The Expression of FLNA and CLU in PBMCs As A Novel Screening Marker for Hepatocellular Carcinoma.

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Abstract

Early detection improves survival and increases curative probability in hepatocellular carcinoma (HCC). Peripheral blood mononuclear cells (PBMCs) can provide an inexpensive, less-invasive and highly accurate method. The objective of this study is to find the potential marker for HCC screening, utilizing gene expression of the PBMCs. Data from the NCBI GEO database of gene expression in HCC patients and healthy donor's PBMCs was collected. As a result, GSE 49515 and GSE 58208 were found. Using both, a statistical significance test was conducted in each gene expression of each data set which resulted in 187 genes. We randomized three selected genes (FLNA, CAP1, and CLU) from the significant p-value group (p-values < 0.001). Then, a total of 76 healthy donors and 153 HCC cases were collected. Quantitative RT-PCR (qRT-PCR) was performed in cDNA from all blood samplesFrom the qRT-PCR, The Cycle threshold (Ct) value of FLNA, CLU, CAP1 of HCC group (28.47±4.43, 28.01±3.75, 29.64±3.90) were lower than healthy group (34.23±3.54, 32.90±4.15, 32.18±5.02) (p-values < 0.0001). The accuracy, sensitivity and specificity of these genes as a screening tool were: FLNA (80.8%, 88.0%, 65.8%), CLU (63.4%, 93.3%, 31.3%), CAP1 (67.2%, 83.3%, 39.1%). The tests were performed in two and three gene combinations. Results demonstrated high accuracy of 86.2%, sensitivity of 85% and specificity of 88.4% in the FLNA and CLU combination. We concluded that FLNA and CLU combination have high potential for being HCC novel markers. Combined with current tumor markers, further research of the gene's expression might help identify more potential markers and improve diagnosis methods.

Introduction

Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-related mortality worldwide.[1] Annually, liver cancer accounts for approximately 840,000 new cases and 780,000 death cases.[2] In Thailand, HCC tends to be diagnosed late and until it becomes worse, for example, 75% of patients diagnosed as HCC have already reached stage B or C in Barcelona Clinic Liver Cancer staging, and 25% of the patients were asymptomatic.[3]

Primary screening for HCC is mainly conducted either by Alpha-Fetoprotein (AFP) level from blood sample or by ultrasound imaging. If an abnormality is found, a contrast-enhanced multiphase Computerized Tomography or Magnetic Resonance Imaging study would be done.[4] However, the AFP blood test produces a wide variation of results with sensitivity ranging from 32–79.5% and specificity ranging from 29.4% – 98.5%.[5–7] Ultrasound is also problematic because it is operator dependent. The results may vary from each operator thus, reducing its capability. Ultrasound test sensitivity for the detection of HCC ranges from 29–100%, whereas its specificity ranges from 94–100%. This means both AFP and ultrasound performance as screening/diagnosis markers are not very satisfactory.[8]

Recently, there is data suggesting that cancer cells can release secretory molecules which affect the gene expressions of the patient's white blood cells (Fig. 1. a).[9] Also, there are experiments utilizing the gene expression on peripheral white blood cells to detect these cancer's influences.[10–14] Therefore, an

invention of markers that are inexpensive, simple, less-invasive and highly accurate can be expected. We focused on the usability of peripheral white blood cells (WBCs), especially peripheral blood mononuclear cells (PBMCs) as a biomarker for liver cancers.[15] The objective of this study is to find the high-performance novel markers for HCC screening, utilizing gene expression of PBMCs *(*Fig. 1.b*)*.

Results

Data summary

Bioinformatic data of the samples

Results of the CU-DREAM program showed a significant p-value in the comparison between GSE49515[16] and GSE58208[17] (p-value = 9.91×10^{-19}, Odd ratio = 2.08, upper 95%CI = 2.46 and lower 95%CI = 1.76). All 187 upregulated genes were classified to identify the highest significant p-value. Then, three genes with high significant p-values including CLU (p-value = 8.16×10^{-5}), FLNA (p-value = 3.35×10^{-5}), and CAP1 (p-value = 2.84×10^{-7}) were selected and applied to observe the gene expression in this study *(*Fig. 1.b, Table 1*)*.

