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Research paper

# GAEC1 mutations and copy number aberration is associated with biological aggressiveness of colorectal cancer

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ABSTRACT

*GAEC1* (gene amplified in oesophageal cancer 1) is a transforming oncogene with tumorigenic potential observed in both oesophageal squamous cell carcinoma and colorectal cancer. Nonetheless, there has been a lack of study done on this gene to understand how this gene exert its oncogenic properties in cancer. This study aims to identify novel mutation sites in *GAEC1*. To do so, seventy-nine matched colorectal cancers were tested for *GAEC1* mutation via Sanger sequencing. The mutations noted were investigated for the correlations with the clinicopathological parameters of the patients with the cancer. Additionally, *GAEC1* copy number aberration (CNA), mRNA and protein expression were determined with the use of droplet digital (dd) polymerase chain reaction (PCR), real-time PCR and Western blot (confirmed with immunofluorescence analysis). *GAEC1* mutation was noted in 8.8% (n = 7/79) of the cancer tissues including one missense mutation, four loss of heterozygosity (LOH) and two substitutions. These mutations were significantly associated with cancer perforation ($p = 0.021$). *GAEC1* mutation is frequently associated with increased *GAEC1* protein expression. Nevertheless, *GAEC1* mRNA and protein are only weakly associated. Taken together, *GAEC1* mutation affects *GAEC1* expression and is associated with poorer clinical outcomes. This further strengthens the role of *GAEC1* as an oncogene.

## 1. Introduction

Gene amplified in esophageal cancer 1 (*GAEC1*) was first reported in the molecular pathogenesis of oesophageal squamous cell carcinoma (Law et al., 2007). *GAEC1* is located within the first intron of SH2 B adaptor protein 2 (*SH2B2*) gene in 7q22 (Tang et al., 2001) and consisted of only a single-exon (Law et al., 2007). Thus, it fitted the criteria as a nested gene (Lee and Chang, 2013; Jovelin and Cutter, 2016). The 2052 bp long *GAEC1* mRNA produces a small nuclear protein of 109 amino acids (~15 kDa) (Law et al., 2007; Wahab et al., 2017).

Overexpression of *GAEC1* in 3T3 fibroblast cells formed undifferentiated sarcoma in athymic nude mice and increased colony formation, indicating its role as a transforming oncogene (Law et al., 2007). On the other hand, knockdown of *GAEC1* fully suppressed xenograft tumour growth in mice (Wahab et al., 2017). In vitro analysis also showed that knockdown of *GAEC1* inhibited cell proliferation, reduced cell migration capacity, decreased clonogenic potentiality and induced apoptosis in both oesophageal squamous cell carcinoma (Chan et al., 2013) and colon cancer cells (Wahab et al., 2017). In addition, knockdown of *GAEC1* was also associated with reduced expression of bcl-2 (B-cell lymphoma 2) and KRAS (Kirsten rat sarcoma virus) and augmented expression of p53 (Wahab et al., 2017).

Despite having preliminary data on genetic variations and *in-vitro/in-vivo* functional properties, the mutation profile of *GAEC1* and their associated pathogenic effects in human cancers are still unknown. Furthermore, no mutations or polymorphisms have been noted in *GAEC1* coding sequence (Law et al., 2007). Thus, for the first time, the current study aims to identify possible mutations and determine the absolute quantification of *GAEC1* copy number aberration in patients with colorectal cancer. Due to the nature of *GAEC1* that had only one exon and a total sequence length of 2052 bp, Sanger sequencing was the preferred method over Next Generation Sequencing (NGS). Additionally, the correlations of *GAEC1* mutations with various clinical and pathological parameters in patients with colorectal cancer were analysed in this study.

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## 2. Material and methods

### 2.1. Recruitment of tissue samples and clinicopathological data

Cancer tissues and matched non-neoplastic mucosae near the surgical resection margin were obtained from the same patient who underwent resection of colorectal carcinomas by a colorectal surgeon (CTL) between the years 2012–2014 in a Queensland-based hospital. The patients were recruited on an ongoing basis without bias during the study period. The patients were excluded from the study if the prospectively collected tissues lacked adequate cancer mass or were lost in clinical follow-up. Ethical approval was obtained for the use of these tissues from Griffith University Human Research Ethics Committee (GU Ref No: MSC/17/10/HREC).

These samples were snap frozen in liquid nitrogen and stored at −80 °C until use. Colorectal carcinomas were graded and typed according to the World Health Organization (WHO) criteria (Hamilton et al., 2010) and staged according to the tumour, lymph node and metastases (TNM) classification (Milburn et al., 2017). Only adenocarcinomas were included in the study. After examination, 79 patients (40 females; 39 males) with colorectal adenocarcinoma were included for the analysis. A schematic summary of the exclusion criteria is shown in Fig. 1.

