RESEARCH ARTICLE

CHEK1 and GFPT1 as Potential Blood-Based Biomarkers for Colorectal Cancer

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Abstract

BACKGROUND: The checkpoint kinase 1 (CHEK1) and glutamine-fructose-6-phosphate aminotransferase 1 (GFPT1) genes have been reported to have a crucial role in carcinogenesis in colorectal cancer (CRC). However, their association with the pathogenesis of CRC remains unclear. This study was conducted to study the expression of CHEK1 and GFPT1 genes in adenoma and adenocarcinoma CRC patients' whole blood samples compared to the healthy controls.

METHODS: A comparative cross-sectional study to examine the expression of CHEK1 and GFPT1 genes were conducted in 6 colorectal adenoma and 6 colorectal adenocarcinoma patients along with 6 healthy controls. Blood samples were taken from subjects, and CHEK1 and GFPT1 genes were analyzed by using reverse transcription quantitative polymerase chain reaction (RT-qPCR).

RESULTS: CHEK1 gene expression in blood samples has the highest in adenoma patients meanwhile for the GFPT1 gene expression has the highest in adenocarcinoma patients. Higher CHEK1 gene expression were also found in adenoma patients compared to healthy controls (p=0.040) and adenocarcinoma patients (p=0.025). Besides, at the 5% level of significance, the median GFPT1 gene expression was higher in colorectal adenocarcinoma patients compared to colorectal adenoma patients and healthy controls.

CONCLUSION: CHEK1 and GFPT1 may function as potential regulators in adenoma and adenocarcinoma and measuring their expression might be a potential tool to determine the CRC progression and could be further explored as blood diagnostic biomarkers for CRC patients.

KEYWORDS: CHEK1, GFPT1, colorectal cancer, adenoma, adenocarcinoma

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Introduction

According to the World Health Organization, colorectal cancer (CRC) is the second leading cancer-related death, accounting for nearly a million deaths each year. Colorectal adenocarcinoma is the most common type of cancer that starts to develop in the large intestine. Most of the CRC cases were diagnosed at advanced or late stages. This situation is worrying since it is quite difficult to treat patients with the late stage of CRC because the cancerous cells usually have metastasized to the other parts of the body, especially the liver.(1) Colorectal adenoma is a kind of polyp or abnormal cell growth that build up in the epithelium of the colon and rectum producing a small clump. This small clump is commonly considered benign. Even though most

colorectal adenomas are harmless, nearly up to 0.43% of them can progressively develop into adenocarcinoma each year which is a serious health issue if untreated earlier.(2,3) Hence, potential biomarkers detecting cancer progression or metastasis at an early stage is very important to prevent tumorigenesis and delayed treatment. This can be done by conducting an immunohistochemistry methods or *in vitro* study which described the elevation in the expression levels of potential target genes by utilising the early and late-stages CRC model cell lines to confirm it as an important metastasis marker for CRC.(4-8)

Most patients refused to fully participate in physical CRC screening procedures such as colonoscopy because it is invasive, irritating, and painful which then leads to delayed treatment.(9) Prior to the colonoscopy procedure, screening was done by using the immunological faecal occult blood test (iFOBT) kit among the asymptomatic patients to detect the presence of blood in their stool. However, lack of knowledge, fear of results and embarrassment from the procedures might greatly influence and discourage citizens from participating in the screening procedure earlier and refusing hospital appointments.(9) Therefore, biomarkers which utilized patients' blood is a better alternative with less invasive procedure which could be a much-preferred screening tool for CRC compared to the currently available screening test.

Currently, carcinoembryonic antigen (CEA) is the blood-based biomarker established for the purpose of monitoring the prognosis, treatment response and recurrence of CRC in patients.(10) This is because the CEA is highly expressed in advanced stages of colorectal malignancies. (11) The elevation of this antigen is usually linked to cancer progression and can represent a relapse after treatment. However, the elevated level of CEA in patients' whole blood is not specific to CRC only because it also can be highly detectable in other inflammatory diseases such as irritable bowel syndrome, pancreatitis, and liver.(11) Therefore, CEA was not applied as the blood-based screening biomarker for CRC patients.

