression of DARPP-32 and t-DARPP as compared with normal samples.

M_r 30,000 (Fig. 3). Western blot analyses were also performed on other gastric epithelial cell lines, xenografted gastric tumors, and normal gastric epithelial tissue, and they demonstrated the clear abundant expression of the two DARPP protein bands in gastric cancers, with no detectable expression in the normal gastric epithelial tissue (Fig. 3). The MKN gastric cancer epithelial cell line exhibited protein expression of only full-length DARPP-32. However, using real-time RT-PCR, we detected a low level of t-DARPP mRNA expression in this cell line.

IHC using the same antibody specific for the common COOH terminus of DARPP (Santa Cruz Biotechnology) was used to identify the cell types responsible for the increased expression of the DARPP molecules in gastric cancers and localize the protein at the cellular level. Staining tissue microarrays containing 92 primary gastric cancers demonstrated moderate to strong immunoreactivity in 58 tumors (63%), whereas the remaining 34 tumors showed no or weak positiv-

ity (Fig. 4). The lower frequency of positive cases for DARPP with IHC (63%) as compared with the frequency of mRNA overexpression (80%) may be attributed to the increased sensitivity of PCR-based methods as compared with staining thresholds of IHC as well as the rapid rate of protein degradation in clinical samples. Small levels of immunoreactivity were also seen at the neck region of glands of the normal stomach below the foveolar pits (Fig. 4). All tumors in which the DARPP transcripts were overexpressed >10-fold also exhibited moderate to strong immunoreactivity levels.

DARPP-32 is a neuronally characterized protein that is centrally involved in dopamine-induced signaling pathways in the brain (10, 11). It orchestrates the degree of phosphorylation in a variety of molecular targets in the cell membrane and cytoplasm (11). The phosphorylation control of gastrointestinal malignancies has been recently reported as an important mechanism for some neoplasms (12, 13). The SHP-2 tyrosine phosphatase has been identified as an intracellular target of *Helicobacter pylori* CagA protein (13). Recently, PRL-3 tyrosine phosphatase was associated with metastasis in colorectal cancer (12). To date, DARPP-32 is the only known bifunctional signal transduction molecule that can, depending on which particular amino residue is phosphorylated, function as either a kinase or a phosphatase inhibitor (14). DARPP-32 contains four phosphorylation sites at Thr^{34}, Thr^{75}, Ser^{102}, and Ser^{137} (11). When phosphorylated by PKA at Thr^{34}, DARPP-32 is converted into a potent inhibitor of PP1. Phosphorylation of Thr^{75} by cyclin-dependent kinase 5 converts DARPP-32 into an inhibitor of PKA, reducing its ability to phosphorylate any substrate including DARPP-32 at Thr^{34} (11, 15). Nucleotide sequence analysis of DARPP-32 exons containing the important regulatory phosphorylation sites, Thr^{34} and Thr^{75}, did not demonstrate any point mutations in the 45 GCs that we studied. Notably, t-DARPP encodes a truncated DARPP-32 protein of 168 amino acids that lacks the Thr^{34} phosphorylation site of DARPP-32 but maintains the other three distal sites of phosphorylation (Fig. 1). Hence, t-DARPP is predicted to inhibit PKA, but not PP1. Interestingly, we examined brain tissue and found both DARPP-32 and t-DARPP to be present, although the level of t-DARPP expression was considerably lower (data not shown). In gastric cancer, both isoforms were abundantly overexpressed. This finding suggests that both DARPP-32 and t-DARPP may be involved in signaling pathways that are important for gastric cancer. Although changing kinase activity alone would result in the altered phosphorylation state of a protein, changing kinase and phosphatase activity in opposite directions would result in a more
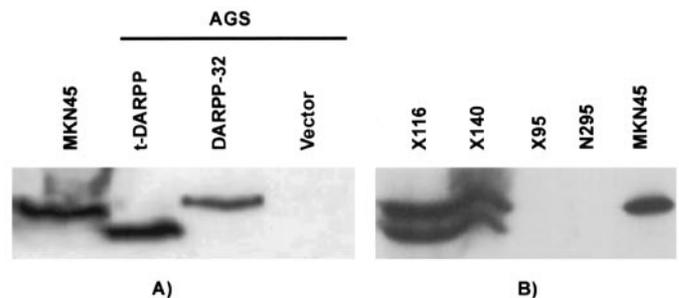

