ORIGINAL ARTICLE

# GFAT1: A Potential Prognostic Biomarker in Colorectal Cancer

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## ABSTRACT

**Introduction:** There is an increasing demand for additional techniques to diagnose and treat cancer including CRC or colorectal cancer effectively. Utilizing antibodies as biomarker could contribute to accurate diagnosis of cancer due to its high specificity and sensitivity. One of the etiologies of CRC progression was proposed as the alterations of hexosamine biosynthetic pathway which could subsequently influence the rate-limiting enzyme, glutamine-fructose-6-phosphate aminotransferase (GFAT1). These increased enzymatic activities resulted in an elevation of glucose uptake that provides nutrients facilitating the progression of cancer cells. Therefore, we attempted to determine the potential of GFAT1 as the biomarker for CRC by correlating its expression with clinicopathological features of the patients. **Methods:** A total of 132 10% formalin-fixed paraffin embedded tissue were retrieved. Immunohistochemistry (IHC) was performed on the tissue sections and digital images were subsequently acquired. All the images were automatedly analyzed using IHC Profiler. GFAT1 immunoreactivity in colorectal tissues was calculated using an adapted H-score formula. Clinicopathological features of the patients were statistically correlated with the status of GFAT1. **Results:** Colorectal adenocarcinoma tissues had the significantly highest GFAT1 H-scores with the mean of 103.18 compared to adenoma and non-tumor tissues. There have been no significant associations between clinicopathological characteristics of the patients and the status of GFAT1 except for tumor size. **Conclusion:** Immunoreactivity of GFAT1 was significantly different between non-tumorous tissues and adenocarcinoma as well as between adenoma and adenocarcinoma tissues. GFAT1 could serve as one of the prognostic biomarkers or useful targets.
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## INTRODUCTION

Colorectal cancer (CRC) has been reported to occur in more than one million new patients and approximately 10% of those patients die due to the disease every year in the world (1). Accurate diagnosis and the use of molecular targeted immunotherapies are considered effective at improving the clinical outcomes and overall survival among CRC patients (2). It is therefore crucial to explore the mechanisms underlying the initiation and prognosis of CRC. Advances in the molecular genetics have led to novel findings of biomarkers and treatments for CRC. Several types of CRC biomarkers were currently being used in pre- or clinical trials (3). Examples of diagnosis biomarkers listed are carcinoembryonic antigen (CEA) and Adenomatous Polyposis Coli (APC) whereas, small non-coding RNA sequences (microRNAs) or short series repeats of DNA sequences (Microsatellites) status as prognosis biomarker and mutated KRAS oncogene or tumor suppressor gene P53 (TP53) were used as the predictive biomarkers. Despite having these biomarkers, there were insufficient evidence and trials which pointed them to be a specific and sensitive biomarker against CRC. Implications towards these limitations have resulted with increasing demands towards novel biomarkers to fully elucidate pathways related to the disease and help reducing the death incidences resulted due to poor diagnostic and treatments choices. One of CRC progressions is also considered the results of prominent biosynthetic pathways and genetic alterations (4,5). Compared to the normal cells, cancer cells generally have an upregulated glucose uptake and underwent glycolysis resulting in increasing yield of intermediate glycolytic metabolites which are utilized as the substrates for the hexosamine biosynthetic pathway (HBP) (6). HBP is initiated by the rate-limiting enzyme, glutamine-fructose-6-phosphate aminotransferase (GFAT1), which converts fructose-6-phosphate to glucosamine-6-phosphate (7). GFAT1

plays a vital role in the cellular glycolysis pathways, where dysregulation in its expression may facilitate the growth of cancer cells (8). GFAT1 was reported to be overexpressed in the pancreatic tumours, chronic pancreatitis patient samples, and hepatocellular cancer (9–11) which were all significantly associated with the worse progression-free survival and overall survival among patients (12). In addition, inhibition of GFAT1 was also reported to reduce the aggressiveness and inhibit the proliferation of pancreatic and hepatocellular carcinoma cells, suggesting that GFAT1 plays a main role in promoting tumorigenesis. However, the protein levels and clinical significance of GFAT1 expression in colorectal cancer have remained unclear. Therefore, we attempted immunohistochemical analysis to determine the expression patterns of GFAT1 in colorectal cancer and correlate the findings with clinicopathological characteristics among patients. These findings could provide evidence to contribute to developing GFAT1 as an additional prognostic biomarker to improve the diagnosis and outcomes of CRC patients.