Table 1
Results from the bioinformatics approach (CU-DREAM program) demonstrated the 187 upregulated genes (p-value = 9.91×10^{-19}, Odd ratio = 2.08, upper 95%CI = 2.46 and lower 95%CI = 1.76) from the intersection of GSE58208 and GSE49515 dataset (level of significance * p-value < 0.001).

GSE58208 / **GSE49515**	**Upregulated**	**Not Upregulated**	**Total**		
Upregulated	187	2,442	2,629		
Not Upregulated	722	19,645	20,367	P-value	9.91E-19
				Odds Ratio	2.08
				Lower 95% CI	1.76
				Upper 95% CI	2.46
Total	909	22,087	22,996		

Characteristics of participants

Age (Mean ± SD) of HCC group was 58.93 ± 9.99 and of Healthy donor group was 48.32 ± 5.16. Gender data shows that our samples had more males than females *(*Table 2*)*; The HCC group has 124 males to 29 females, and the Healthy group has 45 males to 31 females. The staging of HCC from the cancer group showed 12 samples in stage 0, 54 samples in stage A, 61 samples in stage B, 26 samples in stage C, and no samples in stage D according to the BCLC staging system.

Table 2
Demographic data of samples. Healthy Donor (N = 76) and HCC (N = 153) group.

Data	HCC	Healthy
Age	58.93 ± 9.99	48.32 ± 5.16
Mean ± SD	58	48
Median		
Gender	124	45
Male	29	31
Female		
HCC stage (BCLC staging)	12	-
0	54	-
A	61	-
B	26	-
C	0	-
D		
Gene expression(Ct)	28.47 ± 4.43	34.23 ± 3.54
FLNA	28.01 ± 3.75	32.90 ± 4.15
CLU	29.64 ± 3.90	32.18 ± 5.02
CAP1		
p-value	< 0.0001	
FLNA	< 0.0001	
CLU	0.0003	
CAP1		

Quantitative Real-time PCR analysis with $2^{-\Delta\Delta Ct}$ calculation

In the HCC group, The Ct value was 28.47 ± 4.43 for FLNA, 28.01 ± 3.75 for CLU and 29.64 ± 3.90 for CAP1. The level of Ct value in the healthy group was 34.23 ± 3.54 for FLNA, 32.90 ± 4.15 for CLU and 32.18 ± 5.02 for CAP1 *(*Table 2*)*. These results demonstrated that the Ct values in HCC group were significantly lower than the healthy group (p-value < 0.0001 in FLNA, CLU and p-value = 0.0003 in CAP1 *(*Fig. 2.*)*.

Furthermore, we used the $2^{-\Delta\Delta Ct}$ method to calculate expression power compared to the house keeping gene from both HCC and healthy groups. We found that within the HCC group FLNA, CLU, CAP1 gene expressed (Median) 112.7 folds, 134.2 folds, 11.3 folds to the house keeping gene expression, respectively while the Healthy group expressed 1.9 folds, 0.1-fold, 17.1 folds to the house keeping gene, respectively with p-values of < 0.0001, < 0.0001, 0.4663, respectively *(*Fig. 2.*)*.

The performance of genes as a screening test

The performance of results *(*Fig. 3.*)* are reported hereafter. When using the cut-off value of Ct value > 33, the results of accuracy, sensitivity and specificity were FLNA (80.8% accuracy, 88.0% sensitivity, 65.8% specificity) *(*Fig. 3.a*)*, CLU (63.4% accuracy, 93.3% sensitivity, 31.3% specificity) *(*Fig. 3.b*)* and CAP1 (67.2% accuracy, 83.3% sensitivity, 39.1% specificity) *(*Fig. 3.c*)*. Then, the two and three-gene combinations were performed. The results showed that the combination of FLNA & CLU (86.2% accuracy, 85.0% sensitivity, 88.4% specificity) *(*Fig. 3.d*)* demonstrated higher proficiency than the combination of FLNA & CAP1 (74.1% accuracy, 77.5% sensitivity, 68.1% specificity) *(*Fig. 3.e*)*, CLU & CAP1 (76.4% accuracy, 80.8% sensitivity, 67.7% specificity) *(*Fig. 3.f*)*. However, the three-gene combination could not affect the efficiency of the test (80.2% accuracy, 75.8% sensitivity, 88.7% specificity) *(*Fig. 3.g*)*. From all the ROC graphs *(*Fig. 3*)* we showed that the combination of FLNA & CLU has the greatest discriminate capacity than the other tests.

