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Expression and Relative Quantification of B29 Gene in Normal and Cancer Patients

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Abstract: ***B-cell exihibit B cell receptor or BCR, a transmembrane protein located on its surface. BCR comprises of two transmembrane proteins Igα and Igβ. Igβ (CD79B) is encoded by B29 gene. Leukaemic B- cells express low toundetectable Igs, Igβ (CD79B) proteins. Altered and aberrated B29 gene affect expression of Igβ/CD79B protein. One of the main factor of B29 gene alteration is mutation in their coding sequence. We analyzed the quantitative expression of B29 gene in leukaemic samples. Primers for B cell expression was designed using PrimerExpress 3.0 software. Designed primers were used to analyze the expression of B29 gene in different samples through ABI prism 7000 SDS system. Relative quantification of B29 gene in samples was done by 2^-ΔΔCt method. There was under expression of B29 gene in 100% of leukaemic samples. Though reason for lower gene expression in leukaemic samples was not detected yet there are number of papers reporting mutations and methylation as main cause of B29 gen alteration.***

Keywords: B cell receptor, B29, Primer designing, Absolute quantification, 2-ΔΔCt method

1. Introduction

B CLL or B chronic lymphocytic leukemia is the most common form of leukemia. B-lymphocytes in B CLL condition express CD5 [1,2], high Bcl-2 expression and decreased Igs [3-5]. B-lymphocytes have two types of Ig, one is intracellular and other is surface Ig. Intracellular Ig in B CLL is normal to overexpressed [6,7] while surface Ig (sIg) are underexpressed in B CLL [8,9].

B cell exhibit B cell receptor (BCR). Major component of BCR are sIg that covalently bind to heterodimer Igα(CD79a) and Igβ(CD79b) [10,11]. Igα and Igβ proteins are encoded by mb-1 and B29 gene respectively. Expression of these genes are essential for proper expression of BCR [12,13]. These gene play an important role in antigen internalization and induction apoptosis through the BCR pathway [14]. BCR signaling is coordinated by immunoreceptor tyrosine activation motifs (ITAMs) of B29-mb1 heterodimer [15]. Mutation in heterodimer disrupt B29 signal transduction [16-19]. Underexpression or no expression of any of these genes disrupt pre B-cell and B-cell development [20-22]. It was found that B29 deficient mice are blocked at earliest stage of B cell development [22].

B29 gene alteration in CLL patients may be due to either mutation or due to DNA methylation. There are reports on B29 gene mutations in CLL patients [23]. Several mutations in B29 gene were predicted to produce truncated B29 protein. A study of mRNA in B CLL has shown that B29 mRNA was undetectable in half of the B CLL samples whereas mRNA level of B29 was normal in other half of samples. However, B29 gene showed point mutations, insertion, deletion in its transmembrane and cytoplasmic domains in B CLL cells having normal level of mRNA that account for low CD79b surface expression [23].

2. Material and Method

2.1. Material

Normal blood sample were contributed by healthy volunteers. Five cancerous blood samples were collected from local hospital in Noida.

Trizol (Phenol 38 %, Guanidium thiocyanate 0.8 M, Ammonium thiocyanate 0.4 M, Sodium acetate (pH 5) 0.1 M, Glycerol 5%), 0.8 M sodium citrate/1.2 M NaCl, Isopropanol, Chloroform, 70% ethanol, DEPC treated ddH2O, TE buffer(10 mM Tris buffer, 1mM EDTA (pH 8.0)

2.2. Method

2.2.1. RNA extraction

Total RNA was extracted from normal and cancerous blood cells by using the Trizol reagent. 0.1 ml of blood and 1 ml Trizol. 0.2 ml of chloroform (0.2 ml per 1 ml of TRI Reagent) was added to the tubes, shaken vigorously for 30 seconds by hand/vortex mixed and incubated at RT for 10 mins. The samples were centrifuged at 12,000 X g for 15 mins at 4° C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase containing the RNA. The upper aqueous phase was removed without disturbing the interphase and collected in a fresh tube. 0.5 ml isopropyl alcohol per 1 ml of TRI Reagent was added to the tubes. The tubes were then incubated at RT for 10 minutes and then centrifuged at not more than 12,000 x g for 10 minutes at 4 ° C. The supernatant was removed completely. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. The pellet was washed with 1ml of 75% ethanol per 1 ml of TRI Reagent. The samples were mixed by vortexing and centrifuged at no more than 7,500 x g for 5 minutes at 4° C. The pellet was air-dried by keeping the RNA pellet containing tube opened in working bench for 15 mins. The RNA was dissolved in 50 µl DEPC-treated water by passing solution a few times through a pipette tip. The RNA was stored at -20° C for further use or immediately processed for cDNA synthesis.