## MATERIALS AND METHODS

### Tissue samples

This retrospective study was approved by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (MOH) [NMRR-20-1530-53858] and UniSZA Human Research Ethics Committee (UHREC) [UniSZA/UHREC/2021/228]. Briefly, 10% formalin-fixed paraffin embedded (FFPE) tissue blocks including 78, 47 and seven blocks from the patients with colorectal adenocarcinoma, adenoma, and non-tumorous, respectively, from January 2016 until December 2018 were retrieved from Hospital Sultanah Nur Zahirah (HSNZ). Paraffin blocks from the patients with hereditary nonpolyposis colorectal carcinoma, inflammatory bowel disease and incomplete clinical data were excluded. Clinicopathological characteristics of the patients were recorded from the pathology reports deposited in the Laboratory Information System (LIS). Pancreatic carcinoma paraffin block was included as positive control and in negative control of immunostain, primary antibody was omitted. These histological samples were sectioned at three-micron thickness, baked at 60°C for 15 minutes and subsequently stained on the salinized glass slides (MUTO, Japan). Deparaffinization and rehydration were subsequently performed using xylene and ethanol (Leica Biosystems, USA) prior to antigen retrieval.

### Immunohistochemistry (IHC)

IHC was performed using anti-GFAT1 [EPR4854] monoclonal antibody (Abcam, USA) according to the protocol of the manufacturer, EnVision™ FLEX Mini Kit, High pH (Link, Dako, Denmark). The following conditions were employed for the monoclonal antibody with 1:1000 ratio for overnight incubation at 4°C. Heat-based antigen retrieval method was conducted by microwave irradiation set at high power for 20 minutes and using the high pH (pH 9) antigen retrieval solution (Dako, Denmark). Samples were stained for five minutes using the EnVision FLEX DAB + Chromogen (Dako, Denmark) solution to visualize on the antigen-antibody complex. All tissue sections were counterstained with EnVision FLEX Hematoxylin (Link, Dako, Denmark) for five minutes. The sections were then rehydrated, mounted, and viewed under the light microscope for verification of the findings. Brown cytoplasmic staining of GFAT1 is considered as a positive expression. The sections were air dried and stored in slide box until image acquisition.

### Image acquisition

Digital images were captured at five different fields representing the whole tissue sections using the Cell^F software (Olympus, Japan) and Fluorescence Microscope BX43 (Olympus, Japan) with magnification of 40x.

### Automated scoring for immunohistochemical staining

The digital images were analysed for the staining intensity of the cytoplasmic immunoreactivity of GFAT1 using the IHC Profiler plugin of ImageJ (Fiji) downloaded from the Sourceforge website (https://sourceforge.net/projects/ihcprofiler/). The scoring was conducted as reported by Varghese F. and colleagues (13) with modifications. Selected immunopositive cells were highlighted by using the selection tool to draw a line around the selected cells. Histogram profiles generated from the images categorized them as high positive, positive, low positive and negative based on their pixel colour intensities (13). The automated scores generated were tabulated in Microsoft Excel and their mean values were obtained to calculate on the immunoreactivity of GFAT1 using the adapted H-score formula.

Immunoreactivity evaluation

The immunoreactivity score was calculated based on the adapted H-score formula which quantified the cytoplasmic staining intensities between the range of 0-300 scale (14). The relative intensity scores were assigned as follows: high positive=3, positive=2, low positive=1 and negative=0. In this study, the H-score was obtained by adding on the automated value of percentage of pixel intensity score multiplied with the relative intensity scores. The calculated immunoreactivity scores were then presented in a bar chart.

