Title: Expression of *GAEC1* mRNA and protein and its association with clinical and pathological parameters of patients with colorectal adenocarcinoma

Running head: GAEC1 expression in colorectal adenocarcinoma

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Abstract**Aim**

GAEC1 (*Gene amplified in esophageal cancer 1*) is an oncogene with key regulatory roles in the pathogenesis of oesophageal and colorectal carcinomas. The aim of this study was to investigate expression profiles and clinicopathological significance of *GAEC1* mRNA and protein in patients with colorectal carcinomas.

Method

Matched cancer and non-cancer fresh frozen tissues were prospectively collected from 80 patients diagnosed with colorectal adenocarcinoma (39 men and 41 women). The tissues were sectioned for RNA extraction and cDNA conversion and quantified by a real-time polymerase chain reaction. *GAEC1* protein expression was analysed by immunohistochemistry using a custom made *GAEC1* antibody.

Result

GAEC1 mRNA was upregulated in majority (52%, n=42/80) of the colorectal carcinomas when compared to the matched non-neoplastic tissues. High expression of *GAEC1* mRNA as correlated with patients of younger age ($p=0.008$), with lower grade carcinoma ($p=0.028$), presence of synchronous adenocarcinomas ($p=0.034$) and without any associated adenomas ($p=0.047$). In addition, patients with high *GAEC1* mRNA overexpression had a shorter survival time. Furthermore, high *GAEC1* protein expression was noted among patients having perforated colorectal carcinoma ($p=0.04$).

Conclusion

The high expression of *GAEC1* mRNA/protein as well as its correlation with multiple clinicopathological characteristics in patients with colorectal carcinoma strongly suggests that GAEC1 is a key regulator in the initiation of colorectal carcinogenesis.

Keywords: GAEC1; immunohistochemistry; pathology; expression

Introduction

Identification of diagnostic and prognostic biomarkers will ultimately lead to the development of new therapeutic strategies and increase the survival time of patients with colorectal carcinoma [1-5]. *GAEC1* (Gene amplified in esophageal cancer 1) is reported to have tumorigenic potential almost equal to the *Ras* gene family and over expression of this gene played a crucial role in the cancer transformation of oesophageal squamous cell carcinoma [6, 7]. In addition, *GAEC1* DNA was frequently amplified in patients with colorectal carcinomas and were significantly correlated with patient survival [8-9]. Subsequently, cDNA microarray analysis showed downregulation of calpain 10 (CAPN10) and upregulation of trinucleotide repeat containing 6C (TNRC6C) transcripts when *GAEC1* expression was suppressed in oesophageal squamous cell carcinoma [10]. Recently, the oncogenic properties and potential co-regulatory partners of *GAEC1* were demonstrated in-vivo and in-vitro in colon carcinoma [11].

Till to date, the mRNA/protein expression profiles of *GAEC1* and its correlation with different clinical and pathological characteristics in colorectal carcinoma was not known. Thus, in this study, we aimed to analyse the expression patterns, tissue localisation and clinicopathological correlations as well as prognostic implications of *GAEC1* mRNA and protein in a large series of patients with colorectal carcinomas .

Materials and Methods

Recruitment of the tissues and sample selection

Cancer tissue and matched non-cancer mucosae from eighty patients with colorectal adenocarcinoma (39 men and 41 women) were prespectively collected in fresh and snapped frozen at -70⁰C. Formalin fixed paraffin cancer tissue from these patients were also sampled at the time of surgery for *GAEC1* protein expression analysis. This study was approved by the

Human Research Ethics Committee of Griffith University (GU Ref No: MSC/17/10/HREC). A signed consent from each of the participating patients was also obtained.

Clinical and pathological data of the patients

The mean age of the patients was 70 years (range from 24-91 years). The site and size of the cancer were recorded at the time of dissection. The pathological features of the carcinoma was reviewed by the author (AKL). The cancer was graded according to the World Health Organization (WHO) [12]. Staging of the cancer was done according to TNM (Tumour, Lymph node, Metastasis) classification in Cancer staging manual by American Joint Committee on Cancer (AJCC) [13]. The other pathological parameters including the association of the colorectal adenoma, the presence of lymphovascular permeation and peri-neural invasion was noted. Microsatellite Instability (MSI) was tested in these carcinomas by immunohistochemistry on 4 proteins (MLH1, PMS2, MSH2 and MSH6) as described previously [14].

