

GAEC1 and colorectal cancer: A study of the relationships between a novel oncogene and clinicopathological features

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ABSTRACT

Background, GAEC1 is a novel gene located at 7q22.1 which was detected in our previous work in oesophageal cancer. The aims of the present study are to identify the roles of GAEC1 in colorectal pathogenesis and its relationship with the clinicopathological parameters;

Methods, 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 non-neoplastic tissues) using real time PCR.

Results, Copy number level of DNA in colorectal adenocarcinomas was higher in comparison with non-neoplastic colorectum. 79% of the colorectal adenocarcinomas showed amplification and 15% showed deletion of GAEC1 ($p < 0.0001$). Of the adenomas, 90% showed deletion of GAEC1 but none revealed any amplification of GAEC1. The differences in GAEC1 copy number between colorectal adenocarcinoma, colorectal adenoma and non-neoplastic colorectal tissue are significant ($p < 0.0001$). GAEC1 copy number was significantly higher in adenocarcinomas located in distal colorectum compared with proximal colon ($p = 0.03$); **Conclusions,** GAEC1 copy number was significantly different between colorectal adenocarcinomas, adenomas and non-neoplastic colorectal tissues. The copy number level was also related to site of the tumour. These findings imply that GAEC1 is important in the pathogenesis of colorectal adenocarcinoma.

Keywords: Colorectal; adenocarcinoma; adenoma; GAEC1; gene

Introduction

Colorectal cancer (CRC) is amongst the most common malignancies found in developed countries and the mortality rates for this cancer are generally high. The most common histological type of CRC is adenocarcinoma [1]. It is known that CRC develops after a series of genetic mutations, corresponding to the histological progression from normal colonic mucosa to adenoma (dysplasia), adenocarcinoma and finally metastases [2]. Currently, surgery is the main option for treatment, with adjuvant chemotherapy and radiotherapy for patients who belong to some 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 instance, we have documented that p53, p16, p21, aurora kinase, survivin and telomerase activities in the pathogenesis appear either as tumour 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 oesophageal cancer [10]. A frequently amplified sequence of 357 bp was identified in the ISSR-PCR bands for oesophageal cancer and that sequence showed an exact homology to an expressed sequence tag sequence (293 bp) at 7q22 [10]. Our work on GAEC1 indicates that it has tumorigenic potential and over-expression of GAEC1 is a critical step for tumour transformation in oesophageal squamous cell carcinoma [11]. GAEC1 has a full length mRNA of 2052bp and encodes a nuclear protein of 109 amino acids. Expression of GAEC1 has been detected in a range of normal tissues including oesophagus, small intestine and colon [11]. GAEC1 is not the only gene which 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 carcinogenesis of varying malignancies. At present, however, there is a lack of fundamental information of the molecular roles of this novel gene in colorectal cancer. Despite knowledge concerning amplification of the region containing GAEC1, copy number changes or amplification of GAEC1 has not yet been studied in colorectal cancer. Recent molecular studies show that DNA copy number changes within tumours can be a marker for tumour sensitivity to targeted anticancer therapy [16].

Correlation of GAEC1 copy number changes and clinicopathological parameters have also not been previously investigated in cancers other than oesophageal cancer. The aim of this study was to identify the copy number of GAEC1 in different colorectal tissues including tumour, adenomas and non-neoplastic tissues and characterise any links to pathological factors of these tumours.

Material and Methods

Tissue samples

The patients who were chosen for this study had resection for primary colorectal carcinomas, colorectal adenomas and non-neoplastic colorectal tissue between January 2004 and December 2006, in Queensland, Australia. Ethical approval of this study has been obtained from the Griffith university ethic committee. The patients were consecutively chosen and with no selection bias. The resected tissues were fixed in 10 percent formalin and embedded in paraffin wax. Histological sections were cut and stained for haematoxylin and eosin for light microscopic examination. These sections were reviewed by the authors. Both conventional and mucinous adenocarcinomas were included in the study. Non-neoplastic samples including polyps, adjacent normal and inflammatory diseases were recruited to act as control tissue. The carcinomas were graded according to the World Health Organization (WHO) criteria [17].