H-score,
= (3 x percentage of high positive pixel intensity)
+ (2 x percentage of positive pixel intensity)
+ (1 x percentage of low positive pixel intensity)
+ (0 x percentage of negative pixel intensity)

### Statistical analysis

Data were statistically analyzed using Statistical Package for Social Science Version 26.0. Comparisons of GFAT1 expression intensities and GFAT1 immunoreactivity

scores among different types of tissue samples were evaluated using Fisher's Exact test and one-way analysis of variance (ANOVA) followed with post-hoc multiple comparison test Scheffe's procedure respectively. Association between the clinicopathological data against the expression patterns of GFAT1 in the colorectal tissue samples were tested using Fisher's Exact Test, ANOVA, Pearson Chi-square, and Mann-Whitney. The data is considered significant when p-value less than 0.05.

## RESULTS

### Localization, distributions, and patterns of GFAT1 immunoreactivity in colorectal cancer patients

GFAT1 was markedly positive in the cytoplasm of the tumor cells with no nuclear immunoreactivity. The cases were qualitatively categorized according to the relative immunointensity of GFAT1 according to IHC Profiler as summarized in Table I (13). As tabulated, there was a significant difference (p=0.000) between GFAT1 immunointensity of high positive, positive, low positive and negative expression among all types of tissues. About 90% of the adenocarcinomas were immunohistochemically positive for GFAT1.

**Table I: Analysis of GFAT1 stained digital images in different types of colorectal tissue samples**

| GFAT1 staining intensities | Adeno-carcinoma | Adenoma | Non-tumour | *P*-value |
|---|---|---|---|---|
| High Positive | 8 | 0 | 0 | 0.000 |
| Positive | 32 | 9 | 0 | |
| Low Positive | 30 | 16 | 2 | |
| Negative | 8 | 22 | 5 | |

Few representative digital images of the adenocarcinoma tissues representing on the distinct cytoplasmic staining intensities were selected to confirm on the different classification generated by the automated software. Fig. 1 clearly demonstrates that DAB stained the cytoplasmic region in brown whereas hematoxylin stained the nucleus region in blue. These digital images were arranged according to decreasing staining intensities from high positive to positive, low positive and negative. The differences of the morphology and staining intensities detected in the colorectal tissues were displayed along the positive controls in Fig. 2. Cancer cells were morphologically different by having irregular shape with a scarce cytoplasm (15). Darker stains could be observed in the adenocarcinoma tissues as compared to the others showing the upregulation of GFAT1 protein expression. Visible positive staining of positive control tissue also confirmed on the robustness and reproducibility of the techniques employed.

### Scoring of immunohistochemical staining

The mean of immunoreactivity score for the colorectal tissues was summarized in Fig. 3. We used the adapted H-score formula to calculate on the immunoreactivity of GFAT1 by using the scores generated from IHC Profiler. Our data showed that there was increasing immunoreactivity of GFAT1 from non-tumor to adenoma and adenocarcinoma tissues. Results obtained also revealed that adenocarcinoma had the highest immunoreactivity score with the mean of 103.18 followed by adenoma with the mean of 58.18 and non-tumor with the mean of 41.35. At 5% level of significance, the mean H-score calculated was significantly different (p=0.000) among the tissues.

In addition, the mean differences between each type of tissue samples using one-way ANOVA followed with

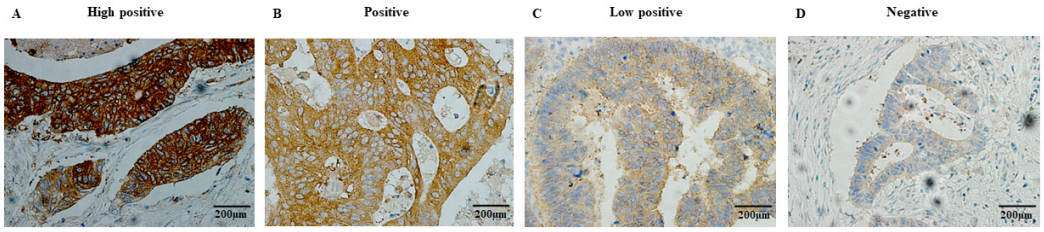


**Figure 1: Digital images of DAB cytoplasmic staining intensities in colorectal adenocarcinoma tissues expressing GFAT1.** (A) High positive staining intensity (B) Positive staining intensity (C) Low positive staining intensity (D) Negative staining intensity. All images were captured at the magnification of 40x.