Discussion

FLNA and CLU gene combination might be a prospective marker for HCC

We demonstrated that the PBMCs are affected by the HCC and the result contained the upregulated FLNA and CLU gene (86.2% accuracy, 85.0% sensitivity, 88.4% specificity) which possess high performance as novel screening markers for discriminating the presence or absence of HCC in patients. Moreover, this test only requires peripheral blood for testing e.g., AFP and is considered one of the least invasive types of testing available. Furthermore, this test is operator independent, unlike ultrasound which require years of training and hours of procedures to be able to produce reliable results.

Individual gene function and expression on HCC cells

The relationship between HCC cells and each candidate gene does exist. There are reports on FLNA gene that it has a complex role on the cytoskeleton[18]. Filamin A has also been reported to be involved in cell migration by prompting transcription factor SRF along with MLK1. It is reported that elevated gene expression in the harvested HCC tissue could be a prediction of recurrence of HCC[19] and could also be a marker for the progression of HCC[20]. It has been shown that CLU (or Clusterin) is involved in cell aggregation in vitro[21], promoting metastasis of HCC[22–23], protecting HCC cells from endoplasmic reticulum stress induced apoptosis[24], and affecting resistance to drugs (such as Sorafenib)[25–26]. A study of the CAP1 gene reported its involvement in the metastasis of hepatocellular carcinoma because it is

related to the control of actin filaments and is also thought to be involved in the localization of cell polarity and mRNA[27]. Another study has reported that CAP1 gene expression increases in other cancers, such as ovarian cancer, and is involved in cell proliferation[28]. It might be unusual to consider it as mere coincidence that the expression of these genes is also upregulated in PBMCs. It may be possible to think that cancer cells reshape the PBMC with some mechanism. An experiment was conducted in our laboratory in breast cancer cells which showed the actual “reshaped” by cancer cell was carried out, although the detailed mechanism is unknown[9]. The same phenomenon seemed to be occurring also in the case of HCC. Future study to show the phenomenon in vitro and to clarify the mechanism are needed.

Benefits of this study (qRT-PCR assay)

Currently, the diagnosis of HCC has been done with imaging tests (Ultrasound, CT, MRI), conventional markers like AFP, or biopsy.[4] However, all of them have limitations: imaging tests are quite expensive and not very suitable for wide screening and some tests are operator dependent; conventional markers have unsatisfiable accuracy; the pathological diagnosis using biopsy is highly invasive. Our FLNA and CLU combination markers, on the contrary, can be attained by less invasive blood test, yield high performance, and could be done with lower cost. Our markers could be expected to contribute in both screening and diagnosis of HCC in future clinical application.

Limitations of the study

This experiment did not compare samples of patients with other related cancer patients or other related diseases such as hepatitis or Cholangiocarcinoma. It is possible that these gene expressions may be similarly upregulated in other related diseases, in which case, the accuracy may be affected in clinical application. Therefore, in the future, investigating whether elevated expression of the gene is specific to patients only with HCC is warranted.

Methods

Method statement

all research was performed in accordance with relevant guidelines and regulations.

