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Original contribution

GAEC1 and colorectal cancer: a study of the relationships between a novel oncogene and clinicopathologic features☆

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Summary *GAEC1* is a novel gene located at *7q22.1* that was detected in our previous work in esophageal cancer. The aims of the present study are to identify the copy number of *GAEC1* in different colorectal tissues including carcinomas, adenomas, and nonneoplastic tissues and characterize any links to pathologic factors. The copy number of *GAEC1* was studied by evaluating the quantitative amplification of *GAEC1* DNA in 259 colorectal tissues (144 adenocarcinomas, 31 adenomas, and 84 nonneoplastic tissues) using real-time polymerase chain reaction. Copy number of *GAEC1* DNA in colorectal adenocarcinomas was higher in comparison with nonneoplastic colorectum. Seventy-nine percent of the colorectal adenocarcinomas showed amplification and 15% showed deletion of *GAEC1* ($P < .0001$). Of the adenomas, 90% showed deletion of *GAEC1*, with the remaining 10% showing normal copy number. The differences in *GAEC1* copy number between colorectal adenocarcinoma, colorectal adenoma, and nonneoplastic colorectal tissue are significant ($P < .0001$). *GAEC1* copy number was significantly higher in adenocarcinomas located in distal colorectum compared with proximal colon ($P = .03$). In conclusion, *GAEC1* copy number was significantly different between colorectal adenocarcinomas, adenomas, and nonneoplastic colorectal tissues. The copy number was also related to the site of the cancer. These findings along with previous work in esophageal cancer imply that *GAEC1* is commonly involved in the pathogenesis of colorectal adenocarcinoma.

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1. Introduction

Colorectal cancer (CRC) is among the most common malignancies found in developed countries, and the mortality rates for this cancer are generally high. It is known that CRC develops after a series of genetic mutations, corresponding to the histologic progression from normal colonic mucosa to adenoma (dysplasia), adenocarcinoma, or other subtype and finally metastases [1]. The most common histologic type of CRC is adenocarcinoma [2]. Currently, surgery is the main option for treatment, with adjuvant chemotherapy and radiotherapy for patients who have developed certain specific subtypes of CRC [3]. It has been proven that research on molecular pathways of cancers directly contributes in advanced care for patients with CRC by more accurately refining prognosis and selecting the most appropriate adjuvant therapy for individual patients with CRC [4]. For example, we have documented that several markers, including p53, p16, p21, aurora kinase, survivin, and telomerase activities in the pathogenesis, appear either as cancer markers or as prognostic markers in CRC [5-9].

GAEC1 (gene amplified in esophageal cancer 1) is a novel gene located at 7q22.1, detected in our work of comparative DNA fingerprinting with inter-simple sequence repeat–polymerase chain reaction (ISSR-PCR), which identified a series of amplifications and deletions in esophageal cancer [10]. A frequently amplified sequence of 357 base pairs (bp) was identified in the ISSR-PCR bands for esophageal cancer, and that sequence showed exact homology to an expressed sequence tag sequence (293 bp) at 7q22 [10]. Our work on *GAEC1* indicates that it has tumorigenic potential approximately equivalent to the *RAS* gene family, and overexpression of *GAEC1* is a critical step for cancer transformation in esophageal squamous cell carcinoma [11]. *GAEC1* has a full-length messenger RNA of 2052 bp and encodes a nuclear protein of 109 amino acids. Expression of *GAEC1* has been detected in a range of normal tissues including esophagus, small intestine, and colon [11]. *GAEC1* is not the only gene that has been identified within the 7q22 locus, which has been found to be amplified in many cancers [12-15]. This itself suggests that genes within the 7q22 locus have an important role in the carcinogenesis of varying malignancies. At present, however, there is a fundamental lack of information about the molecular role of this novel gene in CRC. In addition, despite a certain amount of research examining the amplification of the region containing *GAEC1*, copy number changes or amplification of the gene specifically has not yet been studied in CRC. Although our earlier work examined the correlation of *GAEC1* copy number and clinicopathologic parameters in esophageal cancer, the results of that research may not be completely applicable to CRC. Recent molecular studies have shown that DNA copy number changes within cancers can be a marker for cancer sensitivity to targeted anticancer therapy, so examination of the prevalence of *GAEC1* copy number change may yield good starting data for similar exploration [16]. The aims of this study were to identify the copy number of *GAEC1* in different colorectal tissues including cancers, adenomas, and nonneoplastic tissues and characterize any links to pathologic factors.

