Expression profile of the GA733 gene family in colorectal cancer: correlation with clinicopathological parameters

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ABSTRACT. *GA733-1/-2/-3* genes have been detected in various types of cancer, although their role has not been fully clarified. *GA733-2* and *GA733-1* have been correlated with lymph node metastases in laryngeal cancer and liver metastases, respectively. Only a few studies have elucidated the mechanisms regulating *GA733-1/-2* expression and their effect on colorectal cancer. Therefore, the expression pattern and the role of the aforementioned molecules in colorectal carcinogenesis were evaluated in this study. Tissue samples were obtained from 40 patients with colorectal cancer with no liver metastases. *GA733-1/-2* mRNA levels were evaluated by quantitative real-time polymerase chain reaction. *GA733-1/-2* gene expression in noncancerous/cancerous tissues was also correlated with clinicopathological parameters. The *GA733-1* mRNA levels were very low; however, the *GA733-1* mRNA transcripts were higher in cancerous tissues than in

normal tissues (median ratio, 0.004391/0.00093; range, 0.000001-0.025139/0.000001-0.007761), respectively (P = 0.012). *GA733-2* gene expression was higher in noncancerous tissues than in cancerous tissues (median ratio 273.31/115.64; range, 65.24-1,486.41/11.58-1,189.14; P = 0.0000195). Lower *GA733-2* expression in cancer tissues appeared to correlate with lymph node metastases (P < 0.05). *GA733-1* gene expression was significantly higher in cancerous samples; conversely, the *GA733-2* mRNA levels were higher in noncancerous tissues, and were significantly correlated with lymph node perforation in colorectal cancer (P < 0.05). Therefore, *GA733-1/-2* mRNA expression levels appear to be a potential predictive marker of tumorigenesis.

Key words: GA733 family; Adenocarcinoma; Colorectal cancer; RT-PCR

INTRODUCTION

Based on their molecular structure, GA733 proteins belong to the group of cell adhesion molecules (CAM) (Chothia and Jones, 1997). The cell-to-cell communication, facilitated by the CAM family, plays a vital role in a variety of dynamic cell functions, including cell-movement, differentiation, and division (Basak et al., 1998). The GA733 family contains three different protein members, two of which, GA733-1 and GA733-2, have been studied extensively in animal models and cell cultures (Chong and Speicher, 2001). Each member of the GA733 family has been shown to express different functional characteristics.

The *GA733-1* gene is also known as *TACSTD2*, *TROP2*, *M1S1*, or *EGP*-1, while the *GA733-2* gene is also known as *TACSTD1* (Calabrese et al., 2001), *Ep* (epithelial)-*CAM*, or *KSA*. The *GA733-1* gene shows 49% homology with *GA733-2* (Dayhoff et al., 1983), and both genes encode type-1 transmembrane glycoproteins (35.7 and 38 kDa, respectively) (Strnad et al., 1989; Klein et al., 1990; Linnenbach et al., 1993). The extracellular region of the members of this protein family differs distinctly and consists of at least three easily recognizable structures. The first is an N-terminal domain consisting of a hexamer of cysteine amino acid residues, containing a bisulfate bond characteristic of a GA-733 type 1 protein. The second domain also has a cysteine hexamer and the related bisulfate bonds to thyreoglobulin type-1-A, and is concerned with the GA-733 type 2 protein. The third structure is a free poly-cysteine structure with no apparent homologous structures to other non-GA-733 family proteins, which is characteristic of the third member of the GA-733 protein member, GA-733 type 3 (Chong and Speicher, 2001).