2.2.2. Qualitative Estimation of RNA Concentration by Denaturing Gel Electrophoresis

The extracted RNA was run on a 1% TAE gel at 65 V for ¾ length of gel at 4^0C. Gel was visualized in UV trans-

illuminator of 300 nm.

2.2.3. Quantitative estimation of RNA

The concentration of the extracted total RNA was quantified by measuring the absorbance at 260 nm in a spectrophotometer and calculated by using the formula as given below

Total RNA (µg /ml) = OD260 × 40 × Dilution factor.

2.2.4. cDNA First Strand Synthesis

cDNA first strand was synthesized using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit #K1621. All components of kit were thawed and centrifuged at 2000 rpm for 2 min and placed on ice. RNA concentrations of all samples were optimized to 5 µg in 20 µl reaction mixture. RNA template 5 µg, oligo (dt)18 primer 1 µl, Water: nuclease free added to PCR vials (200 µl). Reaction mixture was gently mixed, centrifuged and incubated at 650C for 5 min. Chilled on ice, spinned down and again place in ice. 5 X Reaction buffer 4 µl, RiboLock RNase Inhibitor 1 µl, 10 mM dNTP Mix 2 µl, RevertAid M-MulV Reverse Transcriptase 1 µl added to reaction mixture. Gently mixed and centrifuged. Incubated for 60 min at 420 C followed by reaction termination by heating at 700 C for 5 min. Reaction product were stored a -200 C.

2.2.5. Primer Designing

All the transcripts of B29/CD79b gene were downloaded from NCBI site. A conserved sequence between the transcripts was retrieved using MAFFT (Multiple sequence alignment tool). Alignment file was visualized in JalView and conserved sequence was downloaded. The retrieved sequence was then used to design primer for real time PCR of B29 gene. Primer for real time PCR was designed using Primer Express 3.0. Primer specificity was checked using Primer Blast tool of NCBI site.

2.2.6 Real Time PCR of B29 gene

Quantitative estimation of the expression of the B29 genes was done via real-time PCR analysis. cDNA was used to analyze the expression of B29 genes along with β-Actin as a house keeping gene.

For qPCR kit Kappa SYBR ® fast Universal (2x) mastermix was used. Mastermix was prepared according to protocol of kit cdna and primers and were then processed on the qPCR. machine (ABI PRISM® 7000 Sequence Detection System)
qPCR Master mix

Table 1: qPCR mastermix preparation

Component	20 µl rxn1	Final conc.
PCR-grade water	Up to 20 µl	N/A
2X KAPA SYBR® FAST qPCR Master Mix Universal	10 µl	1X
10 µM Forward Primer	0.4 µl	200 nM
10 µM Forward Primer	0.4 µl	200 nM
Template DNA	1 µl	<20 ng
50X ROX High	0.4 µl	1X

qPCR program – The first segment is a hot-start phase, which activates the SYBR® green. The second segment is the annealing and elongation phase, as with conventional PCR. The last segment creates a dissociation curve.