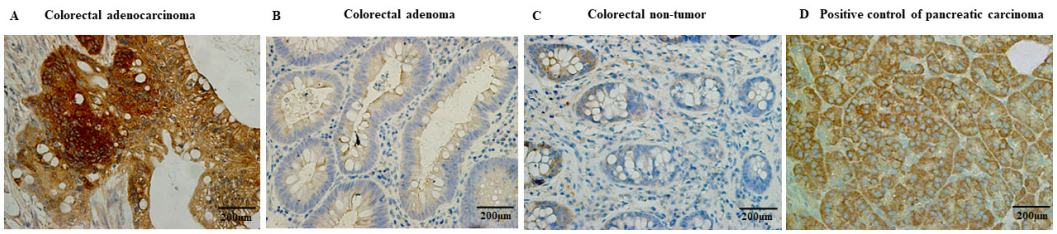


**Figure 2: Immunohistochemical stained digital images of GFAT1 in different types of tissues samples.** (A) Colorectal adenocarcinoma tissue (B) Colorectal adenoma tissue (C) Colorectal non-tumor tissue (D) Positive control of pancreatic carcinoma tissue. These images were captured at magnification of 40x.

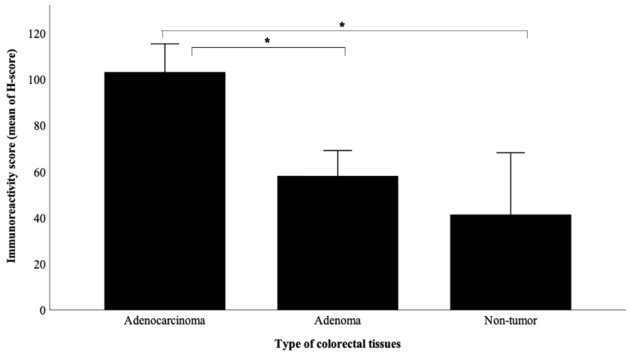


**Figure 3: Immunoreactivity score calculated for different types of colorectal tissues.** The mean difference is considered significant when p<0.05.

post-hoc analysis were summarized in Table II. Results revealed the significant differences (p<0.05) between non-tumor and adenocarcinoma as well as between adenoma and adenocarcinoma tissues. However, there were no significant differences (p=0.687) in the mean H-scores between non-tumor and adenoma tissues. Therefore, results indicated that GFAT1 was highly expressed in malignant tissues compared to benign tissues.

**Table II: Comparison of GFAT1 expression in colorectal tissue samples**

| Type of colorectal tissue samples | Mean difference with 95% confidence interval | *P*-values |
|---|---|---|
| Adenoma vs. Non-tumour | 21.00 (64.84, -31.19) | 0.687 |
| Adenocarcinoma vs. Non-tumour | 93.84 (108.59,15.07) | 0.006 |
| Adenocarcinoma vs. Adenoma | 45.00 (66.88,23.12) | 0.001 |

ANOVA test was applied followed by post-hoc multiple comparison test Scheffe's procedure F (df) = 19.27 (2, 19.15), $p$<0.001.

## Association between GFAT1 and clinicopathological characteristics of colorectal cancer patients

The clinicopathological characteristics of patients' samples retrieved were summarized in Table III. These characteristics were then associated with GFAT1 immunoreactivity in all the cases examined. The digital images categorized with high positive, positive, and low positive staining were grouped as positive GFAT1 group whereas those with negative staining as negative GFAT1 group. Most of the adenocarcinoma tissue were found to be moderately differentiated. Moreover, the adenoma tissues mainly belonged to the low or mild category of dysplasia. Besides that, most of the samples were also found to have already metastasized to the lymph node with cancer stage above pT2. This data further confirms on the trends of late diagnosis among CRC patients. It was also observed that higher staged tumor has much bigger size of nucleus compared to the lower staged tumor. Selected images of adenocarcinoma tissues samples classified at different tumor staging were displayed in Fig. 4. Results from the association test found no significant differences (p>0.05) between GFAT1 expression patterns and the listed clinicopathological characteristic of CRC patients. When Mann-Whitney was applied, the only characteristic that obtained p<0.001 was the association between the tumor size and GFAT1 expression. Tumor with larger mean size of 47.59 mm was identified with positive GFAT1 expression (data not shown). Thus, this data supported on the assumption of GFAT1 being involved with the growth of tumor.