Bioinformatics analysis

In this study, we recruited a bioinformatics approach to narrow the candidate genes for potential markers. In the Gene Expression Omnibus (GEO) repository of NCBI, microarray analysis results submitted by worldwide researchers are made available. From the NCBI database, data sets of gene expressions in PBMC were searched. Search terms were (Homo sapiens) AND (HCC OR (hepatocellular carcinoma)) AND (PBMC OR (peripheral blood mononuclear cell) OR (white blood cell) OR (WBC))”. Inclusion criteria were (1) PBMCs or any other white blood cells’ expression file (2) Including both healthy donor cases and HCC cases (3) Datasets of homo sapiens. As a result, two gene expression datasets that compared between PBMC samples from healthy individuals and HCC patients were selected, GSE49515 and GSE58208. We

conducted t-tests in each gene expression of each dataset using “Connection Up and Down Regulation Expression Analysis of Microarrays (CU-DREAM) http://pioneer.netserv.chula.ac.th/~achatcha/CU-DREAM/)[29]”, to evaluate the intersection genes and obtained 187 upregulated genes from both datasets. Then, three genes with highly significant p-values ($p < 0.001$) were selected and used to observed gene expressions in our samples.

Study Population

All samples were recruited from King Chulalongkorn Memorial Hospital, Bangkok, Thailand and included 2 cohorts as the following:

Cohort 1: Samples were collected from June 2018 to January 2019 and included 83 HCC cases and 52 healthy donors.

Cohort 2: Samples were collected from January 2020 to July 2020 and included 70 HCC cases and 24 healthy donors.

A total of 153 HCC cases and 76 healthy donors participated in this study. Patients with hepatitis viral infection were excluded from this study. HCC staging was recorded according to current BCLC guidelines. All subjects in this study were of Asian descent, further bioinformation is provided in *(Table 2).*

We then used the preliminary results from both GSE 49515 and GSE 58208 to find the appropriate sample size with the following formula:

$$n = [(Z_{\alpha/2} + Z_{\beta})^2(\sigma^2_d)]/(_d)^2$$

n = sample size

d = Different of value in each group

$_d$ = Different of mean in each group

σ^2_d = Different of variance in each group

$Z_{\alpha/2}$ = Standard normal variate for level of significance

Z_{β} = Standard normal variate for power

We calculated and found that the sample size for our study was 24.44 samples, confirming that our study has recruited enough samples for the experimentation.

Blood sampling and PBMC extraction

Two ml of EDTA blood was extracted from all patients. Lymphocyte isolation medium was added to a 15 ml tube and centrifuged at 1600 rpm at 16°C for 12 minutes and the plasma was separated. Whole

blood (diluted 1: 1) with PBS was carefully layered on a tube of lymphocyte separation medium and centrifuged at 2,800 rpm for 15 minutes at 16°C. The cell interface layer was carefully separated into 1.5 ml tubes and cells were washed with 1 ml PBS for 15 minutes at 1700 rpm 16°C and 500 ml PBS for 5 minutes at 4°C. The research methodology employed in this project was approved by The Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No. 108/60 and 438/60). All study subjects provided written informed consent.

RNA extraction

PBMCs were mixed with 1 ml of TRIzol reagent (ThermoFisher Scientific, MA, USA) and incubated at room temperature for 5 minutes, then 200 µl of chloroform was added and incubated at room temperature for 3 minutes. Thereafter, it was separated into three phases by centrifugation at 8,760 rpm at 4°C for 15 minutes. The colorless upper aqueous phase was transferred to a new RNA tube, supplemented with 4 µL of glycogen (20 mg / mL) and 500 µl of 100% isopropanol, incubated for 10 minutes at room temperature, then centrifuged at 8,760 rpm at 4°C for 15 minutes. The supernatants of the centrifuged tubes were discarded, and the RNA pellets were washed with 1 ml of 75% ethanol, mixed by vortexing, and centrifuged at 6,930 rpm at 4°C for 5 minutes. Thereafter, supernatant was discarded again, and RNA pellet was dried by vacuum for 8 minutes and resuspended with 30 µL of DEPC water. RNA concentration and integrity were confirmed by Nanodrop and bioanalyzer.