2. Materials and methods

2.1. Tissue samples

The patients who were chosen for this study had resection for primary colorectal carcinomas, colorectal adenomas, and nonneoplastic colorectal tissue between January 2004 and December 2006 in Queensland, Australia. Ethical approval of this study has been obtained from the Griffith University human research ethics committee. The patients were consecutively chosen and with no selection bias. The resected tissues were fixed in 10% formalin and embedded in paraffin wax. Histologic sections were cut and stained for hematoxylin and eosin for light microscopic examination. These sections were reviewed by the authors. Both conventional and mucinous adenocarcinomas were included in the study. Nonneoplastic samples including surgically removed diverticular disease and inflammatory diseases were recruited to act as control tissue. The carcinomas were graded according to the World Health Organization criteria [17].

The pathologic features of patients with colorectal adenocarcinomas and adenomas were analyzed. These included the histologic variants and pathologic grades by assessing the cellular morphology and extent of mucin production. Presence of lymph node metastases at the time of surgery was also recorded. The carcinomas were staged according to TNM classification [18].

After reviewing the tissue samples, colorectal tissues from 259 patients (153 men and 106 women) were selected for the study. These included 144 colorectal adenocarcinomas, 31 colorectal adenomas, and 84 nonneoplastic tissues. The mean age of the patients was 65 (range, 12-93) years. For the colorectal adenocarcinomas and colorectal adenomas, the site and size (maximum length) of the lesion were recorded. One tissue block from each of these tissues was used for DNA extraction. The tissue block was checked to ensure that it contained a representative cancer area.

2.2. Extraction of DNA

To separate cancer tissue from the surrounding morphologically benign tissue, microdissection was performed. Microdissection was done under the guidance of hematoxylin- and eosin-stained slides from the selected paraffin blocks to ensure that no significant surrounding cell contamination had occurred. Our previous work in breast cancer has shown that this method is effective in allowing discrimination of different gene expression in cancer and immediately surrounding tissue [19]. For each selected

block, ten 10-μm sections were cut for DNA extraction. DNA was extracted and purified with Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's protocol (Qiagen Pty Ltd, Hilden, NRW, Germany). Purity of DNA was obtained by checking the optical density 260/280 ratio. Concentration of DNA was also noted in nanograms per microliter. All DNA extracted was of good quality for the PCR experiment.

2.3. Primers

The primer sets for amplification of *GAEC1* (GenBank accession no. AC005088) and HBD (GenBank accession no. NM_000519) genes were designed using the Primer3plus interface (http://frodo.wi.mit.edu/). The primer sets chosen were 5′-CCTCAGGGAAGAAGCAAGTT-3′ and 5′-TCTTGCATGGTGCCAGTT-3′ with an amplicon of 121 bp for *GAEC1* and 5′-TGGATGAAGTTGGTGGTGAG-3′ and 5′-CAGCATCAGGAGTGGACAGA-3′ with an amplicon of 229 bp for hemoglobin delta (*HBD*), which acted as a reference gene.

2.4. Real-time quantification PCR

Real-time quantitative PCR was performed for detection of *GAEC1* gene copy number changes. IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was used to run real-time quantification PCR.

PCR was performed in a total volume of 20 μL reaction mixture containing 10 μL iQ SYBR Green Supermix (Bio-Rad), 1 μL of each 5-μmol/L primer, 2 μL of DNA at 50 ng/μL, and 6 μL of 0.1% diethylpyrocarbonate-treated water. In the last tube, 2 μL of diethylpyrocarbonate-treated water was added as a nontemplate control. All the samples (unknown and standard) were run in duplicate and accompanied by a nontemplate control. Thermal cycling conditions included initial denaturation in 1 cycle of 3 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 30 seconds at 60.5°C, and 30 seconds at 72°C. Melting curve analysis was also performed using 81 cycles of 30 seconds increasing from 55°C. The melting curves of all final real-time PCR products were analyzed for determination of genuine products and contamination by nonspecific products and primer dimers. To ensure that the correct product was amplified in the reaction, all samples were also separated by agarose gel electrophoresis on a 20-g/L gel. For each tissue sample, the PCR reaction was performed in duplicate to increase the reliability of the results.