CHEK1 is a gene encoded for checkpoint kinase 1 (CHK1) enzyme which is also known as a serine/threonine kinase protein in humans.(12) Generally, the vital role of CHK1 is to regulate DNA damage response (DDR) pathway and cell cycle checkpoint.(7) This complex pathway is activated and induced when there is DNA damaging incidence such as the replication stress.(13) Replication stress has been known to cause serious impacts including genome instability and it is interconnected with pre-tumor and tumor cells.(14) Additionally, CHK1/CHEK1 was reported to be highly expressed in breast, lung, and CRC.(7,15-17)

The GFPT1 gene encodes for the GFAT1 enzyme which mainly regulates the Hexosamine Biosynthetic Pathway (HBP) which is linked to disease and may be used as a therapeutic target.(18) GFAT1/GFPT1 was proven to be highly expressed in different types of cancer such as pancreatic, hepatocellular, and cervical cancer.(19-21) It was described in the previous study that the potential of developing GFAT1 protein expression as a prognostic tissue-based biomarker in CRC by showing a significantly high expression in the patients' adenocarcinoma.(8) The HBP activation would probably cause an elevation towards GFPT1 gene expression which might potentially serve as a novel blood-based biomarker for CRC.

In the present study, we hypothesized that CHEK1 and GFPT1 genes could be potential blood-based biomarkers for CRC which may help in the early screening and diagnosis of CRC's patients. Therefore, the gene expression CHEK1 and GFPT1 in 6 colorectal adenoma, 6 colorectal adenocarcinoma patients and 6 healthy controls were investigated.

Methods

Subjects Recruitment and Sample Collection

A comparative cross-sectional study involving a total of 18 subjects, consisted of 6 colorectal adenoma patients, 6 colorectal adenocarcinoma patients and 6 healthy controls was conducted.(22) Only limited number of subjetcs were recruited due to the study time restriction. Colorectal adenoma and adenocarcinoma subjects were recruited from newly diagnosed patients that was registered under Hospital Sultanah Nur Zahirah in the year of 2022, while 6 healthy control subjects were recruited from Universiti Sultan Zainal Abidin's employees that met the defined inclusion and exclusion criteria.

The inclusion criteria were colorectal adenoma and colorectal adenocarcinoma patients with complete clinical data in their medical records, and have appointments and suggested by the colorectal surgeon (co-researcher).The exclusion criteria were as follows: i) patients clinically suspected to have colorectal adenocarcinoma associated with inflammatory bowel disease; ii) patients with metastasized cancer originated from other types of cancer; iii) patients with insufficient clinical data in their medical records; iv) patients who do not have follow-up consultation at Hospital Sultanah Nur Zahirah, are bed-bounded or

deceased; and v) healthy participants with abnormal medical records related to the digestive system and under treatment or consuming prescribed medications. After adenoma and adenocarcinoma subjects' consultation appointment with the medical specialist, informed consent was obtained from each subjects. About 5 mL of whole blood from each subject was extracted by a trained phlebotomist. Their demographics and clinical data were collected from the Hospital Information System (HIS) of Hospital Sultanah Nur Zahirah. Meanwhile, for the 6 healthy control subjects, the blood samples were extracted by trained Medical Laboratory Technologist staff from Universiti Sultan Zainal Abidin. The protocol of this study was approved by the Universiti Sultan Zainal Abidin Human Research & Ethics Committee (No. UniSZA/UHREC/2021/228) and Medical Research and Ethics Committee (MREC) (No. NMRR ID-22-00518-RMO [IIR]).

RNA Isolation

Total RNA extraction from peripheral blood samples was extracted using SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA), within 3 hours of blood collection according to the manufacturer's protocol. RNA purity and concentration were measured using the Nanodrop spectrophotometer. The RNA integrity was assessed by native agarose gel electrophoresis.