The mean age of patients within this study was 67 years (ranging from 24 to 91). The site and size (length in mm) of the cancers were recorded. Proximal cancers were cancer located in the caecum, ascending colon and transverse colon whereas distal cancers were defined as the cancers found in the region of descending colon, sigmoid colon and rectum. 45.6% of colorectal cancers were found in the proximal colon (n = 36) while 54.4% of colorectal cancers were found in the distal colorectum. Out of the 79 patients, 86.1% (n = 68) had no cancer perforation while the remaining 13.9% (n = 11) patients had perforation. A total of 2.5% (n = 2) patients were diagnosed with stage I carcinomas, 11.4% (n = 9) with stage II carcinomas, 60.8% (n = 48) with stage III carcinomas and 25.3% (n = 20) with stage IV carcinomas.

### 2.2. Clinical management

Clinical management was by the standardised multi-disciplinary protocol. All the patients were discussed in weekly multi-disciplinary team management meeting during the course of their management. The cancer tissues were tested for microsatellite instability (MSI) markers (by immunochemistry) and tested for *BRAF* mutation status (via Sanger sequencing) where MSI proved positive according to the clinical guidelines. Pathological staging was used as a determining factor for post adjuvant therapy. Follow-up period was outlined as the interval between the date of surgical resection and the date of death or end date of the study. The actuarial survival rate of the patients was assessed from the date of surgery to the date of death of last follow-up. Only cancer-related death was accepted as the endpoint in the statistical analysis (Lam et al., 2008). Persistence or recurrence of the disease were also noted.

### 2.3. Cell culture and transfection

Three colon cancer cell lines SW480 (ATCC® CCL-228™), SW48 (ATCC® CCL-231™) and HCT 116 (ATCC® CCL-247™) and one non-neoplastic colon epithelial cell line FHC (ATCC® CRL-1831™) were obtained from the American type culture collection (ATCC) and maintained accordingly.

SW480, SW48 and HCT 116 cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated in a 37 °C incubator supplemented with 5% $CO_2$. FHC cells were cultured in Dulbecco's Modified Eagle Medium (DMEM): F-12 media supplemented with extra buffer HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) for a final concentration of 25 mM, 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL of transferrin, 100 ng/mL transferrin, 10% FBS and 1% penicillin/streptomycin and incubated in a 37 °C incubator supplemented with 5% $CO_2$. All cells were routinely checked for mycoplasma contamination. SW48 cells were transiently transfected using jetPRIME® Polyplus transfection reagent (Polyplus-transfection® SA, Illkirch, France) according to the manufacturer's protocol with *GAEC1* plasmid kindly provided by Law and colleague (Law et al., 2007).

### 2.4. Genomic DNA and RNA extraction

Tissues were sliced to 7 µm sections using a cryotome. Then, the sections were stained with haematoxylin & eosin (H&E) to confirm pathological diagnosis. Tissues were only accepted as samples for further analysis if the cancer comprises 70% of the tissue. The clinical and pathological information of every case were reviewed by the author (AKL). Genomic DNA (gDNA) and RNA were then extracted from the selected tissues with Tissue & Blood extraction kit and miRNeasy Mini kit (Qiagen, Hilden, Germany) respectively according to the

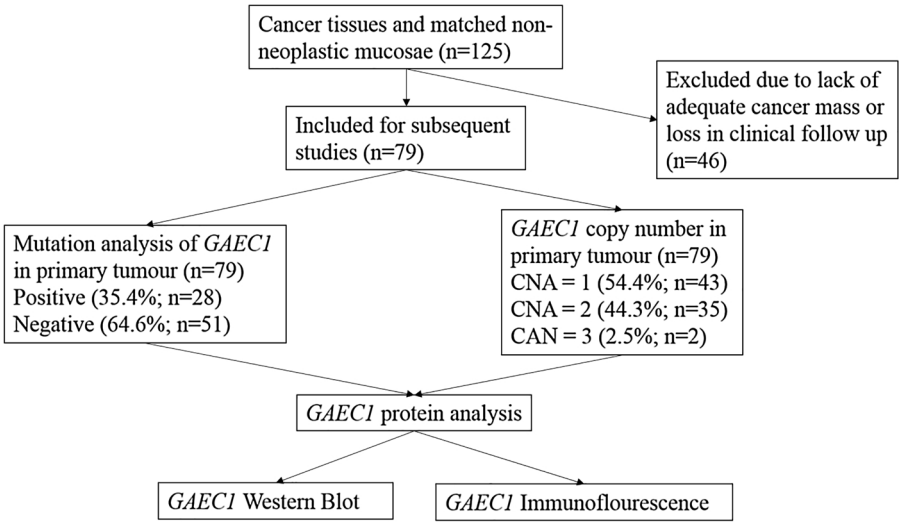


**Fig. 1.** Schematic representation of the methodological flow used. Inclusion criteria – patients with colorectal carcinomas underwent surgical resection by the same surgeon; and, the same pathologist examined all clinicopathological details. Exclusion criteria – patients who were lost to follow up or no adequate cancer tissue collected.