2.5. PCR efficiency and data analysis

A standard curve was constructed from a set of known concentrations of complementary DNA generated from universal human reference RNA (Stratagene, Cedar Creek, TX) for the determination of PCR efficiency. The dilution series consisted of concentrations of 120, 100, 80, 60, 40, 20, and 10 ng/μL. Δ Ct for *GAEC1* and HBD was determined for each concentration, and the slope of the line of best fit was calculated from a plot with Δ Ct on the y-axis and the log of total complementary DNA on the x-axis. Efficiencies for the *GAEC1* and HBD genes were found to be comparable.

For analysis, the ratio was calculated by normalizing the copy of GAEC1 in each sample by dividing obtained *GAEC1* Ct by the Ct obtained for HBD (normalization = Ct *GAEC1* [sample]/Ct HBD [sample]). Normalized values for each duplicate sample were averaged to give the final data used. The fold change in the target gene for the results of quantitative amplification was also calculated for each sample using $2^{-\Delta\Delta Ct}$ method [20], where $\Delta\Delta Ct = (Ct_{GAEC1} - Ct_{HBD})_{cancer} - (Ct_{GAEC1} - Ct_{HBD})_{normal}$.

Ratios were expressed as inverse ratios (1/ratio) to reorient changes in ratio to reflect actual behavior of *GAEC1* (ie, increased ratio = increase in *GAEC1* copy number). A ratio of more than the average ratio of noncancer samples was considered as gain of *GAEC1* copy number/amplification, whereas a ratio less than the reference range for noncancer samples was considered as loss of *GAEC1* copy number/deletion. Normalized final data (inverse Ct ratio) were analyzed using 1-way analysis of variance to determine if there was a significant difference of copy number between colorectal tissue groups. The χ^2 test was used for analysis of categorical variables.

All the data were entered into a computer database. Statistical analysis was performed using the Statistical Package for Social Sciences for Windows (version 17.0; SPSS Inc, Chicago, IL). Significance level was taken at $P < .05$.

3. Results

3.1. Identification of *GAEC1* in colorectal tissues

GAEC1 was detectable in all samples used for quantification. After performing real-time PCR, a 122-bp fragment was observed for *GAEC1* and a 225-bp fragment was observed for HBD (Fig. 1). The mean copy number ratio for *GAEC1* in colorectal adenocarcinoma, colorectal adenoma, and nonneoplastic colorectal tissue (control tissue) was 1.07 (range, 0.88-30), 0.91 (range, 0.69-1), and 0.98 (range, 0.89-1.08), respectively. The characteristics of the colorectal adenocarcinoma and adenoma patients and their relationship with *GAEC1* copy number variations have been described in Table 1.

3.2. Gain of *GAEC1* copy number in CRCs

In colorectal adenocarcinomas, 79% (n = 114) showed some degree of amplification (relative to control tissue) and 15% (n = 21) showed loss of *GAEC1* copies. Six percent (n =

9) of the adenocarcinoma did not show any change in copies compared with the control tissues. The copy number level of DNA in adenocarcinomas was higher in comparison with control tissues and colorectal adenomas ($P < .0001$). A comparison of the relationships between the tissue types has been illustrated in Table 2. Average *GAEC1* copy number in colorectal adenocarcinomas was 3.5-fold higher than that of control tissues and approximately 48-fold higher than that of adenomas (Fig. 2).