Fig. 3. DARPP isoform protein expression. Two gastric cancer cell lines (AGS and MKN), three gastric cancers (X116, X140, and X95), and a normal gastric epithelia sample (N295) were studied (*A*). Whole cell lysates from the MKN cell line and AGS cells stably transfected with pcDNA3.1FLAG-DARPP32, pcDNA3.1t-DARPP, or the empty pcDNA3.1zeo vector (Invitrogen) were separated by SDS-PAGE and analyzed for DARPP protein expression by Western blot with a COOH-terminal DARPP-32 antibody (Santa Cruz Biotechnology). *B,* freshly isolated xenografted gastric tumor samples (X116, X140, and X95), freshly biopsied normal gastric epithelium (N295), and MKN45 cells were lysed directly in boiling SDS sample buffer and analyzed for DARPP isoform expression as described in *A*. The *top band* corresponds to the M_r 32,000 DARPP-32 protein, whereas the *bottom band* corresponds to the M_r 30,000 t-DARPP protein.
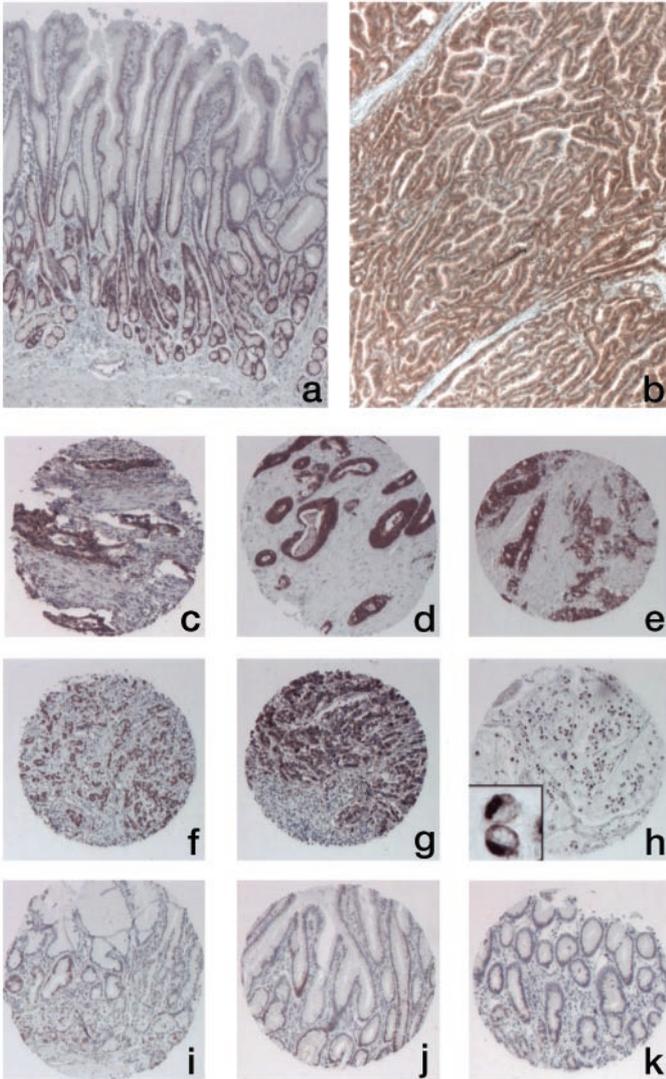
Fig. 4. DARPP-32 IHC in human gastric tissue and GCs. *a,* in sections of benign gastric mucosa, moderately strong immunostaining for DARPP-32 is present only in the epithelial cells of the neck of glands. Foveolar epithelium on the surface of the mucosa and in the foveolar pits shows no staining or weak staining (original magnification, ×40). *b,* this staining pattern is typical of GCs, showing diffuse strong immunoreactivity (original magnification, ×40). *c−g,* representative samples of GCs from the tissue microarrays, with a spectrum of glandular differentiation, all showing diffuse moderate to strong immunoreactivity for DARPP-32. Note that the intervening stromal and inflammatory cells show no staining (original magnification, ×100). *h,* example of signet ring cell carcinoma with strong DARPP-32 immunoreactivity (original magnification, ×100). The *inset* shows both an apparent nuclear and cytoplasmic staining pattern (original magnification, ×1000). The abundant intracytoplasmic mucin vacuoles in this variant of adenocarcinoma show no staining. *i−k,* representative samples of normal gastric mucosa in a tissue microarray showing focal immunoreactivity in the deep glands and foveolar neck regions (original magnification, ×100). The tissue microarrays were also immunostained for cytokeratin (AE1/AE3), which showed a similar level of staining between benign gastric epithelium and malignant carcinoma, as a control of retained tissue immunogenicity (data not shown).

profound effect. The possibility that DARPP-32 and t-DARPP may interact with each other for synergistic regulation of the balance between protein phosphorylation and kinase activity needs to be investigated.

In brain, DARPP-32 is a potent inhibitor of PP1 (11, 14) and can mediate phosphorylation and activation of MAPK and CREB (16). Recently, PP1 and other protein phosphatases that reverse the action of cyclin-dependent kinases are emerging as important cell cycle-regulatory enzymes (17–19). Many oncogenes have been shown to encode proteins that transmit mitogenic signals upstream of the MAPK pathway (20, 21). Phosphorylation of CREB is known to generate signals that inhibit apoptosis (22, 23). However, to understand the potential role of DARPP-32 in human cancer, additional studies and biological assays are required.

In summary, we have identified DARPP-32 as a frequently overexpressed gene in GCs. Additionally, we have identified a novel isoform of DARPP-32, t-DARPP, which is also overexpressed in gastric cancer. Our current knowledge of DARPP-32 signal transduction pathways is largely associated with dopaminergic signaling in neurons, where it is known to influence the phosphorylation and activation of MAPK and CREB and can inhibit PP1. Because DARPP-32 and t-DARPP are overexpressed in gastric cancers, these proteins may provide an important survival advantage to neoplastic cells. Additional studies are under way to investigate the biochemical mechanisms through which DARPP-32 and t-DARPP influence gastric tumorigenesis.

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