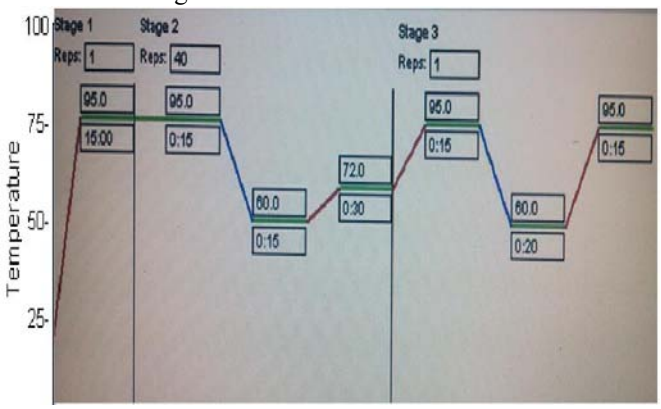


Figure 1: Thermal profile

The first segment is the 'hot-start' segment, while the second segment is the annealing and elongation part, as seen in conventional PCR. In this part a measurement is obtained in each cycle-end, which measures the relative amount of the target gene. Lastly, the third segment measures the dissociation of the product to determine the melting temperature to make a dissociation curve. The thermal profile was set to 95°C for 15 minutes in the first segment and 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 30 seconds in the second segment. The first segment was 1 cycle, while the second segment was set to 40 cycles. The last segment was set to 95°C 15 seconds, 60°C for 30 seconds and 95°C for 30 seconds, which also was 1 cycle.
Dyes ROX was chosen as reference dye. Standard curve was obtained by running the diluted cDNA samples. Dilution series of cDNA was 1x, 3x, 9x, 27x and 81x. Automatic threshold value was obtained by standard curve.

3. Result and Discussion

3.1 Qualitative analysis of RNA

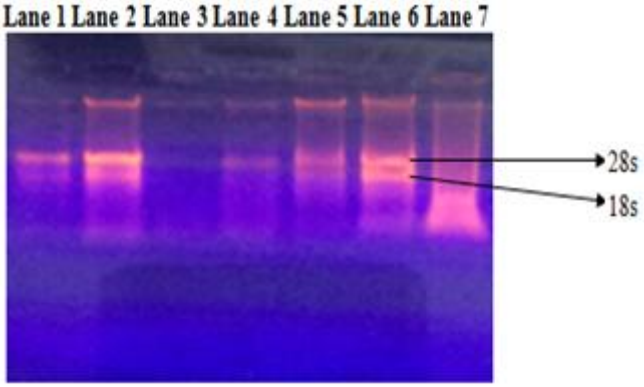


Figure 2: Extracted RNA run on 1% TAE agarose gel

Table 2: Table showing Lane numbers and their respective normal and cancer blood samples. Sample CP1, CP3, CP4, CP5 and CP 6 were selected for further study

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Normal blood RNA	CP1 (Cancer patient 1)	CP2 (Cancer patient 2)	CP3 (Cancer patient 3)	CP4 (Cancer patient 4)	CP 5 (Cancer patient 5)	CP 6 (Cancer patient 6)

3.2 Quantitative Estimation of RNA

Table 3: Concentration of RNA and their yield in different sample

Sample	Absorbance @ 260nm	Absorbance @ 280nm	Abs @260nm / Abs@ 280nm	Conc. of RNA (microgram/ml)	Yield of RNA in 50 microlitre (microgram)
Normal Blood RNA	0.128	0.065	1.94	512	25.6
CP 1	0.086	0.040	2.1	344	17.2
CP 3	0.079	0.043	1.83	316	15.8
CP 4	0.097	0.051	1.90	388	19.4
CP 5	0.132	0.069	1.89	528	26.4
CP 6	0.131	0.070	1.87	524	26.4

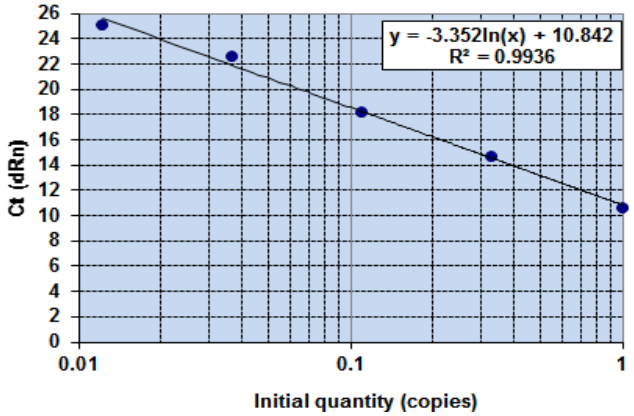
3.3 Table 4: Table showing sequence of Designed primer