Complementary DNA (cDNA) synthesis

After, the total RNA was extracted from PBMCs using TRIzol reagent (Thermo Scientific) according to the manufacturer's protocol. Then, cDNA was synthesized using RevertAid First Strand cDNA Synthesis (Thermo Scientific). The process of cDNA synthesis is as follows: thaw, mix and centrifuge the components of the kit then add the template RNA 0.1 ng - 5 µg, primer 1 µL, nuclease-free water up to 12 µL, 5X reaction buffer 4 µL, Ribolock RNAse inhibitor 1 µL, 10 mM dNTP mix 2 µL, RevertAid M-MuLV RT 1 µL. After mixing and brief centrifuging, the samples were incubated for 5 min at 25°C followed by 60 min at 42°C. Finally, terminate the reaction by heating at 70°C for 5 min. The product of the first strand cDNA synthesis can be used directly in PCR or qPCR.

Primer preparation

Primers were designed using Primer3plus[30] (for FLNA) and Primer Blast[31] (for CAP1 and CLU). Primers were synthesized by BIONEER. Each primer sequence, melting temperature, and product length are shown in *(Table 3)*. Prior to quantitative PCR, conventional PCR and electrophoresis for finding optimal temperature for each primer was conducted.

Table 3
Details of forward and reverse primer sequences of three candidated genes used for qRT-PCR analysis.

Gene	Forward	Reverse	Tm	Product length
CLU	CAGGCCATGGACATCCACTT	GTCATCGTCGCCTTCTCGTA	60.03	78 bp
FLNA	TTTCCGCCAAATGCAGCTTG	ACACCAGTTTGATGCTCTCG	60.32	74 bp
CAP1	GGAACTCTGAGGTGGTCCATTA	ACGGTGCATGTCAGAGGTATG	60.13	108 bp

Quantitative Real-time PCR (qRT-PCR) analysis

The quantitative PCR contained 10µl SensiFast (Bioline), 0.8µl of forward and reverse primers, cDNA(1µl for FLNA, 0.5µl for CLU and CAP1), and 7.4µl distilled water in a total volume of 20µl. The reactions were carried out on QuantStudio 6 (Thermo Fisher Scientific) according to the manufacturer's protocol. PCR conditions were as follows: denaturation at 95°C for 2 mins with 45 cycles, annealing at 59°C, 55°C, 59°C for CLU, FLNA, CAP1, respectively for 30s. Fluorescence signals from the amplified product were detected at the end of the annealing step. Duplications were done on available and unamplified samples. The Ct value was set to 45 if the sample did not show any amplification twice. In this study, the housekeeping gene or the reference gene, that was used is glyceraldehyde 3-phosphate dehydrogenase (GAPDH). We used this gene to test and analyze alongside our interested gene (CLU, FLNA, and CAP1).

The calculation is as follows:

$\Delta\Delta Ct = \Delta Ct$ (a target sample) $- \Delta Ct$ (a reference sample)

The final result is represented in the folds of change (thus, the equation is in the power of 2 or $2^{-\Delta\Delta Ct}$) of the interested gene expression in the sample against the reference sample.[32]

Statistical analysis

Box plot, summary of the dataset (including t-test results of Ct mean of each gene), benchmarks (Accuracy, Sensitivity, and Specificity) heatmaps (confusion matrices) and the Receiver Operating Characteristic (ROC) curves were drawn with python 3.9 program with packages (scipy[33], pandas[34] and matplotlib[35]). Ct values of each gene were included into the dataset. For the evaluation of performance, the entire dataset was used for the test. The p-value cut-off for each test was at <0.05 for results to be statistically significant.

Abbreviations

AFP Alpha-Fetoprotein

CAP1 Cyclase Associated Actin Cytoskeleton Regulatory Protein 1

CLU Clusterin

Ct Cycle threshold

FLNA Filamin A

GAPDH glyceraldehyde 3-phosphate dehydrogenase

GEO Gene Expression Omnibus

HCC Hepatocellular Carcinoma

PBMCs Peripheral Blood Mononuclear Cells

qRT-PCR quantitative Real Time – Polymerase Chain Reaction

ROC receiver operating characteristic

WBCs White Blood Cells

Declarations

Acknowledgement

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Author's contributions

RP – acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis

SR - acquisition of data, analysis and interpretation of data, statistical analysis

PK - acquisition of data, material support

NC - acquisition of data, material support

PT - acquisition of data, material support

AM - study concept and design, obtained funding, administrative support

CP – study concept and design, acquisition of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, technical support, study supervision

Conflicts of interest

All Authors Declare No conflicts of interest exist.

