

Gastric Cancers Overexpress S100A Calcium-binding Proteins¹

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Abstract

Serial analysis of gene expression provides quantitative and comprehensive expression profiling in a given cell population. In our efforts to define the genes overexpressed in carcinoma of the stomach, we performed serial analysis of gene expression analyses on dissected neoplastic and normal gastric epithelia. We identified 91,334 expressed tags, including 26,633 that were unique. The 20 most up-regulated genes ($P < 0.01$) in gastric cancer (GC) compared with normal gastric epithelia included several keratins that are specific for epithelial cells such as keratin 6A, 13, and 17. Interestingly, five calcium-binding proteins (S100A2, S100A7, S100A8, S100A9, and S100A10) were overexpressed. Quantitative real-time PCR on primary GC samples demonstrated overexpression of S100A2 in 18 of 20 tumors (90%). The other calcium-binding proteins were overexpressed in 25–45% of the GC samples that we studied. Our results indicate that S100A proteins may be important for gastric tumorigenesis. Additional investigations are required to elucidate the biological role of calcium-binding proteins in cancer.

Introduction

GC³ is one of the most common malignancies worldwide and is the second most frequent cause of cancer-related death (1). Moreover, gastroesophageal junction tumors have the most rapidly rising incidence of all visceral malignancies in the Western world, for reasons that are not completely clear (2, 3). Although several large-scale gene expression studies have been performed (4–7), there remains a discrepancy of these reports as a result of using different forms of cDNA or oligonucleotide arrays. To date, GC has not been studied using SAGE, an important technology that produces an unlimited comprehensive profile of gene expression in a given cell population, representing the entire transcriptome (8, 9). The most attractive feature of SAGE is its ability to evaluate the expression pattern of thousands of genes in a quantitative manner without prior sequence information. This method has been valuable in studies of several tumor types including adenocarcinomas of the colon (10, 11), prostate (12), pancreas (13), ovary (14), and breast (15). In this study, we report, for the first time, SAGE analysis of GC, and we document the overexpression of several members of the S100A calcium-binding protein family.

Materials and Methods

SAGE. High-quality total RNA (500 μ g) was extracted using RNeasy kit (Qiagen, GmbH, Hilden, Germany) from a dissected gastric adenocarcinoma and from normal gastric epithelium. The GC arose in the gastroesophageal

junction of the stomach and was a moderately differentiated stage III tumor. The normal gastric epithelium consisted of a pool of four different normal gastric epithelia biopsy samples that came from four patients who were referred for endoscopy for dyspepsia or for screening before gastric bypass surgery for obesity. All normal samples had histologically normal-looking mucosa. Importantly, histopathology examination confirmed that none of the normal samples had any areas of inflammation or necrosis. The GC sample had the least amount of necrosis and the fewest contaminating normal inflammatory and stromal cells. Care was taken to avoid areas with normal gastric epithelium. Areas rich in tumor were then cut from the frozen block for extraction of RNA. Hence, the tumor selected for SAGE analysis was estimated to consist of >80% tumor cells. All samples were collected after patient consent in accordance with the Human Investigation Committee regulations at the University of Virginia.

SAGE was performed using SAGE protocol version 1.0e, June 23, 2000, which includes a few modifications of the standard protocol (8). The *Nla*III restriction site used in library construction defined all SAGE tags. A detailed protocol and schematic of the method is available.⁴ Two thousand clones were sequenced for each case by the Cancer Genome Anatomy Project. Tags were extracted from the raw sequence data using SAGE2000 analysis software version 4.12 developed by Dr. Kenneth Kinzler. This software automatically delimits each tag from its adjacent tag and abutting linker sequence and counts the number of copies of each tag (thus, of each mRNA species). The resulting tag libraries were compared with UniGene cluster to SAGE tag “reliable” mapping database,⁵ and the statistical analyses were performed using the SAGE software. Significant changes in levels of expression ($P < 0.01$) were determined. Sequence data from our SAGE libraries are publicly available⁶ with Geo Accession Omnibus numbers GSM757 for GC and GSM784 for normal stomach. The Cancer Genome Anatomy Project maintains the SAGE public database for gene expression in human cancer (16).