Gene Expression Analysis via Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

The gene expression analysis was performed using the StepOne software v2.3 (ThermoFisher Scientific, Waltham, MA, USA). The RT-qPCR method was performed by using GoTaq® 1-Step RT-qPCR System (Promega Corporation). The qPCR machine was programmed according to the suggested parameters to run the cycle once starting from reverse transcription at 37℃ for 15 minutes to reverse transcriptase inactivation and GoTaq® DNA Polymerase activation at 95℃ for 10 minutes. Then the process was followed by 40 cycles of denaturation at 95℃ for 10 seconds, the annealing and data collection at 60℃ for 30 seconds and extension at 72°C for 30 seconds. The housekeeping gene, Beta-actin (β-actin) was included as the reference gene and the relative gene expression levels presented as Delta CT values were normalized to the β-actin. The Snapgene software was used for confirmation of the primer sequences design. The primer sequences (Integrated DNA Technologies, Coralville, IA, USA) for GFPT1, CHEK1 and β-Actin are listed in Table 1.(22-24) All the RT-qPCR reactions were performed in triplicate.

Statistical Analysis

All statistical analyses were performed using SPSS software version 25 (IBM Corporation, Armonk, NY, USA). Kruskal Wallis test followed by Multiple Mann-Whitney test with Bonferroni correction was applied to summarise the median differences of CHEK1 and GFPT1 gene expressions between the healthy controls group and experimental groups and the level of significance was set at 0.017 (based on Bonferroni correction).

Results

Subjects' Demographics and Clinical Characteristics.

The subjects' demographics and clinical characteristics were summarized in Table 2. The study involved a healthy control group that aged between 37-55 years old, and the adenoma patients aged between 55-80 years. Besides, the median age of colorectal adenocarcinoma patients was between 42-73 years old. Moreover, the stage of cancer for adenocarcinoma patients varies into different stages. One of the patients was at stage I. Three of them were at stage III and the rest were at stage IV.

Gene Expression Analysis via RT-qPCR

The results of RT-qPCR analysis were presented in the histograms of Figure 1A and Figure 1B, which indicated the frequencies of the fold change of oligonucleotides and RT-qPCR experiments relative to the mean normalized expression levels of CHEK1 and GFPT1 genes respectively in all 6 samples of healthy controls, adenoma, and adenocarcinoma whole blood samples. The results analysis also demonstrated on the distribution for the data obtained showing a not normally distribution sets of data.

Table 1. List of primer sequences for GFPT1, CHK1 and β-actin genes.

Target Genes	Forward Primers (5'-3')	Reverse Primers (5'-3')	Accession Numbers	References
GFPT1	GCAAGCAGTTGGCACAAGG	CTCCACTGCTTTTTCTTCCAC	NM_001244710	23
CHEK1	GGTGCCTATGGAGAAGTTCAA	TCTACGGCACGCTTCATATC	NM_001114121	24
β-actin	GAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT	NM_007393	22

Table 2. Subjects' demographics and clinical characteristics.

Demographics and Clinical Characteristics	Healthy Control (n=6)	Adenoma (n=6)	Adenocarcinoma (n=6)
Age range (years)	37-55	55-80	42-73
Sex, n (%)			
Male	2 (33.3%)	6 (100%)	4 (66.7%)
Female	4 (66.7%)	-	2 (33.3%)
Race, n (%)			
Malay	6 (100%)	5 (83.3%)	6 (100%)
Chinese	-	1 (16.7%)	-
Cancer staging, n (%)			
I	-	-	-
II	-	-	1 (16.7%)
III	-	-	3 (50.0%)
IV	-	-	2 (33.3%)

CHEK1 was Higher in Colorectal Adenoma Subjects

The median of CHEK1 gene expression in blood samples of healthy controls, adenoma and adenocarcinoma are demonstrated in Figure 2A. The data expression is expressed as median with the interquartile rate. The results revealed that adenoma has the highest CHEK1 gene expression with the median of 2.85 followed by adenocarcinoma with the median of 1.12 and healthy controls with the median of 1.