Primer sequence	Tm	Length	GC %
TGTCCAGCTCTTGCCAAAGG	60.54	20	55
ATCCCCAGAGAACTCCCTCC	60.03	20	60

3.4 Real Time PCR of B29 gene

The gene expression level of B29 gene was detected in five cancerous blood samples.

qPCR data was compared to B29 expression level of healthy human blood, normalized to β-actin [24]. Standard curve with cDNA dilution of 0x, 3x, 9x, 27x and 81x. Ct values of dilution were used to prepare standard curve. Standard curve should always give a slope of -3.3 to -3.6 and PCR efficiency should be between 80-100%. Standard curve for β-actin showed a slope of -3.35 and efficiency of 98.7 % standard curve for B9 showed a slope of -3.404 and efficiency of 96.67 % (Graph 1 and Graph 2 respectively).



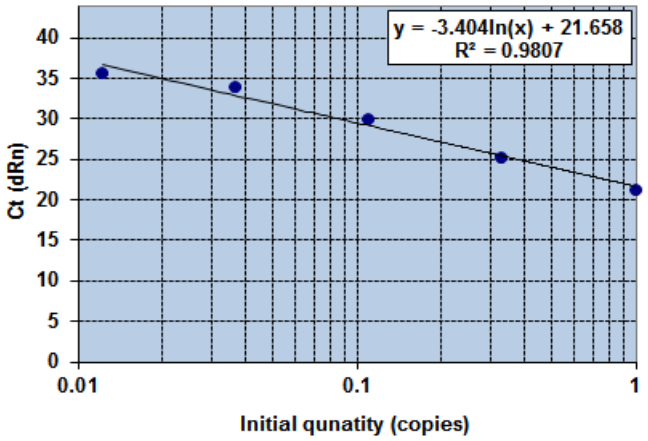
Graph 1: STANDARD CURVE OF B-ACTIN

Standard curve obtained by real time pcr of b-actin gene with normal blood sample. Curve plotted against Ct (dRn) and initial quantity indicted good PCR efficiency. Standard curve analysis is required to predict PCR efficiency and the initial amount of cdna for further quantification and expression of protein in all sample. Ct values of all diluted cdna is given in table no 5. Lower Ct value of b-actin gene indicate higher quantity of initial gene.

Table 5: Ct value of diluted cdna quantity of normal blood sample for β-actin

Dilution	*cDNA (ng or µl)*	*Ct (or CP)*
1x	1	10.59
3x	0.333333333	14.65
9x	0.111111111	18.17
27x	0.037037037	22.6
81x	0.012345679	25.03

Graph 2: STANDARD CURVE OF B29



Standard curve obtained by real time pcr of B29 with normal blood sample. Curve plotted against Ct (dRn) and initial quantity indicted good PCR efficiency. Ct values of all diluted cdna is given in table no. 6. Higher Ct value of B29 gene indicate lower quantity of initial gene.

Table 6: Ct value of diluted cdna quantity of normal blood sample for B29

Dilution	cDNA amount	Ct (or CP)
1x	1	21.23
3x	0.333333333	25.14
9x	0.111111111	29.92
27x	0.037037037	33.8
81x	0.012345679	35.6

Table 7: Threshold values obtained for B29 gene

	Threshold value
B29	0.024

Threshold value for B29 gene was calculated by setting automatic mode of PCR machine while running the qpcr.

3.5 Quantification of Gene

The quantification of gene was obtained by qPCR, which was performed on healthy blood tissue and five cancerous blood samples B29 gene. The data (Ct values) was normalized by calculating Δct followed by ΔΔct. The ΔΔct values were normalized with the formula 2-^ΔΔct

3.5.1 Ct values for β-actin and B29 gene

The qPCR data was compared to the gene expression levels of healthy human blood, normalized to β-actin. The data collected from qPCR was normalized against β-actin according to Livak & Schmittgen (2001). The qPCR results are given as a threshold cycle (Ct) values, which indicate the cross point between the threshold line and the exponential

phase of thefluorescence measurements.