Quantitative Real-Time PCR. For quantitative real-time PCR, 20 primary GCs and 13 normal gastric epithelial samples were collected. All tumors were dissected from frozen tissue specimens and had at least 80% tumor cell content with the least possible amount of contaminating necrotic, normal inflammatory, and stromal cells. The normal gastric mucosal epithelial tissues were carefully examined and were devoid of any inflammatory or necrotic contaminating cells. The collected tumors ranged from well-differentiated to poorly differentiated, stages I to IIIa, and there was a mix of intestinal and diffuse-type tumors. The mRNA was isolated using RNeasy kit (Qiagen). Single-stranded cDNA was synthesized using 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 triplicate, and threshold cycle numbers were averaged. Gene-specific primers for S100A2, S100A7, S100A8, S100A9, and S100A10 were designed. The primers used for RT-PCR were obtained from GeneLink (Hawthorne, NY), and their sequences are available on request. The results were normalized to β -amyloid precursor protein, which had minimal variation in all normal and neoplastic gastric samples that we tested. Fold overexpression was calculated according to the formula $2^{(Rt - Et)/2^{(Rn - En)}}$ as described previously (17), 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 the reference gene observed in the normal

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³ The abbreviations used are: GC, gastric cancer; SAGE, serial analysis of gene expression; RT-PCR, reverse transcription-PCR.

⁴ http://www.sagenet.org/sage_protocol.htm.

⁵ <ftp://ftp.ncbi.nih.gov/pub/sage/map/Hs/Nla3/>.

⁶ <http://www.ncbi.nlm.nih.gov/SAGE/>.

sample, and R_t is the threshold cycle number for the reference gene observed in the tumor sample. R_n and E_n values were an average for the 13 normal samples that were analyzed. Samples that had expression levels ≥ 5 -fold were considered overexpressed.

Results

SAGE. Sequence analyses of SAGE libraries produced a total of 91,334 expressed tags. These tags were compared with Unigene cluster release that identified 26,633 unique SAGE tags. Sequence data from our SAGE libraries are publicly available⁶ with Geo Accession Omnibus numbers GSM757 for GC and GSM784 for normal stomach.

Among the 20 most up-regulated genes ($P < 0.01$), 5 calcium-binding proteins (S100A2, S100A7, S100A8, S100A9, and S100A10) were overexpressed (Table 1). Other genes such as keratins (keratin 6A, 13, and 17), small proline-rich protein 3, and annexin A1 were also overexpressed (Table 1).

Quantitative Real-Time RT-PCR. Real-time RT-PCR confirmed our SAGE findings for S100 proteins and demonstrated that these proteins are differentially overexpressed in GC. The most frequently overexpressed S100A protein was S100A2 (90%). Other S100 proteins were less frequently overexpressed (S100A7, 25%; S100A8, 45%; S100A9, 30%; and S100A10, 35%). Due to the small number of cases analyzed, association with a particular histopathological subtype was not possible to ascertain. The real-time RT-PCR expression data are shown in Fig. 1.

Discussion

SAGE is a novel technology that produces a comprehensive profile of gene expression in a given cell, which represents that cell population's entire transcriptome (9). The most attractive feature of SAGE, unlike hybridization microarray analyses, is its ability to evaluate the expression pattern of thousands of genes in a quantitative manner without prior sequence information. Although several gene expression microarray studies have been performed in GC, they have used different platforms that varied in the number and identity of genes printed on them (4–7).

In this analysis, we report, for the first time, the use of SAGE technology to compare the GC cell transcriptome with that of normal gastric mucosa. The normal gastric expression was evaluated on a pool of normal gastric mucosal samples prepared from endoscopic biopsies. The quality of normal samples to compare findings in the

cancers is important to ensure accurate analysis. The use of endoscopic biopsies to obtain the normal samples has the unique ability to enrich for epithelial cells. Endoscopic biopsy samples are relatively free of stromal elements, with epithelial cells comprising the overwhelming majority of cells.