Higher *GAEC1* copy number was noted in colorectal adenocarcinomas from men compared with those from women. However, the difference was not statistically significant (mean ratio = 1.08 versus 1.05, $P = .07$). *GAEC1* copies were significantly higher in adenocarcinomas located in distal colorectum (including descending colon, sigmoid colon, and rectum) compared with proximal colon (mean ratio = 1.07 versus 1.04, $P = .03$). The DNA copy number of *GAEC1* in the cancer population was found to have no relationship with the age, size, grade, histologic subtypes, or TNM staging of the colorectal adenocarcinomas.

3.3. Loss of *GAEC1* copy number in colorectal adenomas

The *GAEC1* copy number in colorectal adenomas was lower (ie, showing deletion) in comparison with control tissues ($P \leq .0001$). The data also showed that *GAEC1* has reduced copy number in the vast majority of adenomas. Of the 31 adenomas, 90% (n = 28) showed reduced and 10% (n = 3) showed no change in *GAEC1* copies compared with control tissues. *GAEC1* copy number showed an average decrease of 13.7-fold in adenoma compared with control tissues. The copy number of *GAEC1* in adenoma population was found to have no relationship with the age and sex of the patient, nor with the site and size of adenoma.

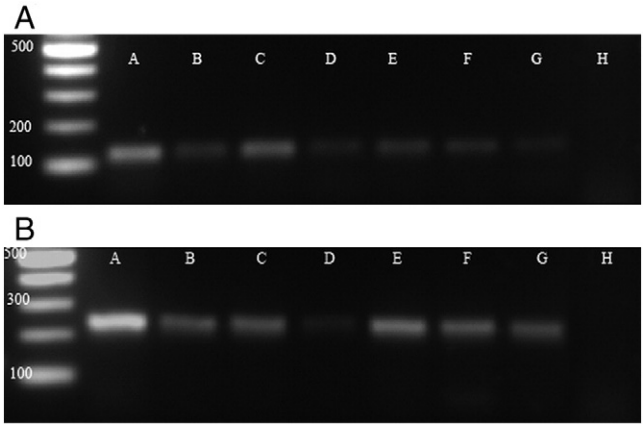

Fig. 1 A, *GAEC1* amplification bands in CRCs after real-time PCR in 2% agarose gel. *GAEC1* fragments of 122 bp were noted in all samples except for the water control (H). One hundred–base pair DNA ladder (M) was used for comparison. B, Control gene (HBD) amplification bands in CRCs after real-time PCR in 2% agarose gel. HBD fragments of 225 bp were noted in all samples except for the water control (H). One hundred–base pair DNA ladder (M) was used for comparison.

4. Discussion

In our previous study, we have documented that *GAEC1* showed amplification and overexpression in primary esophageal cancers. Overexpression of *GAEC1* in mouse fibroblasts also caused enhanced cell proliferation, foci formation, and colony formation in soft agar, and was comparable with the transforming action of H-ras. Injection of *GAEC1*-transfected cells into athymic nude mice formed undifferentiated sarcoma, indicating that *GAEC1* is a transforming oncogene [11]. In this study, we first demonstrated that colorectal adenocarcinomas have higher *GAEC1* copy number than nonneoplastic tissue and adenoma, implying that the expression of the gene is also higher in colorectal adenocarcinoma. This reflects the previously observed transforming power of this oncogene in the development of esophageal cancer and indicates that it may play a similar role in CRC.

The higher copy number of *GAEC1* in cancers compared with that of control tissue and adenomas was highly significant in this study. The high prevalence of *GAEC1* amplification in colorectal adenocarcinomas indicates that it may be an important part of the pathogenesis of many colorectal adenocarcinomas, perhaps in concert with other oncogenic mutations. It is possible that, as the cancer develops, amplification of *GAEC1* may occur simply as a side effect of other mutations associated with the progression, perhaps of other nearby genes. However, because *GAEC1* overexpression alone has been observed to increase cellular proliferation and colony formation in cell lines, it is likely that amplification of *GAEC1* offers a direct growth advantage to cancers and occurs as part of the overall progression of the disease, although it may not necessarily be involved in the early stages of pathogenesis. A small proportion of adenocarcinoma showed either normal *GAEC1* copy number or deletion. Given the likelihood that *GAEC1* functions as a cancer initiator, these samples may represent cancers that have been initiated by a separate oncogenic pathway or where the up-regulation of *GAEC1* is achieved by other molecular events.