In addition, the median differences between the control group and experimental groups were summarised in Table 3 by using Kruskal Wallis Test and it showed a significant result with a p=0.046. Pairwise comparison with the Multiple Mann-Whitney Tests were then applied with a Bonferroni adjusted alpha level set at 0.017. Based on the Bonferroni correction, the gene expression analysis manifested that there was no significant higher of CHEK1 gene expression in adenoma subjects compared to healthy controls (p=0.040) and adenocarcinoma (p=0.025) and that due to the small sample size used in this study. In addition, no significant difference in CHEK1 gene expression can be observed between adenocarcinoma blood samples compared to healthy control blood (p=1.000).

GFPT1 was Higher in Colorectal Adenocarcinoma Subjects

The median of GFPT1 gene expression in healthy controls, adenoma and adenocarcinoma were shown in Figure 2B. The results revealed that adenocarcinoma has the highest

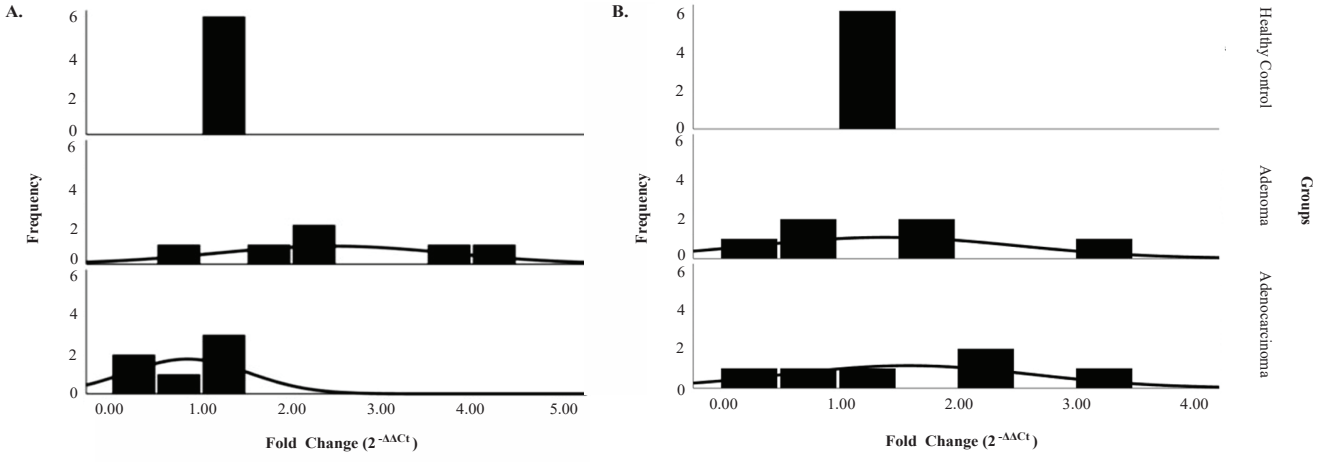

Figure 1. RT-qPCR analysis results. A: A histogram indicating the frequencies of the fold change of oligonucleotides and RT-qPCR experiments relative to the mean normalized expression levels of CHEK1 gene in healthy controls (n=6), adenoma (n=6) and adenocarcinoma (n=6) whole blood samples. B: A histogram indicating the frequencies of the fold change of oligonucleotides and RT-qPCR experiments relative to the mean normalized expression levels of GFPT1 gene in healthy controls (n=6), adenoma (n=6) and adenocarcinoma (n=6) whole blood samples.