We have found that several members of the S100A protein family are overexpressed in GC. By RT-PCR, S100A2 was overexpressed in almost all primary gastric tumors that we studied. Other S100A proteins such as S100A7, S100A8, S100A9, and S100A10 were also overexpressed at varying levels in our tumors. To date, 20 members of this protein family have been identified that are known to regulate intracellular processes such as cell growth and motility, cell cycle regulation, transcription, and differentiation (18).

S100A2 has been described as a potential tumor suppressor gene that is down-regulated in a number of tumors such as lung, breast, and head and neck carcinomas (19–21). On the other hand, tumors such as ovarian cancer melanoma and epithelial tumors of the skin have shown overexpression of S100A2 (22–24). In melanoma, S100A2 was moderately to highly expressed in early lesions, whereas none of the metastases expressed S100A2 mRNA (22). The notion that S100A2 was overexpressed in $>90\%$ of our gastric tumors of various stages suggests that S100A2 overexpression may be an early tumorigenic event rather than a tumor progression marker in gastric carcinogenesis.

S100A7, S100A8, S100A9, and S100A10 were differentially expressed in 25–45% of our gastric tumors. S100A7 has been identified in association with the transition from preinvasive to invasive breast cancer (25). In addition, S100A7 is considered as a potential tumor biomarker for the noninvasive follow-up of patients with urinary bladder squamous cell carcinoma. There is sparse information regarding the role of S100A8, S100A9, and S100A10 in cancer. Typically, S100A8 and S100A9 are known to be differentially expressed at sites of acute and chronic inflammation (18). However, recent reports indicate that S100A8 and S100A9 are overexpressed during skin carcinogenesis and at the invasive margin of colorectal carcinoma (26, 27). S100A10 is an annexin 2 protein ligand (28). Few data are available in literature about this protein. To our knowledge, there is only one report about this gene in human cancer, in which S100A10 is overexpressed in human renal cell carcinoma (29).

Using SAGE analysis, we have identified transcripts likely to be important in gastric tumorigenesis. Moreover, our findings add to the developing literature about the possible role of S100A calcium-bind-

Table 1 Transcripts most elevated in GC ($P < 0.01$)

Tag sequence	Tag count		Unigene ID	Description
	Normal	GC		
GTGGCCACGG	0	334	112405	S100 calcium-binding protein A9
TACCTGCAGA	0	284	100000	S100 calcium-binding protein A8
TTTCTGTGCTC	0	266	139322	Small proline-rich protein 3
TTTCTGTGCTC	0	44	81134	Interleukin 1 receptor antagonist
TAATTTTTGC	0	211	273321	Differentially expressed in hematopoietic lineages
AGAAAGATGT	0	137	78225	Annexin A1
AAAGCACAAG	0	103	334309	Keratin 6A
AGCAGATCAG	1	95	119301	S100 calcium-binding protein A10
CCCAAGCTAG	0	94	76067	Heat shock 27-kDa protein 1
ATCCTGTGCTG	1	87	2621	Cystatin A (stefin A)
GAGCAGCGCC	0	75	112408	S100 calcium-binding protein A7
AAAGCGGGGC	0	73	74070	Keratin 13
TTGAATCCCC	0	62	112341	Protease inhibitor 3, skin-derived (SKALP)
TGGCCCCAGG	0	56	268571	Apolipoprotein C-I
AAGATTGGTG	0	53	1244	CD9 antigen (p24)
CTTCCTTGCC	0	53	2785	Keratin 17
GAGAAATCGT	0	53	234734	Lysozyme (renal amyloidosis)
CGACCCACG	1	51	169401	Apolipoprotein E
ACCGCCGTGG	1	46	68877	Cytochrome b-245, α polypeptide
GATCTTTGG	0	46	38991	S100 calcium-binding protein A2