The pathological features of patients with colorectal adenocarcinomas and adenomas were analysed. These included the histological variants and pathological grades by assessing the cellular morphology and extent of mucin production. Lymph node metastases at the time of surgery were also recorded. The carcinomas were staged according to TNM classification [18].

After reviewing the tissue samples, 259 patients (153 men; 106 women) colorectal tissues were selected for the study. These included 144 colorectal adenocarcinomas, 31 colorectal adenomas and 84 colorectal non-neoplastic tissues. The mean age of the patients was 65 (range 12 to 93). For the colorectal adenocarcinomas and colorectal adenomas, the site and size (maximum length) were recorded. One tissue block from each of these tissues were chosen for DNA extraction.

Extraction of DNA

Haematoxylin and eosin sections were taken from the selected tissue paraffin blocks to choose the area for DNA extraction. In order to first separate cancer tissue from the surrounding morphologically benign tissue, samples underwent microdissection. For each selected block, ten 10 micron 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 (OD) 260/280 ratio. Concentration of DNA also noted in ng/ μ L. All the DNA extracted was of good quality for the PCR experiment.

Primers

The primer sets for amplification of GAEC1 (GenBank accession number AC005088) and HBD (GenBank accession number 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 Haemoglobin delta (HBD), which acted as a reference gene.

Real-time quantification PCR

Real-time quantitative polymerase chain reaction (PCR) was performed for detecting GAEC1 gene copy number changes. IQ5 Multicolour Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA) was used to run real-time quantification PCR.

PCR was performed in a total volume of 20 μ L reaction mixture containing 10 μ L iQ SYBR green supermix (Bio-Rad), 1 μ L of each 5 μ mol/L primer, 2 μ L of DNA at 50ng/ μ L,

and 6 μL of 0.1% diethylpyrocarbonate (DEPC) treated water. In the last tube, 2 μL of DEPC treated water was added as a non template control. All the samples (unknown and standard) were run in duplicate and accompanied by a non template 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 eighty one cycles of 30 seconds increasing from 55°C. The melting curves of all final real-time PCR products were analysed for determination of genuine products and contamination by non-specific products and primer dimers. For each tissue sample, the PCR reaction was performed in triplicate to increase the reliability of the results. All real-time PCR conditions and primers were optimized to produce a single product of the correct gene fragment. To ensure that the correct product was amplified in the reaction, all samples were also separated on 20 g/L agarose gel electrophoresis.

PCR efficiency and Data analysis

Standard curve was constructed from a known concentration of cDNA sample (generated from universal human reference RNA (Stratagene, Cedar Creek, TX, USA) for determination of PCR efficiency. Dilution series of 120, 100, 80, 60, 40, 20 and 10 ng/ μL were used. Delta Ct for GAEC1 and HBD was determined and the slope was calculated from a plot with delta Ct on the Y axis and the log of total cDNA on the X axis. Efficiencies for the GAEC1 and HBD genes were found to be comparable. For analysis, copy number for GAEC1 was normalized in each sample by dividing obtained GAEC1 Ct by the Ct obtained for HBD (normalization = Ct GAEC1 [sample] / Ct HBD [sample]). For PCR efficiency a comparative Δ Ct curve of the GAEC1 and HBD experiments was constructed.

Data was obtained as Ct values according to the manufactures guidelines and ΔCt value ($\Delta\text{Ct} = \text{Ct of the target gene} - \text{Ct of housekeeping gene}$). The fold change in the target

gene for the results of quantitative amplification was calculated for each sample using $2^{-\Delta\Delta Ct}$ method [19], where $\Delta\Delta Ct = (Ct_{GAEC1} - Ct_{HBD})_{CANCER} - (Ct_{GAEC1} - Ct_{HBD})_{NORMAL}$. As mentioned above, this controls for PCR efficiency by normalising the data. Normalised values for each duplicate sample were then averaged to give the final data used. Quantitative amplification data obtained for the target gene was also analysed by expressing it as a ratio of the Ct for GAEC1 to HBD, which had been carried out on the same experimental run for each tumour and control sample. Ratios were then expressed as inverse ratios (1/Ratio) in order to reorient changes in ratio to reflect actual behaviour of GAEC1 (i.e. increased ratio = increase in GAEC1 copy number). A ratio of more than the normal range was considered as gain of GAEC1 copy number / amplification compared to the housekeeping gene. A ratio value less than normal range for non-tumour samples was considered as loss of GAEC1 copy number / deletion. Normalised final data (Inverse Ct ratio) was analysed using one-way Analysis of Variance (ANOVA) to determine if there was a significant difference of copy number between colorectal tissue groups.