The *GA733-1* gene is located at human chromosome 1p32 (Calabrese et al., 2001). The retroposition of *GA733-2* mRNA into chromosome 1 results in the intron-less *GA733-1* gene. The molecule encoded by the *GA733-1* gene is slightly different compared to that encoded by *GA733-2* (Bergsagel et al., 1992; Linnenbach et al., 1993). The GA733-1 molecule is expressed in the epithelial tissue, and is a cell surface receptor that participates in the control of cancer cell development (Fornaro et al., 1995). The exact function of the GA733-1 protein, however, is unknown. It has been proposed that GA733-1 plays a role in the growth of normal or transformed cells (Alberti et al., 1992), which explains the high expression of the *GA733-1* gene in human trophoblast cells as well as ovular and bladder

cancer cells (Lipinski et al., 1981; Fradet et al., 1984; Miotti et al., 1987; Alberti et al., 1992). GA733-1 is also responsible for gelatinous drop-like corneal dystrophy (Tsujikawa et al., 1999).

The *GA733-2* gene encodes the Ep-CAM, and consists of a total of nine exons located on chromosome 2p21 (Chong and Speicher, 2001; Calabrese et al., 2001). Amino acid sequences encoded by exon 1 correspond to a signal peptide sequence, while exons 2 to 6 encode sequences that form the extracellular domain. The transmembrane region is encoded by exon 7, and the 15-amino acid portion of the cytoplasmic domain, including a cluster of six positively charged amino acids, is encoded by exon 8. Exon 9 encodes the remaining 13 amino acids of the cytoplasmic domain, the stop codon, and the 3'-untranslated region (Balzar et al., 1999). GA733-2 plays an important role in the regulation of cell adhesion (Zanna et al., 2007). A previous study has shown that GA733-2 inhibits the invasion of tumor cells in transfected CT-26 mouse colon carcinoma cells (Basak et al., 1998). The same study also reported that the mouse homologue of GA733, mEGP (a homotypic CAM with 82% amino acid sequence identity with GA733), expressed by transfected mouse CT-26 cells (mEGP-negative), caused a significant decrease in cell growth *in vitro* and inhibited metastasis *in vivo*. The mechanism responsible for these two phenomena remains to be elucidated. High levels of GA733-2 are also expressed in various types of cancer (Momburg et al., 1987; Klein et al., 1990), including breast (Tandon et al., 1990; Spizzo et al., 2002), lung (Piyathilake et al., 2000), and colorectal cancer (Girardet et al., 1986). However, there is little information regarding the mechanisms modulating GA733-1/-2 expression in colorectal cancer or the possible differences from normal epithelial cells.

In this study, we have investigated *GA733-1* and *GA733-2* mRNA expression in noncancerous and cancerous tissues, and their correlation with the demographic and histological parameters of the patients with colon cancer and no liver metastases. We have attempted to provide evidence for the differential expression of GA733-1 and GA733-2 in colorectal carcinoma cells from patients with no liver metastases.

MATERIAL AND METHODS

Tissue samples

Cancerous and noncancerous tissues were obtained from 40 patients with colorectal cancer who underwent surgical resection at the Department of Surgery of the University Hospital of Larissa in Greece. Forty patients with colorectal cancer were chosen from the preoperative diagnosis, conducted via screening tests or a thorough evaluation of the medical history. Clinical examination, diagnostic imaging tests, including X-rays, computed tomography scans, magnetic resonance imaging, or rectal ultrasounds, and a colonoscopy, sigmoidoscopy, and a biopsy were performed. The study was approved by the Scientific Committee of the University Hospital of Larissa (Greece), and is in accordance with the 1964 Declaration of Helsinki. Informed consent was obtained from all patients.

A minimum margin of 10 cm between the healthy and cancerous tissue was maintained for all samples to ensure the integrity of the healthy tissue samples. All tissue samples were obtained by a pathologist less than 20 min after tumor resection, and prior to its preservation in formalin.

RNA extraction and cDNA preparation

RNA was extracted from each sample using TRIzol reagent, as detailed in a previous report (Rio et al., 2010). RNA quality was evaluated by agarose gel electrophoresis and absorption spectrophotometry at 260/280 nm. Each RNA sample (1 µg) was reverse transcribed into cDNA using AMV reverse transcriptase (First-Strand cDNA Synthesis Kit, Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer protocols.