All the patients were followed-up clinically according to pre-agreed standardised multidisciplinary protocol. The adjuvant therapy was administered in the postoperative patients based on the pathological stage of carcinoma. The follow-up period was considered from the date of surgery for colorectal cancer to the time of death or closing of the study. Recurrence of cancer after surgery was also recorded.

RNA extraction and cDNA conversion

The fresh frozen colorectal tissues were sectioned using a cryostat and histological analysis was made after staining with hematoxylin and eosin staining to assess the suitability for the study. We required cancer tissue with > 50% proportion of cancer cells for RNA extraction.

Matched adjacent tissues that confirmed by histological analysis with no neoplastic cells were also sectioned. The selected tissues were sectioned and followed by extraction using Qiagen miRNeasy Mini Kit which is specific for purification of the total RNA including mRNA from fresh frozen tissues (Qiagen, Hilden, North Rhine-Westphalia, Germany). The purity of RNA was obtained by checking the optical density (OD) 260/280 ratio and concentration of RNA was noted in ng/ μ l (Spectrophotometer, Thermo Fisher Scientific, Wilmington, USA). Reverse transcription reactions were performed using 1 μ g total RNA in a final reaction volume of 20 μ l. RNA was converted to cDNA using miScript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The sample was stored at -20°C until the polymerase chain reaction (PCR) analysis.

Quantitative Real-time polymerase chain reaction

The expression of *GAEC1* mRNA from the colorectal tissue was examined by using a QuantStudio 6Flex Real-time PCR system (Applied Biosystems, Foster city, CA, USA). Quantitative real time polymerase chain reaction (qPCR) was performed in a total volume of 20 μ l reaction mixture containing 10 μ l of Quantitech Syber Green PCR kits (Qiagen), 1.5 μ l of each 5 μ mol/l, 3 μ l of cDNA at 50 ng/ μ l and 4 μ l of 0.1% diethylpyrocarbonate (DEPC) treated water. All the samples (cancer and non-cancer) were run in triplicates. The amplification efficiencies were normalized to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Sequences of the primers used in this study are detailed in Table 1. The fold change in the target gene for the results of quantitative amplification was calculated for each sample using $2^{-\Delta\Delta CT}$ (fold change) method as previously reported [14, 15, 16]. A fold change more than

2 was considered as high expression and a fold change of less than 1 was regarded as low expression/down regulation of *GAEC1* mRNA.

Immunohistochemistry of GAEC1 in colorectal cancer tissues

The formalin fixed paraffin blocks of colorectal cancer tissue from the same patients were used for GAEC1 protein expression analysis. A high P^H ENVision™ FLEX Mini Kit (Dako, Glostrup, Hovedstaden, Denmark) was used for the immunohistochemical analysis of GAEC1 protein. In brief, deparaffinised and rehydrated sections (4µm) were first processed for antigen retrieval in citrate buffer (at pH 6.0) for 15 minutes in a microwave oven and then blocked with peroxidase blocking reagent for 10 minutes. Tissues were incubated with a mouse monoclonal GAEC1 antibody (Promab, Richmond, CA, USA) for 60 minutes at room temperature. The slides were then incubated with a horseradish peroxidase conjugated secondary antibody (Dako) for 15 minutes at room temperature. Visualization of staining was done by using 3, 3'-diaminobenzidine (DAB) (Dako) to produce a brown colour, and then counterstained with haematoxylin. A block of colonic adenocarcinoma that showed strong cytoplasmic staining was used as a positive control in each run of the experiment. A negative control sample was prepared by the same procedure without GAEC1 antibody. A grading scale ranging from 0 to 3 was used for this assessment, where 0 represented a negative staining, 1 represented weak staining (1-30%), 2 and 3 represented moderate (31 to 70%) and strong staining (>70%) respectively. Assessment of the slides according to the extent and intensity of GAEC1 staining was also taken into consideration for analysis.

Statistical analysis

Statistical analysis was done by using the Statistical Package for Social Sciences (SPSS, version 24, IBM, New York, NY, USA). Chi-square test or likelihood ratio was used for categorical variables. Pearson correlation test was used for continuous variables. Independent t-test was performed for the analysis of continuous variables in categories. A p value of < 0.05 was considered as statistically significant.

Results

GAEC1 mRNA/protein expression

GAEC1 mRNA and protein expressions were noted in all (n=80) colorectal adenocarcinomas and its corresponding non-neoplastic tissues at varied levels. Approximately 52.5% (n=42) of patients with colorectal adenocarcinoma showed higher expression of *GAEC1* mRNA whereas 47.5% (n=38) exhibited lower expression when compared to their matched non-neoplastic tissues. Similarly, ~ 66% (53/80) of colorectal adenocarcinomas showed high *GAEC1* protein expression (positive staining), while the remaining colorectal cancer cases were noted with no *GAEC1* protein (negative) expression. *GAEC1* protein was predominantly located in the cytoplasm of the colorectal adenocarcinomas and showed low to no expression in non-neoplastic large intestinal mucosa (Figure 1).