All the data was 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, USA). Significance level was taken at $P < 0.05$.

Results

Identification of GAEC1 in colorectal tissues

GAEC1 showed copy number variation in all samples 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 non-neoplastic colorectal tissue (control tissue) was 1.07 (range 0.88 to 1.30), 0.91 (range 0.69 to 1) and 0.98 (range, 0.89 to 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.

Gain of GAEC1 copy number in colorectal cancers

In colorectal adenocarcinomas, 79 % (n=114) showed some degree of amplification (relative to control tissue) and 15 % (n=21) showed loss of GAEC1 copies or deletion. 6 % (n=9) of the adenocarcinoma did not show any change in copies compared to the control tissues. The copy number level of DNA in adenocarcinomas was higher in comparison to control tissue ($p < 0.0001$) and colorectal adenomas ($p < 0.0001$). Comparison has been illustrated in Table.2. GAEC1 copy number in colorectal adenocarcinomas was 3.5 fold higher than that of control tissues and approximately 48 fold high than that of adenomas (Fig.2).

Higher DNA copy number of GAEC1 was noted in colorectal adenocarcinomas from men compared to those from women. However, the difference was not statistically significant (mean ratio= 1.08 versus 1.05, $p = 0.07$). GAEC1 copies were significantly higher in adenocarcinomas located in distal colon (including descending colon, sigmoid colon and rectum) compared with proximal colon (mean ratio= 1.07 versus 1.04, $p = 0.03$). The DNA

copy number of GAEC1 in the cancer population was found to have no relationship with the age, size, grade, histological subtypes or TNM staging of the colorectal adenocarcinomas.

Loss of GAEC1 copy number in colorectal adenomas

The copy number of GAEC1 DNA in colorectal adenomas was lower (i.e. showing deletion) in comparison with control tissues ($p = < 0.0001$). The data generated from adenomas in this study showed that GAEC1 has reduced copy number in the majority of adenomas. Of the 31 adenomas, 90% (n=28) showed reduced and 10% (n=3) showed no change in GAEC1 copies compared to control tissues. A 13.7 fold decrease of GAEC1 copy number was noted in adenoma compared to control tissues. The copy number of GAEC1 in adenoma population was found to have no relationship with the age and gender of the patient, nor with the site and size of adenoma.

Discussion

In our previous study, we have documented that GAEC1 showed amplification and over expression in primary oesophageal tumours. Over-expression of GAEC1 in mouse fibroblasts also caused enhanced cell proliferation, foci formation and colony formation in soft agar, and was comparable to the transforming action of H-ras. Injection of GAEC1-transfected cells into athymic nude mice formed undifferentiated sarcoma, indicating the GAEC1 is a transforming oncogene [11]. In this study, we first demonstrated that colorectal adenocarcinomas have higher GAEC1 copy number than non-neoplastic 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 oesophageal cancer and indicates that it may play a similar role in colorectal cancer.

The higher copy number of GAEC1 in cancers compared to 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, since GAEC1 over-expression 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 tumours and occurs as part of the overall progression of the disease, though it may not be involved in the early stages of pathogenesis.

In this study, we also did not find any relationship between GAEC1 amplification and patient age, pathological grades, stages or size of the tumour. However, our results indicated that GAEC1 is more often amplified in tumours located the distal part of the colorectum compared with tumours in proximal colon. This implies different molecular pathogenesis for

GAEC1 in proximal and distal parts of the colorectum, perhaps based on pre-transformation utilisation of GAEC1. These observations are in line with previous studies showing that clinical features and molecular pathways are different in both proximal and distal colorectal cancers [6, 8, 9, 20, 21]. p53 expression in ordinary colorectal adenocarcinoma and p16 expression in mucinous adenocarcinoma were reported to occur more often distal colorectal tumours [8, 22]. Aurora kinase expression and telomerase activity was 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 colon may have implications in specifically targeted therapeutic practice in the future.