Quantitative real-time polymerase chain reaction (PCR) assessment of *GA733-1/-2* mRNA expression

GA733-1 and *-2* mRNA transcripts were estimated by developing and evaluating the real-time fluorescence PCR assays in a Roche LightCycler 1.5 (Roche Diagnostics). cDNA (1:10 dilution; 2 µL) was utilized for each reaction. A separate PCR conducted in the same assay estimated the expression of the human porphobilinogendeaminase (*h-PBGD*) gene, which was used as the reference gene based on a literature review (Higuchi et al., 1993; Wittwer et al., 1997; Sun et al., 2010).

The amplification mixture consisted of 2 µL 10X reaction buffer (FastStart DNA Master HyProbe, Roche Diagnostics), 4 mM $MgCl_2$ for both the *GA733-1* and *-2* genes, 0.6 µM of each oligonucleotide primer, 0.2 µM of each oligonucleotide probe, and 2 µL template cDNA (1:10), in a final volume of 20 µL. Samples were amplified using the following reaction conditions: initial denaturation at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 0 s, annealing at 58°C for 60 s (for both mRNA transcripts), and extension at 72°C for 6 s, and a final extension at 40°C for 30 s. The temperature transition rate was 20°C/s. All experiments were conducted in triplicate.

The DNA oligonucleotide primers and hybridization probes were synthesized by TIB Molbiol GmbH (Berlin, Germany). The adjacent ends of the hybridization probes were labeled with fluorophores. The 5'-end of the first probe was labeled with the acceptor fluorophore LC Red 640, and the 3'-end of the second probe was labeled with the donor fluorescein (FITC-3FL). The 5'-labeled probes were 3'-phosphorylated to block polymerase extension during PCR. The nucleotide sequences of the primers and hybridization probes that were used are as follows: *GA733-1*; primers: sense, 5'-ATGAGCGCCCCCAAGAA-3'; antisense: 5'-GCTGCT CGTAGTGCACGG-3'); probes: 5'-GCCCACCGAGTTCACGCACC-3'FL; 5'-LC640-GCAC ACCGACGTCTGGTTGCACTG-PH. *GA733-2*; primers: sense, 5'-AAGAAAATGGACCTG ACAGTAA-3'; antisense, 5'-CCATCTCCTTTATCTCAGCC-3'; probes: 5'-ATCAACATAAT AAATTAAAGTTTGACCAGG-3'FL; 5'-LC640-TCCAgATCCAgTTgTTCCCCA-PH. PCR products for the *GA733-1* and *-2* genes were 534 and 384 bp long, respectively.

Statistical analysis

The results were assessed statistically using the R Statistical Programming Language (version 2.12.2, Licenses from GNU General Public License v2.0). All data are reported as the median value, and was analyzed statistically. Differences between the groups were estimated using the Student χ^2 test. A P value <0.05 was considered to indicate statistical significance for all tests.

RESULTS

Patient characteristics

This study included 41 patients of Caucasian origin (20 male and 21 female), with a median age of 67 years (range: 52-85). Ten patients (10/40, 25%) were subjected to right colectomy; 2 patients (2/40, 5%) underwent left colectomy, while 29 (29/40, 70%) were subjected to a low anterior resection-sigmoidectomy. Forty members of the patient group were diagnosed with adenocarcinoma, while one was diagnosed with adenoma; since adenoma has different biological behavior from adenocarcinoma, this patient was excluded from the study (Table 1).

Table 1. Demographic and clinical characteristics of the patient group.

Total (N = 40)	
Gender (male/female)	19/21
Age [years, means (SD)]	67 (52-85)
Histological grade	
Good	3
Moderate	27
Poor	10
TNM stage	
Tumor	
T1	3
T2	7
T3	27
T4	3
Node	
N0	27
N1	5
N2	8
Metastasis	
M0	40
M1	0
Lymph node metastasis	
Absent	27
Present	13

T1: tumor invades the submucosa; T2: tumor invades the muscularis propria; T3: tumor invades through the muscularispropria into the subserosa or into non-peritonealized pericolic tissues; T4: tumor directly invades other organs or structures and/or perforates visceral peritoneum; N0: no regional lymph node metastasis; N1: metastasis to one to three regional lymph nodes; N2: metastasis to four or more regional lymph nodes; M0: no distant metastasis; M1: distant metastasis.