Clinical and pathological correlations of *GAEC1* expressions

Table 2 showed the correlation of the mRNA expression of *GAEC1* with the clinical and pathological factors in patients with colorectal adenocarcinoma. High expression of *GAEC1* mRNA was predominantly seen among patients below 60 years when compared to those patients over 60 years of age (78%, versus 44%, $p=0.008$). Patients with synchronous colorectal adenocarcinomas mostly exhibited lower expression of *GAEC1* mRNA when compared with

other patients (86% vs 56%, $p=0.034$). Compared to patients with poorly differentiated colorectal adenocarcinomas (grade III), patients with well and moderately differentiated colorectal adenocarcinomas (grades I or II) showed a higher expression of *GAEC1* mRNA (57% versus 20%, $p=0.028$). In addition, higher *GAEC1* mRNA expression was frequently noted among patients presented without any co-existing adenomas (63% versus 41%, $p=0.047$).

Table 3 showed the correlation of the expression of GAEC1 protein with the clinical and pathological factors in patients with colorectal adenocarcinoma. More than 90% (10/11) of patients with perforated colorectal adenocarcinoma had high expression of GAEC1 protein when compared with those without perforated cancer ($p=0.04$). No significant correlation was observed between GAEC1 protein expressions in colorectal adenocarcinoma with clinicopathological parameters.

Cancer recurrence was not associated with the expression of *GAEC1* mRNA or protein. Patients with high expression of *GAEC1* mRNA appeared to have better survival rates than those with low expression (Figure 2) though the differences did not reach statistical significance ($p > 0.05$).

DISCUSSION

In this study, we have demonstrated the expression profiles of *GAEC1* mRNA/protein in patients with colorectal cancers was associated with different clinicopathological characteristics. The prevalence of high *GAEC1* mRNA and protein levels in patients with colorectal carcinomas as well as the poorer survival of patients with high *GAEC1* mRNA are in line with the previous reports on the oncogenic functions of GAEC1.

The development of colorectal adenocarcinoma could be from the well described adenoma-carcinoma or serrated pathway [17]. The recent Cancer Genome Atlas (TCGA) project

and Consensus Molecular subtype (CMS) consortium further sub classified the different molecular groups in the pathogenesis of colorectal cancer [18]. Nevertheless, the most common pathway in colorectal pathogenesis involves deregulation of DNA synthesis in the epithelial cells of the colorectum. This is followed by the development of an adenoma, with increasing degree of cellular atypia and acquisition of properties leading to tumor invasiveness. Also, age dependent genetic and epigenetic changes are not uncommon in the pathogenesis of colorectal adenocarcinomas especially in the early steps of the adenoma-carcinoma sequence [19, 20, 21]. In this study, patients aged less than 60 years have high expression of *GAEC1* mRNA in their colorectal cancer tissues. Similarly, *GAEC1* mRNA was frequently expressed high in patients with a co-existing adenoma and adenocarcinoma in the large intestine. These age and adenoma-carcinoma associated changes in *GAEC1* mRNA suggests that colorectal carcinogenesis is dependent on different predisposing genomic instability, possibly assisted by *GAEC1*, in patients with different clinical and pathological characteristics. This notion is further strengthened by the additional findings in this study, which has noted high *GAEC1* mRNA levels in patients with well and moderately differentiated colorectal adenocarcinomas when compared to patients with poorly differentiated colorectal carcinomas. Taken together, it can be hypothesised that *GAEC1* exhibits its oncogenic functions during the initiation of colorectal cancer pathogenesis and the downstream partners of *GAEC1* will lead the future genetic progression in these carcinomas.

Synchronous colorectal carcinomas refer to carcinomas with more than one primary carcinoma in the colorectum at the time of the presentation [22]. Also, synchronous colorectal carcinomas have different patterns of chromosomal instability, microsatellite instability, *p53* mutation and *K-ras* mutation status indicating its complex genetic/epigenetic modifications during pathogenesis [23]. The prevalence of high *GAEC1* mRNA expression

noted among patients with non-synchronous colorectal carcinomas imply that synchronous carcinoma had a different pathogenic pathway from the solitary colorectal carcinoma.