In addition to the differences related to site, we also observed a mild difference in GAEC1 copy number between male and female patients with colorectal adenocarcinoma. Though not significant, the observed reduction in amplification of GAEC1 in female patients with colorectal adenocarcinoma could be indicative of a reduced pathogenic effect of GAEC1 in female patients.

The data generated from colorectal adenomas in this study showed that GAEC1 has reduced copy number in most of the adenomas. The observed 13.7 fold deletion in adenomas compared to control tissues was highly significant ($p = < 0.001$). This indicates that the role of GAEC1 may have significant differences in colorectal adenomas compared to adenocarcinomas, perhaps related to modulation of GAEC1 action by interaction with regulatory genes.

7q22 deletion has been reported in benign tumour, namely uterine leiomyoma [23, 24]. It is possible that GAEC1 deletion (and related down-regulation) in colorectal adenomas may offer some initial advantage to further transformation. This may be due to the coincident loss of a gene with tumour suppressing properties nearby in 7q22, followed by re-amplification of GAEC1 later. It may be this loss is specific to certain tumour cells within

the adenoma, and these provide some necessary support to those cells retaining full GAEC1 complement. In addition, adenomas experience 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 proto-oncogenes [25]. Our present study demonstrates that GAEC1 is amplified in 79% of colorectal adenocarcinomas. This is the first study showing significant correlation of GAEC1 with clinicopathological features. The previous study of GAEC1 in oesophageal cancer did not detect any significant relationships between GAEC1 alterations and site of the tumour or gender of the patient. In this study, significant amplification of GAEC1 was noted between different colorectal tissues including non-neoplastic 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 colorectal cancer.

In conclusion, the role of GAEC1 was studied in different colorectal tissues. GAEC1 amplification was significantly increased in colorectal adenocarcinoma compared to adenoma and was associated with tumour 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 imply that GAEC1 is important in the pathogenesis of colorectal adenocarcinoma. The high level of amplification of GAEC1 and its correlation with different tissues samples and sites of tumour may also be important for the development of gene targeting therapies for colorectal cancer. Further research into the precise mechanisms of action of GAEC1 should be encouraged to improve understanding of the role of this novel oncogene.

Competing interests

The whole GAEC1 project was supported by research grants from (1) Griffith Institute of Health and Medical Research (GIHMR) & Gold Coast Hospital Foundation Grant; (2) Research Grant Council (Ref No.562708) of Hong Kong. There is no conflict of interest in the study.

Authors' contributions

VG was involved with the design of the study, acquisition of data, data analysis and drafting of the manuscript. MRN and KY were involved with the design of the study, acquisition of data, data interpretation and critical review of the manuscript. RAS were involved with the design of the study, data interpretation and critical review of the manuscript. AS, SYK, YHH and SW involved in acquisition and analysis of data. AKYL and JCOT contributed to the design of the study, data interpretation, critical review of the manuscript and overall supervision of the work. All authors have read and approved the final version of the manuscript.

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Table1. Clinicopathological features and GAEC1 genetic changes

Characteristics	Number	Amplification	Deletion	No change	P value
Age					
< 60	43 (30%)	32 (74.4%)	7 (16.3%)	4 (9.3%)	0.22
>60	101 (70%)	82 (81.3%)	14 (13.7%)	5 (5.0%)	
Gender					
Male	80 (55.6%)	67 (83.7%)	8 (17.0%)	5 (6.3%)	0.07
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%)	0.46
> 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%)	0.03
DC	94 (65.3%)	77 (82.0%)	12 (12.7%)	5 (5.3%)	
Histological Grade					
Well	14 (9.7%)	9 (64.3%)	4 (28.6%)	1 (7.1%)	0.66
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%)	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%)	

PC= Proximal Colon ; DC= Distal Colon

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%)	< 0.001
Adenoma	31	0 (0%)	28(90.3%)	3 (9.7%)	< 0.001

P-value: compared with non-neoplastic colorectal tissue

Figure Legends



Figure 1a: GAEC1 amplification bands in colorectal cancers after real time PCR in 2 % agarose gel. GAEC1 fragments of 122 base pair were noted in all samples except for the water control (H). 100 base pair DNA ladder (M) was used for comparison.