GA733-1 and *GA733-2* mRNA expression in tumor and normal tissue samples

Quantitative real-time PCR was performed on 40 clinical sample-pairs (tumor-normal tissue samples) to determine the expression of both *GA733-1* and *GA733-2* (Table 2). *GA733-1* mRNA expression was low (or nil) in normal (median ratio *GA733-1/h-PBGD*: 0.00093, range: 0.000001-0.0025139) and significantly higher ($P < 0.012$) in tumor samples (median ratio *GA733-1/h-PBGD* 0.004391, range: 0.000001-0.007761). *GA733-2* mRNA transcript was detected in all 40 adenocarcinoma samples. The median ratio *GA733-2/h-PBGD* mRNA level was 273.31 (range: 65.24-1,486.41) in noncancerous tissue, and 115.64 (range, 11.58-1,189.14) ($P = 0.0000195$) in cancerous tissue. Twenty-three of forty (57.5%) patients showed differential expression of the *GA733-2* mRNA between noncancerous and cancerous tissues.

Specifically, noncancerous tissues expressed *GA733-2/h-PBGD* mRNA transcripts at a median ratio of 329.62 (range: 82.19-1,486.41), as opposed to the median ratio of 89.675 (range: 11.58-724.03) observed in cancer patients (P = 0.0000002384). Similar levels of *GA733-2* mRNA transcripts were detected in the cancerous and noncancerous tissue samples of 15 patient-pairs (15/40, 37.5%; median ratio *GA733-2/h-PBGD*: 242.34 and range: 65.24-844.58) *vs* median ratio of 202.45 and range: 40.30-697.32 in noncancerous and cancerous tissues, respectively; P = 0.02954). In contrast, three of our patients (7.3%) showed overexpression of the *GA733-2* gene in cancerous tissue samples (median ratio *GA733-2/h-PBGD*: 405.69 and range: 309.20-1,189.14 in cancerous tissue *vs* median ratio of 82.56 and range: 75.53-292.47 in noncancerous tissue samples; P = 0.25) (Figure 1).

Table 2. Quantification of the *GA733-1/-2* mRNA levels in the adenocarcinoma cases included in this study (cancerous and noncancerous tissue).

Tissues	*GA733-1/h-PBGD*	*GA733-2/h-PBGD*
Cancerous	Median ratio: 0.004391 Range: 0.000001-0.007761	Median ratio: 115.64 Range: 11.58-1,189.14
Noncancerous	Median ratio: 0.00093 Range: 0.000001-0.0025139	Median ratio: 273.31 Range: 65.24-1,486.41