**Table III: Clinicopathological profiles between GFAT1 expressions in adenocarcinoma, adenoma, and normal colorectal tissue samples**

| Variables | Total (n) | Positive GFAT1 expression | Negative GFAT1 expression | *P*-values |
|---|---|---|---|---|
| Gender[a] | | | | |
| Male | 72 | 52 | 20 | 0.843 |
| Female | 60 | 45 | 15 | |
| Age[a] | | | | |
| <60 | 39 | 25 | 15 | 0.133 |
| ≥60 | 93 | 72 | 21 | |
| Tumour site[b] | | | | |
| Caecum | 2 | 2 | 0 | |
| Ascending Colon | 21 | 12 | 9 | |
| Hepatic flexure | 3 | 3 | 0 | |
| Transverse colon | 13 | 9 | 4 | |
| Splenic flexure | 4 | 4 | 0 | 0.298 |
| Descending colon | 11 | 8 | 3 | |
| Sigmoid colon | 49 | 40 | 9 | |
| Rectosigmoid | 10 | 8 | 2 | |
| Rectum | 7 | 5 | 2 | |
| Colon, unspecified | 12 | 6 | 6 | |
| Tumour differentiation[b] | | | | |
| Poor | 4 | 3 | 1 | |
| Moderate | 48 | 44 | 4 | 0.520 |
| Well | 25 | 23 | 2 | |
| No information | 1 | 1 | 0 | |
| Dysplasia in Adenomas[b] | | | | |
| Low/Mild | 35 | 19 | 16 | 0.787 |
| Moderate | 8 | 3 | 5 | |
| High/Severe | 4 | 2 | 2 | |
| Pathologic Tumour Stage (pT)[b] | | | | |
| T1 | 1 | 0 | 1 | |
| T2 | 16 | 15 | 1 | 0.161 |
| T3 | 40 | 37 | 31 | |
| T4 | 21 | 19 | 2 | |
| Lymph node metastasis[b] | | | | |
| + | 52 | 47 | 5 | 1.000 |
| - | 26 | 24 | 2 | |

[a] Pearson Chi-square
[b] Fisher's Exact

## DISCUSSION

In this immunohistochemical study of GFAT1 in colorectal cancer (CRC) with correlation of the results with the clinicopathological characteristics of CRC patients, a significantly higher cytoplasmic expression of GFAT1 (p<0.05) was detected in adenocarcinoma

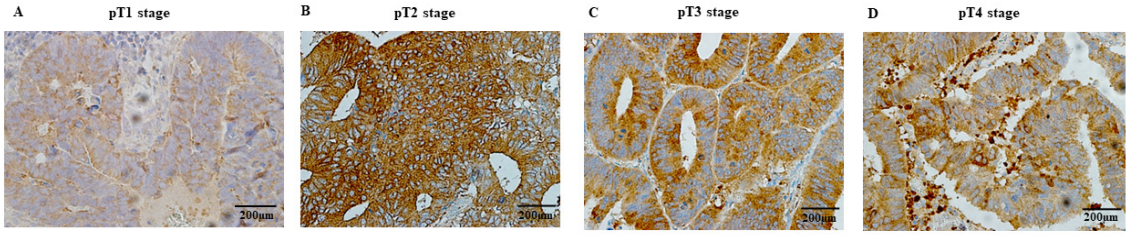


**Figure 4: Digital images of GFAT1 staining of adenocarcinoma tissue samples with different pathologic tumour stage (pT).** (A) pT1 stage (B) pT2 stage (C) pT3 stage (D) pT4 stage. All images were captured at the magnification of 40x.