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Ethical statement

The research methodology employed in this project was approved by The Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No. 108/60 and 438/60). All study subjects provided written informed consent.

Authors statement

All authors had access to the study data and had reviewed and approved the final manuscript.

Method statement

all research was performed in accordance with relevant guidelines and regulations.

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Figures

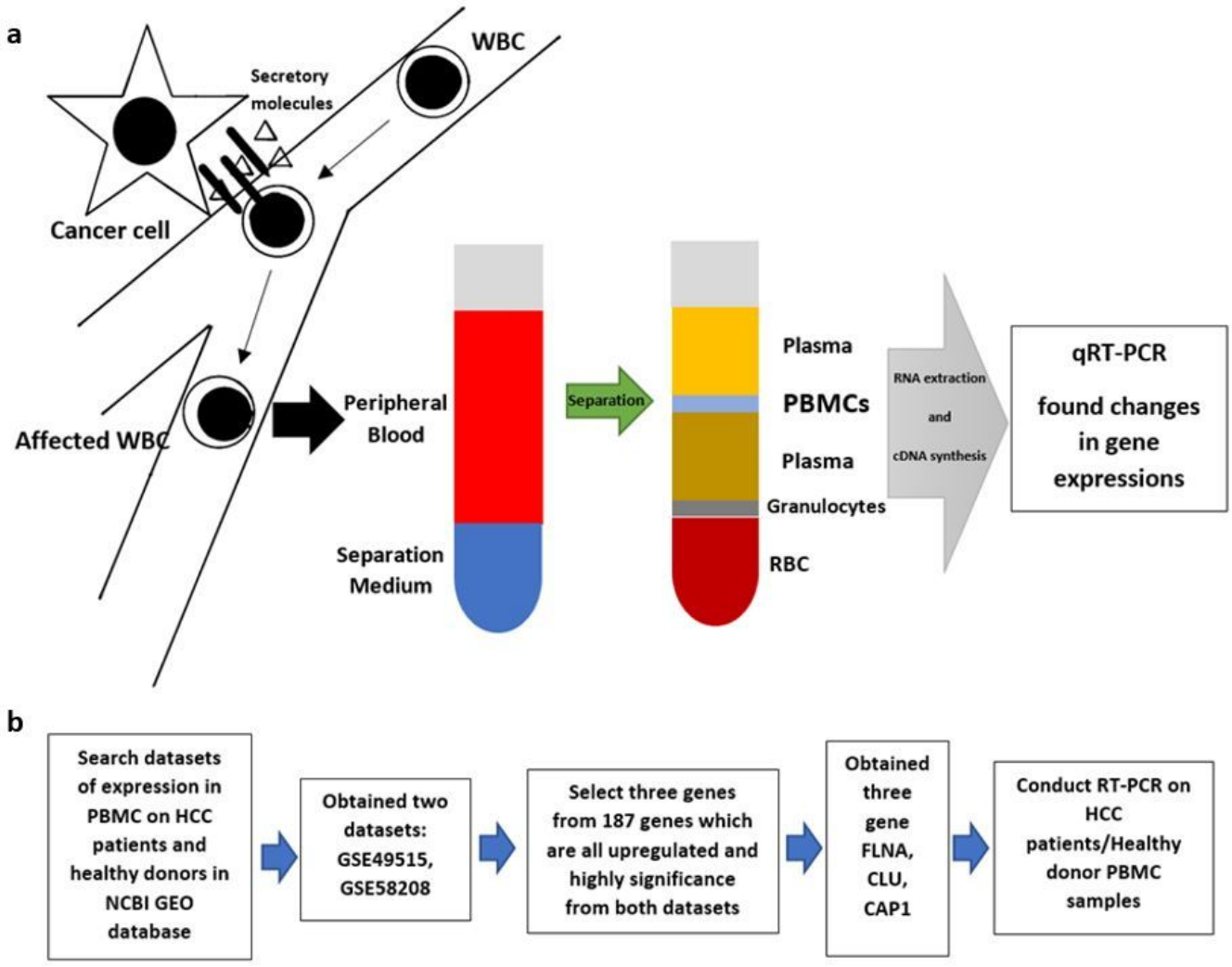

Figure 1

Summary of the experiment. a an illustration of the effect of the secretory molecule from cancer cell that regulated gene expression change in WBC. The study focused on the usability of affected WBC especially for PBMCs which detected from the RT-PCR as a high-performance biomarker for HCC. b a schematic design flow of the experiment following the above illustration