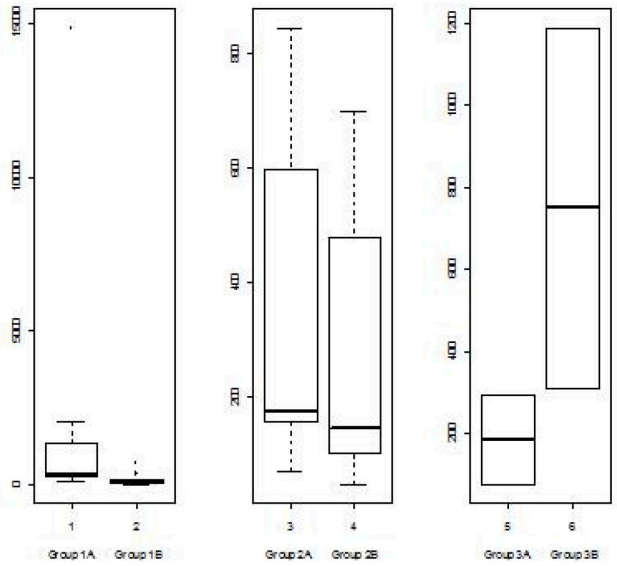

Figure 1. Median relative mRNA expression levels of *GA733-2/h-PBGD* in normal and cancer tissue samples. Group 1, cases with overexpressed *GA733-2* in (A) noncancerous tissues, compared to (B) cancerous tissue samples; Group 2, cases with equal expression of *GA733-2* in both (A) noncancerous and (B) cancerous tissue samples; and Group 3, cases with higher levels of expression of *GA733-2* mRNA in (B) cancerous tissues, compared to (A) noncancerous tissue samples. The data in the box plot graph are reported as boxes representing statistical values. The boundary of the box closest to zero indicates the 25th percentile. A line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90 and 10th percentiles, respectively. The dotted line indicates the mean value, and outlying points display the extreme values. The differences in *GA733-2* expression between subgroups A and B were found to be statistically significant in both Group 1 (P = 0.0000002384) and Group 2 (P = 0.02954).

Furthermore, any possible correlations between the expression patterns of *GA733-1/-2*, and the clinicopathological data were determined. The following parameters were evaluated within this cohort: gender, histological grade, tumor, nodes, and metastasis (TNM) stage, and lymph node metastasis. The extremely low *GA733-1* gene expression could not been correlated to other clinicopathological parameters. On the other hand, the patients were further divided into three subgroups based on the *GA733-2* mRNA expression levels between noncancerous and cancerous tissues. These were the patients overexpressing *GA733-2* mRNA in noncancerous tissues (group 1), cases with equal *GA733-2* mRNA levels in both noncancerous and cancerous tissue samples (group 2), and cases with overexpression of *GA733-2* mRNA transcripts in cancerous tissues (group 3) (Table 3). Statistically significant associations were not identified between *GA733-2* expression patterns and any of the aforementioned parameters, except lymph node perforation (P = 0.008668) and the N stage of the disease (P = 0.0343).

Table 3. Correlation between *GA733-2* mRNA expression levels in noncancerous and cancerous tissues in adenocarcinoma colon samples and the clinicopathological parameters.

Variables	Groups of patients with different *GA733-2/h-PBGD* mRNA levels			
	Group 1*	Group 2*	Group 3*	P > 0.05
Gender				
Male	11	6	2	
Female	12	9	1	
Histological grade				0.7329
Good	2	1	0	
Moderate	14	10	3	
Poor	7	3	0	
TNM stage				
Tumor				0.1658
T1	0	3	0	
T2	5	2	0	
T3	15	9	3	
T4	3	0	0	
Node				0.0343
N0	10	14	3	
N1	5	0	0	
N2	8	0	0	
Metastasis				>0.05
M0	23	14	3	
M1	0	0	0	
Lymph node metastasis				0.008668
Absent	10	14	3	
Present	13	0	0	

*Group 1: cases with overexpression of *GA733-2* in noncancerous tissues, compared to cancerous tissue samples; Group 2: cases with equal expression of *GA733-2* in both noncancerous and cancerous tissue samples, and Group 3: cases with higher levels of expression of *GA733-2* mRNA in cancerous samples, compared to noncancerous tissue samples.

DISCUSSION

Development of malignant tumors is characterized by the ability of the tumor cell to overcome cell-cell adhesion and invade the surrounding tissue (Perl et al., 1998). Many different cell adhesion molecules are implicated in human carcinogenesis. The expression of some adhesion molecules is restricted, whereas the expression of some others is induced, during the transition from normal cells to highly malignant tumor cells (Gao et al., 2013).

The *GA733* gene family consists of different genes encoding proteins that share a 49%

peptide homology (Dayhoff et al., 1983). Based on their molecular structure, these proteins belong to the group of CAMs (Chothia and Jones, 1997). However, their role in healthy cells as well as carcinogenesis remains to be elucidated.