In this study, we first investigated the expression of GAEC1 protein in cancer tissue by immunohistochemistry. We have recently reported the cytoplasmic localisation and stage dependent expression patterns of GAEC1 protein in-vitro [11]. GAEC1 protein was expressed higher in cancer cells from early stage colon carcinomas (Stage II or III) when compared to cancer cells from advanced stage (Stage IV) colon cancer. In the current study, high level of *GAEC1* mRNA expressions in cancer tissues amongst patients diagnosed with early phases of colorectal carcinomas are in agreement with these in-vitro findings. This study has also noted a cytoplasmic and differential expression pattern for GAEC1 protein in colorectal cancer tissues suggesting its regulatory effects on other key interacting proteins in colorectal carcinogenesis [11].

Perforation colorectal adenocarcinoma are considered clinically aggressive, as there is a high chance of peritoneal dissemination of cancer cells, sepsis and multiple organs failure [24]. The current study has noted high protein expression levels of GAEC1 in 90% (10/110) of patients with perforated colorectal adeno carcinoma ($p < 0.05$). The *GAEC1* mRNA levels also exhibited a similar non-significant ($p > 0.05$) trend in expression changes in these patients. However, GAEC1 expressions with mRNA was predominately noted with early stages and lower grade colorectal carcinomas. This contradicting result might be due to the tissue specific changes with GAEC1 protein expression and low number ($n=11$) of colorectal cancer cases with intestinal perforation.

It is worth noting that our most recent study has indicated a co-regulatory effect of GAEC1 with key oncogenes in colorectal carcinogenesis such as K-ras and Bcl-2 [11]. *P53*, a

key tumour suppressor gene, was also negatively regulated by GAEC1 expression [11]. Thus, it can be also hypothesised that as the colorectal cancer progresses to more aggressive phenotype with advanced pathological stages, an increase in the activation of GAEC1 mediated signal transduction pathway occurs.

To conclude, this study has clinically validated the oncogenic function of GAEC1 in a large cohort of patients with colorectal carcinomas. The differential expression pattern of GAEC1 mRNA/protein as well as its correlation with patients with different clinicopathological characteristics have indicated that GAEC1 is a key regulator in the initiation colorectal carcinomas. Further studies are warranted to confirm its potential use as therapeutic target in colorectal carcinomas.

Figure legend

Figure 1: Expression of GAEC1 in colorectal adenocarcinoma and non-neoplastic mucosa (x 25).

1A. strong (3+) staining in cancer; 1B moderate (2+ staining in cancer); 1C weak (1+ staining in cancer); 1D; weak staining in non-neoplastic mucosa.

Figure 2: The relationship between patients' survival and expression levels of GAEC1 mRNA in colorectal carcinoma. Patients with high expression level of GAEC1 mRNA in their colorectal cancer tissue had marginally reduced survival time when compared to patients with low expression level.

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Conflict of interest: None

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Table 1: Primer sequences for quantitative Real-time polymerase chain reaction

Gene	Orientation	Sequence	Amplicon size (Base pairs)
<i>GAEC1</i> (AC005088)	Forward	5'-CCTCAGGGAAGAAGCAAGTT-3'	121
	Reverse	5'-TCTTGCATGGTGCCAGTT-3'	
<i>GAPDH</i> (NM_002046)	Forward	5'-TGCACCACCAACTGCTTAGC-3'	88
	Reverse	5'-GGCATGGACTGTGGTCATGAG-3'	

Table 2: The correlation of GAEC1 mRNA expression level with different clinical and pathological parameters in patients with colorectal carcinoma.