In this study, we also did not find any relationship between *GAEC1* amplification and patient age, sex, histologic subtype, pathologic grade, and stage or size of the cancer. In part, this would agree with its previously observed effect in cell lines because, as a basic-type oncogene, it is likely to be a cancer initiator more than a modulator of later phenotype. However, our results indicated that *GAEC1* is more often amplified in cancers located in the distal part of the colorectum compared with cancers in proximal colon. This implies different molecular

Table 1 Clinicopathologic features and GAEC1 genetic changes

Characteristics	n (%)	Amplification	Deletion	No change	*P* value
Age					
≤60	43 (30%)	32 (74.4%)	7 (16.3%)	4 (9.3%)	.22
>60	101 (70%)	82 (81.3%)	14 (13.7%)	5 (5.0%)	
Sex					
Male	80 (55.6%)	67 (83.7%)	8 (17.0%)	5 (6.3%)	.07
Female	64 (44.4%)	47 (73.4%)	13 (20.3%)	4 (6.3%)	
Size					
≤50 mm	114 (79.2%)	92 (80.7%)	15 (13.2%)	7 (6.1%)	.46
>50 mm	30 (20.8%)	22 (73.3%)	6 (20%)	2 (6.7%)	
Site					
PC	50 (34.7%)	37 (74.0%)	9 (18.0%)	4 (8.0%)	**.03**
DC	94 (65.3%)	77 (82.0%)	12 (12.7%)	5 (5.3%)	
Histologic subtypes					
CA	125 (86.8%)	98 (78.4%)	19 (15.2%)	8 (6.4%)	.83
MA	19 (13.2%)	16 (84.2%)	2 (10.5%)	1 (5.3%)	
Histologic grade					
Well	14 (9.7%)	9 (64.3%)	4 (28.6%)	1 (7.1%)	.66
Moderate	112 (87.5%)	91 (81.2%)	14 (12.5%)	7 (6.3%)	
Poor	18 (12.5%)	14 (77.8%)	3 (16.7%)	1 (5.5%)	
Stage					
Stage 1	38 (26.4%)	31 (81.6%)	4 (10.4%)	3 (8.0%)	.72
Stage 2	57(39.6%)	45 (79.0%)	8 (14.0%)	4 (7.0%)	
Stage 3	44 (30.6%)	36 (81.8%)	7 (16.0%)	1 (2.2%)	
Stage 4	5 (3.5%)	2 (40.0%)	2 (40.0%)	1 (20.0%)	

Abbreviations: PC, proximal colon; DC, distal colorectum; CA, conventional adenocarcinoma; MA, mucinous adenocarcinoma. Bold values indicate a clinical significance of $P < .05$.

pathogenesis for *GAEC1* in proximal and distal parts of the colorectum, perhaps based on pretransformation utilization of *GAEC1*. These observations are in line with previous studies showing that clinical features and molecular pathways are often different in both proximal and distal CRCs and may be the result of more general effects rather than one specific to *GAEC1* [6,8,9,21,22]. For example, p53 expression in ordinary colorectal adenocarcinoma and p16 expression in mucinous adenocarcinoma were noted more often distal CRCs [8,23]. Aurora kinase expression and telomerase activity were also reported more in distal colorectum [6,9]. Our similar findings for the *GAEC1* oncogene indicate that these differential responses in the proximal and distal colorectum may have implications in specifically targeted therapeutic practice in the future.

The data generated from colorectal adenomas in this study showed that *GAEC1* has reduced copy number in the great majority of adenomas. The average 13.7-fold deletion in adenomas compared with control tissues was highly significant ($P < .001$). This indicates that the role of *GAEC1* may have significant differences in colorectal adenomas compared with adenocarcinomas, perhaps related to modulation of *GAEC1* action by interaction with regulatory genes.