Table 8: Ct values for β-actin and B29 of normal and cancerous blood samples

Ct	β-actin	B29
Control	10	14
CP1	12	16.56
CP3	9	16
CP4	12	17.432
CP5	13	17.136
CP6	15	20.794

The values are obtained from qPCR for the samples, by using 3x cDNA dilution. Lower Ct value of b-actin in all sample indicate their amplification start much earlier and gives early fluorescence than the B29 gene. Ct value of b-actin are average of 11.883 indicating early amplification of b-actin. Average Ct values calculated by taking Ct value of all samples of B29 gene is 16.51.

3.5.2 ΔCt values for β-actin and B29

Table 9: ΔCt values for β-actin and B9 from normal and cancerous blood samples

ΔCt	ΔCt B29
CONTROL	4
CP1	4.56
CP3	7
CP4	5.432
CP5	4.136
CP6	5.794

ΔCt of all samples were calculated by subtracting Ct value of gene with Ct value of β-actin in respective sample. Delta Ct shows the difference of expression between 2 genes (B29 proteins and housekeeping gene b-actin in our study).

3.5.3 ΔΔCt values for β-actin and B29.

The data (Ct values) was normalized by calculating ΔCt followed by ΔΔCt. The ΔΔCt values were normalized with the formula 2^ΔΔCt (Livak & Schmittgen, 2001).

Table 10: ΔΔCt values for β-actin and B29 from normal and cancerous blood samples

ΔΔCt	**ΔΔCt B29**
CONTROL	0
CP1	0.56
CP3	3
CP4	1.432
CP5	0.136
CP6	1.794

Specifically, Delta Delta CT assumes that each PCR cycle will exactly double the amount of material in your sample amplification efficiency = 100%. ΔΔCt was calculated using the following formula.

$$\Delta\Delta CT = (CT\ (target, sample) - CT\ (ref, sample)) - (CT\ (target, control - CT\ (ref, control))$$

ΔΔCt of all samples for B29 are positive except CP5 AND CP6.

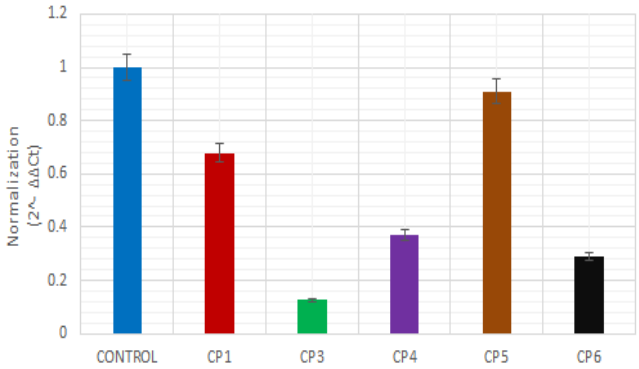
3.5.4 Normalization of β-actin and B29. The data are normalized according to Livak & Schmittgen (2001).

Table 11: Normalization (2^- ΔΔCt) data for B29 gene.

Normalization (2^- ΔΔCt)	B29
CONTROL	1
CP1	0.678302
CP3	0.125
CP4	0.370617
CP5	0.910039
CP6	0.288371

Expression of B29 highest in CP5 followed by CP6>CP1>CP4>CP3. B29 gene overexpressed in CP5 and CP6 sample while under expressed in CP1, CP3 and CP4.

Graph 3: Graph shows expression of B29 gene in different cancer blood samples with the designed specific primer through qPCR



Graph indicate the relative gene expression of B29 gene normalized to β-actin. This method is a convenient way to calculate relative gene expression levels between different samples in that it directly uses the threshold cycles (CTs) generated by the qPCR system for calculation. 2-ΔΔCT of B29gene in sample CP5 is highest indicating their high fold change and high expression in the sample.

4. Conclusion

In our work, we concluded that though mRNA levels of normal and cancer blood samples are near about same, their expression for B29 gene greatly vary between the sample. It was found that B29 gene is under expressed in all leukaemia sample in comparison to normal blood sample.

We selected few samples. Our sample size is small. Sample size can be increased to get more specific data and result predicting B29 gene role and its expression in leukaemia blood samples.

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