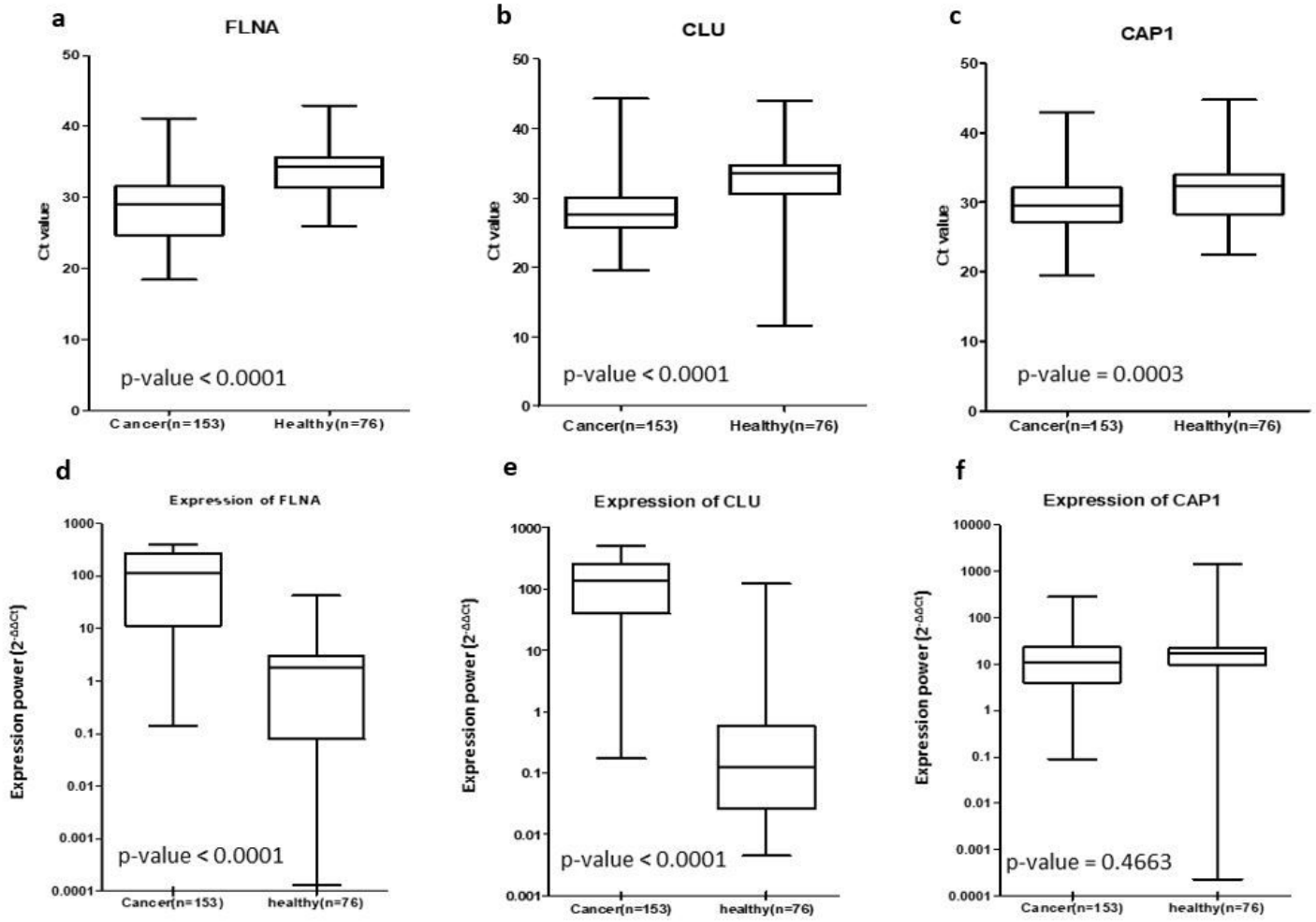

Figure 2

Ct values and expression (2-ΔΔCt) of each gene compare to the housekeeping gene (GAPDH) within Healthy Donor group and HCC cancer group, shown in boxplot (Mean±SD); a Ct value of FLNA gene, b Ct value of CLU gene, c Ct value of CAP1 gene, d Expression of FLNA gene, e Expression of CLU gene, f Expression of CAP1 gene. The Ct values in HCC group were significantly lower than the healthy group (p-value < 0.0001 in FLNA, CLU and p-value = 0.0003 in CAP1). The expression of