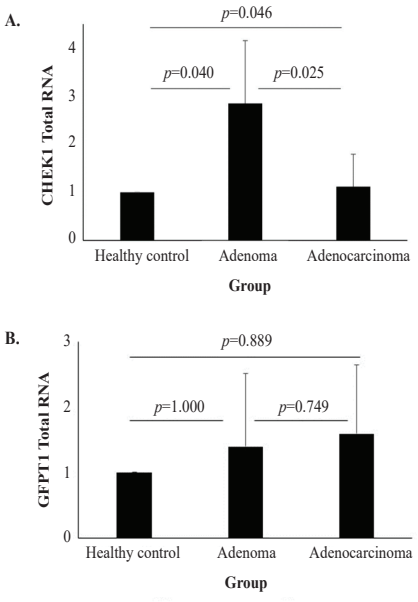

Figure 2. CHEK1 and GFPT1 gene expression analysis. A: CHEK1 total RNA [[a]Kruskal Wallis Test was applied, CHEK1 expression for Healthy control *vs.* Adenoma *vs.* Adenocarcinoma (p=0.046); [b]Multiple Mann-Whitney Test with Bonferroni correction was applied and level of significance was set at p=0.017, CHEK1 expression for Healthy control *vs.* Adenoma (p=0.040), CHEK1 expression for Healthy control *vs.* Adenocarcinoma (p=1.000), and CHEK1 expression for Adenoma *vs.* Adenocarcinoma (p=0.025)]. B: GFPT1 total RNA [[a]Kruskal Wallis Test was applied, GFPT1 expression for Healthy control *vs.* Adenoma *vs.* Adenocarcinoma (p=0.889); [b]Multiple Mann-Whitney Test with Bonferroni correction was applied, and level of significance was set at 0.017, GFPT1 expression for Healthy control *vs.* Adenoma (p=1.000), GFPT1 expression for Healthy control *vs.* Adenocarcinoma (p=0.592), and GFPT1 expression for Adenoma *vs.* Adenocarcinoma (p=0.749)].

GFPT1 gene expression with the median of 1.59 followed by adenoma with the median of 1.19 and healthy controls with the median of 1.

The median differences between the healthy controls group and experimental groups were summarised in Table 3 using Kruskal-Wallis test. The result exhibited no significant difference in GFPT1 gene expression between healthy controls, adenoma, and adenocarcinoma blood samples, with a p=0.889. At the 5% level of significance, the median GFPT1 gene expression was higher in colorectal adenocarcinoma patients compared to colorectal adenoma and healthy controls. Then, the pairwise comparison with the Multiple Mann-Whitney Tests were then applied with a Bonferroni adjusted alpha level set at 0.017. Based on the Bonferroni correction, the gene expression analysis demonstrated an insignificant difference in GFPT1 gene expression when compared to all 3 different groups: healthy controls *vs.* adenoma (p=1.000), healthy controls *vs.* adenocarcinoma (p=0.592), and adenoma *vs.* adenocarcinoma (p=0.749).

Discussion

The number of newly reported colorectal cancer (CRC) incidences and deaths keep on increasing every year despite various modern treatment techniques and approaches. Higher sensitivity and specificity of CRC screening would probably result in better outcomes followed with prompt treatments. Having early screening at younger age based on their individual risk factors could also lengthen survivability and increase the effectiveness of the treatment applied besides reducing the adverse effects and risks of mortality. Better screening techniques and usage of biomarkers would strongly influence patients' willingness to participate and give a higher compliance rate.

The results revealed that whole blood samples of patients with adenoma have the highest CHEK1 gene expression compared to the healthy controls and adenocarcinoma whole blood samples. The higher CHEK1 expression may be considered as a key indicators of the presence of tumor cells which divides at a faster rate and tend to have a higher DNA damage frequency.(15) Previous study has discovered that the CHK1 protein is overexpressed in colon cancer tissues.(7) Tumour cells with higher levels of CHEK1 also have an advantage in terms of survival due to DNA damage response (DDR).(25)

Table 3. Comparison of median CHEK1 and GFPT1 gene expression between healthy control, adenoma, and adenocarcinoma subjects.

Type of Gene Expression	Median (IqR)			X^2-statistic[a] (df)	p-value[a]
	Healthy Control (n=6)	Adenoma (n=6)	Adenocarcinoma (n=6)		
CHEK1	1.00 (0.02)	2.20 (2.61)	1.12 (1.41)	6.171 (2)	0.046
GFPT1	1.00 (0.01)	1.20 (1.51)	1.60 (1.96)	0.236 (2)	0.889

[a]Kruskal Wallis Test was applied.