The initial studies of gene expression in colon cancer cell lines and animal models did not report the expression of *GA733-1* in either cancerous or noncancerous samples (Fornaro et al., 1995; Tsujikawa et al., 1999; Bogenrieder and Herlyn, 2003). On the other hand, some studies have reported the high expression of *GA733-1* mRNA in human cancerous and noncancerous samples, including human ovarian and bladder cancer, and human trophoblast samples (Lipinski et al., 1981; Fradet et al., 1984; Miotti et al., 1987; Fornaro et al., 1995). In this study, the *GA733-1* mRNA levels were evaluated by quantitative real-time PCR in patients without intercurrent hepatic (or other) cancer metastases. The *GA733-1* expression levels were very low in a majority of cancerous and noncancerous patient cells. Furthermore, no statistically significant correlation was observed between the *GA733-1* gene expression and other clinicopathological parameters. Our results are further supported by a recent independent study utilizing two cancer-cell lines, Hct-116-ltp (for low tumorigenic potential) and Hct-116, where the cDNA expression profiles of the two cell lines (using microarrays) showed that one of the largest differences between the two cell lines was a 17-fold decrease in *GA733-1* (*TROP-2*) mRNA levels in Hct-116-ltp cells compared to that in the Hct-116 cells. In addition, Hct-116-ltp cells formed fewer tumors, which were (on average) <25% the size of the tumors formed in Hct-116 cells, when measured 4 weeks after xenografting (Wang et al., 2008). Moreover, the high expression levels of *GA733-1* in breast cancer cells were correlated with lymph node metastasis (Huang et al., 2003). Therefore, our results are in accordance with the results of previous studies, wherein the *GA733-1* gene was proposed to be a driver of tumor progression, and a cancer-related gene correlated with the biological aggressiveness and poor prognosis of colorectal cancer (Ohmachi et al., 2006; Fang et al., 2009; Trerotola et al., 2013).

Previous studies in experimental models (Basak et al., 1998) and human samples have suggested a possible relation between continuous *GA733-2* expression and stimulation of cancer cell growth (Zanna et al., 2007). The level of expression of the *GA733-2* gene has been associated with poor prognosis of the disease (Joo et al., 2005). Based on the study of laryngeal carcinoma, the absence of *GA733-2* expression at the primary tumor site was revealed to be correlated with nodal metastasis (Takes et al., 1997; Zhang et al., 1997). In this study, the decrease in *GA733-2* mRNA expression in colon carcinoma tissue was shown to be correlated with the presence of lymph nodes. This could be attributed to the methylation of CpG islands, which has been previously implicated in *GA733-2* gene silencing in tumor cells (Tai et al., 2007).

A statistically significant correlation was drawn between the reduced *GA733-2* mRNA expression and lymph node perforation, based on the expression profile of *GA733-2* in our samples, and the differences between noncancerous and cancerous colorectal tissues. We hypothesized that the reduction in *GA733-2* mRNA expression in colon cancer cells increases the potential of nodal perforation near the primary non-metastatic cancer site, a phenomenon that has also been observed in laryngeal cancer cells (Takes et al., 1997). This could be explained by the fact that the protein product of the *GA733-2* gene is a cell adhesion molecule (Litvinov et al., 1994; Chothia and Jones, 1997); non-expression or under-expression of the *GA733-2* gene results in low levels of adhesion protein expression, which in turn leads to an increase in cancer cell motility and metastasis potential. Therefore, this increases the potential escape of tumor cells, as well as the possibility of lymph node perforation and successful metastasis (Zhang et al., 1997).

The results of *GA733-1/-2* mRNA measurements in adenocarcinoma colon cancer

cells obtained in this study suggested that *GA733-2* gene expression could be used as an early, sensitive, and reliable predictive marker for metastasis and poor prognosis in early-stage colon adenocarcinoma. This was because of the strong correlation observed between the reduction of *GA733-2* mRNA levels and lymph node perforation. Further studies using a larger series of biological markers in colon cancer may result in the development of significant diagnostic tools for better clinical diagnosis.

Conflicts of interest

The authors declare no conflict of interest

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