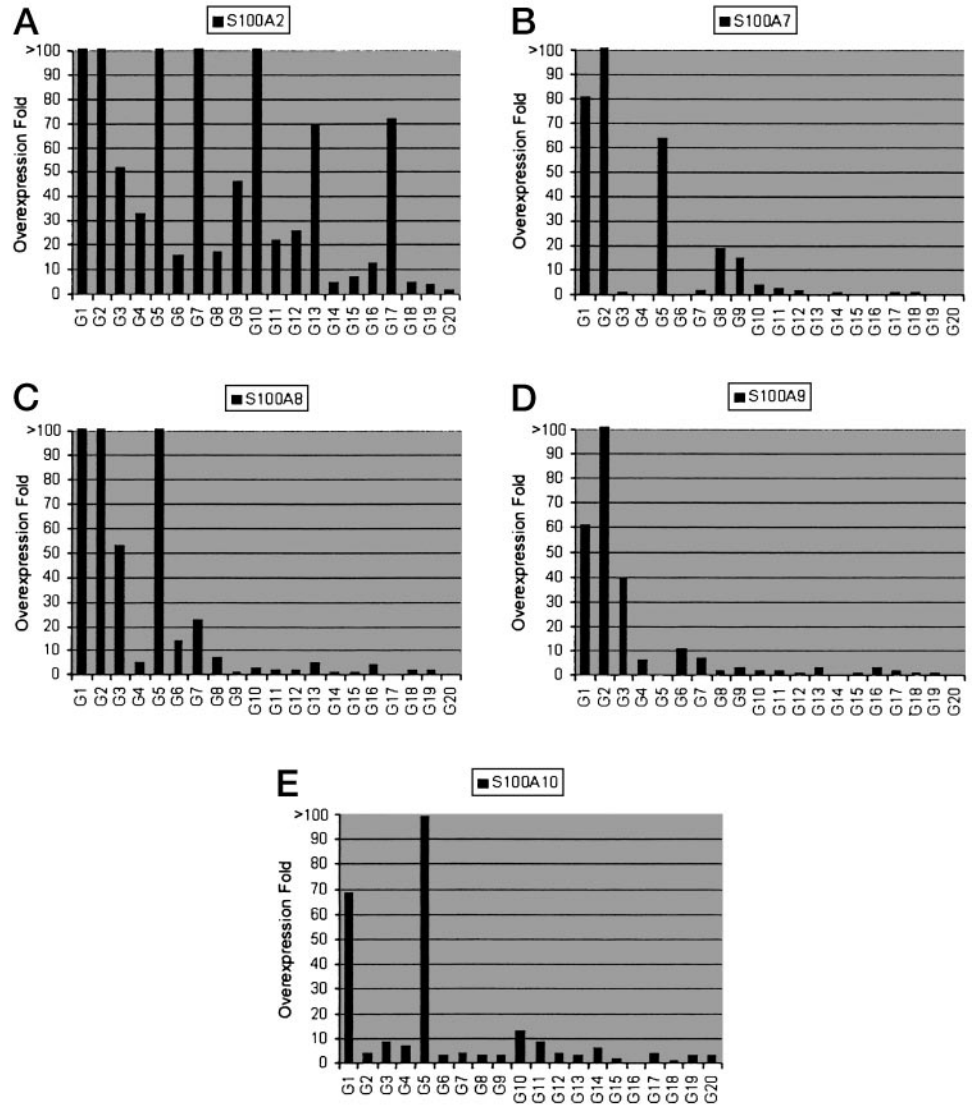


Fig. 1. Quantification of mRNA expression of S100A calcium-binding proteins S100A2, S100A7, S100A8, S100A9, and S100A10 was performed on 20 GC samples using iCycler (Biol-Rad). The expression of S100A calcium-binding protein genes was compared with β -amyloid precursor protein gene, which had minimal variation in all normal and neoplastic gastric samples that we tested.

ing proteins in cancer. The fact that several members of the S100A protein family were differentially overexpressed suggests, for the first time, that these proteins may be critical for the development and/or progression of GC. Additional analyses are needed to elucidate the exact role of S100A proteins in gastric tumorigenesis.

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