It is found that the decrement of CHEK1 expression in solid tumors is usually associated with a poor prognosis in colorectal cancer (CRC) patients.(26) These findings are consistent with the results of the current study where the lower expression of CHEK1 gene in adenocarcinoma patients' blood compared to normal control and adenoma. Therefore, the CHEK1 gene has a good potential impact and may be a blood-based diagnostic biomarker for early screening of CRC. Additionally, the expression level of GFPT1 was found to have the highest in the colorectal adenocarcinoma patients' whole blood compared to colorectal adenoma and normal healthy control. These findings are supported by a previous study in which there was up-regulation of the GFPT1 gene in patients diagnosed with pancreatic ductal adenocarcinoma (PDAC).(27) Besides that, another study revealed that GFPT1 gene can be used to predict the poor prognosis in pancreatic cancer patients.(19) Remarkably, GFPT1/HBP has also been shown to be responsible for coordinating the glucose and glutamine metabolism pathway which is found to be activated in various types of cancer too.(28) Glucose has been described as the main energy provider towards the cancer cells promoting its growth and proliferation due to abnormal metabolism which then resulted in the metastasis of the malignant cells.(29)

A recent study suggested that a signal propagation channel was demonstrated in CRC originating from the early-stage biomarker mitochondrial antiviral signaling (MAVS), an essential immune protein and signalling protein in the mitochondria towards the late-stage biomarker GFPT1 where it was detected at a higher level in the late stage of CRC. While MAVS responded to CRC during its early stages where it subsequently transmitted the illness signal to GFPT1, whose dysfunction sped up the patients' cancer's progression.(30) In addition, the elevation of GFPT1 gene expression also contributed towards the rise in the levels of transforming growth factor-1 (TGF-1).(19) Additionally, it has been reported that lung adenocarcinoma cells overexpressing GFAT1 protein promote the production of a mesenchymal marker, indicating that GFAT1 stimulates the epithelial-mesenchymal transition (EMT) process contributing to cancer metastasis and progression.(31) However, there is still insufficient evidence that linked the prognostic value towards the GFPT1 gene within CRC which monitors the prognosis beginning from adenoma into adenocarcinoma. Therefore, it is suspected that an increase in the level of GFPT1 gene levels could undergo cell transformation which then resulted in the progression of CRC.

In this study, small number of samples was recruited which makes it very challenging to confirm the effectiveness as a blood-based biomarker. A small sample size influences the statistical tests conducted causing the available data not normally distributed. This small number of recruits may influence the research findings in this preliminary study and does not represent the whole sample population in real cases since it was initially performed to understand on the differential gene expression among the whole blood samples. Therefore, it is recommended to conduct additional studies using a larger sample size to establish better outcomes. Even though this study only recruited small number of subject, however it is important to analyze the expression of CHEK1 and GFPT1 because exploring further into the complex interactions and regulations between CHEK1 and GFPT1 genes along with downstream genes within the DDR and HBP pathways and their relationship with other carcinogenesis pathways might provide a better understanding of CRC progression and determine their other potential such as utilizing them as a target gene for CRC gene therapy.(32) Besides, a stronger fundamental knowledge regarding CHEK1 and GFPT1 gene expression involving normal healthy control, colorectal adenoma and adenocarcinoma patients could be established to draw a comprehensive conclusion on the relationships associated between the gene expression patterns and other related variables.

Conclusion

In conclusion, this study demonstrated a significant lower CHEK1 and higher GFPT1 in adenocarcinoma compared to adenoma, which may play a role in the cancer progression among CRC patients. The findings of the present study suggested that the expression of CHEK1 and GFPT1 genes could be potential blood-based colorectal cancer diagnostic and prognostic biomarkers for such patients.

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Authors Contribution

AA, YN, and HS designed and conceptualized the study. Data collection was performed by HF, NSA, NAM and MFAR. The experiment was conducted by HF, NSA, and NAM. Data were analysed by NSA, NAM and HANAJ. The manuscript was drafted by HF, NSA and NAM and reviewed by AA and HANAJ. Supervision was provided by AA and MFAR. The final manuscript has been read and approved by all authors.

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