Figure 1b: Control gene (HBD) amplification bands in colorectal cancers after real time PCR in 2 % agarose gel. HBD fragments of 225 base pair were noted in all samples except for the water control (H). 100 base pair DNA ladder (M) was used for comparison.

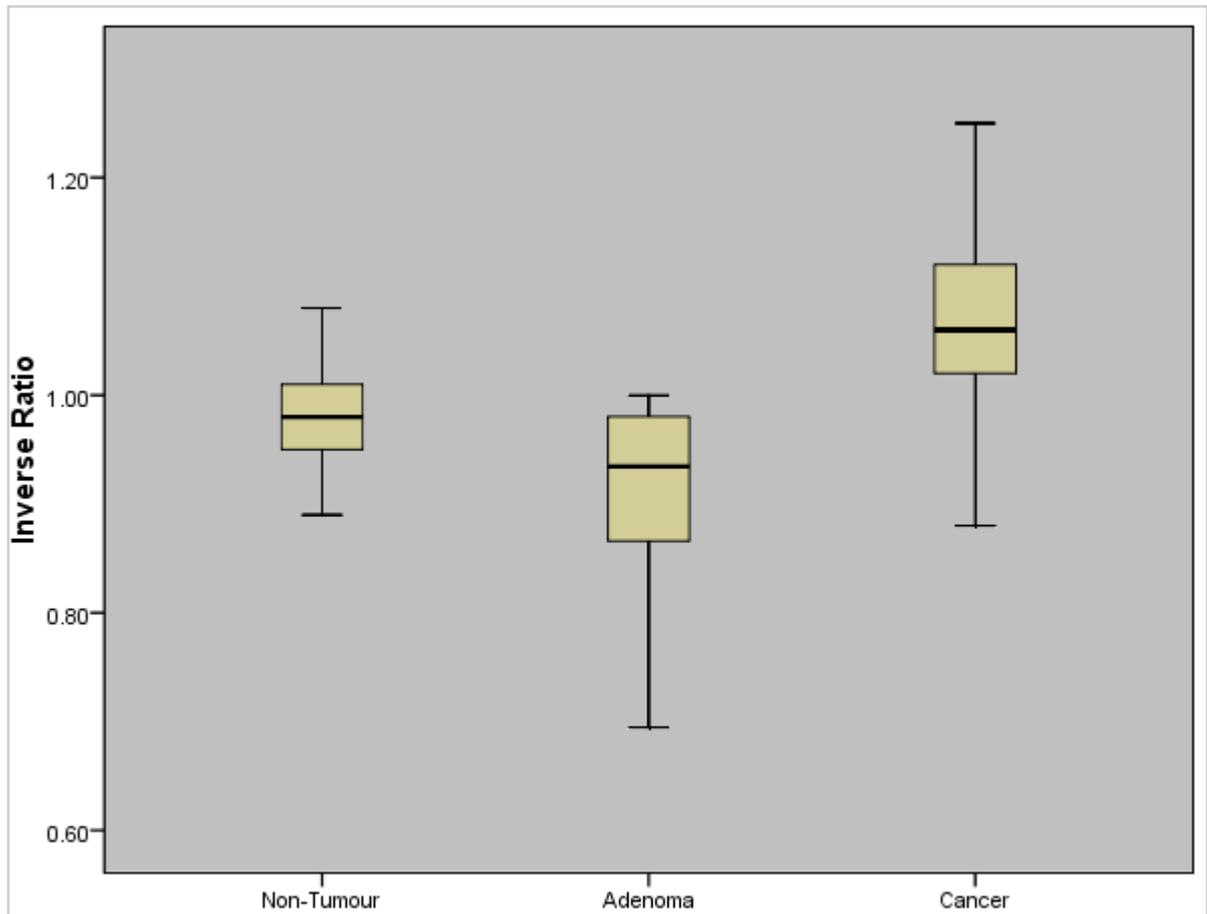


Figure 2: GAEC1 amplification levels in different colorectal samples. Higher level of amplification ratio (inverse ratio) was obtained for cancer samples compared to the adenoma and non-tumour samples.