tissues compared to benign tissues and also significantly associated with the tumor size. The findings were consistent with those of previous studies demonstrating its positive association with cancer progression resulting in poor prognosis in cancer patients (10,16). These studies shed insights on the roles of GFAT1 in carcinogenesis and the clinical outcome of those cancer patients. They have demonstrated that cancer patients had shown an upregulation of glucose uptake and dysregulation of glycolysis which promote cancer cell growth and produces glycolytic metabolites (6,7,17,18). A similar conclusion was reached where CRC tumorigenesis could also be regulated by suppressing this enzymatic end-product expression (19). Interestingly, the result shown in gastric cancer patients showed that loss of GFAT1 expression improves the prognosis of patients. This implied that GFAT1 expression might be associated with tissue specificity (20). Future studies could explore this issue further by elucidating on the mechanisms involved to discover novel biomarkers and potential targets for better diagnosis and treatment of CRC (21).

In our present study we also employed digital pathology in scoring of immunopositive cells identified by DAB colorimetric reaction instead of using the conventional method (14). The application of this method could help reducing bias measurement in scoring immunointensity in tissue samples, which is relatively time consuming and generally requires intensive labour (13). As summarized in this study, the cancer cells had relatively large nuclei and due to rapid growth, they tended to be close to each other which resulted in difficulties of counting each cell manually. It could therefore be difficult to differentiate the immunointensity as reported in the automated software, IHC Profiler due to our eye's limitation. Varghese et al. successfully demonstrated a significant match of 88.6% in the comparison study between the conventional scoring method and automated scoring using IHC Profiler (13). Digital pathology greatly could therefore reduce workload, provide a better approach for data storage encouraging paperless environment and enabling pathologist to connect with physicians and review reports efficiently via network worldwide. This method significantly delivered better classification of histological specimens that could reduce human errors and was designed to provide feasibility in image analysis where it can be performed almost anywhere, shorten the analysis time besides yielding robust results (22–25).

Benefitting from automated scoring we managed to qualitatively classify the cases into positive and negative GFAT1 immunoreactivity and correlated the findings obtained with the clinicopathological characteristics of the patients. It is interesting to note that tumor size was the only characteristic that had a statistically significant association with GFAT1 as reported in other studies (10,26,27). However, we managed to prove on the upregulation of GFAT1 in colorectal adenocarcinoma tissues and demonstrated its clinical significance of correlation with known prognostic factors such as the tumor size. Demographic analysis in our present study demonstrated that 92.7% of the total patients are Malay patients whereas only 6.6% of them Chinese and 0.7% Indian showing disagreement with the Malaysia National Cancer Registry Report 2012-2016 mainly because Malay population dominated at this selected site of investigation being conducted. In addition, the cases examined were mainly isolated from male and senior or elderly patients providing evidence in line with the cancer incidences statistics in Malaysia reported in GLOBOCAN 2020 (28). Based on pathological examination, most of the adenocarcinoma appeared as fungating whereas the adenomas appeared as tubular features. Our results showed that 91% of the total adenocarcinoma cases were immunohistochemically positive for GFAT1. As summarized in Table III, we demonstrated that the majority of the patients examined were categorized as pT3 and pT4 stages, low or mild differentiation and metastasized towards lymph nodes and nearest organs such as the small intestine, uterus, or lungs. These developed or relatively late stage patients could contribute to the rising number of colon cancer incidences and mortality every year in Malaysia (29). However, the regular health check-up at the hospital could enhance the detection of CRC at an earlier stage (29,30). This is important because late-stage cancer patients had fewer selections of suitable treatments and had more complications in recovering from the disease. Therefore, determination on the effectiveness of utilizing GFAT1 as a prognostic biomarker to diagnose cancer prognosis or as potential target to disrupt the prominent carcinogenesis pathways may require additional investigation with a larger patient cohort.

## CONCLUSION

We demonstrated the significantly higher aberrant

expression of GFAT1 in colorectal cancer (CRC) patients indicating the upregulation of the hexosamine biosynthetic pathway resulting in cancer progression showed its potential to serve as the prognosis biomarker or as therapeutic target. Despite the limitations, this study casts a new light on applying digital pathology to accurate diagnosis of cancer by referring to the digitized tissue specimens. Future research should consider recruiting CRC patients to conduct a less invasive blood-based test by using GFAT1 mRNA as a validation tool.

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