Type	Number	High Expression	Low Expression	p value
<u>Age</u>				
≤60	19(23.75%)	15(78.94%)	4 (21.05%)	0.007
>60	61 (76.25%)	27 (44.26%)	34 (55.73%)	
<u>Gender</u>				
Male	39 (48.75%)	19(48.71%)	20(51.22%)	0.50
Female	41 (51.25%)	23(56.09%)	18(43.90%)	
<u>Size</u>				
≤ 40mm	65(81.25%)	37(56.92%)	28(43.07%)	0.09
> 40 mm	15(18.75%)	5(33.33%)	10(66.66%)	
<u>Location</u>				
Colon	64(80%)	32(50%)	32(50%)	0.37
Rectum	16(20%)	10(62.5%)	6(37.5%)	
<u>Synchronous tumour</u>				
Yes	7 (8.75%)	1(14.28%)	6(85.71%)	0.03
No	73(91.25%)	41(56.16%)	32(43.83%)	
<u>Perforation</u>				
Yes	11(13.75%)	8(72.72%)	3(27.27%)	0.14
No	69(86.25%)	34(49.27%)	35(50.72%)	
<u>Grade</u>				
I or II	70(87.5%)	40 (57.14%)	30(42.85%)	0.02
III	10(12.5%)	2(20%)	8(80%)	
<u>Stage</u>				
I	9(11.25%)	5(55.55%)	4(44.44%)	0.34
II	34(42.5%)	14(41.17%)	20(58.82%)	
III	17(21.25%)	10(58.82%)	7(41.17%)	
IV	20(25%)	13(65%)	7(35%)	
<u>Associated adenoma</u>				
Yes	37(46.25%)	15(40.54%)	22(59.45%)	0.04
No	43(53.75%)	27(62.79%)	16(37.20%)	
<u>Lymphovascular permeation</u>				
Yes	17(21.25%)	12(70.58%)	5(29.41%)	0.092
No	63(78.75%)	30(47.61%)	33(52.38%)	
<u>Peri-neural infiltration</u>				
Yes	8(10%)	4(50%)	4(50%)	0.88
No	72(90%)	38(52.77%)	34(47.22%)	
<u>MSI status</u>				
Yes	14(17.5%)	7(50%)	7(50%)	0.83
No	66(82.5%)	35(53.03%)	31(46.96%)	
<u>Recurrence of cancer</u>				
Yes	27(33.75%)	16(59.25%)	11(40.74%)	0.38
No	53(66.25%)	26(49.05%)	27(50.94%)	

Table 3. GAEC1 immunostaining of colorectal adenocarcinoma and the association patients' clinical and pathological characteristics.

	Number (%)	Positive	Negative	p value
<u>Age</u>				
≤60	19(23.75%)	12(63.15%)	7 (36.84%)	0.74
>60	61 (76.25%)	41(67.21%)	20 (32.78%)	
<u>Gender</u>				
Male	39 (48.75%)	25(64.10%)	14(35.89%)	0.69
Female	41 (51.25%)	28(68.29%)	13(31.70%)	
<u>Size</u>				
≤ 40mm	65(81.25%)	44(67.69%)	21(32.30%)	0.57
> 40 mm	15(18.75%)	9(60%)	6(21.42%)	
<u>Location</u>				
Colon	64(80%)	43(67.18%)	21(32.81%)	0.72
Rectum	16(20%)	10(62.5%)	6(37.5%)	
<u>Synchronous tumour</u>				
Yes	7(8.75%)	5(71.42%)	2(28.57%)	0.75
No	73(91.25%)	48(65.75%)	25(34.24%)	
<u>Perforation</u>				
Yes	11(13.75%)	10(90.90%)	1(9.09%)	0.04
No	69(86.25%)	43(62.31)	26(37.68%)	
<u>Grade</u>				
I	8(10%)	5(62.5%)	3(37.5%)	0.61
II	62(77.5%)	40 (64.51%)	22(35.48%)	
III	10(12.5)	8(80%)	2(20%)	
<u>Stage</u>				
I	9(11.25%)	4 (44.44%)	5(55.55%)	0.25
II	34(42.5%)	21 (61.76%)	13(38.23%)	
III	17(21.25)	12 (70.58%)	5(29.41%)	
IV	20(25%)	16 (80%)	4(20%)	
<u>Associated adenoma</u>				
Yes	37(46.25%)	25(67.56%)	12(32.43%)	0.81
No	43(53.75%)	28(65.11%)	15(34.88%)	
<u>Lymphovascular permeation</u>				
Yes	17(21.25%)	13(76.47%)	4(23.52%)	0.31
No	63(78.75%)	40(63.49%)	23(36.50%)	
<u>Peri-neural infiltration</u>				
Yes	8(10%)	6(75%)	2(25%)	0.58
No	72(90%)	47(65.27%)	25(34.72%)	
<u>MSI status</u>				
Yes	14(17.5%)	11(73.33%)	3(21.42%)	0.28
No	66(82.5%)	42(63.63%)	24(36.36%)	

<u>Recurrence of cancer</u>				
Yes	27(33.75%)	19(70.37%)	8(29.62%)	0.57
No	53(66.25%)	34(64.15%)	19(35.84%)

Figure. 1

Figure. 2

Highlights

- *GAEC1* is an oncogene and a key regulator in the initiation of cancer.
- *GAEC1* protein expression was first identified in colorectal cancer
- *GAEC1* mRNA expression was increased in colorectal cancer
- *GAEC1* mRNA expression correlated with clinical and pathological parameters.

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