7q22 deletion has been reported in a benign smooth muscle tumor of uterus [24,25]. It is possible that *GAEC1* deletion (and related down-regulation) in colorectal

Table 2 Genetic changes of GAEC1 in colorectal tumours

Type	n	Amplification	Deletion	No change	*P* value
Carcinoma	144	114 (79.1%)	21 (14.6%)	9 (6.3%)	<.001
Adenoma	31	0 (0.0%)	28 (90.3%)	3 (9.7%)	<.001

NOTE. *P* value: compared with nonneoplastic colorectal tissue.

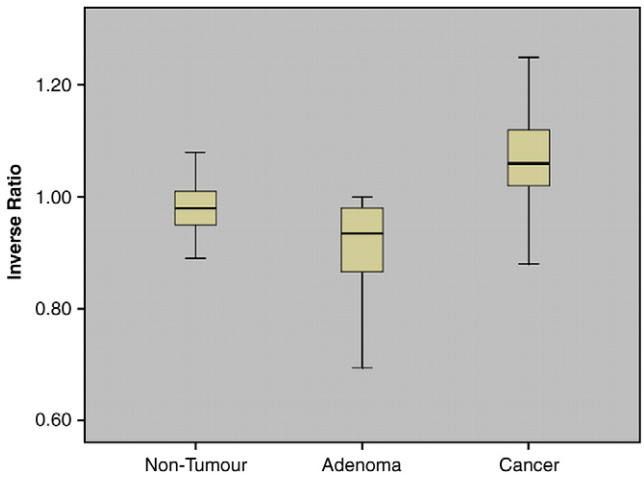

Fig. 2 *GAEC1* amplification levels in different colorectal samples. Higher level of amplification ratio (inverse ratio) was obtained for cancer samples compared with the adenoma and noncancer samples.

adenomas may offer some initial advantage to further transformation. This may be due to the coincident loss of a gene with cancer-suppressing properties nearby in 7q22, followed by reamplification of *GAEC1* later. It may be that this loss is specific to certain cancer cells within the adenoma; and these provide some necessary support to those cells retaining full *GAEC1* complement, perhaps through release of inhibitory pathways mediated through *GAEC1*. This raises the possibility that such cells may remain present within later adenocarcinomas at very small concentrations, in a manner undetectable by this style of analysis. It is also possible that such cells cluster into specific regions of the cancer, leading to potential skewing of the data if these regions make up significant proportions of the cancer that were selected or missed because of the sectioning of tissue. In addition, it is possible that adenomas experiencing *GAEC1* deletion may rarely or never progress to adenocarcinoma, representing a successful change to senescence.

Genomic amplification of cancer-related genes contributes to cancer pathogenesis by activating protooncogenes [26]. Our present study demonstrates that *GAEC1* is amplified in 79% of colorectal adenocarcinomas. This is the first study showing significant correlation of *GAEC1* with clinicopathologic features. The previous study of *GAEC1* in esophageal cancer did not detect any significant relationships between *GAEC1* alterations and site of the cancer or benign neoplasm. In this study, significant amplification of *GAEC1* was noted between different colorectal tissues including nonneoplastic tissue, adenoma, and adenocarcinoma. Our results also support the notion that *GAEC1* is a putative oncogene and that its amplification contributes to the neoplastic phenotype of CRC. Future research into *GAEC1* in CRC should examine the expression of the gene and localization of the protein. It will also be advantageous to begin dissecting the precise function of GAEC1 to search for linkages to known oncogenic pathways and systems.

5. Conclusions

Copy number of *GAEC1* was studied in different colorectal tissues. *GAEC1* amplification was significantly increased in colorectal adenocarcinoma compared with normal tissue and adenoma and was associated with cancer site, potentially representing changes to the significance of *GAEC1* in different areas of the colon. *GAEC1* deletion was noted in colorectal adenoma, which may indicate different molecular pathogenesis of *GAEC1* in colorectal adenoma and adenocarcinomas. The findings coupled to previous work on the gene imply that *GAEC1* is involved in the pathogenesis of colorectal adenocarcinoma. The high level and prevalence of amplification of *GAEC1* in adenocarcinoma and its correlation with sites of cancer may also be important for the development of gene-targeting therapies for CRC. Further research into the precise mechanisms of action of *GAEC1* should be encouraged to improve understanding of the role of this novel oncogene.

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