FLNA, CLU, CAP1 was increase in the HCC group when compare with the healthy group (p-value<0.0001 in FLNA and CLU and p-value = 0.4663).

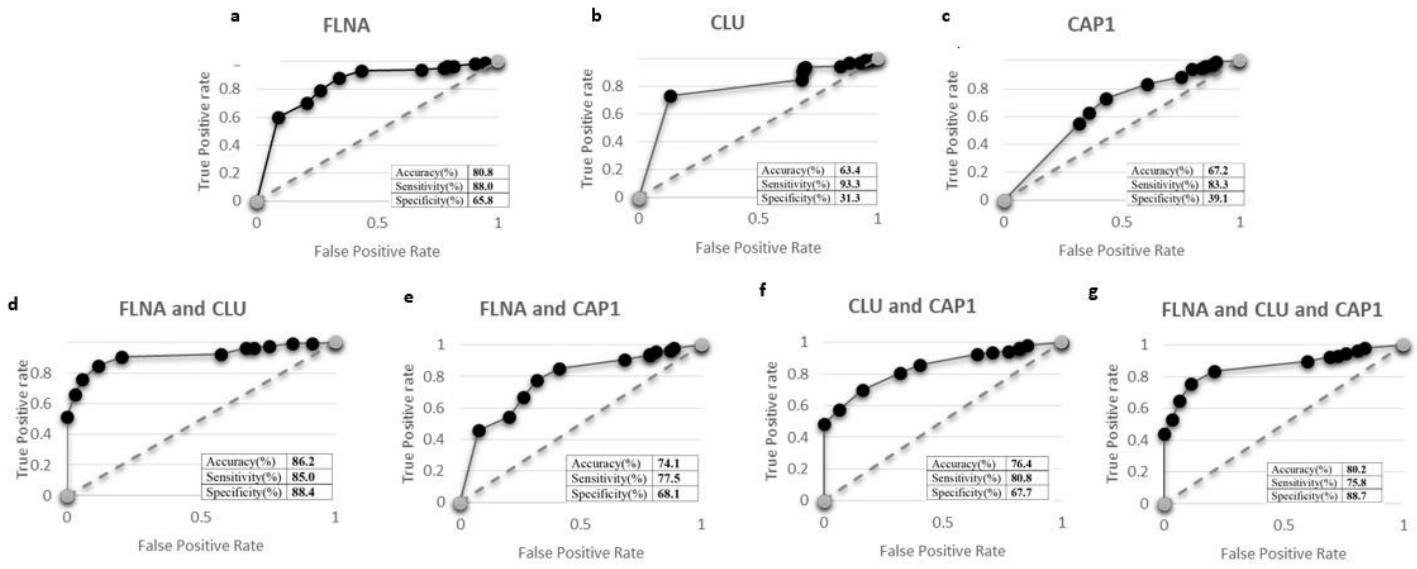

Figure 3

The ROC graph and the result of accuracy, sensitivity, specificity in each combination including; a FLNA, b CLU, c CAP1, d FLNA and CLU, e FLNA and CAP1, f CLU and CAP1, g FLNA and CLU and CAP1. The combination of FLNA and CLU has the greatest discriminate capacity than other tests (86.2%accuracy, 85.0%sensitivity, 88.4%specificity).